The Effects of Gestational and Lactational Bisphenol A Exposure on Rat Pup Morphometric Measurements and on Adrenal Gland Glucocorticoid Receptor Gene Expression

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Abbreviations

The following are terms that have been frequently used throughout the thesis, in full and abbreviated forms.

Full Term	**Abbreviation**
Endocrine Disrupting Chemicals	EDC
Hypothalamic-Pituitary-Adrenal Axis	HPA axis
Bisphenol A	BPA
Estrogen Receptor	ER
Stress Hyporesponsive Period	SHRP
Postnatal Day	PND
Corticotropin Releasing Hormone	CRH
Corticotropin Releasing Hormone Receptor-1	CRHR-1
Adrenocorticotropic Hormone	ACTH
Adrenocorticotropic Hormone Receptor	ACTH-R
Steroidogenic Acute Regulatory Protein	StAR
Corticosterone	CORT
Glucocorticoid Receptor	GR
Corticosteroid Binding Globulin	CBG
Mineralocorticoid Receptor	MR
11-Beta Hydroxysteroid Dehydrogenase	11-βHSD
Glucocorticoid Response Binding Element	GRE
Heat Shock Protein	HSP
Gestational Day	GD
Dihydroepiandrosterone sulfate	DHEA-S
Anogenital Distance	AGD
Crown- Rump Length	CR
Nonmonotonic Dose Response	NMDR

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Abstract

Endocrine Disrupting Chemicals (EDC) are exogenous agents that mimic endogenous hormone activity in the body. EDC exposure during the critical period of neonatal development can potentially cause life-long neurological, behavioural and physiological disease. This thesis focuses on the EDC Bisphenol A (BPA), a synthetic xenoestrogen widely prevalent in everyday materials that has significant environmental relevance given its ubiquitous presence in humans around the world. The central research question of my thesis is: Does perinatal exposure to BPA affect rat pup development?

A rodent model was selected to study the effects of BPA on the adrenal component of the hypothalamic-pituitary-adrenal axis (HPA axis) stress pathway, which has not been extensively studied. Rat dams were divided into five groups (vehicle control (VEH), positive control diethylstilbestrol (DES), BPA 5, BPA 50 and BPA 500 μg/kg bw/day) and dosed daily throughout gestation and for four days of lactation. Rat pups were sacrificed at two time-points at the beginning and the end of the stress hyporesponsive period (SHRP), at postnatal day (PND) 5 and PND 15. Changes in three morphometric parameters (bodyweight, crown-rump (CR) length and anogenital distance (AGD) were assessed based on the factors of Treatment and Sex. Adrenal gland glucocorticoid receptor (GR) and 18SrRNA expression was determined by qPCR in male pups at PND 5 and PND 15.

At PND 5, compared to the VEH group, the BPA 50 pups were significantly heavier (ANOVA, Dunnett's post-hoc) and the DES and BPA 50 pups had significantly longer CR lengths (ANOVA, Dunnetts' post-hoc). At PND 15, xenoestrogen treatment significantly influenced CR length (ANOVA). At both time-points, males had significantly longer AGD than females, as physiologically expected (ANOVA).

Adrenal gland GR expression in male pups was not significantly affected by treatment, but there was an effect of treatment in18SrRNA gene expression at PND 5 (Kruskal-Wallis). Using the △△Ct method to determine GR and 18SrRNA fold changes, we cautiously suggest that our experimental doses resulted in a non-monotonic dose response to BPA in the PND 5 animals and a monotonic dose response to BPA exposure in the PND 15 animals.

This study highly values the importance of investigating the effects of environmentally relevant, low dose BPA exposure during the critical window of development, given the little that is known about potentially permanent alterations to the stress pathway due to exposure during this delicate period of development.

BACKGROUND

Endocrine Disrupting Chemicals (EDC) are exogenous agents that can exert action similar to hormones in the body (Diamanti-Kandarakis et al, 2009). EDC disrupt the normal function of endogenous hormone pathways, thereby contributing to disease and dysfunction (Rudel & Perovich, 2009; Diamanti-Kandarakis et al, 2009). Mechanisms of action include modulation to estrogenic, androgenic, thyroid and steroidogenic enzyme pathways (Diamanti-Kandarakis et al, 2009). Exposure to EDC during neonatal development may have the potential to cause life-long neurological, behavioural and physiological disease (Markey et al, 2003; Henley & Korach, 2006; Rogan & Ragan, 2007). Given the sensitivity of the neonatal period of development to exogenous chemical exposure, it is necessary to consider the effects of putative EDC on endocrine pathway development during this critical period.

My thesis focuses on the EDC Bisphenol A (BPA), an organic compound used to synthesize epoxy resins and polycarbonate plastics (Wetherill, 2007; Doerge et al, 2010a,b.). BPA is widely prevalent in everyday materials such as water bottles, plastic toys and thermal receipts and is ingested and absorbed through the skin (reviewed by Vandenberge et al, 2007). BPA is estimated to be ubiquitously present in over 95% of Canadians (Health Canada, 2012) making it an environmentally relevant EDC to study. Xenoestrogens such as EDC are exogenous compounds that behave physiologically like estrogens (Watson et al, 2007). BPA is a weak xenoestrogen (Lapensee & Ben-Jonathan, 2010; Li et al, 2012; Alonso-Magdalena, 2012) and can influence estrogen-signaling pathways (Naciff et al. 2002; Wetherill et al. 2007; Vandenberg et al. 2009). Both estrogen and xenoestrogens act through the steroid hormone estrogen receptor (ER) and target specific genes in the nucleus to regulate gene expression (Marino, Galuzzo & Ascenzi, 2006). In addition to estrogen's well-established roles in reproduction and development, it is also a modulator of the

hypothalamic-pituitary-adrenal (HPA) axis. The HPA axis is the body's major endocrine axis for stress response regulation and its function is to maintain homeostasis after challenge by a stressor (Smith & Vale, 2006; Stephens, 2012). The HPA axis hormonal cascade culminates in the secretion of glucocorticoids into systemic circulation to exert rapid, nongenomic effects or to transactivate or repress target sequences on genes in cells and tissues (Ranabir & Reetu, 2011). Estrogens can modulate stress pathway components, for example, by increasing or decreasing hormone activity and by altering enzymatic gene expression (Liaw et al, 1992; Serova et al, 2010; Handa, Mani & Uht, 2012). Therefore, it is biologically plausible that the HPA axis would also be perturbed by exposure to a xenoestrogen such as BPA. Examples of established consequences linked to developmental EDC exposure include reproductive tract malformations and carcinomas in males and females (Diamanti-Kandarakis et al, 2009), metabolic syndrome, obesity, type II diabetes (Casals-Casas & Desvergne, 2011), and adverse neurobehavioural effects (vom Saal et al, 2007).

Few studies have examined the susceptibility of the HPA axis to endocrine disruption during the critical period of development. Similarly, assessment of the effects of BPA on HPA axis maturation during fetal and neonatal development has not been well studied. A rat model was chosen for the study as they are a preferred model for in vivo toxicological screening (Parasuraman, 2011), share similarities with the human responses to toxicological exposures (Iannaccone & Jacob, 2009) and allow for the distinctive incorporation of the Stress Hyporesponsive Period (SHRP). This is a unique physiological phenomenon observed in the rodent wherein HPA axis activity is significantly reduced between postnatal day (PND) 4 and 14 to protect the still-developing brain from excessive glucocorticoid exposure. The Long-Evans rat, the particular strain chosen for the project, has a standard gestational period of 21-23 days (White et al, 2011; Anderson et al, 2015) and was selected as it is well characterized in the study of BPA (Adewale et al, 2011; Nanjappa et al, 2012; Sadowski et al, 2014), in studies exploring the SHRP (Kalinichev et al, 2002;

Prusator & Greenwood-Van Meerveld, 2016), and in maternal separation studies (Huot et al, 2002; Ladd et al, 2005).

My investigation of the central research question of my thesis: *Does perinatal exposure to BPA affect rat pup development?* will address these gaps. This is a particularly important question considering the evidence of permanent disruption to endocrine pathways due to xenoestrogen disruption during fetal development, which manifests into various physiological, metabolic, reproductive and behavioural consequences throughout life (Moriyama, 2002; Zoeller, 2005; Diamanti-Kandarakis et al, 2009; Casals-Casas & Desverge, 2011).

I. INTRODUCTION

HPA Axis

The HPA axis is an important endocrine pathway that maintains homeostasis after stressful challenge. Examples of physiological stressors include environmental pollutants (Perez et al, 2013), hypoxia (Perez et al, 2013), aging (Kagias, Nehammer & Pocock, 2012) and foot shock (Van Vuuren, 2005). The body's response to stressors occurs by the controlled secretion of central and peripheral hormonal cascades moderated by negative feedback loops (Smith & Vale, 2006), illustrated in **Figure 1**.The HPA axis is comprised of the hypothalamus, the anterior pituitary and the adrenal glands. This endocrine pathway plays a major role in metabolism and regulates glucocorticoid release from the peripheral adrenal gland to modify the function of other cells and tissues to maintain homeostasis (Smith, 2006).

Hypothalamus

HPA axis activation begins at the hypothalamus where excitatory neurotransmitters stimulate the parvocellular neurons in the hypothalamic paraventricular nuclei to synthesize and secrete corticotropin releasing factor hormone (CRH) into the hypophyseal portal vessels (Vale et al, 1981; Rivier & Vale, 1983; Herman et al, 2012; Stephens & Wand, 2012). CRH travels through the systemic circulation to the anterior pituitary where this neural factor binds to its receptor (CRHR-1) to stimulate the synthesis and release of adrenocorticotropic hormone (ACTH).

Anterior Pituitary

Ligand binding of CRH to CRHR-1 stimulates the synthesis and release of ACTH, which is cleaved from proopiomelanocortin (POMC) prohormone and is released from the corticotropic cells of the anterior

pituitary (Lim et al, 2014). ACTH travels in the systemic circulation to the adrenal gland (Smith & Vale, 2006) where it binds to G-protein coupled adrenocorticotropic hormone receptors (ACTH-R) (Forti, Diaz & Armelin, 2006) on the cells of the *zona fasciculata* and *zona reticularis* of the adrenal cortex (Cone & Mountjoy, 1993; Elias & Clark, 2000). This triggers a hormonal cascade through the second messenger cAMP and results in the expression of the steroidogenic acute regulatory protein (StAR). This protein directs cholesterol to the inner mitochondrial membrane to undergo steroidogenesis and conversion to pregnenolone, a precursor for many steroid hormones including estrogen and glucocorticoids (Lin et al, 1995; Stocco & Clark, 1996; Gallo-Payet & Payet, 2003). As such, ACTH is necessary for the synthesis and release of glucocorticoids.

Adrenal Cortex

After ligand binding of ACTH to ACTH-R, the adrenal cortex produces and releases two corticosteroid hormones: mineralocorticoids and glucocorticoids (Liu, 2010). The bioactive form of glucocorticoid is called "cortisol" in humans (and non-human primates) and "corticosterone" (CORT) in rodents (Smith & Vale, 2006; Kadmiel & Cidlowski, 2013). To regulate the HPA axis and initiate negative feedback, glucocorticoids bind to the glucocorticoid receptors (GR) at the anterior pituitary and hypothalamus, to be discussed below.

In addition to their role in the HPA axis, glucocorticoids play an important role in the modulation of at least three physiological functions: (i) the stress response, (ii) metabolism and gluconeogenesis (Kuo, 2012; Kuo et al, 2015) and (iii) mediation of the immune system and inflammatory response (McKay & Cidlowski, 2003; Liou, 2010). The scope of this thesis will be restricted to the role of glucocorticoids on the neuroendocrine stress response.

Glucocorticoids

Glucocorticoids are steroid hormones with lipophilic properties and require carrier proteins such as corticosteroid binding globulins (CBG) for circulatory transit (Maes et al, 1996). They pass easily through the cell membrane to activate GR in the cytoplasm and in the nucleus (Lowenberg, 2008; Oakley & Cidlowski, 2013). Only unbound (free) glucocorticoids are able to activate the GR, such that carrier protein availability is a mechanism by which the accessibility of active free glucocorticoids is regulated (Kadmiel & Cidlowski, 2013). The mineralocorticoid receptor (MR) and GR are co-expressed in numerous brain areas including the hippocampus (Bradbury et al, 1991), hypothalamus and anterior pituitary (de Kloet et al, 2000; Cota et al, 2007) and both have binding affinity, albeit unequally, for glucocorticoids (Zhe, Fang & Yuxiu, 2008). The MR is expressed in the central nervous system and in epithelial cells (Funder, 1994) and has a ten times higher affinity for glucocorticoid than does the GR (Kolber, Wieczorek & Muglia, 2008). This allows for the maintenance of low circulating blood CORT levels under homeostatic conditions (Aardal-Eriksson et al, 2001). Therefore, the MR maintains the basal tone of the HPA axis while the GR mediates negative feedback control after stress (de Kloet at al, 1998; Smith, 2006; Cota et al, 2007; Herman et al, 2012; Gomez-Sanchez & Gomez-Sanchez, 2014). The MR is a high-affinity binding site for aldosterone, while the GR is a low-affinity binding site for aldosterone (Eberwine, 1999; Stephenson, 2012). Regulation of active CORT in the rat occurs through two main mechanisms. First, CORT circulates at levels several times higher than aldosterone, but is bound preferentially to CBG and is therefore biologically inactive (Eberwine, 1999). Typically, under normal conditions, CBG-binding to CORT allows for priority binding of aldosterone to the MR (Eberwine, 1999).

When glucocorticoid levels increase during a period of stress, glucocorticoid saturates the MR and instead binds to GR (de Kloet et al, 1998; Oitzl et al, 2009; Stephenson, 2012; Romero & Wingfield, 2016). Considering that MR saturation must be reached prior to CORT binding to GR, ligand binding to GR

would indicate that circulating glucocorticoid levels are high (Romero & Wingfield, 2016) and pose a threat to homeostasis. Second, 11β-hydroxysteroid dehydrogenase type II (11βHSD2), an enzyme highly populated in the fetal brain and the placenta, is key for moderating glucocorticoid levels during development as it quickly metabolizes CORT via oxidation to inactive metabolite form (Eberwine, 1999; Wyrwoll, Holmes & Seckl, 2011). Both mechanisms regulate circulating active CORT levels, play a role in the initiation of the negative feedback loop of the HPA axis during times of high stress, as well as maintain low circulating glucocorticoid levels under non-stress conditions.

Glucocorticoid Receptor

The GR is a ligand-activated steroid hormone nuclear receptor (Dostert & Heinzel, 2004) derived from a single gene (Oakley & Cidlowski, 2011). The GR is expressed in virtually all tissues (Planey et al, 2003; Pujols, 2004; Oakley & Cidlowski, 2011; Preedy, 2016) including the hypophysiotropic neurons of the paraventricular nucleus of the hypothalamus (Walls, 2005), the hippocampus (Jacobson & Sapolsky, 1991; McEwen, 2000) and in the prefrontal cortex (Herman, 1993). Membrane-bound GR protein receptors (mGR) are located on the cell membrane and rapid, nongenomic effects can be exerted upon ligand binding (Strehl et al, 2011; Vernocchi et al, 2013). The chemical compound BPA has been observed to bind to the GR with similar affinity to cortisol, dexamethasone, and other GR-binding drugs (Divya, Prasanth & Sadasivan, 2010).

The human and rat GR have two isoforms which bind specific sequences of DNA to control gene transcription. The two isoforms of the GR are the classic, ligand-binding GR-α and the alternatively spliced GR-β (de Castro et al, 1996; Oakley et al, 1996; Fruchter et al, 2005; DuBois et al, 2013). The human GR is encoded by the NR3C1 gene (Turner & Mueller, 2005; Reddy et al, 2009; Palma-Gudiel, 2015) as is the

rat GR (Mark et al, 2009; UniProt, 2016). The NR3C1 has 9 exons with exon 9 encoding the ligand-binding domain (Jain et al, 2014). In the human, the last exon is spliced to produce 9α and 9β (Jain et al, 2014). This alternative splicing of exon 9α and 9β results in the generation of both GR-α and GR-β isoforms (Bamberger et al, 1995; Oakley Sar & Cidlowski, 1996; Yudt & Cidlowski, 2002; Lu & Cidlowski, 2006; Kino et al, 2009; Jain et al, 2014). Splicing to GR-α and GR- β isoforms similarly occurs in rodents, with both isoforms present in the mouse and in the rat (DuBois et al, 2013). The GR-α isoform is widely expressed in human and rat cells and tissues (Pujols et al, 2002,2004; DuBois et al, 2013) and is the biologically relevant receptor for glucocorticoids (Lu & Cidlowski, 2006; Pujols et al, 2007; Jain et al, 2014). It is the longer isoform and contains a fully functional ligand-binding domain, allowing it to act as a receptor for glucocorticoids (Oakley, Sar & Cidlowski, 1996; Jain et al, 2014). The GR- β has a shorter sequence and lacks the ligand binding domain and is therefore not ligand-inducible by glucocorticoid (Oakley, Sar & Cidlowski, 1996; Jain et al, 2014; LS Bio, 2016). There is some evidence that unbound GR- β may competitively inhibit GR-α at the GR response binding element (GRE) (Oakley, Sar & Cidlowski, 1996; Jain et al, 2014; LS Bio, 2016). As I will be investigating BPA's modulation of rat GR expression, I am limiting the scope of my investigations to the effects of BPA on the receptor most relevant to the HPA axis: GR-α, herein referred to as GR.

Glucocorticoids are small and lipophilic; therefore they are able to pass easily through the cell membrane (Vandevyver, Dejager, & Libert, 2012), which allows for binding to cytoplasmic GR-α. This activates the receptor and allows it to translocate into the nucleus and bind to GRE on DNA to mediate gene transactivation or repression. Receptor phosphorylation is essential for the activation of the GR (Krstic et al, 1997; Miller et al, 2005), and phosphorylation at different sites induces site-specific conformational changes that result in differential cofactor recruitment and ultimately affect gene transcription and

translation (Chen et al, 2008). Similar to most members of the nuclear receptor superfamily, GR resides in the cytoplasm as an inactive receptor where it is bound to the heat shock (HSP) HSP90 chaperone heterocomplex, a combination of HSP90, HSP70, p60 and p23 (Whitesell & Cook, 1996; Beato, Truss & Chavez, 1996; Dittmar et al, 1997; Vandevyver, Dejager, & Libert, 2012). The p23 component is important because binding of this protein further stabilizes the HSP90 heterocomplex (Dittmar et al, 1997; Pratt & Toft, 2003) and allows for enhanced stability of the GR. Bound to the heterocomplex, the GR is transcriptionally inactive, but is held in a conformation that allows for high affinity hormone binding (Oakley & Cidlowski, 2013). Binding of a ligand to GR causes two main actions prior to nuclear translocation. First, a conformational change occurs after ligand binding (Oakley & Cidlowski, 2013) which results in the release of the heat shock proteins from the GR (Pratt et al, 2006; Oakley & Cidlowski, 2013) and this shedding of chaperone proteins exposes two nuclear localization signals (Saklatvala, 2002; Oakley & Cidlowski, 2013). All steroid hormone receptors including the GR are already phosphorylated at the DNA-binding domain of GR (Sanchez et al, 1987), but glucocorticoid agonists also promote a subsequent step of hyperphosphorylation (Orti, Hu & Munck, 1993), which occurs after ligand-binding to the receptor (Bodwell et al, 1998; Ismaili & Garabedian, 2004). Hyperphosphorylation occurs via serine/threonine phosphorylation of GR by map kinase (MAPKs), cyclin-dependent kinase (CDKs) (Krstic et al, 1997) and glycogen-synthase kinase 3 (GSK-3beta) (Rogatsky et al, 1998; Galliher-Beckley & Cidlowski, 2008; Ismaili & Garabedian, 2004).at the transactivation domain or N-domain (Oakley & Cidlowski, 2013). This step induces a conformational change that is necessary for the receptor to interact with cofactors, and largely determines subsequent transcriptional activity (Chen et al, 2008). Although the order of the sequence is not established, it is hypothesized that the HSP90 heterocomplex dissociates after ligand-binding to the GR, and this is followed by hyperphosphorylation of the GR (Bolander, 2013). The hyperphosphorylated complex is then translocated to the nucleus (Savory et al, 2001; Vandevyver, Dejager,

& Libert, 2012). Inside the nucleus, the hyperphosphorylated GR binds to the consensus GR sequence GGAACAnnnTGTTCT, which is an imperfect palindrome (Oakley & Cidlowski, 2013). Specifically, one GR subunit binds to each half site, and the triple n (nnn) sequence between the two half sites allows for GR to dimerize (Saklatvala, 2002; Galliher-Beckley & Cidlowski, 2009; Vandevyver et al, 2012;Oakley & Cidlowski, 2013). Therefore, GR homodimerization occurs upon binding to the GRE once inside the nucleus. Transactivation or repression of specific target gene sequences occurs within the promoter regions of glucocorticoid-regulated genes (Pujols et al, 2002; Dostert & Heinzel, 2004; Vandevyver, Dejager, & Libert, 2012; Oakley & Cidlowski, 2013). The mechanism of action of GR activation and transcriptional activity is displayed in **Figure 2.** Examples of glucocorticoid-regulated genes include the nuclear factor *NF-KBIA*, a proinflammatory signaling pathway gene which inhibits inflammation mediated by *NF-KB* (Auphan et al, 1995; Rhen & Cidlowski, 2005), genes involved in apoptosis inhibition such as B-cell lymphoma-2 (*BCL-2)* (Rogatsky et al, 1999) and protein-coding genes involved in metabolism such as Fatty Acid Synthase (*FAS*), (Sul & Wang, 1998).

Negative Regulation of the HPA axis

Inducible physiological systems, such as the HPA axis, are generally regulated by inhibitory signals or 'negative regulation'. The HPA axis is negatively regulated by at least two main mechanisms: (i) GR-mediation at the hypothalamus and anterior pituitary; (ii) GR-mediated autoregulation at the adrenal gland and a third mechanism iii) regulation of CRH by its stimulatory and inhibitory receptors. This latter mechanism (iii) involves the two CRH receptors: CRHR-I and CRHR-II. Despite the CRHR-I being the primary receptor for CRH, stress-induced augmented levels of circulating CRH activates anterior pituitary CRHR-I as well as CRHR-II. Activation of CRHR-II provides a 'brake' or inhibitory modulation of the

CRH action (Hauger et al., 2006). The scope of discussion will focus on the two main GR-mediated regulatory mechanisms.

(i) Central GR-mediated negative feedback at the hypothalamus and anterior pituitary

Glucocorticoid binding to hypothalamic and anterior pituitary-GR initiates HPA axis negative feedback (de Kloet et al, 1998;Saeb-Parsy, 1999; Smith & Vale, 2006). At the hypothalamus, GRE are located in the promoter region of the CRH gene (Guardiola-Diaz, 1996; Roche, 1998) leading to reduced CRH expression (Schwartz, Strack & Dallman, 1997) and CRH secretion (Juruena, Cleare & Pariante, 2004) (refer to **Figure 1**.) upon glucocorticoid activation of hypothalamic GR. Glucocorticoids also decrease anterior pituitary CRHR-I mRNA expression and protein by both inhibition of receptor translation and stimulation of receptor degradation (Hauger et al., 2006). At the anterior pituitary, GR activation inhibits pro-opiomelanocortin (POMC) mRNA synthesis through gene repression (Eberwine & Roberts, 1984; Jameson & de Groot, 2015), thereby reducing circulating ACTH levels (Doniach, 1985; Jameson & deGroot, 2015). Thus, glucocorticoids can mediate the negative feedback loop of the HPA axis by reducing gene expression and by modulating receptors at various levels of this pathway. As BPA binds GR (Divya, Prasanth & Sadasivan, 2010) and may also modulate the HPA axis as a xenoestrogen, it is important to consider all relevant endocrine pathways, both stimulatory and inhibitory, on the modulation of HPA axis activity.

(ii) GR-mediated Regulation at the Adrenal Gland

A secondary GR-mediated mechanism is GR regulation at the level of the adrenal gland (Gummow et al, 2006; Walker et al, 2015). ACTH reduces DAX-1 transcription (Gummow et al, 2006; Walker et al, 2015) thus enabling StAR transcription (Walker et al, 2015) and therefore adrenal gland steroidogenesis including

glucocorticoid synthesis. Glucocorticoids activate adrenal gland GR and induce transcription of the DAX-1 protein that in turn represses StAR transcription (Walker et al, 2015), thereby reducing steroidogenesis and synthesis of glucocorticoids. It is important to note that the reduction in glucocorticoid synthesis and secretion at the adrenal gland minimizes negative feedback at the anterior pituitary and hypothalamus. Therefore, CRH and ACTH will continue to be released despite decreased GR synthesis at the adrenal gland. This mechanism demonstrates that the negative feedback at the anterior pituitary and hypothalamus are essential for a return to homeostasis,

Estrogen Modulation of the HPA Axis

Estrogens are steroid hormones with well-established roles in reproduction (Hess, 1997, 2003; Sharpe, 1998; McCarthy, 2008; Findlay et al, 2010; Schulster, 2016) and in the modulation of brain development (Bakker & Baum, 2007; McCarthy, 2008; Gillies & McArthur, 2010). Estrogens promote sex differences in the brain and result in the production of larger male hippocampal volume (Becker, 2002; Zhang, Konkle, Zup & McCarthy, 2008). Estrogens are aromatized from androgens by the enzyme P-450 aromatase, expressed in several brain regions (McCarthy, 2008). Estrogens bind the ER which is widely expressed in multiple brain regions including the hippocampus, hypothalamus, cerebral cortex, amygdala, pons, cerebellum and medulla (Guo et al, 2001; Perez, Chen & Mufson, 2003; Amateau et al, 2004; Cao & Patisaul, 2011).

The ER is a steroid hormone nuclear receptor (Tetel, 2009) which mediates the expression of specific gene targets after activation by ligand binding (Tetel, 2009). The transcription factors ER-α and ER-β are ER isoforms that are members of the steroid hormone nuclear receptor superfamily (Norman, Mizwicki & Norman, 2004; Huang, Chandra & Rastinejad, 2010). In the mature male and female rat adrenal cortex, the

expression of these two isoforms are equally expressed (Trejter et al, 2015). In the developing rat, both ER-α and ER-β are present in the hypothalamus (Gonzales, Tetel & Wagner, 2008; Gonzales et al, 2012), anterior pituitary (Pasqualini et al, 1999), cerebral cortex and cerebellum (Guo et al, 2001), in the ovary (Mowa & Iwanaga, 2000) and in the male gonads including the prostate (Prins et al, 1997,1998; Lau & To, 2016). Similarly, in the human fetus, ER-α and ER-β have been located in regions including the hippocampus and cortex (Gonzalez et al, 2008; McCarthy, 2008) and in the fetal adrenal and reproductive tracts (Takeyama et al, 2001). The high levels of estrogen-related compounds in the brain during this delicate period of development emphasize the prevalence of estrogen and its significant role in the modulation of brain development, including alterations to the HPA axis and mediation of GR activity (Kinyamu & Archer, 2003; Zhang et. al, 2009; Weiser & Handa, 2010).

Estrogen plays an important role in the modulation of circulating glucocorticoid levels and in turn, the HPA axis **(Figure 3).** In the rat, estradiol and ER agonists have been shown to increase plasma CORT levels compared to baseline (Liu et al, 2012) and can increase the amount of GR binding sites (Ferrini, Lima & Nicola, 1990; Ferrini & Nicola, 1991; Chirino et al, 1992). Estrogen binding to the ER-α at the paraventricular nucleus of the hypothalamus inhibits the ability of CORT to promote negative feedback to GR in this brain region resulting in the inability to reduce ACTH and CORT secretion (Weiser & Handa, 2009).

Estrogen seems to generally inhibit GR-mediated negative regulation of the HPA axis through two main mechanisms: i) inhibition of GR activation (Zhang et al, 2009) and ii) proteasomal degradation of the GR (Kinyamu & Archer, 2003) **(Figure 3).** As described, GR phosphorylation is an important step for receptor activation. Estrogen increases expression of protein phosphatase 5 (PP5), responsible for GR

dephosphorylation thereby inhibiting GR activation (Zhang et al, 2009). Dephosphorylated GR prevents binding of CORT, thereby inhibiting GR activity including transactivation, repression and gene transcription (Sengupta et al, 2000; Ganguli et al, 2002; Kinyamu & Archer, 2003). The second mechanism by which estrogen downregulates GR is proteasomal degradation **(Figure 3).** This mechanism involves the p53 transcription factor (Sengupta et al, 2000; Sengupta & Wasylyk, 2001), which is an important tumor suppressor (van Boxtel et al, 2011) involved in activation of DNA repair pathways and in the induction of apoptosis (Arai, Harmon & Gobe, 1996). The p53 transcription factor is negatively regulated by complexing with mouse double-minute 2 (Mdm2), an estriol (E3) ubiquitin ligase (Haupt et al, 1997; Riley & Lozano, 2012; Shi & Gu, 2012). The complexing of Mdm2 targets p53 for proteasomal degradation, thereby preventing its role in transcription and induction of apoptosis (Haupt et al, 1997; Riley & Lozano, 2012; Shi & Gu, 2012). Similarly, GR complexed with both p53 and Mdm2 also becomes a target for degradation (Sengupta & Wasylyk, 2001). The increased expression of both p53 and Mdm2 by estrogens is therefore thought to be associated with increased proteasomal degradation of the GR (Kinyamu & Archer, 2003)

The glucocorticoid-mediated negative regulation of the HPA axis requires functional GR in three main locations: the hypothalamus, anterior pituitary and the adrenal glands. Estrogens prevent the functional activation of the GR through dephosphorylation or through receptor degradation, which down-regulates GR protein levels and results in reduced GR activity, as discussed above. Thus, estrogens impair GR-mediated negative regulation of the HPA axis. Estrogen agonists including 17β-estradiol, the xenoestrogens BPA, diethylstilbestrol (DES) and genistein (Kinyamu & Archer, 2003) have all been purported to mediate the inhibition of GR transcription activity by reducing GR expression levels (Kinyamu & Archer, 2003) in multiple cells throughout the body (Zhang et al, 2009; Panagiotidou, 2014). This evidence supports the

relevance of the proposed investigations of the modulatory effects of the xenoestrogen BPA on HPA axis development in the rat during the critical period.

Perinatal Development of HPA axis

Given that my thesis will investigate BPA's modulation of the developing rat HPA axis, it is important to note that the components of the HPA axis form early in gestation and continue developing after birth. Consequently, the prenatal and perinatal periods are potentially susceptible to environmental perturbations including exposures to xenoestrogens and other EDC. Coordination of fetal maturation is modulated by the placenta- an endocrine organ that plays a significant role in the fetal HPA response.

Maturation timing of the HPA axis relative to birth is species-specific and although components of the HPA axis do develop during gestation in the rat pup, much of the neuroendocrine response and pathway development continues after birth during the hyporesponsive period (Kapoor et al, 2006), which will be explored further below. In the fetal rat, CORT and GR mRNA are detectable in the hypothalamus, hippocampus, and anterior pituitary (Kitraki et al, 1996; Diaz et al, 1998) at gestational day (GD) 13 before the adrenal gland has fully matured (Stojanoski et al, 2012). In the neonatal rat, CRH gene expression in the paraventricular nucleus of the hypothalamus is detected at GD 17 and increases to adult levels around PND 4 (Baram & Lerner, 1991). ACTH secretion has also been noted towards the end of gestation, at GD 18 (Ohkawa et al, 1991). Despite the fact that multiple components of the HPA axis are detectable in the developing rat, circadian patterns and feedback regulation still remain immature (Vazquez & Akhil, 1993). Towards the end of gestation, hormonal functions of the fetal HPA axis are enhanced in coordination with heightened hormonal signals from the placenta (Snegovskikh et al, 2006), which eventually trigger parturition. Further, GR levels finally reach their adult levels by PND 30 (Bohn et al, 1994; Yi, Masters &

Baram, 1994; Vazquez et al, 1998; Edwards & Burnham, 2001), suggesting that functionality of the axis occurs after PND 15, but that the complete maturation of the axis where hormone levels are secreted at adult levels is finalized a couple of weeks after the end of the SHRP.

Fetal Stress Response During Pregnancy

Psychological stress and adverse environmental factors are two factors that contribute to the maternal experience of stress and greatly affect healthy fetal maturation. Rat models of psychogenic stress are associated with significant impacts to postnatal development including heavier offspring (Amugongo and Hlusko, 2013), enhanced neuroendocrine responses to stress (Darnaudery & Maccari, 2008; Maccari et al., 2014), increased levels of anxious and depressive-like behaviours, cognitive impairments (Maccari et al., 2014), altered circadian rhythms including sleep, and behavioural disturbances (Darnaudery & Maccari, 2008). In the rat model of anxiety, several physiological effects have been attributed to fetal HPA axis disruption, and these include altered levels of receptors (e.g. serotonin) involved in anxiety disorders (van Den Hove et al, 2006), low exploratory behaviours and escape to novelty (Vallee et al, 1997), alterations in play behaviour (Morley-Fletcher, 2003) and increased anxiety behaviour after maze tests (Grundwald & Brunton, 2015). Low perinatal stress in the rat may be adaptive, producing offspring with greater capacities for learning and reduced thresholds for anxiety-like behaviour and fear (Catalani et al., 2011). Rodent models suggest that effects of perinatal stress may be sex-dependent (Mueller & Bale, 2008; Sickmann et al, 2015). Some studies have reported that male offspring experience more significant long-term impairments, such as the study by Darnaudery & Maccari (2008) which demonstrated reduced hippocampal plasticity in male rats after restraint stress. Perinatal stress may also have long-term consequences for the mother, impairing her ability to care and nurture her offspring, consequently affecting pup GR expression levels (Francis et al, 1999). Examples of stressful influences in the rat include social

stress, such as crowding, isolation and social defeat, and various mild stressors (Koolhaas et al, 1997; Blanchard et al, 1998; Beery & Kaufer, 2015). Thus, neonatal stress response development may be modulated by both prenatal neuroendocrine factors and postnatal aberrations in maternal care. An important determinant of development is maternal nurturing, as described in more detail below.

Several traumatic events during gestation have been reported to negatively impact normal human development. Traumatic psychological stressors induced by war-related events (e.g. bombings, fleeing from one's home) have been reported to result in shorter gestation and smaller fetal head circumference (Lederman et al, 2004; Engel et al, 2005; Liu et al, 2016). Environmental catastrophes causing radiation exposure (e.g. the nuclear explosions in Chernobyl and Hiroshima) have been reported to cause microcephaly and intrauterine growth restriction (Nadakavukaren, 2011; Chen et al, 2014). Sudden-onset disasters (floods, tsunamis and hurricanes) result in high maternal stress and consequently affect fine and gross motor development including crawling and walking, as reported in the Queensland Flood Study (King et al, 2015). Famine during mid to late gestation has been reported to cause reduced birth weight observed in the Dutch famine study (Roseboom et al, 2006; Schulz, 2010). These are only some examples of the perturbations to healthy development caused by adverse environmental factors. Prenatal exposure to stress in both animal and human research models can result in multigenerational epigenetic changes (Weaver et al, 2004; Anway et al, 2005; Harris & Seckl, 2011; Pembry, Saffery & Bygren, 2014) including alteration to GR expression in specific tissues such as brain, liver and adipose (Shanks, Laroque & Meaney, 1995; Harris & Seckl, 2011). Therefore, it is well established in both rodent and human research that the presence of stressors (either directly affecting the mother or indirectly affecting the fetal environment) perturbs normal stress pathway development. As described, exposures to estrogenic EDC have the potential

to modulate the HPA axis. Therefore we consider the perinatal exposure to BPA, central in the design of this thesis, to induce similar disruptions to the HPA axis as the psychogenic models described above.

Role of the Placenta in Fetal HPA Development

The placenta is an endocrine organ that serves as the connection between the fetus and the uterus (Feng et al, 2016). Placental hormone secretion and fetal growth occur in tandem, and the labyrinth zone (the area of maternal and fetal exchange) is a critical area for endocrine coordination (Mark et al, 2009). The placenta is highly vascularized and exposure to high levels of glucocorticoid at the end of pregnancy such as maternal malnutrition and glucocorticoid overexposure (Cottrell & Seckl, 2009), maternal psychological stress (Rondo et al, 2003) and uterine dexamethasone-induction (Hewitt, Mark & Waddell, 2006) can compromise placental vascularity and can negatively impact fetal growth (Hewitt, Mark & Waddell, 2006). Placental endocrine function is highly regulated by enzymatic activity to protect the developing fetus. The 11-βHSD enzymes are a critical component of placental function. These enzymes are expressed in the rat placenta (Jensen Pena, Monk & Champagne, 2012) and moderate active glucocorticoid levels in circulation: enzymatic activity of 11-βHSD1 converts cortisone to cortisol through an oxo-reductase mechanism which uses NADP(H) as a cofactor for cortisol synthesis (Tomlinson & Stewart, 2001) while 11-βHSD2 converts cortisol to cortisone (Walker & Stewart, 2003; Stewart & Draper, 2005) using an NAD-dependent dehydrogenase (Tomlinson & Stewart, 2001). This regulation is crucial as the fetal-placental interaction is highly dynamic and normal fetal growth can be compromised by exposure to excess glucocorticoids (Smith & Waddell, 2000). The rodent placenta also synthesizes and secretes several steroid hormones that are key in fetal HPA axis development, including ACTH (Izumi et al, 2004; Simamura et al, 2011). Placental ACTH secretion to the fetus is triggered by the maternal leukemia inhibitory factor (LIF) , which in turn promotes fetal LIF secretion from fetal red blood cells (Simamura et al, 2011). The maternal-

fetal ACTH-LIF cycle ultimately promotes fetal neurogenesis (Simamura et al, 2011). The placenta also produces androstenedione and testosterone (Warshaw et al, 1986), as well as small levels of progesterone (Warshaw et al, 1986; Itoh et al, 2009; Sun et al, 2012). In contrast to the human, the rodent placenta does not produce CRH (Robinson et al, 1989; Avishai-Eliner et al, 2002). The placenta does produce CORT (Mark et al, 2009) which increases towards the end of gestation in coordination with fetal organ maturation (Mark et al, 2009). The HPA-related receptors present in the placenta include the ACTH-R in the placental labyrinth (Izumi et al, 2004; Nimura et al, 2006), CRH-R (Nakamura et al, 2000) and GR which is ubiquitously expressed in the placental labyrinth zone (Korgun et al, 2011). The interaction between maternal hormones and enzymes and fetal hormone activity are essential for fetal growth as evidenced by the GR/CORT dynamic. At the end of gestation, placental GR mRNA increases three fold in the placental labyrinth with a concomitant ten-fold increase in CORT (Mark et al, 2009). These harmonized increases are due to an increase in 11-βHSD1 (activates CORT) and a decrease in 11- βHSD2 (inactivates CORT) around mid-gestation (Brown et al, 1996). The altered ratio of these enzymes occurring in coordination with hormone and receptor surges, demonstrate the carefully conserved dynamic between placenta and modulation of fetal growth, specifically neurogenesis and fetal organ maturation **(Figure 4).** Increases in placental CORT allow for fetal organ maturation prior to parturition and is essential for maturation of organ systems including neuroendocrine and brain development (Mark et al, 2009). The toxic effect of synthetic glucocorticoid exposure on rodent neonatal neurodevelopment (Uno et al, 1990) emphasizes the vulnerability during the critical window and the permanence of HPA axis disruption in the rodent neonate, which highlights the relevance of my research question.

Exogenous estrogen, such as xenoestrogens, can impact the delicate placental-fetal endocrine dynamic and can ultimately disrupt fetal development (Nishikawa et al, 2010). Both ER-α and ER-β are detected in the

rat placenta at GD 16 (Al-Bader, 2006). Estradiol levels are high at the end of gestation (Medlock et al, 1991; Drummond, Baillie & Findlay, 1999), however just prior to parturition, levels of both ER isoforms are decreased, indicating that the placenta is less responsive to estrogen (Al-Bader, 2006). Introduction of exogenous estrogen such as 17 β-estradiol-3-benzoate have been reported by Furukawa et al. (2013) to reduce both utero-placental blood flow and fetal growth (Furukawa et al, 2013). Exogenous estrogens (e.g. xenoestrogens) can penetrate the placenta and modulate fetal development (Nishikawa et al, 2010). For example, glucuronidated (inactivated) BPA (BPA-GA) in the placenta can cross the placental barrier and can be re-activated in the fetus (Nishikawa et al, 2010), therefore exposing the fetus to high levels of active chemical. Further, due to the inefficient metabolism in the fetus, the BPA-metabolizing UGT enzyme is found at low levels in the fetus (Nishikawa et al, 2010). The presence of exogenous estrogens in the placenta reduces blood flow between the uterus and placenta, provokes hypoplasia, and reduces artery development (Furukawa et al, 2013).

It is important to note that the rodent placenta cannot produce estrogen, as it does not express aromatase (Sun et al, 2012), the enzyme which converts androgen to estrogen (McCarthy, 2008). Endogenous estrogen is synthesized in the rodent using placental testosterone as the substrate for estrogen production in the corpus luteum of the dam ovary (Durkee et al, 1992). The predominant source of estrogen synthesis in the pregnant rat occurs in the ovary, as aromatase is highly expressed in the rat dam ovarian follicle and corpus luteum (Purba & Bhatnagar, 1990; Stocco, 2008). This makes the rat placenta indirectly responsible for ovarian estrogen production. Estradiol is able to cross the placental barrier (Henry & Miller, 1986), and both the rat embryo and fetal rat have established ER activity specifically in the reproductive organs (Nguyen et al, 1988). As the fetal rat matures, it is able to synthesize estrogen in both the testes and the ovary by GD 17 (Weniger, Zeis & Chouraqui, 1993; Weniger, 1993). Induced increases in placental

estrogen transfer to the fetal rat can be lethal at high doses (Bartholomeusz, Bruce & Lynch, 1999), which emphasizes the importance of controlled estrogen exposure in tandem with organ growth and maturation during fetal development.

Mechanisms to Protect Fetus during Pregnancy and Perinatal Stress

Even without the additional neuroendocrine changes brought about by perinatal stressors, pregnancy is a hypercortisolic state characterized by maternal adrenal gland hypertrophy (Mastorakos & Ilias, 2003) and high levels of glucocorticoid in circulation. As described, perinatal stress can adversely affect fetal development, such that biological mechanisms have developed to protect the developing fetus from high levels of circulating glucocorticoid. The two major mechanisms involve (i) CRH binding protein and (ii) placental expression and activity of 11-βHSD2.

CRH- binding protein

The corticotropin-releasing hormone-binding protein (CRH-BP) is found in vertebrate mammals including the human and the rat (Chatzaki, Margioris & Gravanis, 2002; Seasholtz et al., 2002; Mastorakos & Ilias, 2003). It is a binding protein found in the circulation (Linton et al, 1993) and inhibits the actions of CRH including CRH-dependent ACTH release from the anterior pituitary (McClennen, Cortright & Seaholtz, 1998). The role of CRH-BP is critical in the rat as its actions modulate CRH activity, which has implications for HPA axis pathway function (McClennen, Cortright & Seaholtz, 1998; Wang et al, 2004). Rats exhibit tissue-specific expression of CRH-BP; whereas CRH-BP does not exist in the circulatory system of rodents (Trainer et al, 1998), it is present in brain areas including the hippocampus (Wang et al, 2004), amygdala (Timofeeva et al, 1999) anterior pituitary (Behan et al, 1995; McClennen, Cortright & Seaholtz, 1998; Timofeeva et al, 1999; Seasholtz et al., 2002) and adrenal glands (Chatzaki et al., 2002)

where the majority is produced in adrenomedullary chromaffin cells (Chatzaki, Margioris & Gravanis, 2002). CRH-BP binds to CRH and renders it biologically inactive, preventing it from binding to its receptor CRH-R (Seaholtz et al, 2001; Chatzaki et al., 2002). In the developing neonatal rat hippocampus, expression of both CRH and CRH-BP are downregulated upon stimulation of the HPA axis by systemic ACTH treatment (Wang et al., 2004). This is noteworthy as HPA axis activity is significantly reduced in the neonate to protect the still-developing brain. It is likely that expression of CRH-BP in the fetal rat is induced by heightened fetal glucocorticoid production (Chatzaki et al., 2002) in response to the hypercortisolic state of pregnancy, although the role of CRH-BP remains to be fully elucidated in the rat during pregnancy.

11β-hydroxysteroid dehydrogenase

Placental estrogens significantly modulate the fetal HPA axis, and placental 11-βHSD2 is a major mechanism to protect the developing rat fetus from high maternal glucocorticoids (Diaz, Brown & Seckl, 1998; Langley-Evans, 2004). The enzyme is highly expressed in the central nervous system, the hypothalamus and pituitary gland of the fetal rat (Diaz, Brown & Seckl, 1998) and in the kidney (Diaz, Brown & Seckl, 1998). The MR and GR are co-expressed in most cells (Cota et al, 2007), and the MR is expressed in the fetal rat brain including the hippocampus, hypothalamus and pituitary (Diaz, Brown & Seckl, 1998).

In the human, fetal dihydroepiandrosterone (DHEA-S) is an essential precursor required by the placenta to synthesize estriol, the major estrogen circulating during pregnancy (Kota et al, 2013) **(Figure 5).** Serum cortisone triggers fetal ACTH production, which in turn increases synthesis of DHEA-S (Ng, 2000; Albrecht, Aberdeen & Pepe, 2005; Kaludjerovic and Ward, 2012). High levels of cortisol trigger negative

feedback and high levels of plasma estrogen can degrade the GR, thereby inhibiting ACTH release from the pituitary and resulting in downregulation of the fetal HPA axis (Kaludjerovic and Ward, 2012). Late gestation is accompanied by increased placental 11-βHSD2 synthesis (Cottrell et al, 2014; Wyrwoll et al, 2015), which counteracts the high levels of circulating maternal cortisol prior to parturition. The 11-βHSD2 enzyme subsequently declines between gestational weeks 38-40 to allow for the final surge of glucocorticoid before parturition (Murphy & Clifton, 2003). In humans, reduced expression of 11-βHSD2 is associated with intrauterine fetal growth restriction (Tomlinson & Stewart, 2001; Challis et al, 2001) and compromised fetal HPA programming (Tannin et al, 1991; Zhou et al, 1995; Duthie & Reynolds, 2013). In the rat, maternal stressors including hypertension and nutritional deficiencies are associated with decreased expression of 11-βHSD2, resulting in higher CORT levels (Bertram et al., 2001; Mazancova et al., 2003; Huang et al., 2016). Our approach in investigating the effects of the xenoestrogen BPA on perinatal HPA axis development is therefore appropriate, given the roles of maternal and placenta estrogens in fetal HPA axis regulation. As such, the xenoestrogen BPA may modulate bioavailability of cortisol to the developing fetus.

Maternal care and pup HPA axis Hyporesponsiveness

The newborn rat pup development is highly dependent on maternal care and nurturing. Pup HPA axis responsiveness to a given stressor is multifactorial and may be countered by the level of maternal care (Walker et al, 1991; Walker & Dallman, 1993; Meaney et al, 1994). Maternal influence on HPA axis suppression in pups has been widely reported. Maternal licking and grooming appears to reduce pup ACTH secretion in response to stress, while feeding maintains low circulating CORT levels (Suchecki, 1993; van Oers, 1998). Maternal presence inhibits pups from exhibiting a stress response. After PND 10 pups are able to release CORT in response to a severe stressor such as a shock, but this response is inhibited by the

presence of the dam (Moriceau et al, 2006). A full day of maternal separation from the dam can increase pup baseline CORT (McCormick et al, 1998; Ryu et al, 2008; Jahng et al, 2010) and ACTH levels (Workel et al, 1997, 2001; Penke et al, 2001) and can result in reduced GR binding in the hypothalamus (Workel et al, 2001). The increase in pup CORT levels is correlated with the duration of maternal separation (Dent et al, 2000). Thus, the importance of maternal presence and care is pivotal in mediating pup HPA axis activity in developing pups. Throughout our study, we recognize that maternal stress both during gestation and post-parturition are potential confounders for pup HPA axis development. As described in our methodology, we selected minimally-invasive mechanisms of maternal BPA administration, minimized maternal separation and ensured that pups remained with their mothers until the time of sacrifice thereby lessening both maternal and pup stress.

Stress Hyporesponsive Period

The SHRP is an important physiological phenomenon that occurs in select species and is characterized by a reduced stress response for a short period after birth. The SHRP is beneficial as it protects the still-developing brain from the potentially damaging effects of high circulating glucocorticoids (Sapolsky & Meaney, 1986; Lupien et al, 2009; Rincon-Cortes & Sullivan, 2014; Nishi et al, 2014). The SHRP has been well characterized in the rat (Walker & Vrana, 1993; Levine, 1994; Schmidt et al, 2003; Mitev, 2003; Vazequez & Levine, 2005; Dent, 2007; Brunton, Russell & Douglas, 2008) and has also been documented in the tree shrew (Lai & McCobb, 2006; Brunton et al, 2007). In the rat, the SHRP is observed between PND 4 and PND 14 (Dent et al, 2007; Nishi et al, 2013; Herman et al, 2016), is characterized by a reduced ACTH response (Walker, 1991; Levine, 2000; Vazquez & Levine, 2005) and reduced CORT secretion after reaction to a stressful stimulus (Rosenfeld et al, 1991; Walker & Vrana, 1993; Schmidt et al, 2003,

Vazquez & Levine, 2005). During the SHRP in the rodent, GR protein levels are only half of those present in the adult brain (Levine, 1994).

During rodent development, CORT levels are important moderators of the fear response and alterations to these hormone levels during the SHRP can result in altered neuroendocrine stress reactivity (Moriceau et al, 2004). The maintenance of low CORT hormone during the SHRP is important for moderating the pace of development of the HPA axis system (Moriceau et al, 2004). Low basal levels of CORT circulating during the SHRP are not yet able to activate the amygdala to elicit a fear response- this maintains the low responsiveness seen during this critical period (Moriceau et al, 2004; Moriceau, Roth & Sullivan, 2010). Although the stress response is suppressed during the SHRP, it can be evoked in reaction to external stressors (Moriceau et al, 2004). Several hypotheses have been proposed to explain the mechanism of the SHRP, described below.

Corticosterone- Binding Globulin Hypothesis

The CBG hypothesis suggests that the SHRP is maintained in the rat neonate by the decreased biological availability of CORT ('free' CORT) and the decreased expression of CBG (Yehuda & Daskalakis, 2015), reported to be essentially absent during the SHRP (Henning, 1978). The free hormone hypothesis states that the unbound free CORT is only bioactive if not bound to CBG (Breuner & Orchinik, 2002; van Eekelen et al, 2012). As CORT is lipophilic (Saaltink & Vreugdenhil, 2014) it cannot get into the cell to bind to the GR if it is bound to the plasma protein. The onset of the SHRP results in the significant fall in CORT levels (Henning, 1978) and serum CBG (Viau et al., 1996). Levels of both CORT and CBG synthesis increase very rapidly after the SHRP (Viau et al., 1996; Claflin, Greenfield & Hennessy, 2014). The absence of CBG during the SHRP indicates the hyporesponsive state of the HPA axis during this critical period of development. Stress has been shown to reduce CBG levels, resulting in an increase in free

CORT (Fleshner et al, 1995; Tannenbaum et al, 1997). The low circulating levels of CBG (which transport lipophilic hormones through the bloodstream) during this hyporesponsive state emphasize the vulnerability to stress perturbation in the developing rodent during the critical period. The coordinated increases in CORT and CBG after the SHRP indicate that physiological mechanisms are put in place during the SHRP to protect the developing brain from glucocorticoid excess during the SHRP.

Anterior Pituitary Hypothesis

As described, the HPA axis is negatively regulated by glucocorticoids through GR expression at both the hypothalamus and anterior pituitary. It has been reported that the rat hypothalamus remains active during the SHRP, while the pituitary and the adrenal glands are less responsive (Dent et al, 1999), suggesting that some mechanism at the pituitary or adrenal glands generates the SHRP. It is hypothesized that during the SHRP, levels of ACTH, CRH, and baseline CORT levels are decreased (Rosenfeld, Suchecki & Levine, 1992;Schmidt et al, 2003). Basal CRH is only secreted at 16-42% of adult levels during the SHRP (Widmaier, 1989; Zelena et al, 2008) but increases upon saline injection and cold stress (Walker et al, 1991; Yi & Baram, 1994; Dent et al, 2000). The low amount of basal CRH secretion during the SHRP may be related to hyporesponsivity at the anterior pituitary, to be discussed below.

The first hyporesponsive location is thought to be at the level of the anterior pituitary (Sapolsky & Meaney, 1986; Walker et al, 1986; Levine, 2000; Schmidt et al, 2009).). In hypothalamus, hippocampus, and prefrontal cortex, GR gene and protein expression levels were decreased in 10-day old rats compared to adult rats (Varga et al, 2013). High GR expression in the pituitary suggests that this is the primary area for SHRP maintenance; negative feedback mediated by the GR at the anterior pituitary has been hypothesized to be a primary mechanism of regulation during the SHRP (Lai & Huang, 2011). Complete GR deletion at

the pituitary has been shown to be fatal in pups (Erdmann, Schutz & Berger, 2008). The anterior pituitary has been demonstrated to be fully functional during the SHRP as an induced GR-blockade resulted in upregulation of POMC, ACTH and CORT levels (Schmidt et al, 2005). Stimulation of neonatal pituitary cells with CRH can yield ACTH secretion levels similar to the adult rat, which confirms that robust secretion of this hormone is physiologically possible in the neonate (Walker & Vrana, 1993; Witeck-Januseck, 1998; van Oers et al, 1998) when presented with an exogenous hormone or forced stress through experimental manipulations. Therefore, low circulating ACTH and CORT during the SHRP has also been purported to be maintained by a GR-mediated negative feedback signal at the anterior pituitary (Lai & Huang, 2011) and that decreased anterior pituitary GR expression would then result in hyperactivity of the HPA axis (Schmidt et al, 2005, 2009). The anterior pituitary hypothesis may be contradicted by the CBG hypothesis. This emphasizes the lack of certainty surrounding the endocrine mechanisms occurring during the SHRP.

Adrenal Cortex Hypothesis

The second hyporesponsive location is hypothesized to be at the adrenal cortex (Widmaier, 1989). At this stage the adrenal gland is not yet mature and has a reduced sensitivity for ACTH, which is theorized to be a factor in adrenal gland hyporesponsiveness to ACTH (Witek-Janusek, 1988; Walker, 1995; Zilz et al, 1999; Yoshimura et al, 2003). Adrenal gland hyporesponsiveness results in decreased steroidogenesis and therefore, reduced CORT. The peripheral benzodiazepine receptor (PBR) transports cholesterol across mitochondrial membranes and is required for adrenal gland steroidogenesis (Zilz et al, 1999). During the SHRP, PBR expression is reduced, along with concomitant inhibition of steroidogenesis (Zilz et al, 1999). ACTH appears to regulate PBR expression (Lee et al, 2004), such that decreased circulating levels of

ACTH during SHRP results in decreased PBR expression and similarly, decreased steroidogenesis including CORT.

The receptors for CRH and ACTH are present in the developing rat during the SHRP: CRH-R is detected in pituitary (Pihoker et al, 1993), cortex, hippocampus, amygdala (Brunson et al, 2002) and ACTH-R is detected in the adrenal glands (Chatelain et al, 1989). Studies have shown that induced stressors (e.g. hypoglycemia) can increase ACTH but not CORT secretion (Widmaier, 1989). Similarly, ACTH injection has been reported not to have an effect on CORT secretion during the SHRP, while it does increase CORT in rats outside of the SHRP (Dent et al, 2001). The low circulating basal ACTH levels during the SHRP have been thought to contribute to adrenal gland underdevelopment, due to the lack of stimulation of the gland at this stage (Muret et al, 1992). The individual components of the HPA axis demonstrate functionality when injected or manipulated during experiments, yet the axis as a whole remains physiologically blunted during this critical period of development.

It has also been widely reported that maternal care plays a critical role in maintaining HPA axis hyporesponsiveness in pups during the SHRP (Sucheki et al, 1995; Mayer & Saper, 2000; Levine et al, 2002; Schmidt et al, 2005). Pups that experience maternal separation have higher CRH gene expression and CORT levels (Rosenfeld et al, 1991; Francis et al, 1999). Conversely, licking and grooming has been shown to reduce ACTH, CORT, and CRH mRNA and enhances glucocorticoid feedback in later life (Liu et al, 1997). This signifies the importance of maternal care in the healthy development of the pup HPA axis. Impaired negative feedback and subsequent consequences to coping to stress later in life were observed in pups that experienced early life stress (Francis et al, 1999; Koehl et al, 1999; Schmidt et al,

2009) and in pups whose pituitary GR levels had been modulated with stress exposure as neonates (Schmidt et al, 2009).

Despite the fact that numerous rodent studies have investigated CORT and ACTH levels before, during and after the SHRP, all have involved physical stressors such as water-exposure or restraint (Khanaeva et al, 2008; Miklos & Kovacs, 2012) and did not investigate stress levels based solely on one perturbation such as EDC. Simply investigating the effect of the xenoestrogen BPA on modulations to HPA axis development during the SHRP in the rat (without additional factors that may also potentially alter the stress response) is a uniquely focused area of exploration into the critical period of development.

The Human SHRP

In humans, the SHRP is thought to begin in infancy and last throughout childhood, although there are no studies that have elucidated the exact hyporesponsive period (Gunnar & Donzella, 2002; Lupien et al, 2009; Hostinar et al, 2014). It is estimated that the SHRP may last from six months to one year after birth and that disruption during this time may chronically affect the function of the HPA axis (Gunnar & Quevedo, 2007), similar to rodent model outcomes. In the human pituitary, ACTH is present and detectable a quarter of the way through gestation, at eight weeks (Rao, 2015). In human newborns, a sharp increase in glucocorticoid levels due to physical examination or to a heel lance has been observed (Lupien et al, 2009). However, throughout the first year of life, the human HPA axis is thought to be less reactive to stressors (Lupien et al, 2009). After the third month of life, cortisol activity in response to physical examination decreases (Gunnar, 1992) followed by a decrease in HPA axis responsiveness throughout the first year of life (Lupien et al, 2009). HPA axis hyporesponsiveness is also purported to be mediated by maternal care

(Lupien et al, 2009), similarly to the rat. Knowledge about the human SHRP is limited as it has not been extensively studied, and this presents a significant gap in developmental neuroendocrine literature.

Recently, more studies have emerged supporting a role for BPA in the dysregulation of the rat HPA axis (Panagiotidou et al, 2014; Chen et al, 2015; Zhou et al, 2015) although none have examined the SHRP. Many studies have reported adverse effects of low-dose BPA during gestation on both HPA and hypothalamic-pituitary-gonadal axes later on in life (Ramos et al., 2003; Poimenova et al, 2010), but the unique SHRP period remains a component that has not yet been explored. For this reason, the focus of my study on the SHRP provides a novel approach in the exploration of perturbations of the developing HPA axis post-xenoestrogen exposure.

Estrogenic Endocrine Disrupting Chemicals

General and estrogenic EDC are present in a wide variety of substances, are both manufactured and naturally occurring, and include pesticides, industrial chemicals and fuels (Diamanti-Kandarakis et al, 2009), plant phytoestrogens, biphenyls (PCB), phthalate plasticizers, BPA, fungicides, and pharmacological agents (DES) (Diamanti-Kandarakis et al, 2009; Meeker, 2012). Routes of exposure to EDC are most commonly through ingestion, inhalation or absorption and typically associated with diet, workplace and leisure activities (Meeker, 2012). Common sources of exposure in the home include food packaging, plastic toys and children's products, and clothing detergent, kitchenware, cosmetics and fragrances. Notably, estrogenic plasticizers including BPA are present in medical equipment, with high exposure rates for infants in neonatal intensive care units (Calafat et al, 2009). General mechanisms by which EDC disrupt normal endocrine function include activation and/or repression of EDC-sensitive genes (Murray et al, 2001), antagonism of metabolic pathways (Moriyama, 2002; Zoeller, 2005), disruption of

hormone synthesis and transport (Diamanti-Kandarakis et al, 2009) and epigenetic modifications (Hung et al, 2003; Newbold, 2008). Consequences of endocrine disruption may be most significant during developmental periods during which alterations in normal tissue function (Henley & Korach, 2006) may lead to permanent perturbations to endocrine pathway activity (Markey et al, 2003; Rogan & Ragan, 2007). As the general term EDC refers to agents representing diverse chemical groups with different toxicological profiles, mechanisms of action are dependent on individual EDC.

Xenoestrogens

Xenoestrogens are exogenous chemicals that exert effects similar to estrogens produced in the body (Korach et al, 1997), have varying affinity for the ER, and examples include DES, plant estrogens (genistein, phytoestrogens) and BPA. At least four receptors can carry out xenoestrogenic activity with tissue-dependent distribution (Kuiper et al, 1997; Lee et al, 2012): these are the estrogen receptor subtypes ER-α and ER-β, (Kuiper et al, 1997), GPR 30 (Prossnitz et al, 2008; Maggiolini & Picard, 2010; Acconia, 2015) and ERR-ϒ (Liu et al, 2007; Okada et al, 2008; Vandenberg et al, 2009; Bouskine et al, 2009; Chevalier et al, 2012). As previously described, there are several mechanisms by which estrogens modulate the HPA axis, and the established estrogenic effects of BPA support the use of this chemical in our investigation of the role of the toxicant in perturbation of the rat HPA axis during development.

Diethylstilbestrol (DES)

The environmental estrogen DES is a potent synthetic estrogen that was prescribed to pregnant women in the 1950's to manage morning sickness (Palmer et al, 2001; Rubin, 2007; Newbold, 2008). It was infamously banned after it was associated with the development of rare vaginal cancers in the daughters of these women (Schraeger & Potter, 2004). It is an established carcinogen in both humans and animals

(Newbold, 2004; Chen et al, 2007; Hilakivi-Clarke, 2014) and it is one of few carcinogens to act through the placenta (Williams, James & Roberts, 2003). DES is perhaps one of the most potent xenoestrogens, with higher binding affinity for the ER compared to 17-β estradiol (Blair et al, 2000; van Lipzig et al, 2004). DES is between 100-1000 times more potent than BPA (vom Saal & Welshons, 2006; Richter et al, 2007) making the DES 5 μg/kg/day dose approximately equivalent to a dosage of BPA between 500-5000 μg/kg/day. In males and females, negative outcomes include reproductive and genital tract malformations (Herbst, 2000). Among the adverse effects on female reproduction are infertility, miscarriage (Palmer et al, 2001; Hoover et al, 2011), uncommon vaginal adenocarcinomas (Williams, James & Roberts, 2003) and breast cancer (Palmer et al, 2006; Troisi et al, 2007). Women who have been gestationally exposed to DES (DES daughters) have twice the risk of developing breast cancer after age 40 compared to unexposed women of the same age (Palmer et al, 2006). This powerful xenoestrogen has a well-established affinity for estrogen receptors and its chemical structure closely resembles that of BPA (Newbold et al, 2009). It has been reported to be five times more potent than endogenous estrogen (IARC, 2012; Reed & Fenton, 2013) and can also bind to ER-α in a similar manner (Korach, Metzler & McLachlin, 1978; Reed & Fenton, 2013). In rodents, DES is metabolized, carried in the bile and excreted in feces (IARC, 2012; Reed & Fenton, 2013) and in humans, it is metabolized by enterohepatic recirculation and excreted in the urine (Giusti, Imamoto & Hatch, 1995).

Bisphenol A

Each year over eight billion pounds of BPA is produced and 100 tons are released into the atmosphere-making it one of the highest volume chemicals produced worldwide (Burridge, 2003; vom Saal & Hughes, 2005; Vandenberge et al, 2007). There continues to be minimal information concerning the effects of BPA on the developing nervous system (Stump et al., 2010; Perera et al, 2012). The blood-brain barrier and the

ability to effectively metabolize substances such as BPA remain underdeveloped in the fetus (Inadera, 2015). Alarmingly, BPA has been detected in the bloodstream of neonates as well as in umbilical cord blood (Ikezuki et al, 2002; Kuroda et al, 2003; Lee et al, 2008). BPA has been detected in the urine of over 95% of Canadian samples taken between 2009-2011 (Statistics Canada, 2015) and in 95% of samples taken from a representative US population (Calafat et al, 2005). Similar body burdens have been reported globally (Ikezuki et al, 2002; Schönfelder et al, 2002; Calafat et al, 2008). In its free or active form, BPA can be found in adipose and lipid-rich breast milk, while the inactive or hydrophilic form can be detected in urine and feces (Health Canada, 2012). The evidence of exposure to BPA during development paired with the need for further investigation on low dose effects of BPA in humans resulted in a change of legislation. In 2008, Canada became the first nation in the world to ban BPA in both the manufacture and importation of baby bottles (Health Canada, 2008; Environmental Defense Canada, 2012). The government adopted the "As Low As Reasonably Achievable" (ALARA) precautionary principle for items and packaging targeting newborns and infants to attempt to minimize exposure levels in this vulnerable population (Health Canada, 2008).

Bisphenol A Mechanism

BPA is aqueous-soluble and lipophilic (Doerge et al, 2010 a,b) and is an ER agonist (Matsushimo & Shimohigashi, 2007) that acts on the ER to influence estrogen-signaling pathways (Naciff et al. 2002; Wetherill et al. 2007; Vandenberg et al. 2009). It is able to bind to ER-α and ER-β, and can also bind to the ERR-γ (Whetherill et al, 2007; Alonso-Magdalena et al, 2008; Bolli et al, 2010; Gertz et al, 2012; Gao et al, 2015). There are many factors that influence the estrogenic effects of BPA: the nature of the estrogen response binding element (ERE) and the ratio of ER-α to ER-β both affect ER gene responsiveness, as the isoform ratio is variable between cells and tissues (Pennie, Aldridge & Brooks, 1998). It has been reported

that BPA has a higher affinity for ER-β (Kuiper et al, 1997; Matthew et al, 2000; Matthews, Twomey & Zacharewski, 2001; Stossi et al, 2014). In addition to acting as an estrogen agonist on various endocrine pathways, BPA is also a strong androgen antagonist (Prasanth, Divya & Sadasivan, 2010). It can bind to the androgen receptor and inhibits its nuclear localization (Sohoni, 1998; Lee et al, 2003).

BPA effects observed in multiple rodent studies include bodyweight changes, precocious sexual maturation (Howdeshell et al. 1999; Honma et al. 2002; Nikaido et al. 2004), decreased testosterone in plasma (Kawai et al. 2003; Akingbemi et al. 2004), compromised immune function (Yoshino et al. 2003, 2004), and the development of behavioural traits related to behavioural disorders including aggression and hyperactivity (Farabollini et al. 2002; Ishido et al. 2004). The expansive list of these types of reports emphasizes the environmental and physiological relevance of BPA, and investigating this chemical is crucial considering its adverse effects either at or below the reference dose of 50 μg/kg/day (Adriani, 2003; Adewale, 2009; Aldad, 2011).

Bisphenol A Metabolism

The absorption of BPA after oral ingestion is the lowest bioavailable route (Pottenger et al, 2000) compared to the subcutaneous route of exposure. At maximum concentration, the parent compound of BPA in blood after oral administration is 2-8% of the initial dose, compared to 27-51% after intraperitoneal introduction or to 65-76% when administered subcutaneously (SCF, 2002). As discussed, we chose the oral administration route as it is most typical and most relevant for humans. Internal exposure levels to active BPA are very similar for adult rodents, non-human primates and humans (European Union, 2003; Doerge et al, 2010 a,b; Hengstler et al., 2011). A body-weight normalized dose of BPA in the rodent is equivalent to a much higher dose in humans due to differences in metabolism (Witorsch, 2002), illustrated in **Figure**

6. In the human, active BPA is transported from the small intestine into the liver where it is glucuronidated (inactivated) by the main enzyme UGT isoform UGT2B15 (Hanioka et al, 2008). This occurs by first pass metabolism, and the glucuronidate is predominantly excreted through the kidney in the urine (Tominaga et al, 2006). The half- life of BPA in the adult human body is less than 6 hours (Health Canada, 2008). Active BPA can be further metabolized in the body to form a metabolite known as Bisphenol A-Sulphate (Health Canada, 2008), which is a minor conjugation pathway in rats (Doerge et al., 2010a). There is no direct information regarding human fetal metabolism of BPA (Doerge et al, 2010 a,b). However, fetal levels are very similar to maternal circulatory levels due to underdeveloped UGT activity (Coughtrie et al., 1988; Edington & Ritter, 2008; Doerge et al, 2010a). While the enzymes required to form the BPA-glucuronide conjugate are not present in neonates, they do increase with age (Edington & Ritter, 2008).

BPA metabolism in the rodent occurs by enterohepatic recirculation, characterized by cycling between the inactive and active forms of BPA. BPA is absorbed in the gastrointestinal tract, travels to the liver for glucuronidation, and is released into the bile as BPA-glucuronide (Nishikawa et al, 2010). This process is much less efficient during pregnancy (Inoue et al, 2005). From the bile duct, BPA-glucuronide travels to the small intestine and is reactivated by enzyme cleaving and the active form enters the circulatory system. Active BPA in the cecum is reabsorbed through the colon wall into the liver where it is reattached to glucuronic acid and deactivated (Sakamoto et al, 2002; Doerge et al, 2010a). Some elimination is detected in urine but the predominant routes are through biliary and fecal excretion (Kurebayashi, 2003, 2005). Rodent metabolism and elimination of BPA and its conjugate form is thus slower compared to the human due to this cycling (EFSA, 2006).

Numerous studies have reported high levels of active BPA in the fetus as well as fetal cycling between aglycone BPA and its conjugate form (Miyakoda et al, 1999; Moors et al, 2006; Nishikawa et al, 2010). However, the exact mechanism of BPA transfer from dam to fetus is still unknown (Nishikawa et al, 2010) and conjugation of BPA varies throughout gestation (Cao et al, 2012). Active BPA levels in the fetus are similar to maternal tissue levels, and the majority of transferred BPA is in the active form (Doerge et al, 2010 a,b). This is significant as fetal liver microsomes catalyse BPA-GA at much slower rates than maternal microsomes (Doerge et al, 2010 a,b), which prolongs elimination half-life (Nishikawa et al, 2010). This ultimately exposes the fetus to higher levels of active BPA and accentuates the lack of sophisticated fetal drug metabolism as well as the lack of a fetal-placental BPA barrier (Takahashi & Oishi, 2000). Despite the fact that metabolism of BPA in the fetal rat is efficient and increases with age (Domoradzki et al, 2003), long-term studies have shown that exposure to BPA at this critical time in development has long-lasting effects on metabolism and bodyweights (Rubin et al, 2001, 2009; Nikaido et al, 2004). In our study, it was hypothesized that pup HPA axes would be developmentally programmed by early xenoestrogen exposure.

Canadian Exposure to BPA

Low doses of BPA can cause effects in animal models at doses in the human exposure range, indicating that it can act at much lower doses than previously expected (Richter et al. 2007; Vandenberg et al. 2007; vom Saal et al. 2007; Wetherill et al., 2007). It has continuously been recorded in human urine, indicating a low but constant exposure (Bushnik et al, 2010; Health Canada, 2013). More recent large-scale studies have been conducted on total urinary BPA levels in the Canadian population. In cycle 1 of the Canadian Health Measure survey analysis, data collected between 2007-2009 was analyzed for total urinary BPA concentrations and was presented in “The Second Report on Human Biomonitoring of Environmental

Chemicals in Canada". This analysis detected BPA in the urine of over 90% of Canadian participants (Bushnik et al, 2010). Cycle 2 of the same survey featured the addition of a younger demographic of 3-5 year olds and is the most recently conducted Canadian exposure model for probabilistic total dietary BPA exposure. Despite this ubiquitous prevalence, the health effects associated with BPA exposure at this level remain currently unknown, which is explored further in the Interdisciplinary Perceptions section of the thesis.

Significance of BPA

Regardless of evidence of widespread BPA exposure in the general population, knowledge of its potential impacts upon reproduction and pregnancy in humans remains limited (Cantonwine et al, 2013). The xenoestrogen has been detected in serum, umbilical cord blood, amniotic fluid and fetal tissues at various concentrations (Calafat et al, 2005, 2008). Health Canada has stated that there continues to be a lack of information on the effects of BPA on the developing nervous system (Stump et al., 2010). Animal experiments have shown that the critical periods of fetal development, infancy and puberty have a higher sensitivity to low doses of hormones than do adult tissues (Vom Saal and Moyer, 1985; Selevan et al., 2000; Doerge et al, 2010 a,b), and this is significant as it would suggest a higher susceptibility to hormonal disruption. Reports in neonatal primates, in humans and in the rodent have revealed immaturity in renal function, which may lead to higher internal exposures to BPA in young babies (Coughtrie et al., 1988; Hines, 1988; Ligi, Boubred, Grandvuillemin & Simeoni, 2013). Overall, the ubiquitous nature of this mass-produced endocrine disrupting chemical, its reported effects on hormone disruption, its presence in neonates and the gaps of information on neurodevelopmental effects emphasizes the urgent need for continued research.

Research Question

It is well established that BPA has adverse effects on fertility, urogenital development and cancer (Markey et al., 2001,2003; Honma et al., 2002; Heindel, 2005; Heindel and Levin, 2005; Susiarjo et al., 2007; Durando et al., 2007; Adewale et al., 2009; Patisaul and Adewale, 2009; Vandenberg et al., 2009). However, there are gaps in the literature regarding the role of BPA and the development of the HPA axis. To address these little-known areas in the field, my research question is the following: *Does perinatal exposure to BPA affect rat pup development?*

Three sub-questions were also addressed in the study:

1) *Does perinatal BPA exposure induce sex-specific effects on rat pup development?*

2) *Does perinatal BPA exposure modulate adrenal gland GR gene expression in male pups?*

3) *Does perinatal BPA exposure affect development during the SHRP to modulate adrenal GR expression in male pups?*

To attempt to answer these questions, my research project consisted of three main objectives:

(1) To carry out a BPA-dose series exposure study in timed-pregnant Long-Evans rats.
(2) To assess gross morphology parameters in the BPA- exposed rat pups at two time points post-birth (PND 5 and 15).
(3) To characterize the molecular effects of BPA on GR expression levels in PND 5 and PND 15 male adrenal glands, a key component of the HPA axis.

II. MATERIALS AND METHODS

1.0 Animals

1.1 Strain Selection

Rats were selected as the animal model for this study given the investigators' experience with the species, the abundance of environmental toxicology literature using this model and its suitability for assessment of the SHRP. Selection of the appropriate rat strain was carefully considered. The Sprague-Dawley rat choice was eliminated as a previous study had noted that the large history of engineered breeding within these rats had resulted in a reduced sensitivity to environmental toxicants (Putz et al, 2001). An outbred rat strain of the *Rattus norvegicus* species, the hooded Long-Evans rat, was selected as previously discussed, for the experiment. Again, this strain has been reported to be a good model for multipurpose use and particularly for neuroscience and toxicology studies (Hankenson, 2013; Charles River, 2016; Janvier, 2016).

1.2 Ethics Approval

All experimental procedures met the guidelines of the ethical treatment of animal subjects in experimental research set forth by the University of Ottawa. Prior to the commencement of animal work, the ethics approval request #1705 was approved and assigned the protocol number of #ME-1705-A1 by the University of Ottawa Animal Care Committee.

1.3 Animal Husbandry

Thirty untimed-pregnant Long-Evans rat dams were obtained from Charles River (St-Constant, QC), a commercial supplier. Dams were housed in individual cages containing corn-cob bedding. The room was kept on a regular 12 hour light/dark cycle schedule with lights on at 7am. Dry chow and water in glass bottles were provided *ad libitum*. Upon arrival, dams were given 24 hours to acclimatize to the animal care facilities prior to experimental exposures. Dams were checked daily for birth and approximate time of birth was recorded. Two days post-birth, pups were sexed via gross assessment of anogenital distance (AGD) and numbered using a Sharpie marker on their back.

Pregnancy staging upon arrival was estimated using the following formula: 21 days- parturition date. Using this calculation, conception is assumed to occur on Day 1, birth on Day 22. Stage of pregnancy upon first exposure could then be estimated with exposure for some rats as early as GD 6 of pregnancy or as late as GD 14. The determination of pregnancy staging is outlined in **Table 1**. The total duration of gestational exposure was estimated using the following formula: parturition date – date of first exposure.

2.0 Exposure Model

2.1 Dosing

An exposure model **(Figure 7)** was designed to assess the effects of gestational and lactational exposure to BPA. Three doses of BPA were used to investigate potential dose-response relationships. The US National Toxicology Program established a lowest observed adverse effect level (LOAEL) for BPA at 50 mg/kg/day (or 50,000 µg/kg/day) after a 103-week rat study. In a 2014 hazard assessment by the FDA, a no observed

adverse effect level (NOAEL) of 5 mg/kg/day (or 5000 μg/kg/day) for BPA was reported based on two multigenerational rodent studies (Tyl et al 2002, 2008). Thus, the doses selected for this study (BPA 5, BPA 50, BPA 500 μg/kg/day) are low, can be considered environmentally relevant, and are well within the range of regulatory concern (Delclos et al, 2014). Numerous BPA toxicological studies (Farabollini et al, 1999;2002; Dessi-Fulgheri, 2002; Adriani et al, 2003; Sadowski et al, 2014; Rebuli et al, 2014; Panagiotidou et al, 2014; Jiang et al, 2016) have reported adverse effects within our selected experimental range.

The established xenoestrogen DES was selected as a positive control at a dose of 5 μg/kg bw/day and is considered to be the standard positive control for studies involving oral BPA dosing according to the US National Toxicology Program (Richter et al, 2007). DES exposure at similar doses is associated with decreased sperm counts (Jiang et al, 2016), decreased expression of sexual maturation and differentiation-related genes (LaRocca et al, 2011) and decreased litter sizes at birth (LaRocca et al, 2011).

2.2 Exposure Solutions

BPA (Aldrich) and DES (Sigma) in powder form were dissolved in ethanol at the appropriate experimental dosing concentrations. A vehicle control solution of 1% sucrose (SigmaAldrich, SigmaUltra, 95.5%) containing1% ethanol (Commerical Alcohols, 99%, Anhydrous Ethyl Alcohol) was prepared. A 1% ethanol, 1% sucrose BPA stock solution was prepared and diluted to solutions at 0.1% and 0.01% ethanol in 1% sucrose. These dilutions correspond to BPA dosages of 50 μg/kg bw/day and 5 μg/kg bw/day. The BPA 500 dosage (500 μg/kg bw/day) was obtained directly from the stock solution. BPA solutions therefore contained 0.01-1% ethanol. A 1% ethanol, 1 % sucrose DES stock solution was similarly

prepared and diluted to a working solution at 0.01% ethanol in 1% sucrose. This corresponds to a DES dosage of 5 μg/kg bw/day. Treatment groups are shown in **Figure 7**.

2.3 Exposure Protocol

BPA metabolism and bioavailability in the rat is dependent on the route of administration (Tominaga et al, 2009; Doerge et al, 2010a, 2011). As oral administration of doses in the rat are more physiologically and environmentally relevant to mimic human exposure to BPA, which is primarily through oral exposure in food and drink, dose administration involved oral feeding via a needle-free syringe. Injections and oral gavage were not considered as these methods would have placed further stress on the rats (Cagena et al, 1999) and could have confounded the study. Dams were weighed daily, allowing for appropriate daily dosing calculations and were then administered BPA, DES or vehicle-control. Daily dosing was performed regularly in the morning by the same experimenter (myself) to ensure consistent technique. Dams were dosed daily through parturition, and continued to PND 4. The experimental dosing timeline from gestation through sacrifice is presented in **Figure 8.**

2.4 Sacrifice

On PND 5, approximately half of the male and half of the female pups were sacrificed. Selected animals were held in a separate room to reduce potential stress from blood exposure and were transported individually into the necropsy room at the time of sacrifice. Decapitation with sharp scissors without anesthesia was selected, as rats do not show HPA axis activation from this method (Leary et al, 2013). Further, rats are not usually properly anesthetized with typically used isoflurane at this age (Seubert et al, 2013).

On PND 15, both pups and dams were sacrificed in the laboratory. The fume hood was used as an anesthetizing station, in compliance with Animal Care and Veterinary Service recommendations. Each animal was placed in a plastic chamber with isofluorane gas (50 mL, CDMV, Benson Medical Industries) and anesthetized prior to decapitation with a guillotine.

Due to illness, often characterized by weight loss and fur piling, premature euthanasia of affected animals was required as per Animal Care and Veterinary Service protocol. Illness was presumed not be related to the study protocol or BPA/DES exposures. Acute BPA toxicity has been reported to occur at much higher doses (100,000 μg/kg/day range) than doses used here (5-500 μg/kg/day) (Goodman et al, 2006; Chapin et al, 2008; WHO, 2011; Pant & Deshpande, 2012). Further, DES toxicity occurs in the 1000 μg/kg/day range (Odum et al, 2002), while our dose was 5 μg/kg/day. Two sick dams presented symptoms thought to be the result of miscarriage or infection, and one dam had not been gaining weight and was thought to have miscarried. The sudden illness in these two dams and the discovery that the other dam was not pregnant resulted in humane euthanasia and exclusion from the experimental study.

3.0 Morphometric Measurements

3.1 Dams

Daily dam weights were recorded from the first day of exposure, throughout gestation and up to PND 15, when dams were sacrificed.

3.2 Offspring

Litter Size, Sex Ratio, Bodyweights

As early as PND 2, pups were sexed based on gross AGD and litter size, and sex ratios were noted. Sex was confirmed at the time of sacrifice by assessing AGD length, which is typically twice as long in males (Rajeshwari, 2009). The day of ear pinning and eye opening were tracked and recorded.

Crown-Rump Length

Crown-rump (CR) length measurements were made in triplicate at the time of sacrifice. To obtain CR measurements, the top Vernier caliper prong was placed at the top of the head between the ears and the bottom prong was placed at the base of the tail paying careful attention not to stretch the animal (Hood et al, 2016), (see **Figure 9**). The pup was kept in place by holding either side of the trunk. PND 5 pups were measured awake while PND 15 pups were measured under anesthesia, as their larger size and higher activity level would have complicated measurements.

Anogenital Distance

The AGD was measured in triplicate post-sacrifice using Vernier calipers (see **Figure 9**). To obtain AGD, the distance between the cranial edge of the anus and the genital tubercle was measured. Without stretching the animal, the middle finger was placed on the midsection of the pup, and thumb was placed on the tail to gain a better visual of the target points. The top caliper prong was placed at the base of the anogenital aperture (in males) or at the base of the urinary aperture (in females), and the bottom prong was placed at

the cranial edge of the anus (Hood et al, 2016). The AGD measurements were obtained post-mortem for PND 5 pups and under anesthesia for PND 15 pups.

3.3 Data Analysis

Noting the range of gestational exposures generated from our experimental protocol **(Table 1),** a more precise developmental window of 9-11 days of gestational exposure (see Discussion for details) was selected for the morphometric analyses. Both male and female pups were selected from this window for statistical analysis. Animals sacrificed on PND 5 and PND 15 were analyzed separately. Analyses were conducted using IBM SPSS Statistics version 21 (2012). A p-value less than 0.05 was considered statistically significant.

Dam Bodyweight Data Analysis

The average body weight per treatment group per day was also calculated for the dams; this included data from the day of sacrifice. To better compare gestational days across animals, body weight data used for each dam began six days prior to birth. Changes in mean dam weights over time and across treatment groups were assessed using a mixed factorial ANCOVA, with Treatment (five levels: VEH, DES, BPA 5, BPA 50, BPA 500) as the independent factor, Time (21 levels) as the repeated factor and BW-6 (body weight at six days prior to giving birth) as the covariate. Appropriate post-hoc analyses were conducted where deemed necessary.

Offspring

Litter Size Data Analysis

The litter size for each dam as well as the number of litters per treatment group were totaled and recorded. Two dead pups were observed in the DES litters but this did not result in the euthanasia of the entire litter or of the dam.

Sex Distribution Data Analysis

The distribution of male and female pups delivered per treatment group were analyzed to determine if differences in sex were statistically significant across treatment groups. The number of males and the number of females born in each treatment group were totaled and a Chi-squared test of independence was used as the method for statistical analysis, using Treatment (5 levels) and Sex (2 levels) as the two factors.

Morphometric Data Analysis

The variables of Treatment and Sex were analyzed in terms of the specific sacrifice day developmental stage. PND 5 and PND 15 animals were analyzed separately to determine if there was a significant interaction between Treatment and Sex at each developmental stage. The PND 5 and PND 15 developmental stages were not compared as the animals grow between PND 5 and PND 15, thus necessarily affecting these morphometric measures. To begin data transformation, bodyweight was corrected to litter size, CR length was corrected to the corrected bodyweight, and AGD was corrected to the corrected CR length. Therefore, all morphometric data were corrected indirectly to litter size to account for the variation in experimental litter sizes. Following the correction to litter size, the square root

transformation was applied to these values due to heterogeneity of variances (Levene's test). Data transformation is reported to be valid and statistically accepted (Manikandan, 2010). Selection of the square root method is typically accepted with proportional variance and mean (Manikandan, 2010). The use of corrected data in the following analyses are represented with an asterisk (BW*, AGD*, CR*, and square root corrected data are represented with a double asterisk (BW**, AGD**, CR**).

Pup Bodyweight Data Analysis

Pup bodyweights, measured on the day of sacrifice (PND 5 or PND 15) were corrected to their respective litter size (Festing, 2006; Hood, et al, 2016) by dividing the weight of the pup on the day of sacrifice by the size of the litter from which it originated. A 2-way ANOVA was used to assess an interaction between Sex (two levels) and Treatment (five levels). A significant interaction was further explored via simple effects analyses. Main effects were then analyzed followed by a Dunnett's post-hoc test to the Vehicle group.

Pup Bodyweight Progression Data Analysis

To analyze pup bodyweight progressions, daily weights were corrected to respective litter size.
We assessed whether bodyweight progression throughout postnatal life was affected by interactions between the sex of the pup, the treatment it had received, or over time. PND 5 pup analysis was conducted separately from that of the PND 15 animals. A 3-way ANCOVA was done with the independent variables of Treatment (five levels), Sex (two levels) and Time (a repeated factor with 3 levels for PND 5 and 13 levels for PND 15 pups). The fixed covariate consisted of corrected bodyweight on PND 2; PND 2 represented a time when all animals could be confidently sexed, thus, body weights were taken systematically as of PND 2 for all animals. Significant interactions, if applicable, were further explored via simple effects analyses followed by complex comparisons, using an adjusted alpha level to take into

account multiple pairwise comparisons. Main effects for each factor were then assessed if no interactions were found. In order to circumvent any violations to the assumption of sphericity (differences in variance between all group combinations were not equal (Lund Research, 2013), degrees of freedom for the repeated measures factor (Time) was corrected for using the Huynh-Feldt procedure (Huynh & Feldt, 1976; Abdi, 2010).

Crown-Rump Length Data Analysis

The CR lengths were measured in triplicate and raw values were averaged for each pup. This average value was then corrected to the corrected body weight for each pup (Festing, 2006; Hood, et al, 2016). The corrected value was obtained by dividing the average raw CR length for each pup by its corrected bodyweight. Considering the varied litter sizes across the experiment, this correction was thought to be an appropriate way to account for litter size differences, as it is possible that a large or small litter may impact individual pup size.

A 2-way ANOVA was used to determine if there was an interaction between Sex (two levels) and Treatment (five levels). A significant interaction was further explored via simple effects analyses. Non-significant interactions were followed by analysis of the main effects; a Dunnett's post-hoc test to the Vehicle group was then conducted to decipher any significant main effects.

Anogenital Distance Data Analysis

The AGD lengths were measured in triplicate and values were averaged for each pup. The average AGD was then adjusted to the corrected CR length for each pup (Festing, 2006; Hood, et al, 2016). The corrected value was obtained by dividing the average AGD by the corrected CR length for each pup. This step was

necessary to account for differences in litter sizes, bodyweight and CR length. Similar to CR length, the data were analyzed using a 2-way ANOVA using the factors Sex (two levels) and Treatment (five levels). Main effects were analyzed with appropriate Dunnett's post-hoc test to the Vehicle group, if required.

4.0 Molecular Characterization of Glucocorticoid Receptor

4.1 Tissue Collection

At the time of sacrifice, tissues from both rat dams and pups were collected to facilitate molecular characterization of GR expression and other targets. Brain tissues collected included the hypothalamus, pituitary, frontal cortex, hippocampus and cerebellum. Pup tissues collected included kidney, liver, adrenal glands, ovaries, prostates, and testes. Dam tissues (brain, kidney, liver, adrenal glands, ovaries, uterine horns, adipose tissue) were also collected and similarly processed for future molecular investigations. Tissues were preserved in RNAlater (Qiagen) or snap frozen using dry ice and stored at -80°C. Blood samples collected from both pups and dams were centrifuged to isolate plasma which was also stored at -80°C for future analysis.

4.2 RNA Isolation

Molecular studies were restricted to male rat pups that had received 9-11 days of gestational exposure (plus five days of lactational exposure) to vehicle, BPA or DES. PND 5 male adrenal glands were pooled to obtain sufficient tissue for RNA isolation. A single sample (n=1) was comprised of four adrenal glands: bilateral glands from two male pups from the same litter. As PND 15 adrenal glands are larger and did not necessitate pooling of samples, PND 15 adrenal samples were comprised of the bilateral adrenal glands from an individual male pup. Adrenal gland samples were individually homogenized using a

TissueRupter, with rotors sterilized between each use as per manufacturer's instructions. The Qiagen RNeasy kit (Qiagen) was used to extract whole tissue RNA from these adrenal gland homogenates following the Qiagen Protocol. Isolated RNA was quantified, as described below, and was adjusted to a quantity of 500 ng total RNA prior to reverse transcription.

4.3 RNA Concentration and Quality

A spectrophotometer (Thermoscientific Nanodrop 2000, serial number: 2145) was used to determine RNA concentration (ng/µL) and the absorbance ratios (260/280 and 260/230) in triplicate. The RNA concentration and absorbance level readings are displayed in **Table 2.** The absorbance ratios are indicative of RNA quality (Kennedy, 2011; Qiagen, 2016). The 260/280 ratio verifies that no protein contamination of the RNA sample has occurred; ideal absorbance readings are between 1.8-2.2 (Qiagen, 2016; ThermoScientific, 2016). The 260/230 ratio of greater than 2 verifies the absence of salts and contaminants in the RNA sample (Saha & Blunwald, 2014; Qiagen, 2016; ThermoScientific, 2016). Following quantification of RNA, samples were stored at -20°C.

4.4 RNA Gel Electrophoresis

RNA denaturing gels were done to confirm that the isolated RNA samples were intact and of good quality for a range of concentrations. A 2:1 ratio (28SrRNA band intensity: 18SrRNA band intensity) demonstrates intact RNA (Imbeaud, 2005; Wieczorek et al, 2012; ThermoScientific, 2016). Northern blot denaturing agarose gel electrophoresis was performed. Briefly, samples were heat-denatured for 15 minutes and loaded onto the gel using a volume of 5 µL of sample: 2.5 µL NorthernMax (NMAX) formaldehyde load dye. A high-range Riboruler RNA ladder (containing between 200-6000 bases) of purified single-stranded RNA fragments was also run on the gel as an intact comparison tool to approximate the size the RNA

sample fragments (ThermoScientific, 2016). In addition, a non-denatured RNA sample was run on the gel as a control (see gels in Results). Gel visualization and capture was obtained using the Gel AlphaImager Mini Program (AlphaInnotech). The lowest detectable RNA concentration with appropriate 2:1 ratio (28S-rRNA band intensity: 18S-rRNA band intensity) was 47.9 ng/μL. Concentrations of RNA used in entire series of gels ranged from 9.63 ng/μL- 867.3 ng/μL. Only RNA samples with concentrations greater than 47.9 ng/μL were used for qPCR after reverse transcription.

4.5 Reverse Transcription

Reverse transcription is a necessary step for synthesizing the single, complimentary strand of DNA, or cDNA, from a single strand of RNA (ThermoFisher Scientific, 2016) to be amplified in qPCR cycling. All reactions were prepared on ice and RNA samples were converted to cDNA with the Quantitect kit (cat no. 205310, Qiagen) following the Quantitect protocol. As described, all RNA samples were set to a standard RNA quantity of 500 ng prior to cDNA conversion. gDNA wipeout buffer was added to the RNA samples as an initial step to eliminate genomic DNA contamination prior to reverse transcription. Each cDNA sample (at a quantity of 500 ng assuming 100% conversion to cDNA) was then stored at -20°C until qPCR analysis.

4.6 Design of Target Gene Primers

Reference genes regulate basic functions in each cell and serve as the internal control for sample normalization to be able to compare gene expression between different samples (Tong et al, 2009). This constant expression will allow the concentration of the target gene expression in the experiment to be quantified using the known concentration provided by the reference gene expression. Two of the most commonly used reference genes for both humans and rat have been reported in the literature to be GAPDH

and 18SrRNA (Chen et al, 2005; Brattelid et al, 2010) (Ashby& Odum. 2004; de Francesco et al, 2013; Luo et al, 2014). After a literature review, GAPDH and 18SrRNA were selected as the reference genes for this study.

The GR-α ("GR") was selected for molecular investigations of the glucocorticoid receptor, as it is the primary isoform generated by splicing, is biologically relevant, and is the isoform most highly expressed (Pujols et al, 2002). Primer validation of the GR target gene was a lengthy process requiring evaluation of multiple GR gene sequences prior to selection of the final optimal GR primer sequence using NCBI BLAST (**Table 3; parameters described below).** The qPCR amplification of adrenal gland tissue using GR primers generated single-peak melt curves, with efficiency ("E" between 90-110%) and correlation coefficient ("R^2">.98) within the appropriate ranges (BioRad, 2006; Taqman Life Technologies, 2012; ThermoFisher Scientific, 2016).

4.7 Reference Gene Primer Design

Using the BLAST program, specific primer sequences were custom-designed to amplify regions of interest in the two rat reference genes (GAPDH, 18SrRNA). For both reference and target gene primers, only sequences specific to the genes of interest, with no cross-matching to non-specific gene targets were designed. Primer sequences were short (20 bp), with a poly-A tail and no introns. The poly-A tail is represented by a single line of adenines at the end of the 3' sequence (Wahle & Keller, 1992; Chang et al, 2014), which confirms the absence of introns (Lodish et al, 2000). The presence of introns would break up the gene sequence being targeted and prevent the identification of the full target gene sequence. Other characteristics for consideration during design included selecting melt temperatures below or less than 65°C to prevent secondary annealing (BioSoft, 2016), and an appropriate GC ratio (40-60%)

(ThermoScientific, 2016) both of which were confirmed. Primer sequences were ordered custom-made from Invitrogen. Ideally, both reference genes and target genes would share similar qPCR parameters. Unfortunately, the limited availability of gene sequence data for the rat made it challenging to design reference and target genes with similar melt temperature parameters (**Table 3**). As described below, it became necessary to perform separate qPCR runs for reference versus target gene assays.

The creation of a standard curve optimizes the qPCR assay (BioRad, 2006; Sigma-Aldrich, 2016). Three biological replicates and a 5-series dilution are required for optimization testing (ThermoFisher Scientific, 2016; Sigma-Aldrich, 2016). Specific to our experimental assays, a 5-series dilution was used with technical replicates loaded in triplicate. Running varying dilutions of a cDNA sample on the same plate allowed for the creation of a standard curve. For example: the 5 dilution factors used to create our experimental standard curves were x=10^{-1}, 10^{-2}, 10^{-3}, 10-^{4}, 10^{-5}. Based on the starting amount of cDNA present, each diluted version surpassed the threshold of detection at varying Ct values. The log of the dilution factor of the cDNA sample versus its Ct value were plotted to create the curve (BioRad, 2006) as well as the linear regression which demonstrated data point variations (BioRad, 2006). In addition, E and R^2 readouts were generated which indicated whether the run had been optimized. The line of best fit created (the R^2 value) was based on the data on the curve (BioRad, 2006). Standard curves were produced in triplicate using 18SrRNA, GAPDH and GR gene primers to amplify expression in the selected PND 5 and PND 15 male adrenal gland samples.

During Phase I of qPCR (the exponential phase), the amount of product doubles per cycle (BioRad, 2006). During Phase II (the plateau phase), some reagents have been completely consumed and the reaction stops doubling every cycle (BioRad, 2006). The E value is obtained using the slope of the standard curve for

each run (ThermoFisher Scientific, 2016). The following formula would result in an ideal efficiency of 100% with an E value of 2: % Efficiency= (E-1)x100 (BioRad 2006).

A melt curve is generated after sample amplification (Sigma-Aldrich, 2008), or after the 40-cycle run specific to our experimental setup. The curve is created by increasing the temperature until the DNA has melted and results in decreased fluorescence (BioRad, 2006). Given that SYBR green is a fluorescence marker that only associates with double stranded DNA, fluorescence can be tracked throughout the melting cycle. A decrease in fluorescence is the result of SYBR green dissociating from the amplicon (ThermoFisher Scientific, 2016) and indicates that the double stranded amplicons have become single stranded (ThermoFisher Scientific, 2016). If multiple products are present in the sample, they will melt at different temperatures based on variables including length and structure (Sigma-Aldrich, 2008). Therefore, the melt curve should only have one peak to indicate purity, as multiple peaks indicate that sequences other than the target gene sequence have been amplified (Sigma-Aldrich, 2008). To determine that the primers only amplified a single product, the melt curve generated in each run was studied. To confirm that the primers did not adhere to each other, primers with appropriate annealing temperature were designed and selected.

4.8 Primer Validation

To validate gene primers, standard curves were generated for each gene target and each treatment group (Qiagen, 2016), which ensures that the qPCR assay has been optimized in terms of run cycles, temperatures, cDNA dilution and primer design. Optimally, DNA is amplified in a detectable range within the run-time/number of cycles and doubles every cycle. Analysis of standard curves, which include generated melt curves, allows for assessment of primers including contamination, formation of primer-

dimers or non-specific structures (BioRad, 2000). Finally, by completing standard curves for every treatment group, the suitability of selected reference genes can be determined. By definition, reference gene expression should remain stable given the treatments. Our experimental standard curves generally produced E and R^2 values within optimal ranges, all melt curves generated single peaks, and sample integrity was further verified using Northern blot gel electrophoresis, which produced the appropriate 2:1 band ratios.

A cycle threshold (Ct) value, indicates the point in the PCR cycle at which the sample has surpassed the threshold, resulting in detectable fluorescence signals (Wong & Medrano, 2005; BioRad, 2006). Samples with a lower Ct value have a higher amount of initial DNA and surpass threshold faster than samples with a lower starting amount of cDNA and a higher Ct value (Wong & Medrano, 2005).

4.9 Quantitative Polymerase Chain Reaction (qPCR)

Every cell has a certain amount of gene expression and Quantitative Polymerase Chain Reaction seeks to amplify specific sequences in a sample of DNA. *Real time* quantitative PCR (qPCR) is the gold standard for accurate, sensitive gene expression quantification (Derveau et al, 2010). This method allows for a minuscule amount of initial DNA or cDNA from a sample to be amplified millions of times over (Biosoft, 2014). qPCR measures DNA amplification in *real time* and includes three main events which cycle repeatedly (between 25-40 cycles): Denaturation, Annealing and Extension. A double stranded DNA sequence is first separated with heat, primers anneal to specific locations on the complementary strand, and this strand is then extended by DNA polymerase (Biosoft, 2014). *Real-time* 40-cycle qPCR runs were used in the experiments.

This process exponentially increases the amount of sample DNA as the method repeats itself for each new double strand of DNA created. Amplification during PCR cycling creates an exponential number of DNA strands and a high yield of genetic amplification at the end of cycling. Specific to this experiment, qPCR run cycles were the following: Step 1. 98°C for 2 min, Step 2. 98°C for 2 sec, Step 3. Gene-specific annealing temperature (55°C or 60°C) for 5 sec. Steps 2,3 were repeated 39 more times. This was followed by a constant increase in temperate to generate a melt curve. Temperature was increased in 0.2°C increments from 75°C to 95°C during the final phase of the run, followed by a plate read.

The amount of DNA being produced in *real-time* qPCR is measured and indicated after each cycle with the help of fluorescent markers. So-FastEva Green Supermix from BioRad is the reporter dye used to monitor the amplifying DNA. Fluorescence is proportional to the amount of product molecules, called amplicons, produced during the cycles (Invitrogen, 2008).

4.10 qPCR Plate Setup

An important control for plate contamination is the no template control (NTC), which does not contain any cDNA. The NTC well in the plate includes PCR master mix and primer, but no source DNA, and therefore should not generate amplification peaks. Amplification in the NTC well may indicate contamination from neighbouring wells, improper technique or primer-dimer formation. Only qPCR runs with no amplification of the NTC samples were used for analysis.

A positive control was included on each standard curve qPCR plate and represented undiluted cDNA (at a quantity of 500 ng). This control was used to verify the amplification of sample cDNA during selection of

the appropriate dilution for the experiment. All experimental qPCR runs included template cDNA diluted at 10^{-1}. The standard curve plate setup is displayed in **Figure 10**.

A biological replicate is defined as biologically different samples that serve to capture random variation, while a technical replicate is the repeated measure of a sample (Blainey, Krzywinski & Altman, 2014). In total, up to five biological replicates per treatment group were run in the PCR reactions, and technical replicates were performed in triplicate per biological sample per plate. Each plate featured a representative sample from each treatment group: one biological replicate per treatment group were all run on the same plate (ex: VEH sample 1, DES sample 1, BPA 5 sample 1, BPA 50 sample 1, BPA 500 sample 1). The experimental plates were set-up as illustrated in **Figure 11** for the reference genes GAPDH and 18SrRNA, and in **Figure 12** for the target gene GR. Due to the varied melt temperature (Tm) specifications for the reference and target genes, target and reference genes could not be run on the same plate. 18SrRNA and GAPDH genes were run on the same plate with a Tm of 55°C, and the GR target gene was run on a separate plate at a Tm of 60°C. This is considered to be acceptable (Pfaffl et al, 2004; Hellemans et al, 2007; Rieu, 2009) and the values were normalized to the reference gene upon analysis (Qiagen, 2016). Optimal standard curves per gene are displayed in **Figures 13-15**.

5.0 qPCR Data Analysis

The 18SrRNA gene was initially selected as a reference gene based on a literature review but preliminary results suggested that the gene had been influenced by the experimental xenoestrogen treatment. To confirm, a preliminary single-factor ANOVA determined there were significant differences between the raw Ct values across treatment groups (1-way ANOVA, $F(4,73)=7.9058$, $p<0.05$). Raw PND 5 Ct values are presented in **Appendix 1.1** and raw PND 15 Ct values are presented in **Appendix 1.2.** Given this

outcome, the gene was no longer suitable for use as a reference, thus GAPDH was retained as the sole reference gene for statistical analysis. Previous study supports both our choice of GAPDH as an appropriate reference gene in addition to its use as the single endogenous control used to normalize the gene expression levels of the target gene GR (Kazemi et al, 2016). Given the apparent sensitivity of 18SrRNA to our BPA treatment, 18SrRNA was analyzed as a second target gene.

5.1 Fold Change ($2^{-\Delta\Delta Ct}$)

The fold change is one of the most used techniques to calculate concentration differences between samples and normalizes target gene values to a single reference gene, as reported in the MIQE guidelines (Livak & Schmittigen, 2001; Bustin et al, 2009). This method has been employed in numerous studies (Livak & Schmittgen, 2001; Schmittgen & Livak, 2008; Perkins et al, 2012) and was therefore selected as the technique for target GR gene normalization to the GAPDH reference gene. Steps for Ct and delta Ct (ΔCt) value calculations are illustrated in **Table 4** and steps for delta delta Ct (ΔΔCt) value and fold change calculations are illustrated in **Table 5.**

The fold change analyzes gene expression changes by comparing the Ct values generated by the reference gene and the target gene after normalization (Livak & Schmittgen, 2001; Rico et al, 2014). The average of the Ct values for each PND 5 male sample (taken in triplicate) per gene are presented in **Appendix 2.1**, and in **Appendix 2.2** for the PND 15 male samples. These values were then used for the ΔCt statistical analysis and for the fold change calculations and graphs, to be later discussed. Typically, positive fold changes indicate gene upregulation and negative fold changes indicate downregulation (Livak & Schmittgen, 2001; Goni et al, 2009). Although fold change cut-offs are arbitrary in the literature (with ranges between 1.3-2.0) (DeRisi et al, 1996; Schena et al, 1996; Peart et al, 2005; Raouf et al, 2008;

Huggins et al, 2008), the most stringent criteria for fold change cutoff has been reported to be greater or equal to 2, which is an acceptable cut-off (McCarthy & Smyth, 2009; Dalman et al, 2012). Specific fold change cut-offs of 0.5 and below and 2.0 and above have been reported as appropriate indicators of significant fold change (Shuster et al, 2013; Dembele & Kastner, 2014; Chanda et al., 2016; Karmaus et al, 2016). Based on the literature, we used fold changes of 2.0 and above as the threshold for biologically-relevant gene upregulation and fold changes of 0.5 and below as the threshold for gene downregulation (Shuster et al, 2013) As fold-changes are the established presentation of qPCR results (MIQE guidelines) (Bustin, 2009), we have placed greater emphasis on these findings.

The fold changes do not enable detailed statistics, and thus a second approach to the evaluation of the qPCR findings was used to confirm homogeneity of variance. Differences between experimental groups were determined by using the ΔCt values of the target genes after normalization to the reference gene. The ΔCt values were calculated by subtracting the average Ct value per experimental group for both 18SrRNA and GR from the average GAPDH Ct value per experimental group. The ΔCt calculation determines the difference between average Ct values of target and reference genes, specifically by calculating the difference between average Ct values between each target and reference gene per treatment group. Levene's test was first run on ΔCt values within each experimental group to test for homogeneity of variance, which proved to be significant. A significant Levene's test confirmed that the ΔCt data per experimental group were not normally distributed and indicated that a nonparametric test was required for further analysis (Nahm, 2016); further transformation of the data was deemed inappropriate thus the use of non-parametric analyses. Nonparametric testing is advantageous as it bases statistical analysis on ranking and is considered a conservative method (Nahm, 2016). The Kruskal-Wallis ANOVA was then chosen as

the nonparametric statistical method of choice. It was used to determine if the median ΔCt values of our five experimental groups were different (Kahn, 2016). Later, fold change expressions were graphed.

III. RESULTS

1.0 Morphometric Measurements, Litter Analyses

1.1 Dam Bodyweight Progression

Dam bodyweights progressively increased throughout gestation, peaked immediately before birth, and sharply decreased after birth as expected (**Figure 16**). Changes in bodyweight prior to birth up to and including sacrifice on PND 15 were analyzed with a mixed-factorial ANOVA, accounting for differences in starting weights using 6th day pre-birth as the covariate. All dams that carried pups to term were included in the analysis. The progression of dam bodyweights was not significantly affected by Treatment at any specific Time (2-way ANOVA, $F(22,114)=0.894$, $p>0.05$). Further, dam bodyweights were not significantly affected by Treatment during pregnancy (2-way ANOVA, $F(4,21)=0.511$, $p>0.05$). The progression of dam bodyweights during gestation was consistent with typical pregnancy with a significant difference over Time ($F(5, 114) = 4.24$; $p=0.001$).

1.2 Litter Sizes and Sex Ratios

Large variations in litter size were observed. Across all dams in the study, litter size ranged from 5 to 19 pups per litter. When separated by treatment, litters were between 8-15 pups (VEH), 6-14 pups (DES), 9-17 pups (BPA 5), 5-13 (BPA 50), and 8-19 (BPA 500). All dams were included in the analysis of litter sizes if at least one pup was alive at birth.

The following descriptions and statistical analysis pertain to the narrower, specific window of 9-11 days of gestational BPA exposure, as described in the Methods. Litter sizes across all of the treatment groups in this specific window ranged from 5-19 pups (**Figure 17**). As noted in the Methods, three pups died and were subsequently excluded from tissue collection, but they were included in litter size calculations. However, as these pups did not survive to necropsy, they were not included in later morphometric calculations. A Chi-square test of independence conducted on the average litter size per treatment group indicated that litter sizes were not significantly different between treatment groups: X^2 (4, N=?) = 0.000, $p>0.05$. Therefore, treatments did not affect litter size, nor were they expected to, given the low doses used and the timing of exposure.

To determine whether treatment produced different numbers of males/female pups, sex distributions using the average number of males and females born to each treatment group were analyzed **(Figure 18)** using a Chi-square test of independence. Treatment did not significantly influence the number of male and female pups born in each litter (X^2 (4, N = 58) = 0.101, $p > 0.05$), again an anticipated outcome given the low dose concentrations and exposure timing.

2.0 Morphometric Measurements

The second phase of data analysis focused on the morphometric measurements of the male and female pups. There were some instances of heterogeneous variances between treatment groups, as determined by Levene's test. In these cases, the square root transformations of the corrected sacrifice day measurements (BW*=BW/litter size, CR*=CR/BW*, AGD=AGD/CR*) were used for the statistical analyses. In cases where homogeneity of variance was not in jeopardy (again by Levene's test), the corrected data (BW*, CR*, AGD*) were used for the statistical analyses.

2.1 Bodyweights

PND 5 Final Bodyweight

To assess whether Treatment affected bodyweight of pups at PND 5 differently based on Sex , we first performed a Levene's test on the corrected data, resulting in the detection of heterogeneous variances ($F(9,124)=2.085$, $p<0.05$). We therefore applied a square root transformation to the dataset, which resulted in homogeneous variances. As there was no significant interaction between the factors of Treatment and Sex (2-way ANOVA, $F(4,124)=0.587$, $p>0.05$), we proceeded with the analysis of main effects. Treatment influenced pup bodyweights (1-way ANOVA, $F(4,124)=3.817$, $p<0.05$): the BPA 50 treatment group was significantly heavier compared to Vehicle PND 5 pups (Dunnett's post-hoc, $p<0.05$). We report no influence of Sex on pup bodyweights (1-way ANOVA, $F(1,124)= 1.838$, $p>0.05$).

PND 15 Final Bodyweight

To analyze whether Treatment or Sex altered PND 15 pup bodyweight significantly, corrected bodyweight data were used and homogeneity of variance was shown not to be in jeopardy after conducting a Levene's test ($F(9, 79)= 1.763$, $p>0.05$). There was no significant interaction between Treatment and Sex (2-way ANOVA, $F(4,79)= 0.551$, $p>0.05$) on bodyweight. Treatment did not have an influence on PND 15 pup bodyweights ($F(4,79)=2.476$, $p>0.05$), Similarly, Sex did not have a significant influence on PND 15 pup bodyweight (1-way ANOVA $F(1,79)= 2.410$, $p>0.05$). Raw PND 5 and PND 15 pup bodyweight data are presented in **Figure 19** and in **Figure 20**.

2.2 PND 5 and PND 15 Bodyweight Progression

The progression of pup bodyweights were analyzed to determine whether bodyweight changes throughout postnatal life were affected by the sex of the pup, the treatment it had received, over time, or an interaction of these factors. It was important to account for variability in starting weights (using a selected fixed covariate) to eliminate a potential confound between the litters, and PND 2 was chosen in order to account for differences in starting bodyweights. Data used in the analysis were corrected bodyweight data (BW*). We did not find a significant interaction between the three factors of interest (Treatment, Sex and Time) on bodyweight progression in the PND 5 animals ($F(8,174) = 0.799$, p=0.604), or in the PND 15 animals ($F(13, 149) = 0.746$, p=0.712.). The interaction between Sex and Time was not significant on pup bodyweight for the PND 5 animals ($F(2,174) = 1.718$, p=0.182), but there was a significant interaction between Treatment and Time on PND 5 pup bodyweight progression ($F(8,174) = 3.602$, p=0.001). There was no significant interaction between Treatment and Sex on PND 5 bodyweight progression, even when accounting for differing starting weights at PND 2 ($F(4,87) = 0.623$, p=0.647). When analyzed separately, the main effect of Treatment (F(4,87)= 1.109, p=0.358) or Sex (F(1,87)= 1.866, p=0.176) did not significantly modulate pup bodyweights. Therefore, this analysis demonstrated that PND 5 pup bodyweight progressed unperturbed despite exposure to xenoestrogens, and that the sole significant influence in bodyweight changes was attributed to normal pup development over time, as expected. Bodyweight progressions (corrected to litter size) are displayed in **Figure 21 and statistics are presented in Table 6.**

Corrected bodyweight data were also used for pup bodyweight progression analysis in the PND 15 animals. Using the same steps of data analysis as in the above paragraph, analysis revealed no significant interaction between the three factors of interest (Treatment, Sex and Time) on PND 15 pup bodyweight progression

($F(13, 149) = 0.746$, p=0.712). The two-factor analyses that followed indicated no significant interaction between Sex and Time ($F(3, 149) = 0.824$, p=0.488), between Treatment and Time ($F(13, 149) = 1.116$, p=0.350), nor between Treatment and Sex ($F(4,47) = 0.424$, p=0.791) on PND 15 pup bodyweight progression, even when accounting for potential differing starting weights by using PND 2 as the covariate. The analysis did reveal a main effect of Time ($F(3,149) =3.127$, p=0.025) on pup bodyweight progression, with body weights increasing over time; this was confirmed by showing a significant linear trend ($F(1,47)=9.128$, p=0.004), which was to be expected over time. When analyzed separately, Treatment ($F(4,47)=1.525$, p=0.210) or Sex ($F(1,47)= 0.025$, p=0.876) did not significantly modulate pup bodyweights. Therefore, despite developmental exposure to xenoestrogens, pup bodyweights were only modulated by time. Corrected bodyweight progressions (corrected to litter size) are displayed in **Figure 22** and statistics are presented in **Table 7.**

2.3 Crown-Rump Lengths and Anogenital Distances

PND 5 Crown-Rump Length

The next phase of analysis investigated whether CR length was modulated by sex of the pup or by exposure to xenoestrogen. A Levene's test indicated that corrected data variances were heterogeneous ($F(9,122)=3.117$, $p<0.05$). Using the square root corrected data, we found that Treatment and Sex did not significantly interact (2-way ANOVA $F(4,122)= 0.597$, $p>0.05$) to influence CR** length. Treatment did significantly modulate CR** lengths (1-way ANOVA, $F(4,122)=3.760$, $p<0.05$): DES and BPA 50 groups had significantly longer CR** lengths compared to the Vehicle group (Dunnetts' post-hoc, $p<0.05$). Sex did not influence CR** lengths (1-way ANOVA sex $F(1,122)= 2.520$, $p>0.05$). Therefore, Treatment significantly increased CR** length at the PND 5 developmental stage. On PND 5, CR was not expected to

be a sexually dimorphic trait due to the ultra-low doses of BPA administered. Overall, male rodents have a larger body sizes and CR than females (Madeira et al, 1993; Cabello, 2006; Fukushima, 2015) therefore, considering bodyweight and body length are correlated, it can be hypothesized that CR is a sexually dimorphic trait. However, it may not be evident yet at PND 5, as the differences in body size are more prominent later in maturity (Fukushima, 2015).

PND 15 Crown-Rump Length

The same method as described above was used to determine what factors significantly altered PND 15 pup CR lengths using the corrected CR length data (CR*). Treatment and Sex were verified to have equal variability, confirmed by a Levenes' test ($F(9, 79)= 1.672, p>0.05$) demonstrating homogeneity of variance. The corrected CR data continued to be used for the two-way analysis. There was no significant interaction between the factors of Treatment and Sex (2-way ANOVA,$F(4,79)= 0.048, p>0.05$) on CR* length at PND 15. However, Treatment did significantly influence CR* length (1-way ANOVA, $F(4,79)= 3.192, p<0.05$) but no group in particular had significantly longer or shorter CR* lengths when compared to the Vehicle group (Dunnetts' post-hoc, $p>0.05$). Sex was not found to influence CR* length (1-way ANOVA, $F(1,79)= 0.558, p>0.05$). Thus, Treatment was again the sole factor able to modulate crown-rump length at the PND 15 developmental stage. Due to the enhanced maturity of the pups at PND 15, sexual dimorphism of the CR is likely not yet evident, as body size differences are the most emphasized in adulthood (JHU, n.d). Raw CR length data for PND 5 and PND 15 pups are presented in **Figure 23** and **Figure 24**.

PND 5 Anogenital Distance

To determine whether AGD were modified by Treatment or by Sex, square root corrected data were used in the analysis given that a Levene's test on the corrected data indicated heterogeneous variances ($F=(9,122)=3.144$, $p<0.05$). The two factors of Treatment and Sex did not significantly influence each other (2-way ANOVA $F(4,122)= 0.978$, $p>0.05$). When analyzed separately, Treatment did not significantly affect AGD** (1-way ANOVA, $F(4,122)= 1.870$, $p>0.05$), while Sex did have a significant influence on AGD** (1-way ANOVA, $F(1,122)= 56.439$, $p<0.05$); male animals had significantly longer AGD** than female animals, as physiologically expected. Therefore, gestational and lactational exposure to xenoestrogens did not alter AGD**.

PND 15 Anogenital Distance

A Levene's test using the corrected AGD data indicated heterogeneous variances ($F(9,79)=2.519$, $p<0.05$). Similarly as in the previous cohort, square root corrected data were then used in the analysis for PND 15 animals. There was no significant interaction between the factors of Treatment and Sex (2-way ANOVA, $F(4,79)= 0.328$, $p>0.05$). Further analysis indicated that Treatment did not significantly affect AGD* (1-way ANOVA, $F(4,79)= 2.291$, $p>0.05$), however, Sex did significantly influence AGD* (1-way ANOVA, $F(1,79)= 44.59$, $p<0.05$) with males still showing longer AGD than females. Therefore, despite developmental exposure to xenoestrogens, sex remained the sole influence on AGD* length.

Raw AGD values are presented for PND 5 pups **in Figure 25** and for PND 15 pups in **Figure 26**. This distance is typically twice as long in male pups compared to females (Rajeshwari, 2009). The experimental

AGD ratio calculations between males and females indicated that male AGD lengths were approximately 1.5 times longer than females.

3.0 Analysis of Glucocorticoid Receptor Gene Expression in Male Adrenal Glands

The next phase of analysis characterized the molecular effects of prenatal BPA exposure on GR gene expression in male pup adrenal glands in the selected window of 9-11 gestational days of xenoestrogen exposure.

3.1 RNA integrity

RNA integrity from the male adrenal gland samples was assessed using both spectrophotometry and RNA gel electrophoresis prior to cDNA conversion. Isolated adrenal gland RNA from PND 5 pups produced RNA concentrations in the range of 47.9-476.6 ng/μL, much lower than for PND 15 adrenal gland tissues (range of 92.8-867.3 ng/μL). RNA quality 260/280 absorbance ratios for PND 5 and PND 15 adrenal gland samples ranged from 1.90-2.14 and 1.65-2.17 respectively. RNA quality 260/230 absorbance ratios for PND 5 and PND 15 samples ranged from 0.20-2.16 and from 0.25-2.12 respectively. No obvious differences between RNA qualities were observed across treatment groups. The integrity and concentration values obtained here were used to reject sub-par samples in accordance with accepted absorbance ratio quality (see Methods). Northern blot gel electrophoresis allowed for identification of the minimum RNA concentration value to be used for the qPCR results, described below.

The RNA concentration and absorbance level readings are displayed in **Table 2**. Low values reported in the 260/230 absorbance columns were hypothesized to be the result of the spectrophotometer and were not thought to be reflective of the RNA quality. To demonstrate that the RNA was intact, a series of Northern

blot gel electrophoresis runs were performed on male adrenal gland RNA samples, representing each treatment group, with varying RNA concentrations (9.63-152.9 ng/µL, 368.1-867.3 ng/µL, and 206.1-476.6 ng/µL) (**Figures 27-29**). These RNA concentration ranges were selected for PND 5 and PND 15 animals and represented the range of low to high concentrations of RNA within the adrenal gland samples. The limit of detection was at least 47.9 ng/µL. For all subsequent qPCR, only RNA samples of concentrations greater than 47.9 ng/µL were used. A 2:1 28SrRNA:18SrRNA band reading on these gels indicated that the RNA was intact.

4.0 qPCR: Efficiency and Correlation Coefficient Values

The standard curves generated had acceptable E and R^2 values for all gene primers, as presented in the **Appendix 3.1** for PND 5 samples and in **Appendix 3.2.** for PND 15 samples.

4.1 GR Expression- Use of ΔΔCt Method to calculate Fold Change ($2^{-\Delta\Delta Ct}$)

This phase of the analysis assessed whether male adrenal gland GR gene expression was up- or down-regulated following developmental exposure to xenoestrogens. After normalization to the reference gene GAPDH, the fold changes (calculations described in Methods) were analyzed for each experimental group, at both PND 5 and PND 15. Downregulation of male adrenal gland GR expression (<0.5 fold change) was observed in both PND 5 and PND 15 DES groups. Upregulation of adrenal gland GR expression (>2.0 fold change) was observed in the PND 5 BPA 50 and PND 15 BPA 500 samples. Fold change GR expression for both PND 5 and PND 15 are presented in **Figure 30.**

4.2 GR Expression- Evaluation of ΔCt values

A second evaluation of GR expression was conducting using ΔCt values. GR Ct values were normalized to the reference gene GAPDH (ΔCt =Ct ^{GR} (sample)- Ct ^{GAPDH} (sample)) and were statistically analyzed. The PND 5 ΔCt values were not normally distributed as determined by Levene's test, necessitating non-parametric analysis (Qualls, Pallin & Shuur, 2010; Nahm, 2016). Results of the Kruskal-Wallis test indicated that the adrenal gland GR ΔCt values for PND 5 pups were not affected by Treatment: H(4,N=19)=8.274739, p=0.0820. Similarly for the PND 15 cohort, nonparametric analysis showed that Treatment also had no effect on ΔCt values, as indicated by Kruskal-Wallis test: H(4, N=26)=2.716524, p= 0.6063. Average ΔCt values are presented for both PND 5 and PND 15 GR in **Figure 31.** Overall, these analyses indicated that Treatment did not have any effect on ΔCt values or GR expression when animals had received between 9-11 days of dosing during gestation.

4.3 18SrRNA Expression- Use of ΔΔCt Method to calculate Fold Change ($2^{-\Delta\Delta Ct}$)

Similarly to the approach for evaluating normalized GR gene expression fold changes, we assessed whether male adrenal gland 18SrRNA gene expression was up- or down-regulated following developmental exposure to xenoestrogens. After normalization to the reference gene GAPDH, down-regulation of male adrenal gland 18SrRNA expression (<0.5 fold change) was observed in the PND 5 DES, BPA 5 and BPA 500 groups. Up-regulation of adrenal gland 18SrRNA expression (>2.0 fold change) was observed in the PND 5 BPA 50 group. No effects were observed at PND 15 for 18SrRNA fold change expression. Fold changes for PND 5 and PND 15 18SrRNA are presented in **Figure 32.**

4.4 18SrRNA Expression- Evaluation of ΔCt values

A second evaluation of 18SrRNA expression was conducted on the ΔCt values, and Levene's test was again used to determine homogeneity of variance. Nonparametric statistical analyses (Qualls, Pallin & Shuur, 2010; Nahm, 2016) were used at both developmental stages- PND 5 and PND 15 due to heterogeneous variances. Normalized to GAPDH, the PND 5 ΔCt values were significantly affected by Treatment (Kruskal-Wallis: $H(4, N=19)= 9.75$, $p=0.0449$), although no group in particular was significantly different compared to the Vehicle group (Mann-Whitney U, $p>0.05$). However, the ΔCt values for 18SrRNA at PND 15 were not significantly affected by Treatment: $H(4, N=22)= 0.5158103$, $p= 0.9719$. Average ΔCt values for both PND 5 and PND 15 18SrRNA are presented in **Figure 33.**

IV. DISCUSSION

Perinatal BPA exposure in the rat produced morphological and molecular effects that were dependent on dose and developmental stage. Significant morphological changes were observed for pup bodyweights and CR lengths. Our findings seem to support a non-linear, non-monotonic dose-dependent BPA effect on pup morphometric parameters. The interpretation of these major findings requires evaluation and discussion of on the four elements included in the study design: (i) timing of exposure, (ii) dose, (iii) sex and (iv) the SHRP. Each of these will be discussed in terms of the study's outcomes.

i) Onset of Perinatal Exposure

Pups were exposed to BPA and DES during both gestation and lactation. The potential for BPA to disrupt rat pup development is dependent on the onset of exposure and on the embryonic/fetal structures and organ systems under development at that time. As described, gestational periods were not harmonized which resulted in different exposures, including both onset of exposure and duration. Consequently, we chose to narrow the window of exposure onset to 9-11 days of gestational exposure. This 9-11 exposure window is consistent with exposure models used in rodent gestational low-dose BPA literature (Nikaido et al, 2004; Ikeda, Tanaka & Esaki, 2008; Alonso-Magdalena et al, 2010). It has been reported that late gestation is the most critical time for EDC perturbation (Kaludjerovic & Ward, 2012), as characterized by a DES exposure study in the rat where dosing between GD 18-20 induced the most significant effects (Zimmerman et al, 1991). Therefore, our dosing model (GD 9-11) is considered effective to observe EDC effects. The Witschi stages of rat development describes major developmental milestones during GD 9-11 such as attachment of the ectoplacenta, allantoic stalk, ectochorionic cyst, the disappearance of the primitive streak,

organization of the tail bud and formation of recognizable arm and leg buds (Hill, 2016). Importantly, gestational exposure to xenoestrogens was initiated before the development of the hypothalamus, pituitary and maturation of the adrenal gland (GD 16) (O'Rahilly & Muller, 1999; Kota et al, 2013). Gestational dosing onset occurred during the embryonic phase prior to transition to the fetal phase (GD 17) (Hill, 2016). Most importantly, xenoestrogen dosing occurred before the formation of the HPA axis and prior to secretion of relevant hormones and prior to detection of GR mRNA levels in the hypothalamus, hippocampus and pituitary after GD 13 (Diaz et al, 1998). Therefore, introduction of xenoestrogen exposure for 9-11 days during gestation was appropriate to assess perturbations to HPA axis growth, given that the components of the axis were developing after xenoestrogen introduction.

The onset of gestational exposure to BPA occurred following fertilization (sex determination) and implantation (litter size determination), thus BPA was not anticipated to alter these parameters. Indeed, we report no effect of xenoestrogens (BPA, DES) on litter sizes or sex distribution, consistent with the majority of the literature reviewed (Cagen, 1999; Tinwell et al, 2002; Chapin et al 2008; Stump et al, 2010; Ryan et al, 2010; LaRocca et al, 2011; Kobayashi et al, 2012; Roy, Bauer & Lawrence, 2012). Although it may be possible to detect changes in litter size with our BPA exposure timing due to gestational losses, it was not anticipated due to the low dose range used. Similarly, changes to sex ratio could also happen post-fertilization due to selective-abortion of male versus female fetuses, but was not likely at our dose range.

It is well established that low-dose BPA exposure during gestational and lactational periods is associated with increased bodyweights, among other metabolic consequences, in rodent offspring (Howdeshell et al, 1999; Rubin et al, 2001; Somm et al, 2009; Patisaul & Adewale, 2009; Rubin et al, 2009; Wei et al, 2011; vom Saal et al, 2012; Garcia-Arevalo et al, 2014). Therefore, exposure to BPA in our selected window (GD

9-11) was sufficiently early to disrupt morphology, but no major effects were anticipated at our ultra-low doses. Increases in bodyweight after exposure to xenoestrogens was observed in the PND 5 BPA 50 group in our study, consistent with reports from previous studies (Patisaul & Bateman, 2008; Wei et al, 2011). We found no obvious structural anomalies in the pups throughout the experiment (e.g. limb defects, organ defects, spine abnormalities), which were again not expected considering the ultra low doses used in the study.

Physiologically, AGD is the classic measurement technique for sex determination after birth (Welsh et al, 2008; Dean & Sharpe, 2013; Welsh, Suzuki & Yamada, 2014). The CR length is an established parameter of toxicological studies, and provides an assessment of body size to compare with body weight (Collins et al, 2006; Kang et al, 2015; Hood, 2016), both of which are correlated (Hoberman & Lewis, 2011). Both CR length and AGD are sexually dimorphic characteristics with typically longer lengths in males (Rajeshwari, 2009). In response to gestational BPA exposure, the rodent literature is inconsistent, and studies have reported dose-dependent responses to CR lengths. Low dose BPA has been reported to increase CR lengths with differences disappearing in adulthood (Ryan et al, 2010). I observed significantly longer CR lengths in the DES and BPA 50 treatment groups at the PND 5 developmental stage, which is consistent with the literature.

Normal AGD development in the rodent is dependent on androgen production and is regulated by the natural androgen receptor agonist dihydrotestosterone (DHT), which promotes longer AGD in the male animal (Bowman et al, 2003; de Jesus-Tran et al, 2006). Given that androgenic mechanisms are required for normal AGD lengths to develop, it can be hypothesized that estrogen exposure as well as exposure to anti-androgenic substances during development would result in a reduction in AGD. A strong indicator of

phthalate exposure is the relative change in AGD length in exposed versus unexposed animals due to the anti-androgenic effects of the chemical (Sharpe, 2005; Mitchell et al, 2015). It is well established that phthalates reduce AGD (Mylchreest, 2000; van den Driesche, 2001; Ema & Miyawaki, 2001; Foster, 2005; LaRocca et al, 2011) with an anti-androgenic mechanism of action (Foster et al, 2001; Noriega et al, 2009). Studies have demonstrated that developmental exposure to estrogenic substances such as styrenes (Ohyama et al, 2007) and genistein (Musameh, Zin & Kassim, 2014) reduce male rat AGD through estrogenic mechanisms, by binding to ER-α and ER-β to exert weak estrogenic effects (Musameh, Zin & Kassim, 2014). Longer AGD has been observed in the rodent after gestational low-dose BPA (e.g. 50 μg/kg bw/day) and low-dose DES exposure (e.g. under 2 μg/kg bw/day) (Gupta, 2000; Honma et al., 2002), while other studies have reported decreased AGD after high dose exposure to the potent estrogen DES (e.g. 200 μg/kg bw/day) (Gupta, 2000; Palanza, Parmigiani & vom Saal, 2001) and due to low-dose BPA exposure (Williams et al, 2001; Li et al, 2001; Murray et al, 2007; Christiansen et al, 2014; Boudalia et al, 2014). Alternatively, no effects of low dose BPA on AGD have also been observed (Cagen, 1999; Tinwell et al, 2002; Kobayashi et al, 2002; Howdeshell et al, 2008; LaRocca et al, 2011). In our study, we did not observe significant changes in AGD due to DES exposure and given the ultra low-doses and onset of xenoestrogen exposure timing, it was not expected that DES would have induced gross morphometric changes in the animals at either developmental stage. While DES acts in the same manner as estradiol, BPA can exert both estrogenic (Kato et al, 2003; Richter et al, 2007) and anti-androgenic (Lee et al, 2003) effects on reproductive organs. Further, the ability of BPA to act via multiple pathways makes it difficult to elucidate an exact mechanism or explanation for our observed results.

ii) Dose-Dependent Effects of BPA

A non-monotonic dose response (NMDR) suggests non-linear responses to different doses of chemical (Kohn & Melnick, 2002). Variations in receptor affinity, inhibition of receptor function, differential receptor responses and cytotoxicity to different doses are among the hypothesized mechanisms of action (Welshons et al, 2003; Ismail & Nawaz, 2005; Lagarde et al, 2015). The NMDR of various EDC have been widely reported in human and animal literature (Andersen et al., 1999; Calabrese, 2001; Calabrese and Baldwin, 2003; Sheehan, 2006; Vandenberg et al., 2014; Lagarde et al, 2015). Although dose responses are complex and dependent on multiple factors including species, cell type and receptor type, NMDR commonly occur with low-dose range BPA (Bergman et al, 2012; Belcher et al, 2012; Vandenberg, 2012,2013,2014; Lagarde et al, 2015) more frequently compared to pesticides, DES, PCBs, phytoestrogens and endogenous hormones (Lagarde et al, 2015). By design, this thesis used multiple doses within an ultra-low dose range to allow for the observation of possible dose-dependent effects on morphology or GR expression, while remaining at environmentally relevant concentrations.

Receptor affinities can vary, as specific receptor types may be more or less sensitive to a certain dose of hormone or chemical, which causes them to be activated or inactivated when exposed to the exact same dose as another receptor (Toppari et al., 1996; Vandenberg et al., 2009). High doses of hormone can promote cell proliferation or heighten negative feedback (Costa-e-Sousa & Hollenberg, 2012), and desensitization at high doses is another hypothesized mechanism of action (Palanza, Parmigiani & vom Saal, 2001; Incerpi et al, 2003; Inagaki, Gautreaux & Luine, 2010). Conversely, low doses of the same hormone may actually reduce cell numbers (Welshons et al 2003;Vandenberg, 2014). Therefore, the so-

called inverted U-shaped dose response curve indicates a greater response at a middle dose, and small response with a low or high dose (Welshons et al 2003;Vandenberg, 2014).

Dose-response analysis in both morphometric and molecular study is complex considering the parameters involved. Given the varied reports pertaining to NMDR observations, my findings should be interpreted cautiously. The molecular results seem to suggest a NMDR in the PND 5 animals: adrenal gland GR levels were upregulated in the BPA 50 group, and less so at BPA 5 or BPA 500. In the PND 15 animals, GR upregulation was only observed at BPA 500, suggesting a NMDR. Understanding the dose response is complicated by multiple receptors having tissue-specific effects, and the regulation of morphometric parameters and molecular GR expression levels by multiple endocrine pathways. Endocrine pathways typically exert cross talk with each other and with numerous receptor subtypes, which can be simultaneously responsive to estrogenic ligands and susceptible to negative regulation by estrogenic substances. Given the multiple tissue-dependent receptors that can exert xenoestrogenic activity (Kuiper et al, 1997; Lee et al, 2012) and this variable cross talk, this makes it quite difficult to determine a single dose response behavior. Collectively, these factors may explain the PND 5 NMDR and the PND 15 monotonic dose response upon analysis of adrenal gland GR expression levels.

If the study were repeated, I would have also added a fourth and fifth experimental group. Considering that the LOAEL and the low-dose range of BPA in rat exposure studies is under 50, 000 μg/kg bw/day (Shelby/NTP, 2008; FAO/WHO, 2011) I would have selected BPA 5000 μg/kg/day and BPA 50 000 μg/kg/day doses to maintain the ten-fold increases between each dose, while reaching the highest dose of the low-dose range. This would allow us to fully explore the relationships between dose-response, morphometry and GR expression due to low-dose BPA exposure.

iii) Sex-Dependent Differences on Morphometric Parameters and on Adrenal Gland GR Expression

The existence of sexual dimorphisms in HPA axis activity and function in males and female rodents is established, as illustrated by sexually dimorphic gene expression (Cao et al, 2012; 2013) and brain differentiation, which occurs during the perinatal period up to PND 10 (Young, Goy & Phoinis, 1964; Grady, Phoenix & Young, 1965; Bonthuis et al, 2010). The selection of a rat model for EDC study is quite advantageous due to the established sex differences in behaviour due to EDC exposure in the rat. Sex-dependent behavioural traits such as exploratory tendencies, sex-specific changes to brain GR expression, and hypothalamic reorganization- of particular relevance in my study- are all influenced by EDC exposure (Kubo et al, 2003; Fujimoto, Kubo & Aou, 2006; Patisaul et al, 2006; Poimenova et al, 2010; & Patisaul, 2011; Cao et al, 2012; Kundakovic et al, 2013, Jasarevic et al, 2011; 2013; Panagiotidou et al, 2014; Lichtensteiger et al, 2015). Perinatal exposure to 40 μg/kg/day BPA increased basal CORT levels and decreased hypothalamic GR levels in females (Panagiotidou et al, 2014), while males exhibited an increased response to CORT compared to females, and an increase in pro-opiomelanocortin mRNA levels compared to control males (Panagiotidou et al 2014) thus demonstrating a sex-specific effect. Perinatal BPA exposure also adversely affects sexual behavior in male rats in adulthood (Jones, Shimell & Watson, 2011).

Perinatal BPA exposure also alters brain aromatase expression differentially in male and female rodents (Xu et al, 2010; He et al, 2012; McCaffrey et al, 2013; Sadowski et al, 2014), modulates epigenetic programming of ER expression (Kundakovic et al, 2013), and can masculinize or feminize the preoptic nucleus, an area filled with nuclear receptors (Bai et al, 2011; McCaffrey et al, 2013) we well as other brain areas (Kubo et al, 2003; Tando et al, 2007). In addition, BPA has been shown to have sex-dependent

effects on brain GR levels: specifically, by increasing GR expression in females and downregulating GR expression in males (Zhou et al., 2015, Chen et al., 2015). As BPA is the HPA axis modulator in the study, current and future experiments will attempt to elucidate the sexual differences in multiple brain areas related to the HPA axis pathway post-BPA exposure. This will be feasible considering the large amount of brain and organ tissues, and blood and plasma collected in the study. Currently, experiments are being conducted on the female rat adrenal glands to evaluate if there is a sex difference in GR expression levels due to xenoestrogen exposure. Toxicological sex differences will likely be apparent given that they are shown to occur after developmental xenoestrogen exposure, as evidenced in the above studies.

At a molecular level, sex differences in HPA axis activity after a stressful experience during gestation have been observed in the rodent, resulting in sex differences in HPA axis responsiveness (Mueller & Bale, 2008). Post-SHRP, the adrenal gland is more responsive to ACTH in female rats (Yoshimura et al, 2003) while testicular androgens have been hypothesized to inhibit the function of ACTH at the adrenal gland in the neonatal rat pup (Yoshimura et al, 2003). Compared to males, females have higher hypothalamic GR expression levels (Zavala et al, 2011; Bourke, Harrel & Neigh, 2012) and a faster decrease in ACTH and cortisol hormone levels after a stressful response (Keck et al 2002; Bourke, Harrel & Neigh, 2012). During puberty, stressed female rats also exhibit a less rigorous CORT response compared to control females, and downregulated hypothalamic GR expression with no change in GR expression in response to stress (Panagiotidou et al., 2014). Although the study by Panagiotidou et al (2014) did not examine adrenal GR expression in pups, the long-term effects of perinatal BPA exposure indicate modulation of the HPA axis. A different group used the same oral dose (40 μg/kg/day) administered to dams during gestation and lactation and reported that perinatal exposure to low-dose BPA resulted in adolescent offspring with hyperactive HPA axes, following impaired GR-mediated negative feedback via hippocampal GR downregulation, and reduced hippocampal glutamate receptor (mGlu 2/3), both of which are shown to

contribute to anxiety behaviours (Zhou et al., 2015). Yet another low-dose exposure study commenced subcutaneous BPA exposure (2 μg/kg/day) from gestation day 10 to PND 7 to evaluate adult rat offspring HPA axis parameters, similar to the timing of our study. Once again, a sex-difference was apparent in hippocampal and hypothalamic expression of GR, with upregulation in females and downregulation in males (Chen et al., 2014). These findings highlight the sex differences in HPA axis activity after a stressful response in neonatal rodents.

Although molecular characterization of the GR was limited to male adrenal glands, effects of BPA on both male and female morphometric parameters were assessed. As with most animals, rats exhibit sexual dimorphisms with males being typically heavier and longer in adulthood (Tateishi et al, 1997; Swislocki, 2003) and having characteristically longer AGD (Thankamony et al, 2009; Rajeshwari, 2009). The only significant sex difference observed in the morphometric component of the study was longer AGD lengths in the male animals at both developmental stages-PND 5 and PND 15. This was not due to toxicological sex difference from low-dose BPA exposure, but rather to the established anatomical sexual dimorphisms between the sexes. There were no significant sex differences reported between CR lengths, bodyweights or bodyweight progressions either. It had been hypothesized that male animals would be heavier than the females given the sexual dimorphisms present in rodent bodyweights, with males being typically heavier and longer (Tateishi et al, 1997; Swislocki, 2003), but this was not observed. This may be attributed to the timing of dosing.

iv) Morphometric Parameter Comparisons before and after the SHRP

As described, investigation of the SHRP and its sensitivity to BPA exposure was a major component of the design of the study. Morphometric parameters were compared at two developmental stages (PND 5, PND

15) to determine whether pups were differentially sensitive to BPA effects. Both PND 5 and PND 15 pups had identical exposures in terms of timing, dose and duration. PND 15 pups have not only completed the SHRP, but have ten additional days of development, producing heavier, larger and more mature animals. Activity levels and exploratory behaviours of the PND 15 animals were much higher as the animals became more independent. I was expecting to see major physiological changes in the pups between the two developmental stages, and observed how rapidly growth progressed in the rat over a ten-day period. The contrast in size, activity level and appearance (e.g. fur growth) between the two stages of development emphasized the extensive changes occurring both developmentally and physically during the perinatal period. The PND 15 animals were more than triple the weight of the PND 5 pups, had body lengths approximately 1.5 times longer, and had AGD about twice the size of their younger counterparts. In our study, BPA increased bodyweights at PND 5 but not PND 15. Gestational BPA exposure has been shown to increase bodyweights long-term into adulthood (Howdeshell et al, 1999,2000; Takai et al, 2001; Rubin et al, 2001; Nikaido et al, 2004; Alonso-Magdalena et al, 2010; Ryan et al, 2010). As extensive study has been conducted on bodyweights later in adulthood, it can be hypothesized that exposure to BPA during the critical period of development alters the developmental programming occurring during the SHRP and both factors likely result in increased bodyweights in the animals later in life. Based on the literature, it is highly likely that the higher bodyweights observed at the beginning of the SHRP would have still been observed in the animals in adulthood.

The AGD is typically twice as long in male rodents (Steingraber, 2013) and this difference should be conserved over development (pup through to adulthood) (Steingraber, 2013; Ackerman et al, 2016). In our study, exposure to xenoestrogen did not modify absolute AGD, however examination of the ratio of male: female AGD should be stable in both PND 5 and PND 15 animals (~2). AGD plasticity has been observed

after adult exposure to EDC such as flutamide and DES, both of which reduced AGD length in the male adult rodent, but after cessation of exposure, AGD returned to normal lengths (Mitchell et al, 2015). This emphasizes that modulation during development elicits permanent programming effects of AGD versus introduction of chemical in adulthood. Therefore, it can be estimated that, given the lack of effect of xenoestrogens on absolute AGD in the PND 5 and PND 15 animals, the male: female AGD ratios would presumably be conserved over development and into adulthood.

Exposure to xenoestrogen increased CR lengths at each developmental stage. Given that the analysis of CR accounted for changes in bodyweight, CR lengths should presumably be conserved over development, as a function of bodyweight.

Molecular parameter comparisons before and after the SHRP

To reiterate, the SHRP serves to protect the delicate developing brain from high circulation of glucocorticoids (Sapolsky & Meaney, 1986; Lupien et al, 2009; Rincon-Cortes & Sullivan, 2014) by reducing the responsiveness of the HPA axis to non life-threatening stressors between PND 4 - PND 14 (Dent et al, 2007; Nishi et al, 2012; Herman et al, 2016). Reductions in ACTH response (Walker, 1991; Levine, 2000; Vazquez & Levine, 2005) and CORT secretion in response to a stressful stimulus are characteristic of the SHRP (Rosenfeld et al, 1991; Walker & Vrana, 1993; Schmidt et al, 2003, Vazquez & Levine, 2005). Dams exposed to stress during gestation had offspring with hyperactive HPA axis activity, characterized by reduced CRH and hippocampal GR expression levels and elevated CORT levels when tested as adults (Mueller & Bale, 2008).

Assessment of the SHRP effects required evaluation of one or more of the components of the HPA-axis, which are presented here as the effects of xenoestrogen exposure on rat adrenal GR expression. The following discussion will focus on the effects of both the SHRP and xenoestrogen exposure (BPA) on GR expression. To assess if xenoestrogen exposure caused developmental programming disruption throughout the SHRP, GR expression levels in the adrenal glands were used as a molecular marker of perturbation. Little is known about the development of GR expression and maturation throughout the SHRP. The increase in HPA axis activity after the SHRP in the rodent includes high GR levels (Schmidt et al, 2003). We found no studies examining rat adrenal gland GR expression over the SHRP, so it is unknown if the GR is universally up or down-regulated in response to this critical window. Mechanistically, by PND 15 the HPA axis is able to exert a mature stress pathway response, which would suggest optimal GR expression in all related organs, including adrenal glands (Cohen 2006), as adrenal gland GR expression attains adult levels by PND 10. Given the increased size and development of the animals at PND 15 and the exit from the repressed stress response (Schmidt et al, 2003; Navailles, Zimnsiky & Schmauss, 2010; Nishi et al, 2014), GR expression levels should theoretically increase with developmental maturation and emergence from the SHRP at PND 15 after developmental xenoestrogen exposure. It is perhaps most conservative to first consider the vehicle control group in our experimental evaluation, as this group was not confounded by BPA or DES exposure. Future analysis will evaluate GR expression in vehicle-exposed pups before and after the SHRP.

It is established that 18SrRNA levels remain constant in the rodent brain throughout gestation (Al-Bader & Al- Sarraf, 2005) as well as throughout life which is why this ribosomal protein is one of the most stable and therefore most used reference genes (Bas et al, 2004; Svingen et al, 2015). Therefore, it can be

hypothesized that 18SrRNA gene expression levels would remain constant throughout the SHRP, an analysis which will be performed in future.

Molecular studies were limited by small sample size for the BPA 50 group (PND 5), and unanticipated estrogen-sensitivity of one of the reference genes- 18SrRNA. Both fold-changes and statistical analysis of ΔCt values were performed with different outcomes **(see Figures 31 and 32)**. The ΔCt analysis is a more general, conservative method for analyzing qPCR results. The ΔCt analysis compares the average target gene expression per treatment group to the average reference gene expression specific to that treatment group. In contrast, the ΔΔCt values are determined by normalizing target gene per treatment group to only the reference gene expression from the Vehicle treatment group (e.g. Ct^{GR}(DES)- Ct^{GAPDH}(VEH)). Therefore, the ΔCt calculation compares each target treatment group value to that of its specific reference gene treatment group value, while the ΔΔCt calculation compares each reference gene treatment group value to only that of the reference gene Vehicle treatment group value.

As fold-changes are the established presentation of qPCR results, following the ΔΔCt method (MIQE) (Bustin, 2009), we have placed greater emphasis on these findings. A more traditional monotonic dose response relationship was produced in the PND 15 pups: GR expression was upregulated at BPA 500 but no effects were observed at BPA 5 or BPA 50. Exposure to DES reduced GR gene expression levels in the male adrenal glands at both PND 5 and PND 15 developmental stages.

I had anticipated that exposure to BPA throughout gestation and lactation would result in reduced GR expression levels in the adrenal glands at both age cohorts, considering BPA and estrogens' established negative regulation of the GR (Sengupta et al, 2000; Ganguli et al, 2002; Kinyamu & Archer, 2003; Weiser

& Handa, 2009). Certainly DES, the more potent estrogen, did seem to produce downregulation of GR at both PND 5 and PND 15 (**Figure 31**), consistent with the negative regulation (**Figure 3**). The potent estrogen DES can negatively regulate GR gene expression and can downregulate the GR through proteasomal degradation (Kinyamu & Archer, 2003). As DES has demonstrated estrogenic effects at our dose of 5 μg/kg/day (Marselos & Tomatis, 1992; Nagel, 2001; Richter et al, 2007), and produced consistent effects on the morphology of rat pups, our finding that DES-induced downregulation of adrenal gland GR expression is biologically plausible given the established negative regulation of GR by estrogen (Kinyamu & Archer, 2003; Kalra & Ishmael, 2014). The consistent reduction in adrenal gland GR expression after DES exposure in both PND 5 and PND 15 male pups therefore suggests that this potent estrogen reduced GR gene expression independent of the SHRP. Although the studies reviewed did not compare effects pre- and post- SHRP, it can still be hypothesized that gestational exposure to BPA would modulate pup HPA axis development in the same way-interfering with GR levels as discussed, and contributing to axis hyperactivity.

Differential dose responses to BPA between the developmental stages were observed and may be explained by the developmental timing of the SHRP. One of the main mechanisms hypothesized to maintain low HPA axis activity during the SHRP is the inhibition of ACTH secretion at the pituitary (Schmidt et al, 2003). This endocrine inhibition between the pituitary and adrenal gland is actively occurring in the younger cohort. Conversely, the PND 15 animals would be transitioning out of the SHRP at the time of sacrifice (Claflin, Grenfield & Hennessy, 2014) and may no longer experience this inhibition. Although we hypothesized reduced GR levels in both cohorts due to the SHRP and also due to xenoestrogen exposure, I did not expect the full extent of disruption to be completely evident in the PND 5 animals at this point. I

anticipated that the reduction would be more apparent in the PND 15 animals, based on their more advanced development and emergence from the SHRP.

As both dose exposure and the SHRP were both factors in the modulation of the HPA axis, the analysis of our molecular results is complex. Fold change analysis at PND 5, the beginning of the SHRP, reported that BPA 50 (n=2) upregulated GR expression while at PND 15, after the SHRP, the highest BPA 500 dose upregulated GR expression. It is possible that BPA 500 is actually ineffective, if we assume a NMDR, consistent with our morphology parameters. This can perhaps be attributed to the ultra-low doses of BPA administered resulting in the incomplete or partial degradation of the GR. The fact that the lowest oral dose of BPA 5 did not result in significant changes in adrenal gland GR expression in both PND 5 and PND 15 animals is most likely due to the differences in the absorption of BPA ingested orally, which is the lowest bioavailable route (Pottenger et al, 2000).

Our experimental xenoestrogen BPA at three doses may produce more subtle effects through negative regulation of the GR, enabling an SHRP effect to be demonstrated. Further, the potent estrogenic DES effect may have masked any developmental change in the GR due to the SHRP. The effect of the SHRP on GR gene expression in the male adrenal glands was markedly different in terms of dose responses, eliciting either monotonic or NMDR effects that were developmentally-dependent. The two very different observed effects of BPA at the two developmental stages may indicate the following. Apparent upregulation of adrenal gland GR expression on PND 15 may actually be due to the SHRP, and not due to the ineffective and incomplete action of BPA in the negative regulation of the GR. The question remains: why did we not observe a biologically significant effect of BPA 50 on PND 15 adrenal gland GR expression? This may be due to the relatively weaker potency of BPA compared with DES, as

demonstrated by BPA's inconsistent upregulation of fold changes, which indicate the inability to completely downregulate GR gene expression during the SHRP. It is evident that perinatal BPA exposure modulates HPA axis development with sustained effects through to adolescence and adulthood (Zhou et al., 2015, Chen et al., 2015). None of the reviewed studies had been specifically designed to examine the effects of perinatal BPA exposure on the SHRP, nor to measure adrenal GR expression. Therefore, although limited by sample size, my findings are novel. Finally, as described below, several methodological constraints including small sample size in the BPA 50 PND 5 group, use of only a single reference gene and technical limitations may have produced too much variation in the experimental groups.

V. Limitations/Implications/Conclusions

The thesis was complicated by several limitations. First, due to the unavailability of precisely timed-pregnant rats, pregnancies were unstaged. This necessitated variability in the first day of exposure and subsequently in the duration of gestational exposure, resulting in longer or shorter exposure periods to BPA. We attempted to correct for this limitation by selecting a narrow window of 9-11 days of gestational exposure for both morphometric and molecular data analysis. As described, the 9-11 days of gestational dosing window was selected for the thesis as xenoestrogen dosing was initiated prior to formation of HPA axis components (O'Rahilly & Muller, 1999; Kota et al, 2013), pathway functionality, and hormone secretion (Mastorakos & Ilias, 2003). Further, sex-dependent differences in adrenal cortex function after dose introduction at GD 14 have been reported (Chen et al, 2014). Our selected window of dose introduction began in early gestation (Meehan et al, 2016) and other studies have commenced dosing within this range (Gioiosa et al, 2015) and even after this window in mid gestation (Nikaido et al, 2004).

Second, pregnancy can induce or reveal unforeseen medical complications in purchased animals. Three

dams were eliminated from the experiment along with their litters. One DES dam presented with fur piling and was euthanized. Her litter was not healthy: two pups were born dead and the two remaining were the smallest pups throughout all of the litters. A BPA 5 dam was not pregnant and as such was euthanized. A BPA 50 dam presented with fur piling and was euthanized, and it was hypothesized that she had miscarried and had become sick due to infection. It is not possible to determine whether illness/pregnancy complications were caused by exposure, environmental factors or previous illness prior to purchase. As described, major morbidity and mortality was not anticipated at our ultra-low dose range. The method of exposure did not involve procedures that would cause pain, stress or injury to animals. Therefore, a decision was made to exclude these dams/litters from the study.

Use of a rat model was imperative to study the SHRP. However, rats are not used as frequently for molecular studies compared to mice. Consequently, primer design for qPCR was limited, necessitating selection of primers with different qPCR parameters. It was therefore not possible to run both reference gene and GR samples simultaneously, resulting in manual calculations to analyze the data. Consequently, plate to plate variation between the reference and target genes was unavoidable, however, we attempted to keep the plate setup as uniform as possible using consistent preparatory and experimental procedures.

18SrRNA was originally selected as a reference gene, as it is one of the most widely used in the literature (Shroder, Pelch & Nagel, 2009; Wang & Xu, 2010; Liu et al, 2015) and its use and validity has been reported in rodent xenoestrogen study (Shroder, Pelch & Nagel, 2009). There was an effect of Treatment observed in the PND 5 pups with 18SrRNA, measured by fold change corrected to the reference gene. This was surprising, as it was not anticipated that 18SrRNA would be sensitive to estrogen. In a previous study, estrogen did not influence 18SrRNA gene expression in the rat (Rhen & Cidlowski, 2005). However, it is possible that the stage of development and the age of the animal can impact the number of transcripts

expressed on a gene (Kozera & Rapacz, 2013). As such, it is possible that the early developmental stages (PND5 and PND15) selected for investigation in our study may have been a factor in the sensitivity of the 18SrRNA to xenoestrogens.

The use of very low doses of BPA and DES during the critical period of development to investigate perturbations to GR levels in the final component of the HPA axis resulted in a unique experimental approach. The scope of the thesis was focused on the effects of gestational and lactational BPA exposure on: i) three key morphometric measurements and ii) assessment of GR levels in the male pup adrenal glands. Potential confounding factors throughout the experiment were carefully considered and minimized or eliminated where possible. As the HPA axis is a stress axis, the project focused on BPA as the sole HPA modulator and we were conscientious to minimize other potential modulators such as environmental stress or maternal separation. For example, dams and pups remained together and were only separated immediately prior to sacrifice. To avoid further additional stress to the animals, oral dosing via syringe provided an option to combine a quantitative measurement of ingested dose while minimizing additional stress to the dams. We avoided more invasive methods such as gavage, while maintaining the most relevant route of exposure seen in the environment. We handled the pups and dams very minimally, only holding them once a day to dose and weigh each animal.

Implications

The potential for gestational xenoestrogen exposure to produce long-term changes to adult physiology is consistent with models such as the Developmental Origins of Health and Disease (DOHaD) hypothesis, which describes in utero exposure to environmental or maternal stress as instrumental for the manifestation of later-onset diseases (Silveira et al, 2007; McMillen et al, 2008; Wadhwa et al, 2009; Uauy, Kain &

Corvelan, 2011). Models that suggest a causative link between the gestational environment and long-term offspring health outcomes have important implications pertaining to the study of perinatal xenoestrogen exposure. EDC have been linked to the development of anxiety and depression symptoms in rodents, as observed through forced swimming and maze tests (Fujimoto, Kubo & Aou, 2006; Ryan & Vandenberg, 2006; Cox et al, 2010; Patisaul et al, 2012; Xu et al, 2012; Kundakovic et al, 2013). Accordingly, long-lasting behavioural alterations have been evidenced in rodent studies post-exposure to hormone disrupting chemicals. These models provide a well-established link between EDC exposure and alterations to hormonal pathways resulting in the progression of physiological and psychological disease, such as interference to developing molecular pathways resulting in receptor up or downregulation, hormonal imbalance, and the development of psychological disease. Most importantly, they emphasize the permanence of alterations caused by toxicants during the critical period.

BPA exposure during this developmental period has the capacity to permanently increase rodent bodyweight both weeks after birth and in adulthood after gestational (Howdeshell et al, 1999; Takai et al, 2001; Rubin et al, 2001; Nikaido et al, 2004) and perinatal (Howdeshell et al, 2000; Alonso-Magdalena et al, 2010; Ryan et al, 2010) exposure. These findings have implications on bodyweight changes later in life- it is possible that bodyweights in our study animals could have been altered at developmental stages past those selected for our study. However, this exploration was not within the scope of the thesis but is important to take into consideration.

Gestational and lactational exposure to an endocrine disrupting chemical can have serious consequences not only on the healthy development of exposed animals, as previously discussed, but also on multiple generations of offspring, as explored through the DOHaD lens above. Numerous multigenerational rodent

studies have investigated the effect of BPA exposure on bodyweight, litter sizes, reproductive tract development and fertility. Low doses of DES and BPA (2 μg/kg DES and 50, 500 μg/kg BPA) did not significantly alter bodyweights but did reduce litter sizes of DES-dosed dams (LaRocca et al, 2011). Alternatively, some studies have reported increased bodyweights (Boudalia et al, 2013) while others have reported no offspring bodyweight changes post low-dose BPA exposure (Ema et al, 2001; Tinwell et al, 2002; Chitra et al, 2003; Chapin et al 2008; Kobayashi et al, 2012; Roy, Bauer & Lawrence, 2012; Kobayashi et al, 2002; 2012; Ling et al, 2016). At high doses of BPA, dam bodyweights have been shown to increase while offspring pup bodyweights decrease (Tyl et al, 2002, 2008; Stump et al, 2010). Thus, BPA can induce long-lasting metabolic effects throughout multiple generations. Both low and high doses of BPA have been linked to metabolic deficiencies and lowered glucose tolerance (Angle et al, 2013; Li et al, 2014). Multigenerational effects pertaining to reproductive carcinogenesis have also been observed after low DES exposure (Walker & Haven, 1997; Newbold et al., 1998, 2000,2006). The multigenerational effects observed in animal models are hypothesized to be due to epigenetic changes elicited by EDC in both BPA (Wisniewski et al., 2003; Sun et al., 2004; Ruden et al., 2005; Crews & McLachlan, 2006; Newbold, 2006) and DES (Li et al., 2003; Newbold, 2006; Gore, 2008) exposure studies. As the focus of the project was on the critical window of development and early postnatal life, it was not within the scope of the thesis to investigate changes in adulthood.

Conclusion

Evidence of molecular level changes in the adrenal gland during the critical period again requires a cautious interpretation that BPA exposure can and does elicit cellular changes in the HPA axis pathway, which has the potential to cause lifelong consequences in the physiological stress response. The variation in observed time-dependent dose responses suggests that two factors were thought to affect dose response: the

SHRP and exposure to xenoestrogens. The observed changes in gene expression in the adrenal gland indicate that it was evidently susceptible to estrogen-exposure, which has implications for endocrine programming following developmental EDC exposure. Unexpected estrogen-sensitivity to the 18SrRNA gene is a novel finding considering the widespread use of this common reference gene for molecular and toxicology studies. Ultimately, this study serves to illustrate the potential for disruptions to developing endocrine systems caused by exposures to environmentally-relevant, omnipresent low doses of BPA; currently deemed safe globally, including in Canada.

Future Directions

It will be interesting to continue the investigation into the role of sex differences in BPA exposure responses. I anticipate completion of the molecular analysis of GR levels in the female adrenal glands in 2017, enabling comparison of male and female adrenal gland expression at both PND 5 and PND15. Future investigations will include all three components of the HPA axis, made possible by my thesis - which included collection of several key organs, brain areas (including the pituitary and hypothalamus) as well as blood and serum of all experimental animals. This project has provided me with invaluable experiences and has allowed me to gain a multitude of newly acquired skills in wet laboratory research. I am now passionate about environmental toxicology and am fascinated by maternal-fetal health as well as have a newfound appreciation for the intersection between the fields of toxicology, neuroendocrinology and reproductive health. I am very eager to continue this work by expanding on this project to contribute to a better understanding of the intersection between xenoestrogen exposure, critical periods of development and the maternal-fetal dyad.

BPA: Interdisciplinary Perspectives

As described, EDC are exogenous agents that can exert action similar to hormones in the body and are widely prevalent in the home and environment. Routes of human exposure to EDC are most commonly ingestion, inhalation or absorption; typically with exposures associated with diet, workplace and leisure activities. Such widespread routes of exposure underscore the need for fundamental research regarding the human health risks of these agents, and development of subsequent regulation and policy. The study of these chemicals is complex and involves the intersection of many research areas including environmental toxicology, reproductive health, maternal-fetal health, neuroscience, and psychology. Similarly, regulation of environmental health risks requires cooperation of agencies, government and non-governmental organizations representing different geographic, political and legal jurisdictions. These include Health Canada, Environment Canada, the European Union, the European Food Safety Authority (EFSA), the US National Institute of Environmental Health Sciences (NIEHS), the US FDA, and the United Nations' Food and Agriculture Organization (FAO) Food Directorate's Bureau of Chemical Safety. Any discussion of environmental risks must also include behaviours, attitudes and risk perceptions regarding exposures to these agents. Here I will provide an overview of five topics related to EDC and reproductive health: (i) BPA and the low-dose effects controversy (ii) Canadian exposure to BPA (iii) Future Directions in Environmental Health Policy iv) BPA-Free Green Marketing and v) Canadian environmental risk perceptions.

i) BPA and the low-dose effects controversy

Low-dose BPA exposure is hypothesized to cause adverse health outcomes including risks to reproductive health (Richter et al, 2007; Signorile et al, 2010; Varayoud et al, 2011; Fernandez et al, 2010; Ali et al, 2014). Adverse health effects may be most significant following exposures to BPA during the developmental period (Vandenberg et al, 2013). In the mouse, low-dose BPA exposure during late-stage oocyte development disrupts meiosis, oocyte development and growth (Hunt et al, 2003; Ali et al, 2014). Other low-dose BPA studies in the rodent similarly reported adverse health effects in the late 1990s: Colerangle and Roy (1997), Nagel et al., (1997), Steinmetz et al., (1997), Steinmetz et al., (1998), vom Saal et al., (1998).

Evidence from such low-dose BPA studies prompted policy makers to re-evaluate the safety of BPA, weighing the evidence of studies conducted by industry, academia and government. Two major studies funded by the plastics industry (the Tyl et al, 2002, 2008 studies) formed the basis for the US FDA and EFSA decisions that declared that BPA was safe at the current exposure levels in various consumer products including canned food and drink (Aungst, 2014; Aungst & Anderson, 2014; EFSA, 2015). The US FDA and EFSA maintained that their reliance on the Tyl studies was due to the Good Laboratory Practice (GLP) standards used, determining that other studies were less acceptable (Myers et al, 2009).

Weighing the quality of a study depends on its potential for extrapolation to other species and its ability to form the basis for generalizable theories and models. At the heart of the BPA low-dose effects controversy are issues related to the quality of published studies and the potential bias of study funding. All research studies are limited by methodological or application factors that may be used to weigh study quality. There

are many limitations of toxicology research including lack of human exposure data (vom Saal et al, 2008), extrapolating findings from animal models to humans (vom Saal et al, 2008) animal strain selection, dose administration routes, lifespan differences (Van Leeuwen & Vermeire, 2007; vom Saal, 2008) and in vitro versus in vivo testing (Castell & Gmez-Lechn, 1996). Despite the fact that many EDC have been characterized, only a fraction of these have actually been tested for endocrine disrupting properties (WHO, 2012; Bergman et al, 2013). Low-dose levels of EDC may have additive effects in chemical mixtures (Mu &Leblanc, 2004; Kortenkamp, 2007; Diamanti-Kandarakis et al, 2009). As humans are commonly exposed to such chemical mixtures in the form of air pollution (Jonker et al, 2004), pesticides and water sanitation by-products (Feron & Groten, 2002), the approach to exposure modelling in toxicology research relies on both single chemical testing and evaluating chemical mixtures at high doses to laboratory animals (Jonker et al, 2004).

Vom Saal, a prominent researcher studying and reporting the risks of low-dose BPA, has demonstrated adverse reproductive and developmental effects in rodents exposed to low-dose BPA (vom Saal & Hughes, 2005; vom Saal & Welshons, 2006; Welshons, Nagel & vom Saal, 2006; Richter et al, 2007; vom Saal et al, 2007). He is among the researchers that have questioned the establishment of safe BPA levels for human exposure and was among the 38 experts in the field who collaborated to publish The Chapel Hill Consensus Statement which opposed the FDA's finding that BPA was safe (vom Saal et al. 2007). There are now hundreds of published studies reporting low-dose BPA effects in a range of animal species (Vandenberg et al, 2007; Sekizawa, 2008). Increasingly, it is becoming evident that BPA does not adhere to the previously assumed monotonic dose-response relationship (vom Saal & Sheehan, 1998; Vogel, 2009; Myers et al, 2009), as described in the Discussion. Although individual studies may be flawed, as the field of research

grows the collective weight of the evidence supports the hypothesis that certain agents including BPA do have effects at low-doses.

The decision made by the FDA establishing BPA as "safe" was based on studies that had followed good laboratory practice (GLP) regulation. However, the GLP regulations are not the guidelines used in academic research, and a review by Myers et al (2009) stated that the GLP standards are not necessarily correlated with validity and reliability. Further, the review doubts the tenacity of these regulations by iterating that key components (repeatability and up to date assays) are not included as part of these study standard criteria (Myers et al, 2009).

Beyond study quality, another major issue that emerged was the source of research funding. A meta-analysis conducted by vom Saal & Hughes (2005) reviewed numerous scientific studies to determine whether the source of study funding was related to the observation of adverse effects due to low-dose BPA exposure. This analysis reported that over 90 % of government-funded BPA studies had observed adverse low-dose effects in comparison to no measured effects by any industry-funded studies (vom Saal & Hughes, 2005). The issue of the origin of study funds and its effect on regulatory decision-making is acknowledged in the review conducted by Myers et al. (2009). Here, Myers et al. describe the inadequacies in GLP standards and discuss the apparent conflict of interest between study funding source and the reporting of significant results in low-dose BPA research (Myers et al., 2009). Thus, the BPA controversy has prompted a reflection within the field of environmental health regarding the quality of research and the source of funding. Further, the field is demanding the standardization of environmental health and toxicology assessments and greater accessibility to raw data (Borgert, 2007; Schreider, 2010). The issue is prevalent across all areas of scientific research and prompted UNESCO to release a report in 2010 calling

for the creation of a standard across scientific journals in order to manage these increasing conflicts of interest. Scientific journals have responded to the BPA controversy and other similar cases by requiring more explicit conflict of interest statements and declarations of study funding (Braff, 2010; Ruff, 2015). Finally, it is possible that the significant conflicts of interest and the ensuing controversy in low-dose BPA study ignited this more recent action and in part drove this conflict of interest policy that is now standard.

ii) Canadian Contamination: Exposure to BPA in Canada

To better ascertain the levels of exposure experienced across a nation, there is a need for large-scale population-based studies that include wide ranges of ages, measurements and a robust representation of the population. Prominent examples include the Canadian Health Measures surveys (a cross-sectional design) (Statistics Canada, 2016), the Canadian House Dust study (Rasmussen et al, 2011) (random selection samples from over a thousand urban homes), and the National Health and Nutrition Examination survey (Curtin et al, 2013), which increased Hispanic and senior samples to adequately represent the US population. Notwithstanding the considerable amount of studies reporting low-dose effects, the FDA maintains its position that BPA is safe at current exposure levels (Aungst, 2014; Aungst & Anderson, 2014). Despite this position, numerous large-scale research platforms continued to investigate. The National Toxicology Program (NTP) is a research program that operates at the federal level through the National Institute of Environmental Health Sciences. The evaluation of the safety of BPA was run through the NTP Center for the Evaluation of Risks to Human Reproduction (CERHR), where a scientific panel convenes to peer review global scientific literature specific to the chemical of study (NIEHS, 2008). In 2006, the Canadian Chemical Management Plan was implemented to prioritize the investigation of hundreds of potentially human-harming chemicals (including BPA) in order to determine how to proceed with their management (Health Canada, 2011). Just two years later, the NTP review (2008) was the first

large-scale low-dose BPA review to cause legitimate concern regarding the critical window. After conducting a major human exposure assessment study on low-dose BPA in 2008, Canada became the first government to officially ban BPA from baby bottles that same year (Health Canada, 2010; Kiss, 2014). The government also conducted large-scale exposure studies on rodents, and weighed the findings of the 2008 NTP review heavily. As a result, Canada was quite proactive in attempting to reduce Canadians' risk by focusing on legislation to protect the infant and baby population (Health Canada, 2014) and through the adoption of the ALARA (As Low As Reasonably Achievable) principle to reduce exposure risk as much as possible (Health Canada, 2014). In 2010, the WHO summit hosted in Ottawa announced that further low-dose BPA research would be required based on reported effects at doses much lower than the current safe levels (Health Canada, 2014). As of December 2014, the most updated report by Health Canada confirmed that BPA was not detected in any liquid infant formula sold in the country and also estimated that it would eventually become eradicated in canned products affecting all Canadians (Health Canada, 2014). BPA-containing packaging for liquid infant formula was monitored and was completely phased out in December 2014 (Health Canada, 2014).

To determine probabilistic exposure, the concentration of BPA in every food item was applied to the reported rates of the food items consumed by each individual participant in order to achieve average estimates of exposure for each subgroup of the population. The average probabilistic estimate for the general population was reported to be 0.055 μg/kg bw/day (Health Canada, 2012), and **Table 8** illustrates average subpopulation exposure levels in the general Canadian population. Between 2009 and 2011, data were collected on the general Canadian population and included Canadians aged 3 to 79 years old. Total BPA in urine was measured and was detected in over 95% of Canadian participants, at an average concentration of 1.2 μg/L (Health Canada, 2013; Statistics Canada, 2015). A similar report was generated

for the infant population based on data collected between 2008-2011. Included in the analysis were both liquid and powder-form baby formula and baby food contained in glass jars with metal lids to again determine probabilistic dietary exposure. The average daily exposure observed in Canadian infants was the highest out of all the population, largely due to their small size and amount of food intake (Health Canada, 2012), and **Table 9** illustrates the average probabilistic dietary exposure levels in Canadian infant subpopulations. The copyright permission letter for use and reproduction of this information is found in **Appendix 4.0**. It is estimated that Canadian infants between 0 and one month old are subject to daily BPA exposure levels of 0.50µg/kg bw/day. Exposure for 12-18 month old infants is at a level of 0.27µg/kg bw/day. As infants have a higher bodyweight to food intake ratio, they are the most highly exposed population- infant exposure to BPA is more than twice as high as that in adults: an average level of exposure in adults in Canada is 0.05 μg/kg per day, versus an average infant exposure of 0.118 μg/kg per day (Health Canada, 2012) **(Table 9).** The chemical is present in plastic toys that children frequently suck on, and it is evident that banning baby bottles will not ensure protection from oral exposure to BPA in this vulnerable population.

One of the most significant controversies surrounding the safety of BPA concerns inconsistencies in experimental design, dosage method and animal model selection across BPA research experiments, as well as the failure to replicate results (Goodman et al, 2006; Richter et al, 2007; Palanza et al, 2008). Determining the exact levels of safe exposure is therefore of utmost importance considering this most critical period of development and the fact that the health related effects of BPA during the neonatal period continue to be widely disputed (Snijder et al, 2013).

iii) Environmental Health Policy: Moving forward

The Prioritization of Risks strategy encompasses many facets of risk management (e.g. financial collapse, disaster) but its International Organization for Standardization (ISO 31000) sub-category provides a customized interpretation for toxicology (ISO, 2009). The strategy scores chemicals in four categories: persistence, bioaccumulation, toxicity and quantity to assess the chemical's risk (Arnot & MacKay, 2008). An example of a globally applicable environmental health risk is the ubiquitous EDC BPA, which is widely prevalent in the environment, and whose presence poses a risk to developing organisms, animals and humans, as previously discussed. To manage risks to prevalent chemicals such as BPA, strategies such as The Precautionary Principle are applied when health is threatened, even if the full extent of harm is not actually known (SEHN, 1998). The Precautionary Principle is called the Paralyzing Principle by opposers (Sunstein, 2005). If there is any doubt whatsoever of potential health effects of a chemical such as BPA and other related EDC for the Canadian population, I believe that the Precautionary Principle should absolutely be applied. The adverse health effects of BPA reported in rodent studies at low doses quite similar to human exposure levels (vom Saal et al, 2007; Vandenberg et al, 2007; Richter et al, 2007; Soto et al, 2008; Vandenberg et al, 2009), the widespread prevalence of BPA (Vandenberg et la, 2007), and the fact that Canadian exposure levels are consistent with other countries (Calafat et al, 2008; Becker et al, 2009; He et al, 2009;Vandenberg et al, 2010; Bushnik, 2010) indicate that the consequences of allowing exposure to continue should be carefully considered.

iv) BPA-Free- Green Marketing

The term "BPA-free" has been used as a marketing touchstone, effectively capitalizing on the current phase of green living (Ottman, 2011). Companies must adapt to the public's desire to focus on green energy and

green policy and tailor both products and marketing strategies to satisfy the demands of a more environmentally-aware, socially-conscious and accountable generation of consumers (Ottman, 2011). Large multinational companies are purchasing sustainable brands and re-branding to satisfy consumer demand (Ottman, 2011). Major manufacturers (e.g. Nalgene and Playtex) and retailers (e.g. Toys R Us, Walmart and Whole Foods) are among the businesses that have adopted a cautious approach by gradually eliminating sales of bottles made with BPA (Bailin et al, 2008; Houlihan, Lunder & Jacob, 2008). The strategy of a cautious approach may just be smart marketing, given increased consumer consciousness towards everyday chemical exposure (Ottman, 2011). The label "BPA Free" emerged after the year 2008 when large-scale government-funded studies (ie: Shelby/NTP, 2008; Health Canada, 2008) and the cooperative study between the US Work Group for Safe Markets and Environmental Defence Canada (vom Saal & Hughes, 2005; vom Saal 2007; Wilson et al, 2007; Shelby/NTP, 2008) confirmed that BPA was leaching out of bottles and baby bottles.

Consumers want to avoid risk and are accordingly more likely to select alternative products which are BPA-free, at a significant price (Logomasini, 2010) despite not necessarily knowing anything about the chemical used to replace BPA in the new product (Scherer et al, 2014). Consumers may be unaware that the term BPA-free does not mean estrogen-free as, most products labeled BPA-free still have estrogenic activity (Yang et al, 2011; Bittner et al, 2014). Further, BPA-consumer products represent only one source of BPA exposure- the xenoestrogen is almost ubiquitous and dust particles (Chao et al, 2015), thermal receipt paper (Hormann et al, 2004), cosmetics (Larsson et al, 2014) and household appliances (Zhang et al, 2012) are only a few examples of source exposure to BPA. Ultimately, manufacturers and retailers are perhaps taking advantage of consumers' environmental risk perception and environmentally conscious buying habits. The increased consumer consciousness to toxicant exposure paired with the low-dose

controversy surrounding chemicals such as BPA provides a perfect opportunity for large manufacturers and household name brands to capitalize on the current state of attitudes towards EDC.

v) Canadian environmental risk perceptions

Risk perception is a highly individualized process (Brown, 2014) based on each person's unique life experience and attitudes (Hughes, 2003). The word "chemical" is a fear-inducing word in the public (Slovic et al, 1995; Starr et al, 2000) and the "risk perception gap" is a term used to describe the failure to prioritize risks (Slovic, 2000; Gray & Ropeik, 2002). In 2004, the National Health Risk Perception survey (Krewski, 2006) used a representative sample of the Canadian population to assess chemical risk hazard and information sources. The media, the dread factor and the understanding of the issue were important factors in risk perception (Krewski, 2006) with stress, air pollution and pesticides perceived as high risk (Leiss, 2001). Different priorities and risk perceptions are evident between genders (Starr et al, 2000; Gustafson, 1998; Slovic, 1999; Finucaine et al, 2000), age groups, nationality, and socioeconomic status (Fulford et al, 2013; Daniluk & Koert, 2013; Lundsberg et al, 2014). Women and people with higher education were more knowledgeable about BPA (Brewer & Ley, 2011) and older and less educated women had a higher risk perception (Robichaud et al, 2003; Day & Livingstone, 2003).

Of significant consideration is the risk posed to young children and babies, as they tend to play with plastic toys and frequently put objects in their mouths. This younger population spends more time crawling or on the ground and can also easily ingest particles of dust full of these chemicals. Childrens' developmental immaturity and consequential higher exposure to toxicants requires parents and caregivers to be familiar with EDC (Tyshenko et al, 2007; Schlotz & Phillips, 2009). As mothers have the traditional care role for infants and children, they are the most likely to implement appropriate avoidance practices (Ho, Davidson

& Ghea, 2005; Thirlaway & Heggs, 2005; Crighton, 2013) and the role of public health nurses in educating this specific demographic would be advantageous (Stanhope & Lancaster, 2014). Therefore, an educational strategy would also be beneficial for risk management.

Awareness about EDC is greatly influenced by several determinants of health including socioeconomic status and education, as well as family dynamic and language barriers. The "double burden" of housing situation and lack of contaminant knowledge greatly affects low income and/or new immigrants (Chen, Matthews & Boyce, 2002; Marshall, 2004; Matthews & Gallo, 2011). Money and lack of control are two main factors in attitudes towards chemicals (Floyd, Prentice-Dunn, Rogers, 2000). This is significant as there is a correlation between low socioeconomic status and higher exposure (Chen, Matthews & Boyce, 2002; Frohlich, Ross & Richmond, 2006). In addition, a higher perceived risk perception corresponds to a lower initiative for reducing risk, likely due to lack of control (Lee et al, 2005; Ho, Davidson & Ghea, 2005; Thoolen et al, 2008). In Canada, language (not minority or immigrant status) serves as the barrier to risk perception (LaFerriere et al, 2014). Considering the increased vulnerabilities to EDC exposure along with a lack of both monetary resources and feelings of control (Zala & Penn, 2004), it is important to focus on public health promotion (Koren, Bologa & Pastuszak, 1991; Meeker, 2012) in these specific populations to reduce EDC exposure as much as possible.

The Social Amplification of Risk framework proposes that repetitive stories with high dread factors conveyed through media, activists, personal anecdotes, social networks and public agencies essentially heighten public fear (Kasperson et al, 1988). Risk perception is most enhanced in relation to issues affecting the health and wellbeing of fetuses, infants and children; who are perceived as being most vulnerable to risk (Starr et al, 2000). Online educational websites can be an important tool to mediate the

translational gap between scientists and the public (Leiss, 2001), and there is a need for collaboration between media and the experts in the field to ensure correct information distribution. The media is the most prevalent source of health information but can also be the most problematic as it can easily relay erroneous information (Morrone, 2011; Lampi, 2011). Ultimately, health promotion and education can help improve scientific literacy enabling individuals to meaningfully participate in their health decisions, and be proactive in their own health and safety (Fulford et al, 2013).

In terms of shaping new environmental health policy, I would recommend that we need more robust studies to examine the relationships between low SES, poverty, poor housing situation and higher exposure to EDC and resulting adverse health outcomes. Building on these studies, development and implementation of strategies to increase the environmental health of the poor, the standards of their homes and neighborhoods and to promote education of these vulnerable populations. This strategy is similar to the WHO's plan of action (2010) on EDC which promotes data collection and analysis, research, advocacy and education and awareness. EDC are widely detected across the Canadian environmental landscape (Health Canada, 2008; Eyles et al, 2011) and still present a significant cause for concern (Health Canada, 2012). Heightened governmental regulatory protocols for commercial plants and facilities (Health Canada, 2008) are a step in the right direction for reducing environmental EDC exposure in our country. Promotion of open communication and ongoing collaboration among scientists, advocacy groups, government and public health officials should be promoted and encouraged. Finally, Canadians must be engaged in environmental health debates and have access to emerging research findings. The focus on media use is pivotal in the relay of accurate and updated information, as it serves as the link between research scientists and experts in the field with the general public. Scientific findings must be relayed to the general public accurately and in a timely, accessible and relatable manner. Given the ubiquitous nature and deleterious effects of low-dose

EDC, it is crucial that scientific researchers, community leaders and the general public become integrated in the efforts to minimize future EDC exposure risk in the country.

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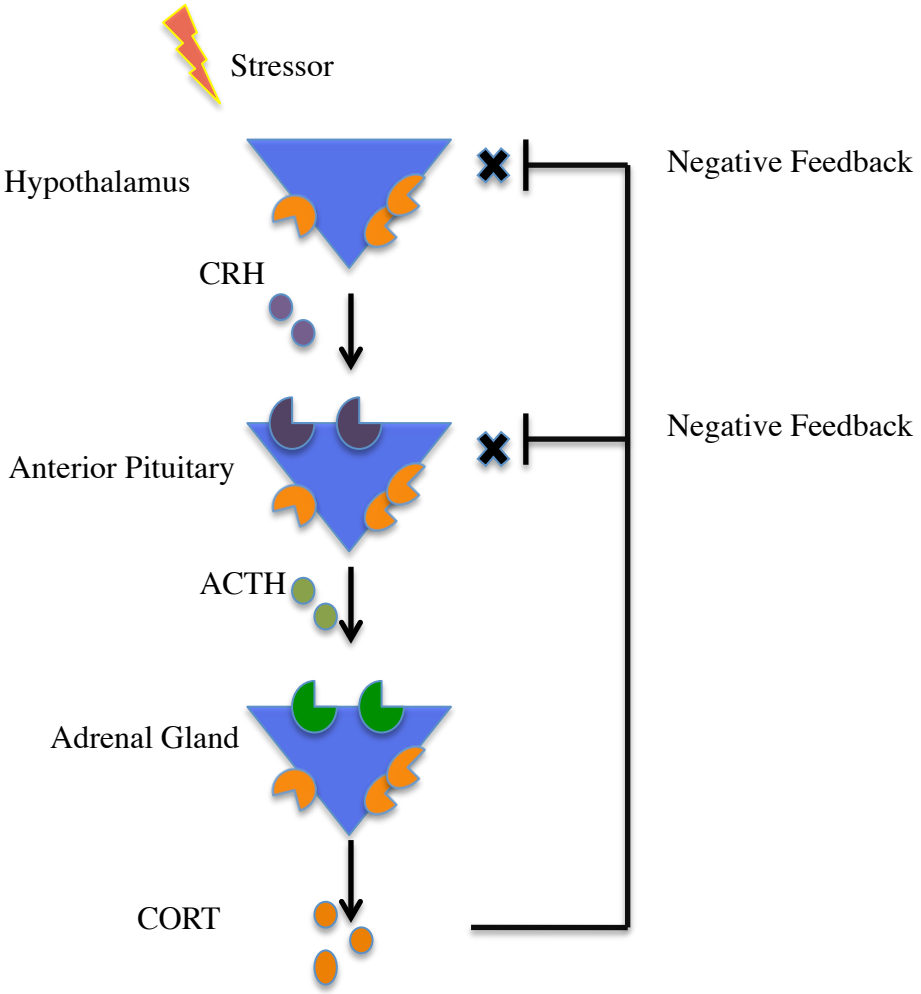


Figure 1. HPA axis. In response to a stressor, CRH is secreted from the hypothalamus and binds to its receptors (CRH-R) on the anterior pituitary. This gland secretes ACTH which travels through the bloodstream and binds to its receptor (ACTH-R) on the adrenal gland. Binding here results in the release of glucocorticoid, which then travels throughout the body to elicit action in a multitude of target cells including cardiac, smooth and skeletal muscle via the glucocorticoid receptor (GR). After the removal of the stressor, glucocorticoids bind anterior pituitary and hypothalamic GR to inhibit further pathway activation (de Kloet et al, 1998;Saeb-Parsy, 1999; Smith & Vale, 2006).

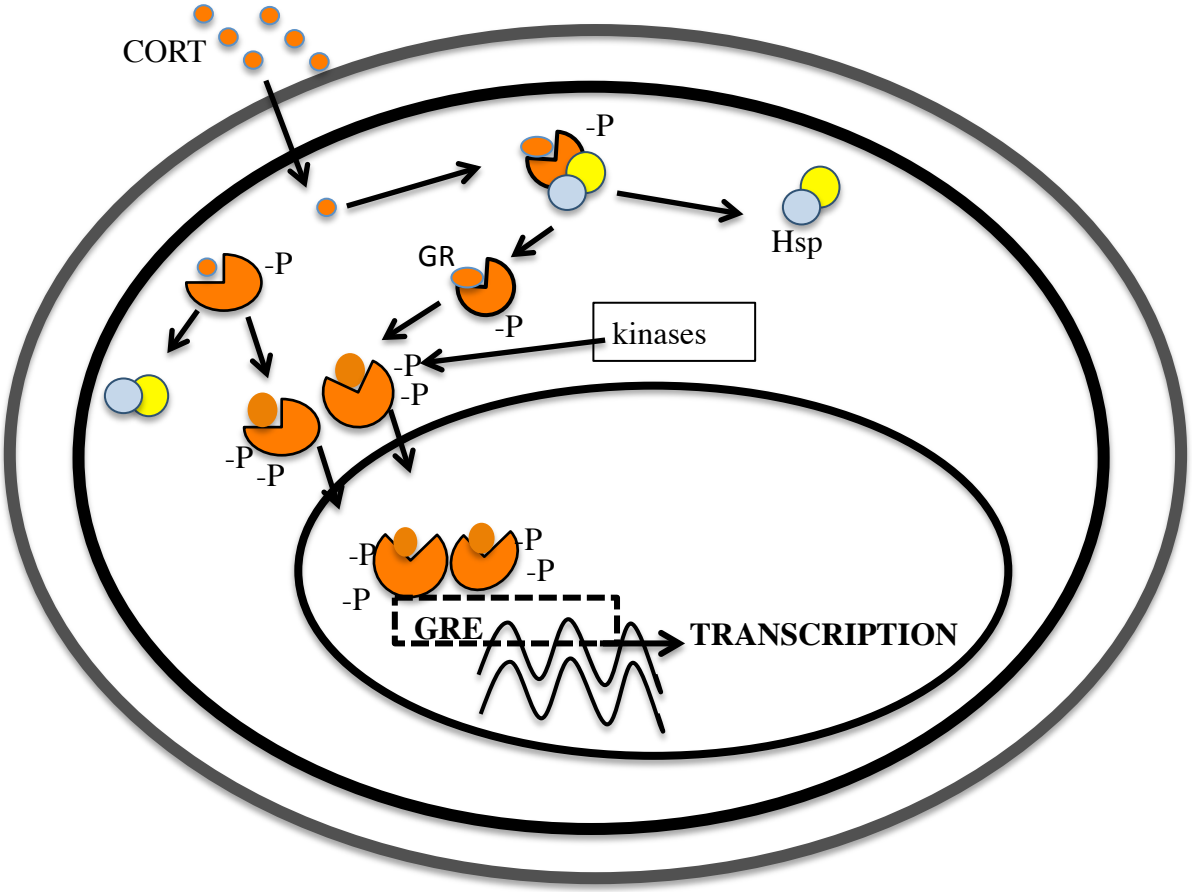


Figure 2. **Glucocorticoid Receptor Activation**. After glucocorticoid ligand binding, the GR is released from its hsp90 heterocomplex (Hsp90, Hsp70, p60, p23), is hyperphosphorylated, and enters the nucleus (Orti, Hu & Munck, 1993; Bodwell et al, 1998; Ismaili & Garabedian, 2004). One GR subunit attaches to one half site on the consensus GRE sequence on the DNA, and the two GR subunits homodimerize (Oakley & Cidlowski, 2013). The homodimer then modulates either positive or negative cell action by transcription (Pujols et al, 2002; Dostert & Heinzel, 2004; Vandevyver, Dejager, & Libert, 2012; Oakley & Cidlowski, 2013). -P: phosphate group

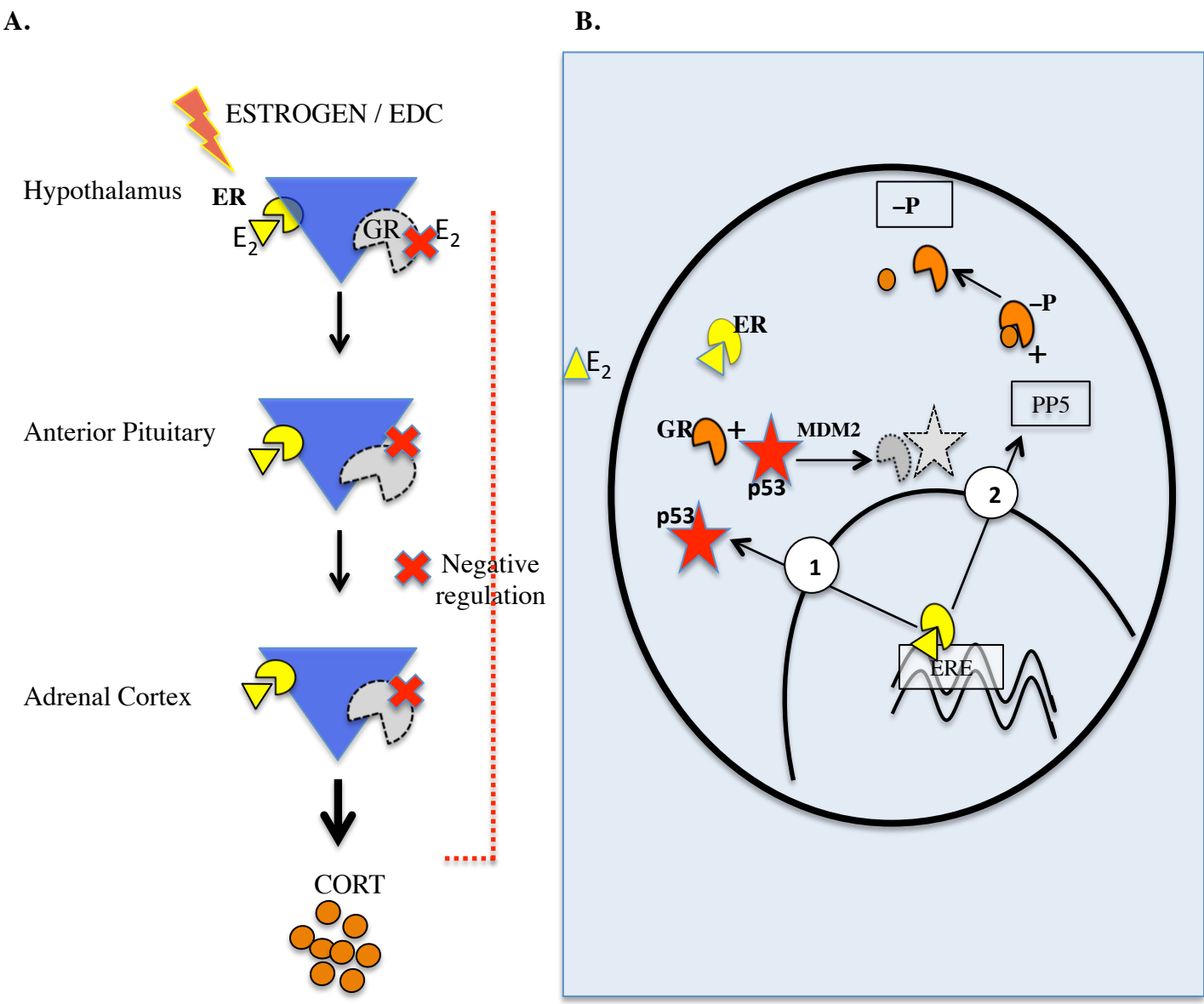


Figure 3. Estrogenic modulation of the HPA axis. **A.** *HPA axis upregulation.* Estrogenic endocrine disruptors such as BPA can upregulate HPA axis activity resulting in high circulating CORT levels. Estradiol (E_2) and xenoestrogens counter the negative regulation at the hypothalamus and anterior pituitary, resulting in hyperactivation of the HPA axis. **B.** *Negative regulation of GR.* Xenoestrogens negatively regulate GR activity by at least two mechanisms: (1) the degradation of GR, following estrogen-dependent upregulation of p53 and activation of the Mdm-2 ubiquitination pathway. (2) Dephosphorylation of the GR, following estrogen-dependent upregulation of protein phosphatase 5 (PP5) (Zhang et al, 2009)

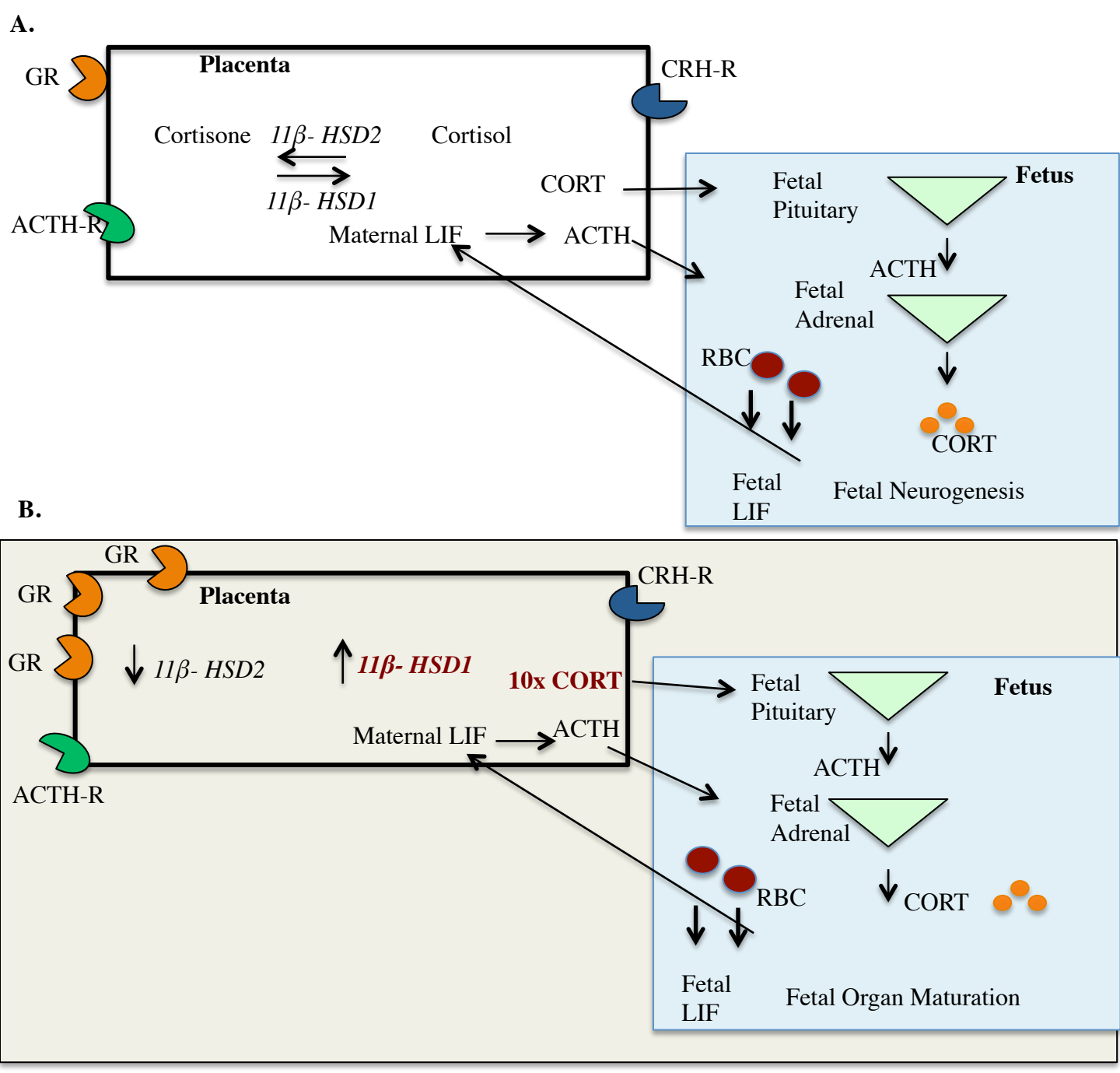


Figure 4. Fetal Neurogenesis and Fetal Organ Maturation in the Rat. A. *Early Gestation*. In the rat, placental endocrine function modulates the development of the fetal HPA axis. Secretion of maternal leukemia inhibitory factor (LIF) promotes placental synthesis and secretion of ACTH, which travels to the fetus and results in LIF secretion from fetal red blood cells (RBC). The maternal ACTH-fetal LIF dynamic promotes the neurogenesis of neuronal progenitor cells in the rat cerebrum (Simamura et al, 2010). **B.** *Mid-Late Gestation*. The shift in the ratio of 11β-HSD isoforms towards the end of gestation results in an increase of placental CORT secretion which promotes fetal organ maturation.

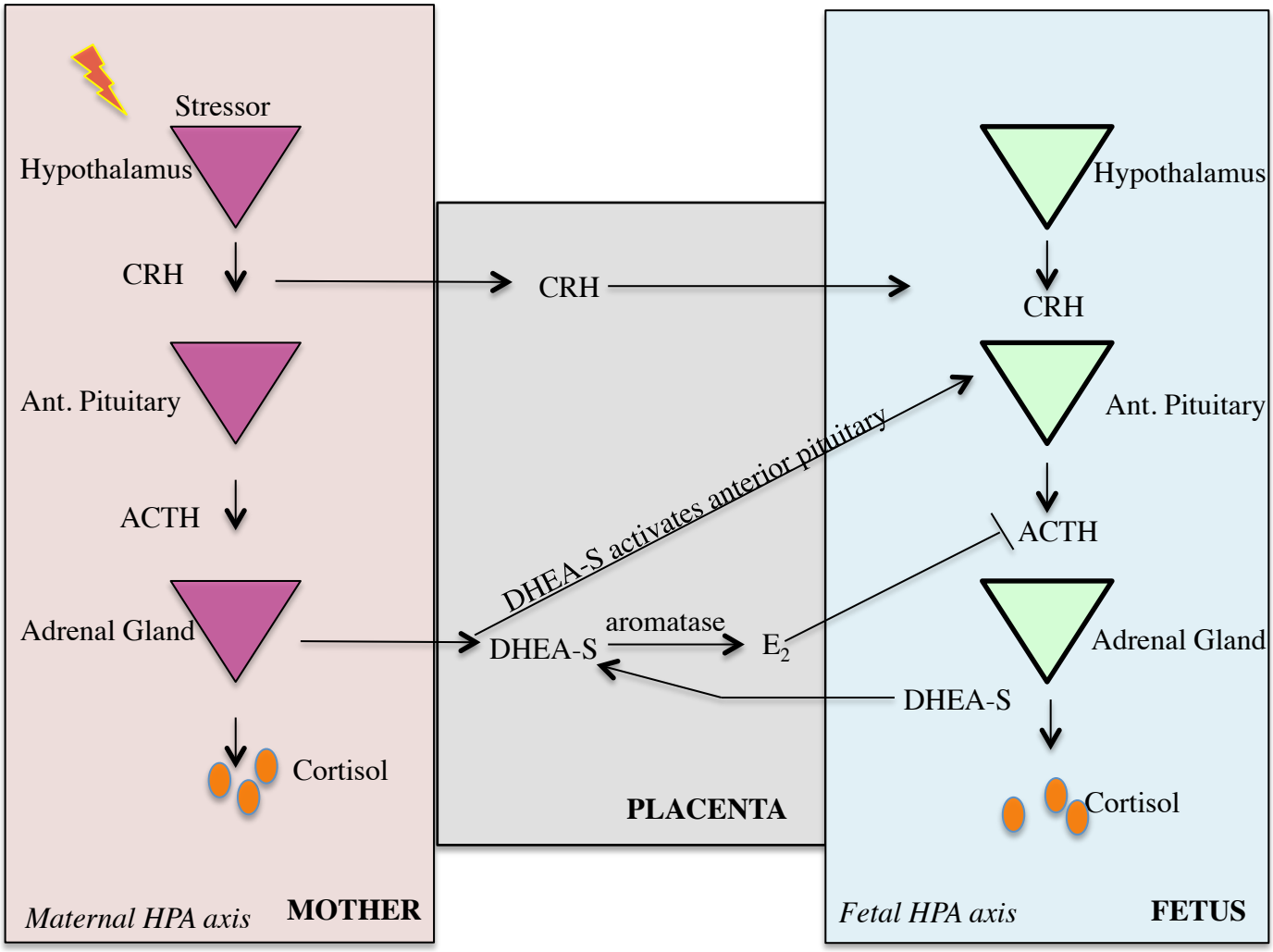


Figure 5. Human Maternal-Fetal HPA Axis Dynamic. In humans, maternal HPA axis activation by a stressor produces dihydroepiandrosterone sulfate (DHEA-S) which crosses the placenta to activate the fetal anterior pituitary. Fetal HPA activation, including maternal CRH stimulation, also produces DHEA-S and glucocorticoids. Both maternal and fetal androgen DHEA-S provides the placenta with the substrate for estrogen synthesis, catalyzed by the aromatase enzyme. Placental estrogen inhibits ACTH release from the fetal anterior pituitary, thereby reducing activation of the fetal HPA axis.

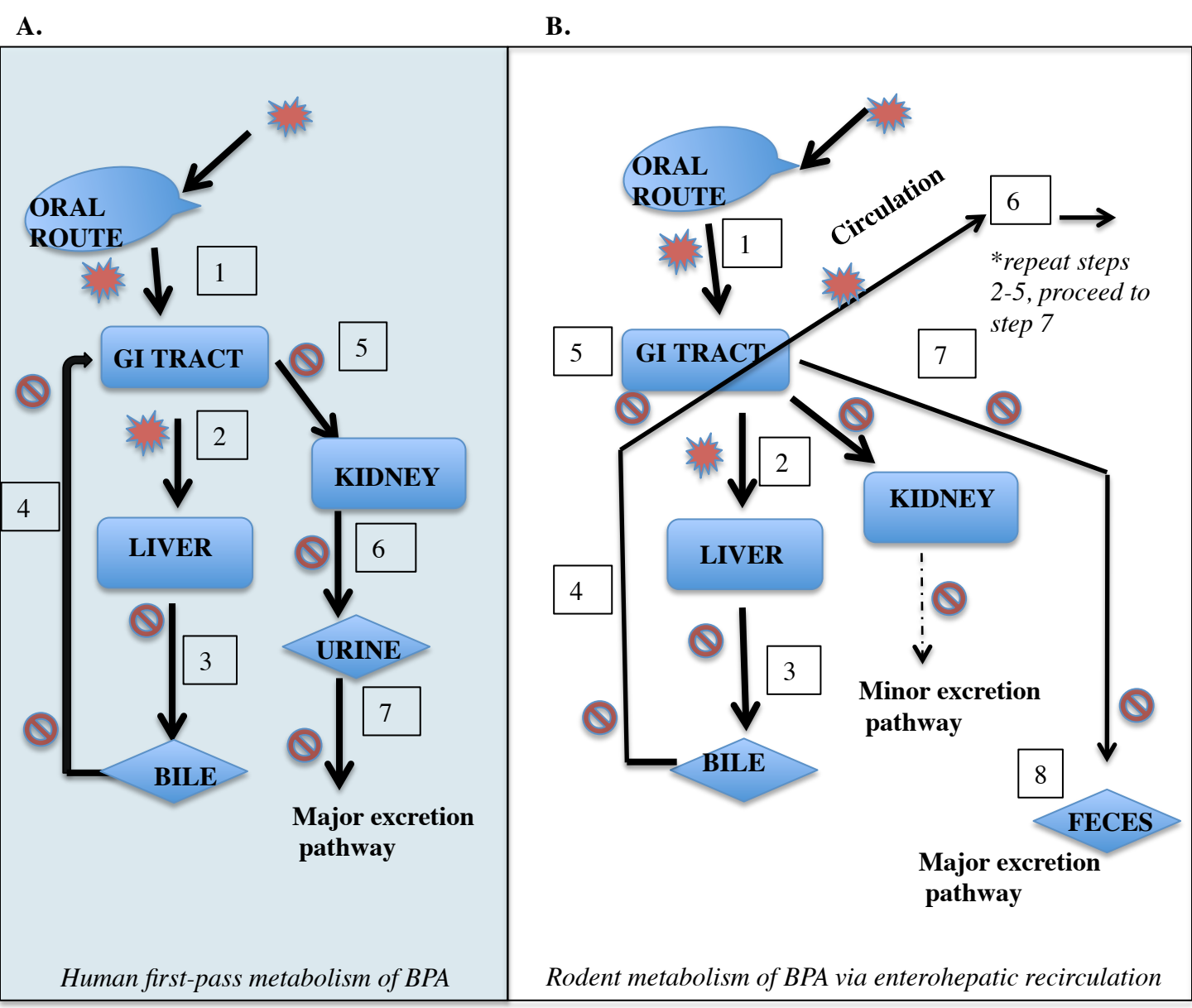


Figure 6. BPA metabolism: Human vs. Rodent. **A**. *Human first-pass metabolism of BPA*. After oral ingestion, BPA is inactivated in the liver to BPA-glucuronide and eliminated through the kidney in urine. **B.** *Rodent metabolism of BPA via enterohepatic recirculation*. After oral ingestion, BPA is inactivated in the liver to BPA-glucuronide, is present in the bile, is reactivated in the GI tract and sent through the circulation. It is again inactivated in the liver, travels in the bile through the GI tract and is finally eliminated in the feces. A minor excretion pathway includes excretion of the inactivated form in the urine.

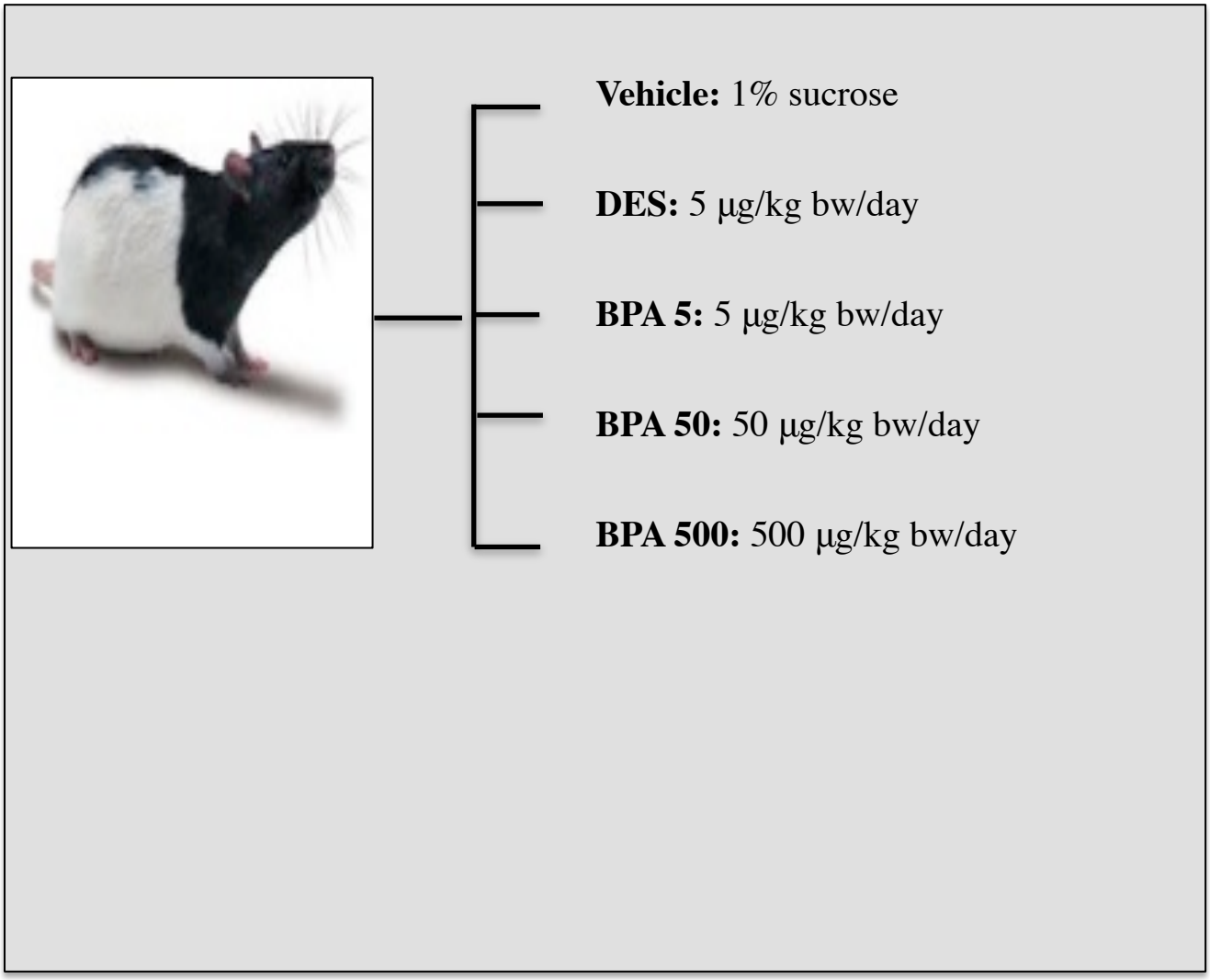
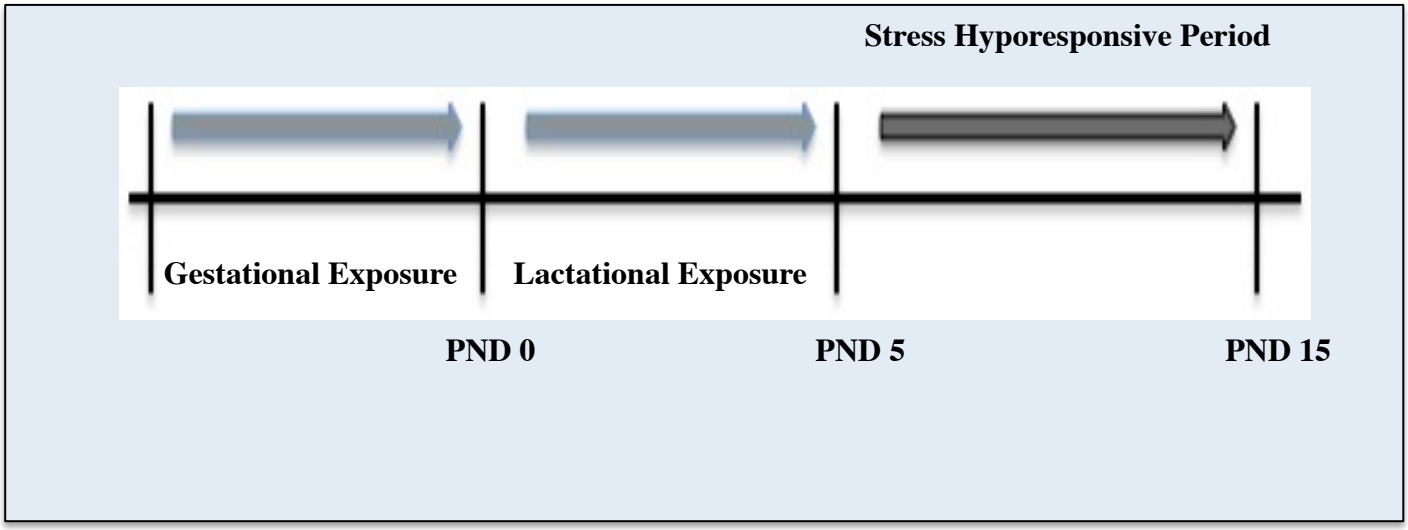


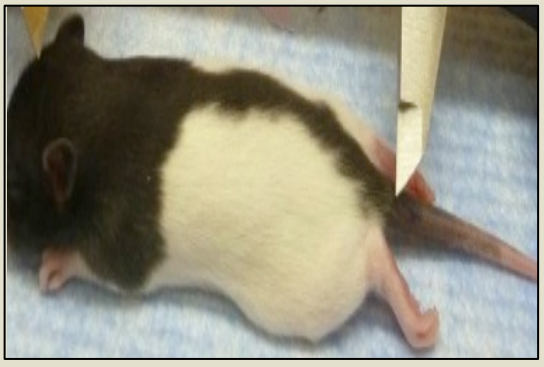
Figure 7. Treatment Groups. Three low doses of Bisphenol A (5, 50 and 500 μg/kg bw/day), a 1% sucrose, 1% EtOH negative control and a 5 μg/kg bw/day Diethylstilbestrol positive control were used as the experimental doses. Dams were weighed daily and dosed accordingly.

A.



B.

PND 5 pup pre-sacrifice



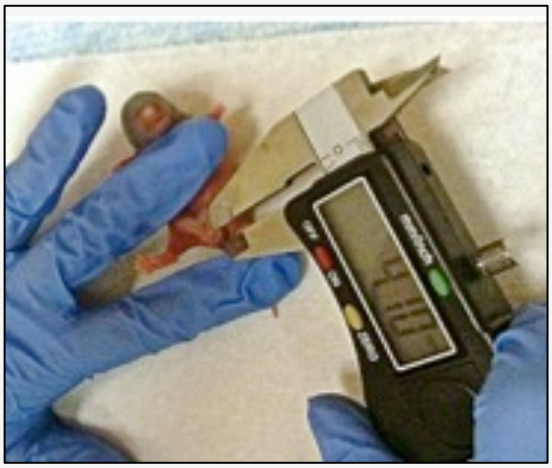
PND 15 pup pre-sacrifice

Figure 8. Experimental Timeline A. *Experiment Timeline*. The study timeline is illustrated, beginning with dam gestational exposure, followed by the birth of pups at PND 0, the transition to lactational exposure up to PND 4, followed by sacrifice at two time-points (PND 5 and PND 15), corresponding to the beginning and to the end of the SHRP. **B**. *Pups at sacrifice*. PND 5 (left) and PND 15 (right) pups are shown at time of sacrifice. Note the differences in size and fur growth.

A.

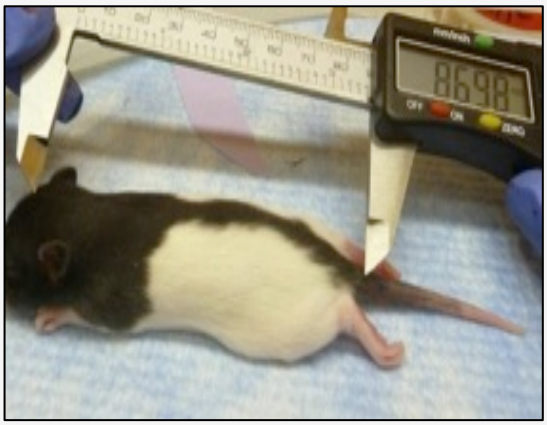
PND 5 Crown-Rump Length

B.



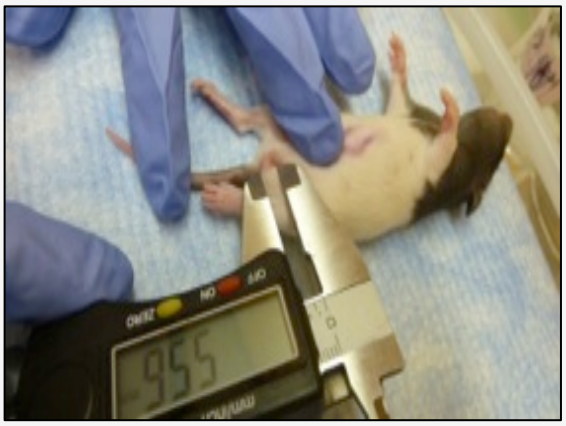
PND 5 Anogenital Distance

C.



PND 15 Crown-Rump Length

D.



PND 15 Anogenital Distance

Figure 9. Pup Morphometric Measurements. Crown-Rump length **(A., C.)** and Anogenital Distance **(B.. D.)** measurements were performed on both PND 5 and PND 15 experimental animals in triplicate on the day of sacrifice using Vernier calipers.

1	---	3	5	7	8	9	10	11	12
NTC			+ve 2 µL	10^{-1} VEH Sample 1	10^{-2} VEH Sample 1	10^{-3} VEH Sample 1	10^{-4} VEH Sample 1	10^{-5} VEH Sample 1	
NTC			+ve 2 µL	10^{-1} VEH Sample 1	10^{-2} VEH Sample 1	10^{-3} VEH Sample 1	10^{-4} VEH Sample 1	10^{-5} VEH Sample 1	
NTC			+ve 2 µL	10^{-1} VEH Sample 1	10^{-2} VEH Sample 1	10^{-3} VEH Sample 1	10^{-4} VEH Sample 1	10^{-5} VEH Sample 1	

NTC: no template control
+ve: positive control

Figure 10. Standard curve plate set-up. Illustration of the plate setup for serial dilution run. Samples were loaded from left to right, A1-A12, B1-B12, C1-C12 at a total well volume of 20 µL (18 µL Master Mix+ 2µL sample). VEH sample 1 used as example.

1	---	3	5	7	9	11	12
		10^{-1} VEH Sample 1	10^{-1} DES Sample 1	10^{-1} BPA5 Sample 1	10^{-1} BPA50 Sample 1	10^{-1} BPA500 Sample 1	
NTC		10^{-1} VEH Sample 1	10^{-1} DES Sample 1	10^{-1} BPA5 Sample 1	10^{-1} BPA50 Sample 1	10^{-1} BPA500 Sample 1	
NTC		10^{-1} VEH Sample 1	10^{-1} DES Sample 1	10^{-1} BPA5 Sample 1	10^{-1} BPA50 Sample 1	10^{-1} BPA500 Sample 1	
NTC							
		10^{-1} VEH Sample 1	10^{-1} DES Sample 1	10^{-1} BPA5 Sample 1	10^{-1} BPA50 Sample 1	10^{-1} BPA500 Sample 1	
		10^{-1} VEH Sample 1	10^{-1} DES Sample 1	10^{-1} BPA5 Sample 1	10^{-1} BPA50 Sample 1	10^{-1} BPA500 Sample 1	
		10^{-1} VEH Sample 1	10^{-1} DES Sample 1	10^{-1} BPA5 Sample 1	10^{-1} BPA50 Sample 1	10^{-1} BPA500 Sample 1	

NTC: no template control

Figure 11. Experimental plate setup for reference gene. Experimental samples were loaded in triplicate with a representative sample from each exposure group on the plate. Total volume per well was 10 µL (9 µL Master Mix+ 1µL sample). Tm was set to 55°C for reference gene plates. Reference genes were run on the same plate.

1	---	3	5	7	9	11	12
		10^{-1} VEH Sample 1	10^{-1} DES Sample 1	10^{-1} BPA5 Sample 1	10^{-1} BPA50 Sample 1	10^{-1} BPA500 Sample 1	
NTC		10^{-1} VEH Sample 1	10^{-1} DES Sample 1	10^{-1} BPA5 Sample 1	10^{-1} BPA50 Sample 1	10^{-1} BPA500 Sample 1	
NTC		10^{-1} VEH Sample 1	10^{-1} DES Sample 1	10^{-1} BPA5 Sample 1	10^{-1} BPA50 Sample 1	10^{-1} BPA500 Sample 1	
NTC							
		10^{-1} VEH Sample 2	10^{-1} DES Sample 2	10^{-1} BPA5 Sample 2	10^{-1} BPA50 Sample 2	10^{-1} BPA500 Sample 2	
		10^{-1} VEH Sample 2	10^{-1} DES Sample 2	10^{-1} BPA5 Sample 2	10^{-1} BPA50 Sample 2	10^{-1} BPA500 Sample 2	
		10^{1} VEH Sample 2	10^{1} DES Sample 2	10^{1} BPA5 Sample 2	10^{1} BPA50 Sample 2	10^{1} BPA500 Sample 2	

NTC: no template control

Figure 12. Experimental plate setup for target gene (GR) plate. Experimental samples were loaded in triplicate with a representative sample from each exposure group on the plate. Total volume per well was 10 µL (9 µL Master Mix+ 1µL sample). Tm was set to 60°C for target gene plates.

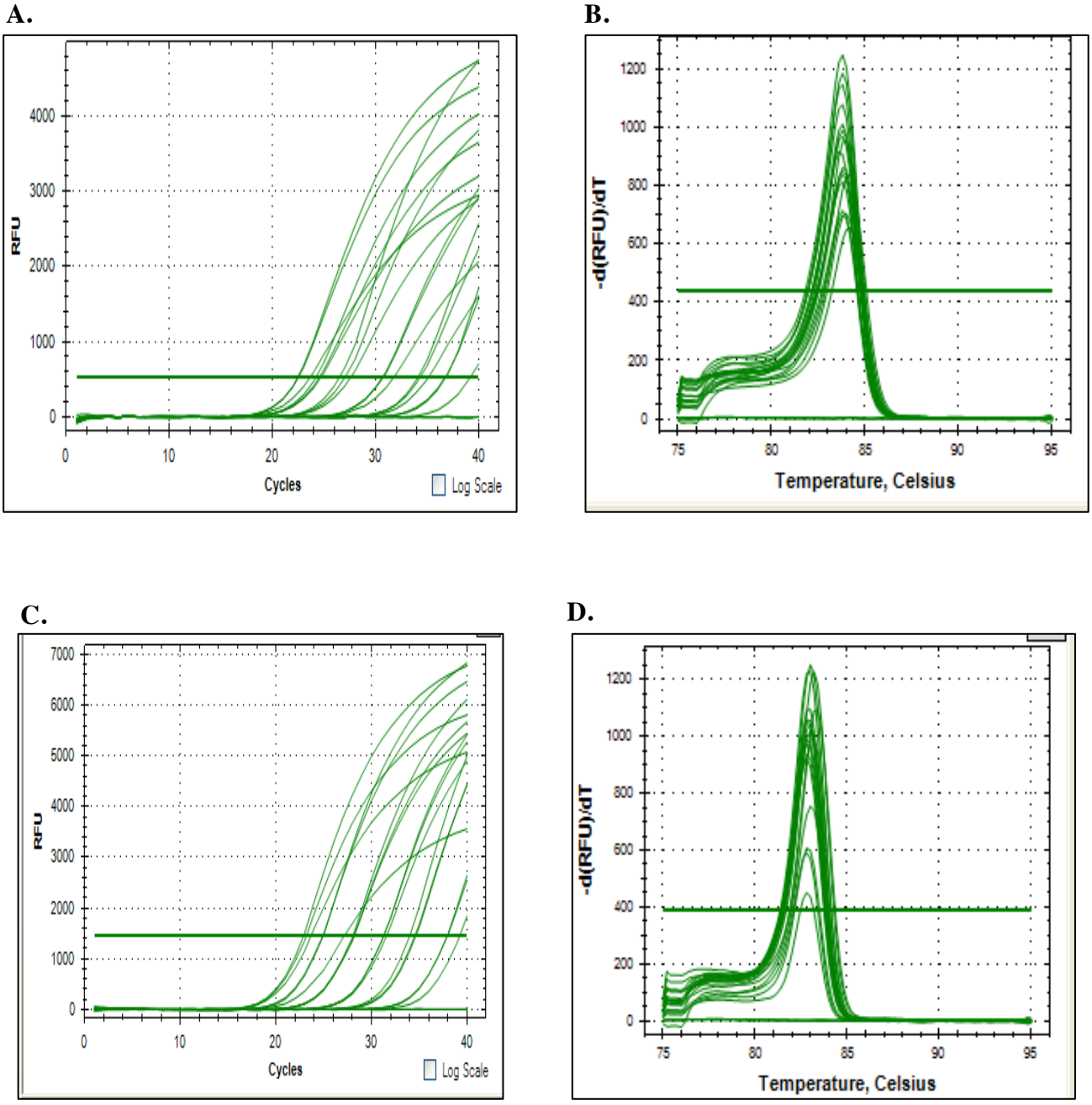


Figure 13. Examples of Reference Gene experimental standard and melt curves. A. *18SrRNA standard curve*. Representative standard curve using PND 5 BPA 50 cDNA experimental sample amplified with 18SrRNA reference gene primer. Satisfactory Efficiency (E=106.6%) and Correlation Coefficient (R^2=0.99) values were generated. **B.** *18SrRNA melt curve*. A single melt peak indicates amplification of a single product and no primer dimers. ***C. GAPDH standard curve.*** Representative PND 5 BPA 500 cDNA experimental sample amplified with GAPDH reference gene primer. Satisfactory Efficiency (E=107%) and Correlation Coefficient (R^2= 0.979) values were generated. **D.** *GAPDH melt curve*. A single melt peak indicates amplification of a single product and no primer dimers.

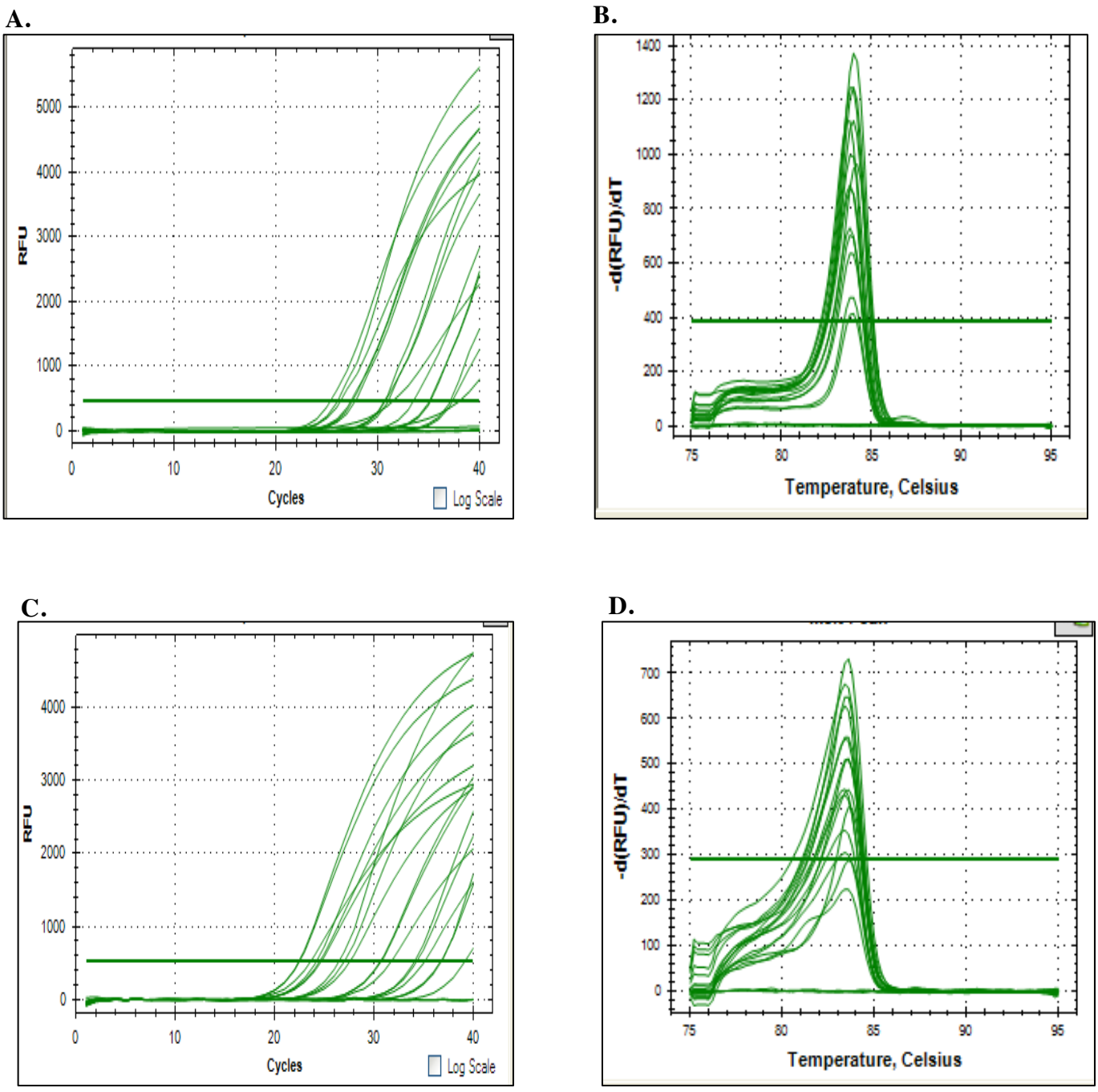


Figure 14. Examples of Reference Gene experimental standard and melt curves. A. *Glucocorticoid receptor (GR) standard curve*. Representative standard curve using PND 5 BPA 500 cDNA experimental sample amplified with GR target gene primer. Satisfactory Efficiency (E=100.3%) and Correlation Coefficient (R^2= 0.981) values were generated. **B.** *GR melt curve*. A single melt peak indicates amplification of a single product and no primer dimers. **C.** *18SrRNA standard curve***.** Representative PND 15 BPA 5 experimental sample was amplified with the 18SrRNA gene primer. Satisfactory Efficiency (E= 98.2%) and Correlation Coefficient (R^2= 0.979) values were generated. **D.** *18SrRNA melt curve***.** A single melt peak indicates amplification of a single product and no primer dimers.

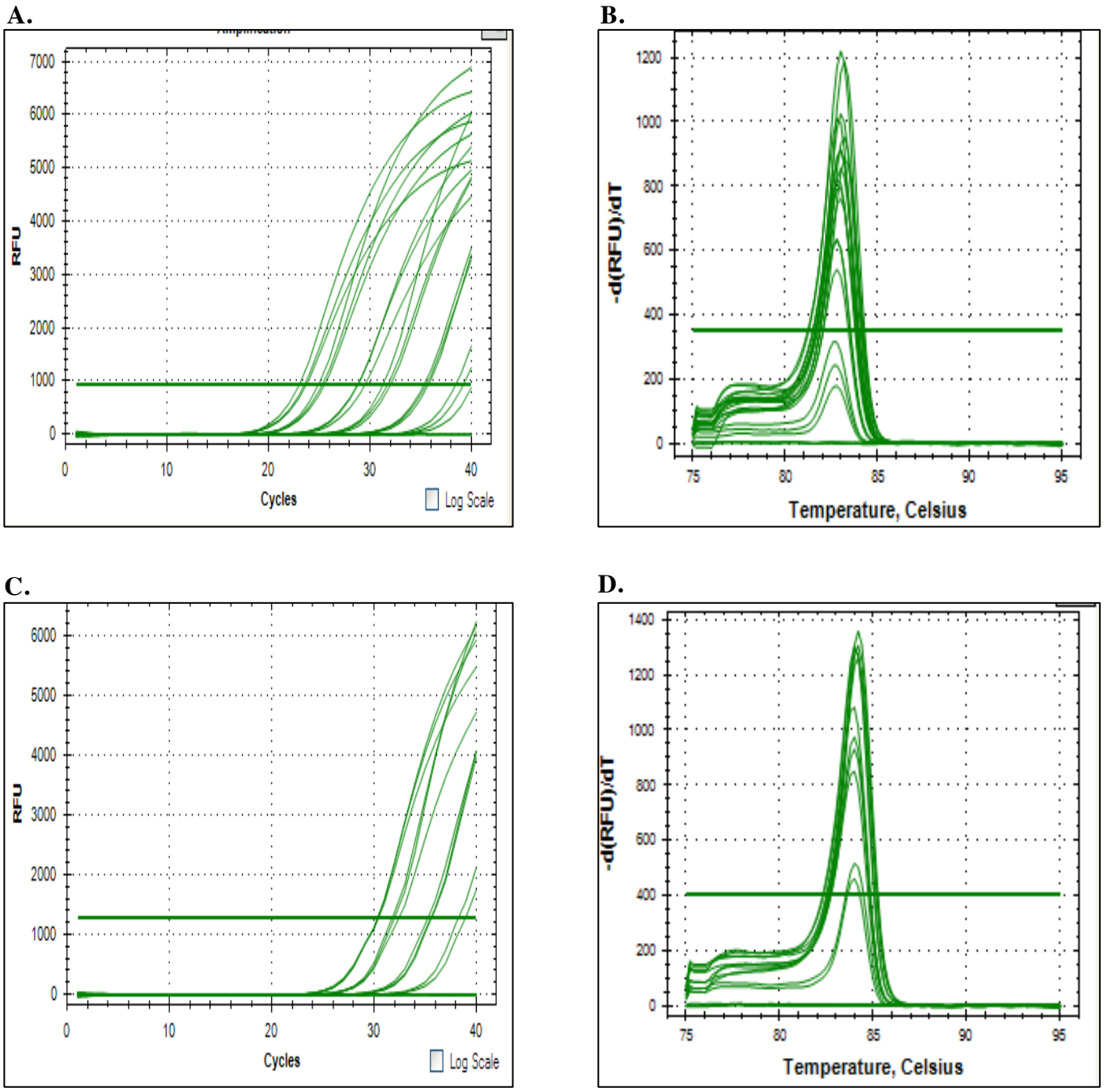


Figure 15. Examples of Reference Gene experimental standard and melt curves. A. *GAPDH standard curve***.** Representative standard curve using PND 15 Vehicle cDNA experimental sample amplified with GAPDH reference gene primer. Satisfactory Efficiency (E= 98.1 %) and Correlation Coefficient (R^2:=0.99) values were generated. **B.** *GAPDH melt curve*. A single melt peak indicates amplification of a single product and no primer dimers. **C.** *GR Standard Curve*. Representative PND 15 DES experimental sample was amplified with the GR target gene primer. Satisfactory Efficiency (E=101.9 %) and Correlation Coefficient (R^2= 0.989) values were generated. **D.** *GR melt curve*. A single melt peak indicates amplification of a single product and no primer dimers.

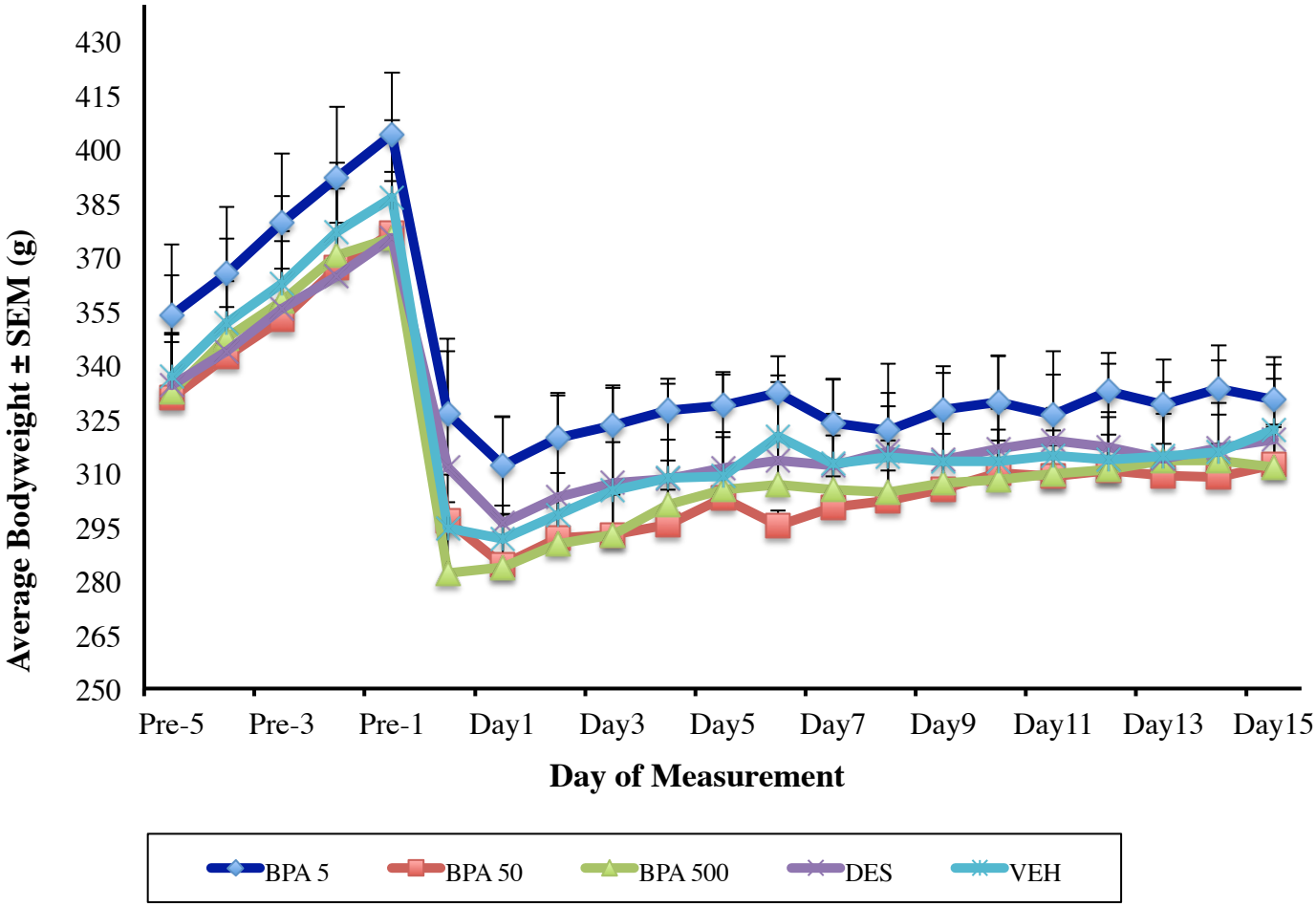


Figure 16. Dam Bodyweight (BW) Progression from Gestation to Postnatal Day 15. Each data point per day represents the average daily dam weight per treatment group. The groups included all dams irrespective of exposure ranges. N values represent the number of dams per treatment group: VEH n=5, DES n=6, BPA 5 n=5, BPA 50 n=5, BPA 500 n=6. Error bars represent the standard error of mean dam BW. Dam weights peaked consistently for all treatment groups immediately prior to birth. There was no significant interaction between Treatment and Time (2-way ANOVA, $F(22,114)=0.894$, $p>0.05$). Treatment did not influence dam BW progressions(1-way ANOVA, $F(4,21)=0.511$, $p>0.05$). Time was a significant influence on dam BW progressions (1-way ANOVA, $F(5,114)=4.24$, $p<0.05$).

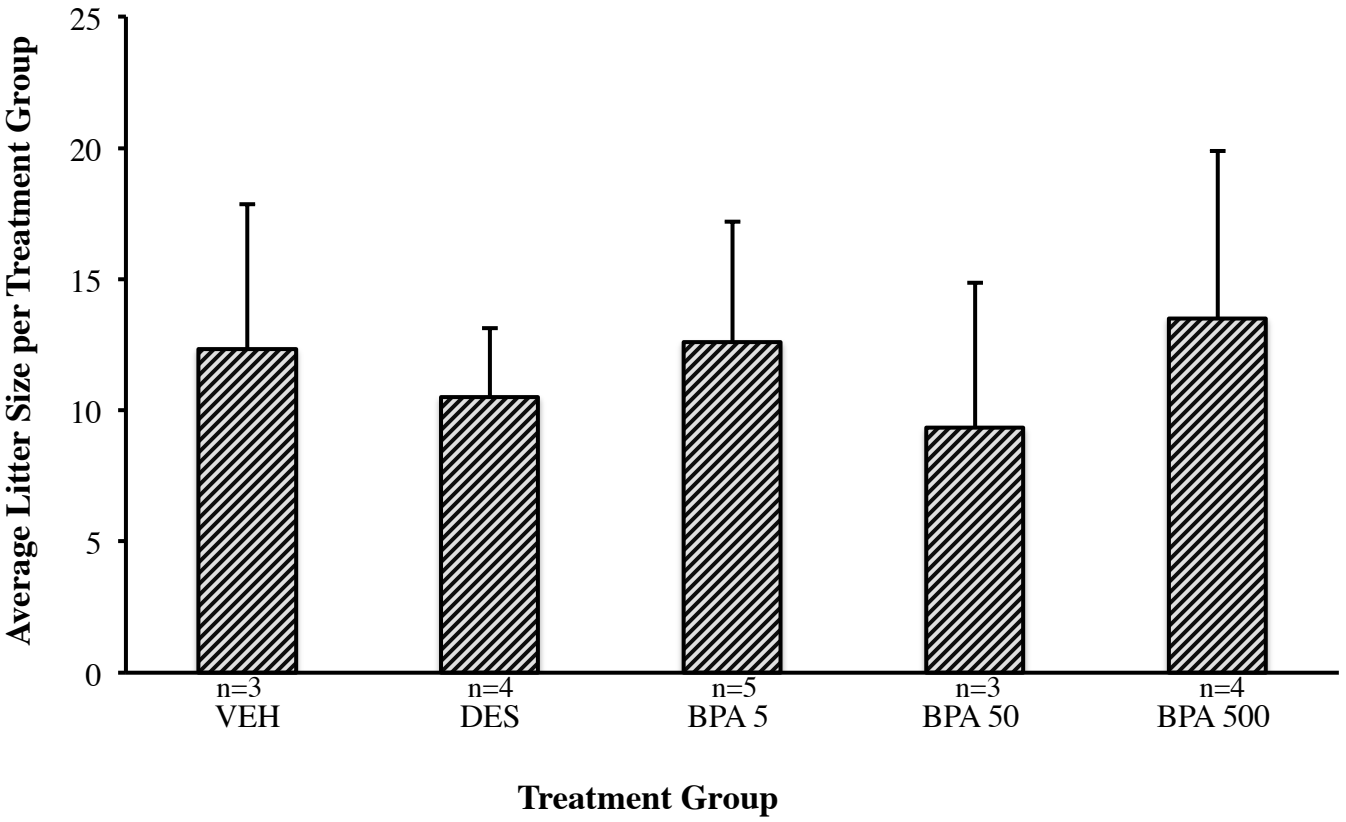


Figure 17. Average Litter Size per Treatment Group. Shown, average litter size per treatment group. N values represent the number of litters per treatment group. Error bars represent the standard error of Litter Size. A Chi-square test of independence conducted on the average values indicated that litter sizes were not significantly different between treatment groups: X^2 (4,4) = 0.000, $p>0.05$. Treatment duration was between 9-11 days of gestational BPA or DES exposure.

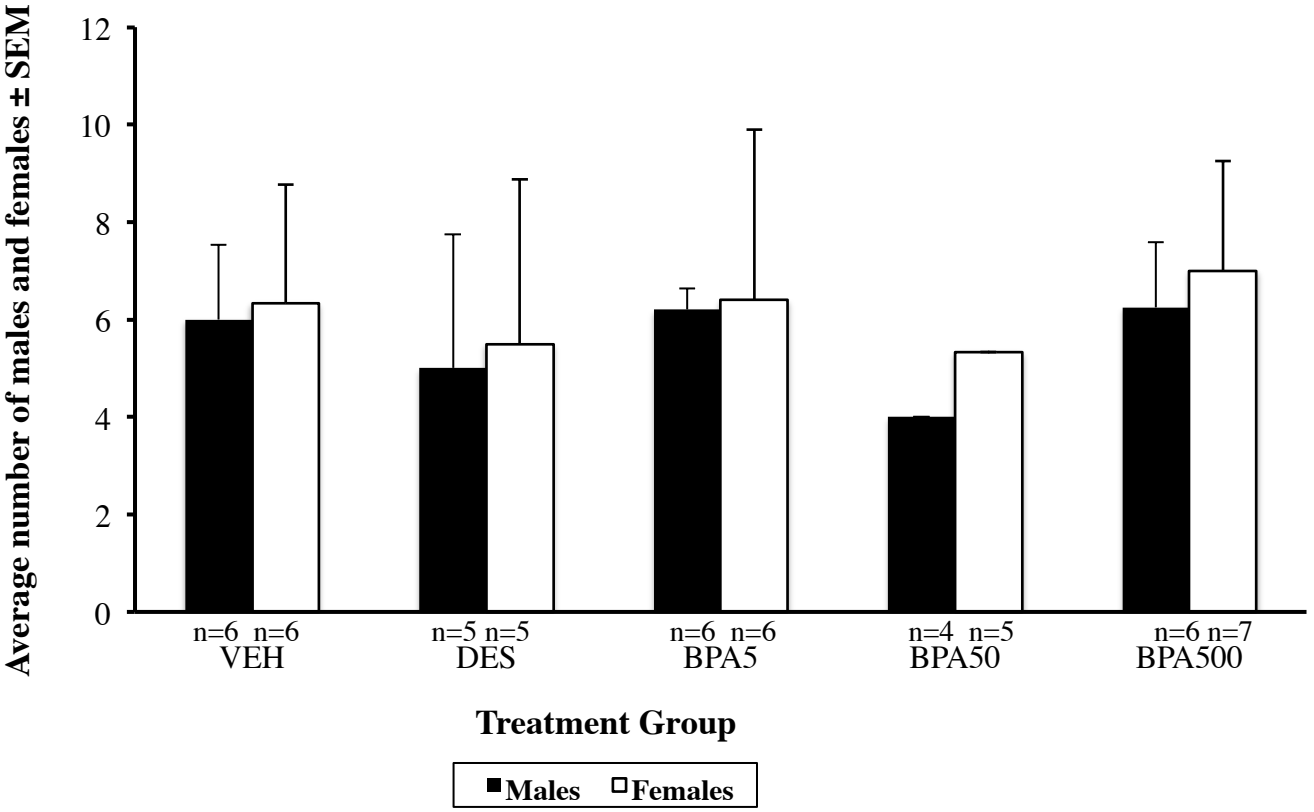
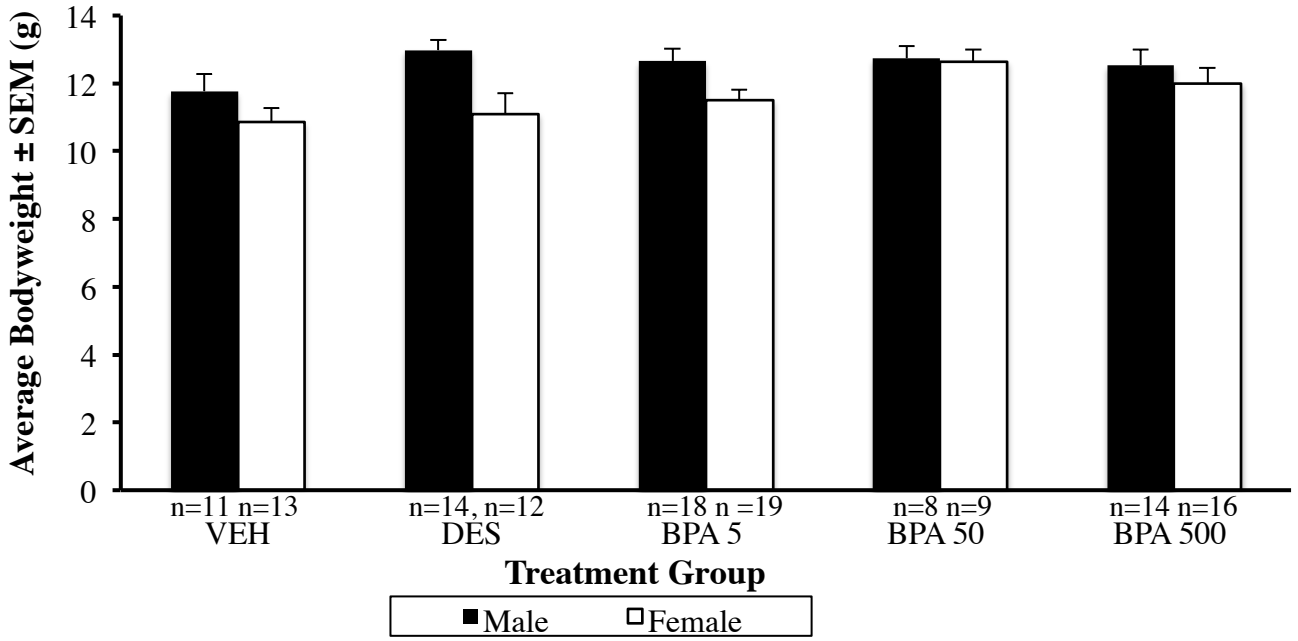


Figure 18. Average Sex Distribution per Treatment Group. Shown, the average number of male and female pups born in each treatment group. N values represent the average number of male and female pups born in each treatment group, obtained by combining the total number of pups (per sex) across all litters in each specific treatment group and calculating this average. Error bars represent standard error of male and female sex distribution means per treatment group. A Chi-square goodness of fit test indicated that sex distribution was not significantly different between the treatment groups: X^2 (4, N = 58) = 0.101, $p > 0.1$.

A.



B.

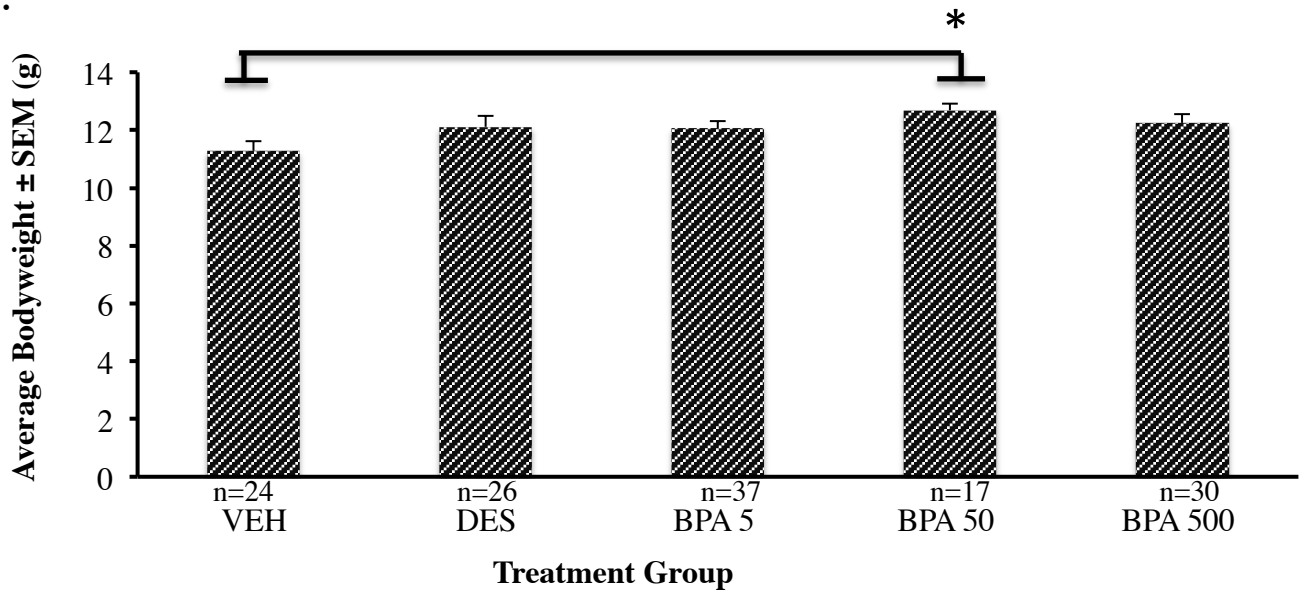


Figure 19. PND 5 Average Bodyweights (BW). Graphs represent the average BW calculated from raw BW values. N values represent the number of animals that were weighed and sacrificed on PND 5 per treatment group for both graphs. Error bars represent the standard error of mean BW values for both graphs. Statistical analysis were conducted on square root of corrected BW: square root of (BW/litter size)= BW** for both graphs**. A. PND 5 Average BW per Sex per Treatment Group**. Sex was not a significant influence on pup BW** (1-way ANOVA, $F(1,124)= 1.838$, $p>0.05$). **B. PND 5 Average BW per Treatment Group**. Treatment significantly affected BW** (1-way ANOVA, $F(4,124)=3.817$, $p<0.05$), and the BPA 50 treatment group was significantly heavier compared to Vehicle pups (Dunnett's post-hoc, $p<0.05$).
* indicates $p<0.05$.

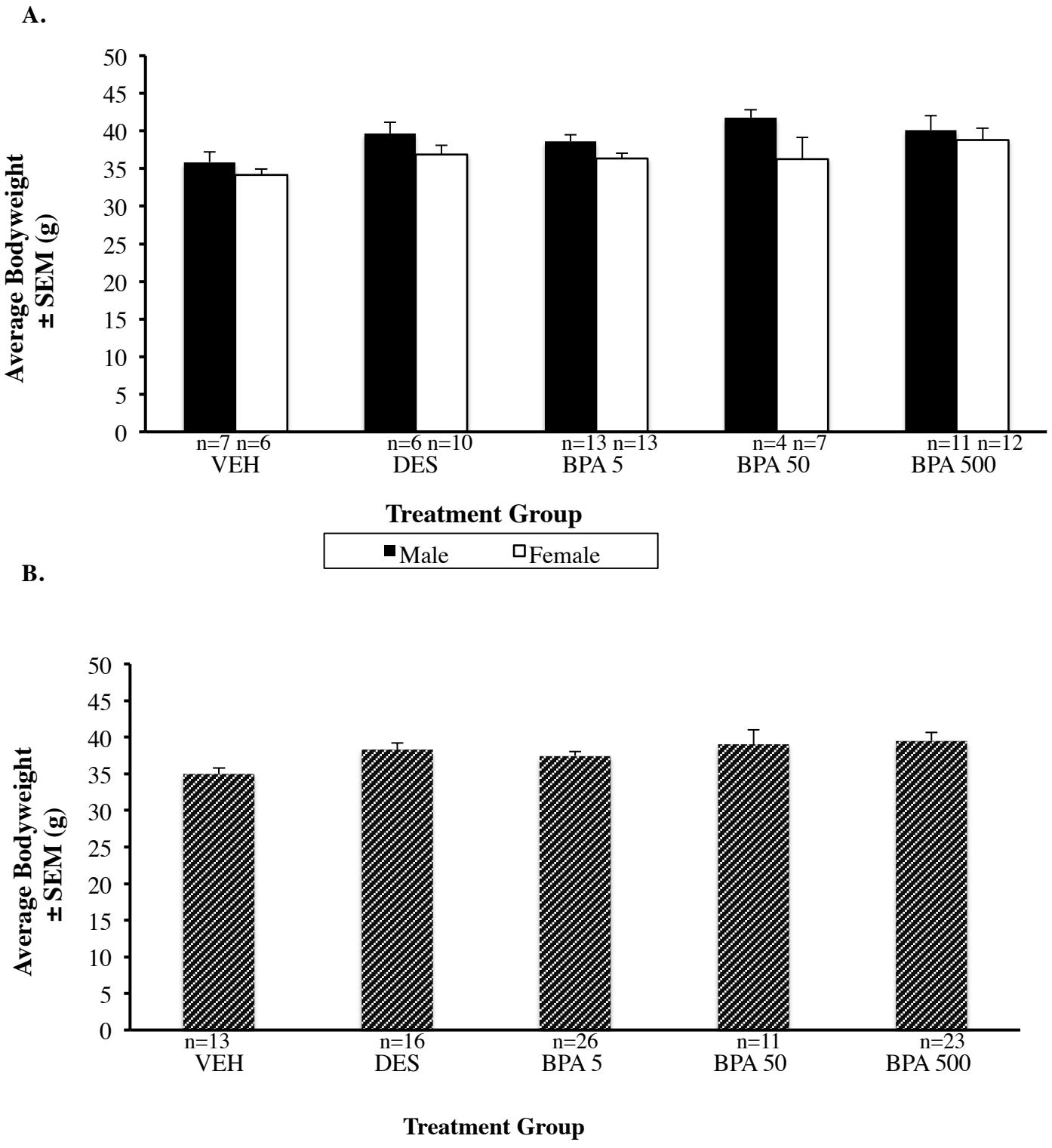


Figure 20. **PND 15 Average Bodyweights (BW).** Graphs represent the average BW calculated from raw BW values. N values represent the number of animals that were weighed and sacrificed on PND 15. Error bars represent the standard error of mean BW values for both graphs. Statistical analyses were conducted on corrected BW data: (BW/litter size) = BW* for both graphs. **A. PND 15 Average BW per Sex per Treatment group.** Sex did not significantly influence corrected pup BW*: (1-way ANOVA $F(1,79)= 2.410$, $p>0.05$). **B. PND 15 Average BW per Treatment Group.** Treatment did not significantly affect pup BW* ($F(4,79)=2.476$, $p>0.05$).

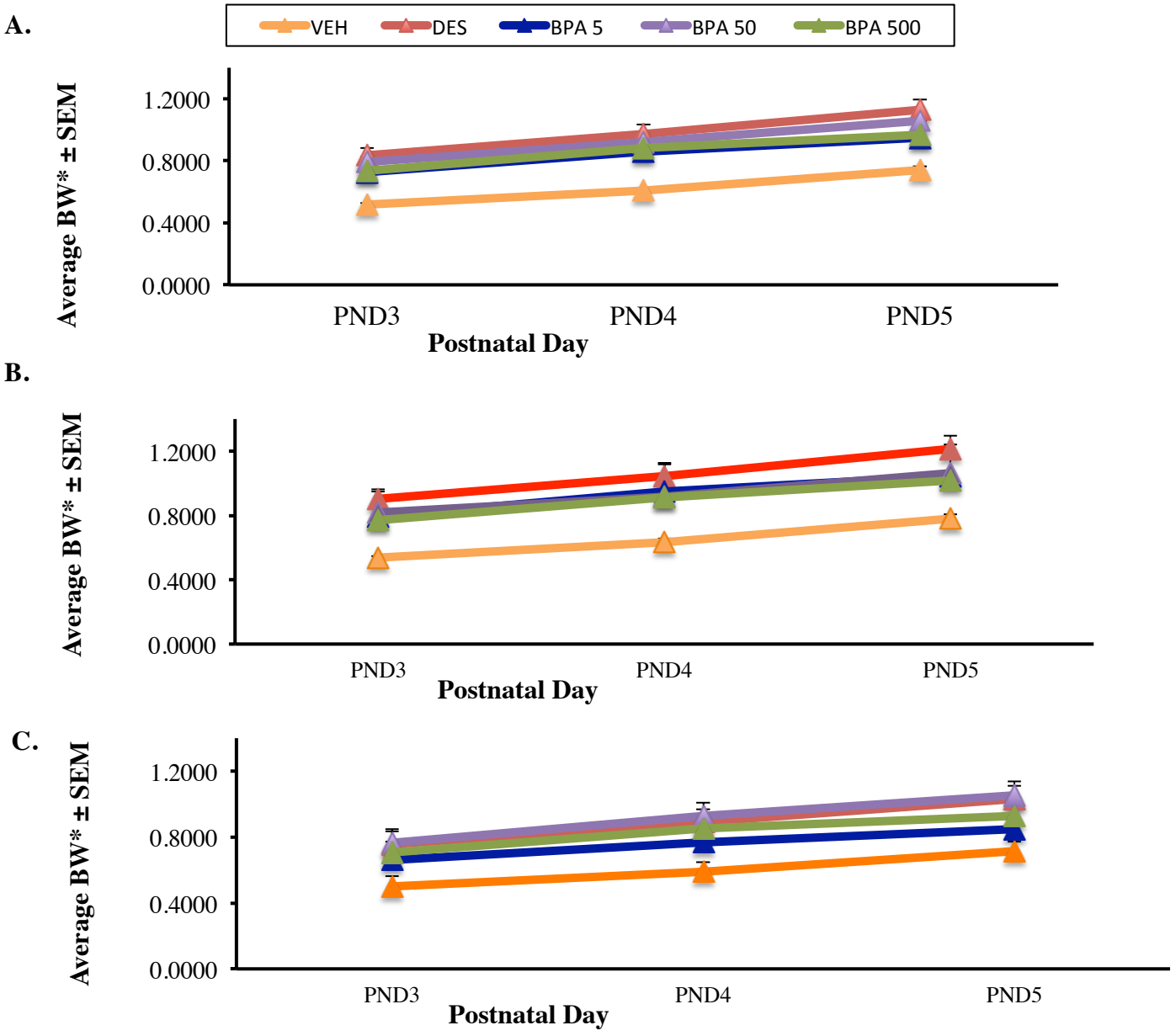


Figure 21. PND 3 – PND 5 Bodyweight (BW) Progressions. Total of male, female corrected BW progressions (BW/litter size): BW* are displayed. Error bars represent standard error of BW* for all graphs. **A. Total BW Progressions from PND 3 to PND 5.** Total of male, female BW* are displayed per treatment group per day. 2-factor analysis reported no significant interaction between Sex and Time on pup BW* progression (2-way ANOVA, $F(2,174)=1.718$, $p>0.05$). However, Treatment and Time did significantly affect pup BW* progression (2-way ANOVA, $F(8,174)= 3.602$, $p=0.001$). The main effect of Time had a significant effect on pup BW* progression (1-way ANOVA, $F(2,174)=3.342$, $p=0.038$). N values represent the total number of males and females: VEH n=20, DES n=26, BPA 5 n=14, BPA 50 n=14, BPA 500 n=24. **B. Male BW Progressions from PND 3 to PND 5.** Depiction of BW* progression from PND 3 to PND 5 for male pups sacrificed at PND 5. Sex did not significantly influence pup BW* progression (1-way ANOVA, $F(1,87)= 2.147$, $p>0.05$). N values: VEH n=9, DES n=14, BPA 5 n=7, BPA 50 n=7, BPA 500n=11. **C. Female BW Progressions from PND 3 to PND 5.** Depiction of BW* progression from PND 3 to PND 5 for female pups sacrificed at PND 5. Sex did not significantly influence pup BW* progression (1-way ANOVA, $F(1,87)= 2.147$, $p>0.05$). N values: VEH n=11, DES n=12, BPA 5 n=7, BPA 50 n=7, BPA 500 n=13.

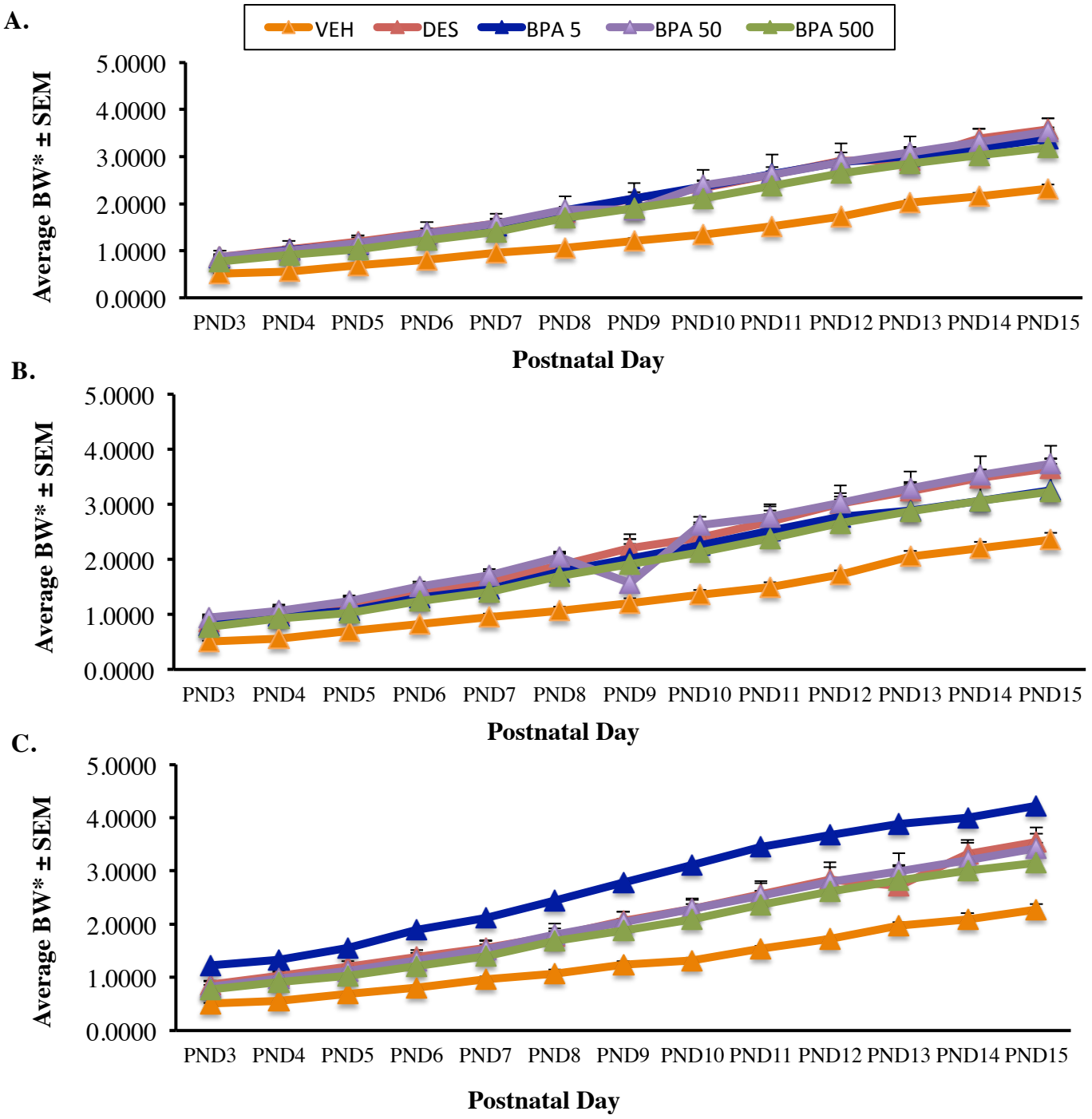


Figure 22. PND 3- PND 15 Bodyweight (BW) Progressions. Total of male, female corrected BW progressions (BW/litter size): BW* are displayed. Error bars represent standard error of BW* for all graphs. **A. Total BW Progressions from PND 3-PND 15.** Total of male, female BW* progressions are displayed per treatment group per day. 2-factor analysis between Sex and Time did not significantly affect pup BW* progression (2-way ANOVA, $F(3,149)=0.824$, $p>0.05$). The factor of Time significantly affected pup BW* progression as expected (1-way ANOVA, $F(3,149)=3.127$, $p=0.025$). N values represent the total number of males and females: VEH n=9, DES n=16, BPA 5 n=8, BPA 50 n=9, BPA 500 n=16.

B. Male Bodyweight Progressions from PND 3-PND 15. Depiction of BW* progression from PND 3 to PND 15 for male pups sacrificed at PND 15. Sex did not significantly affect pup BW* progression (1-way ANOVA, $F(1,47)= 0.025$, $p>0.05$). N values: VEH n=5, DES n=6, BPA 5 n=7, BPA 50 n=3, BPA 500 n=8.

C. Female Bodyweight Progressions from PND 3- PND 15. Depiction of BW* progression from PND 3 to PND 15 for female pups sacrificed at PND 15. N values: VEH n=4, DES n=10, BPA 5 n=1, BPA 50 n= 6, BPA 500 n=8.

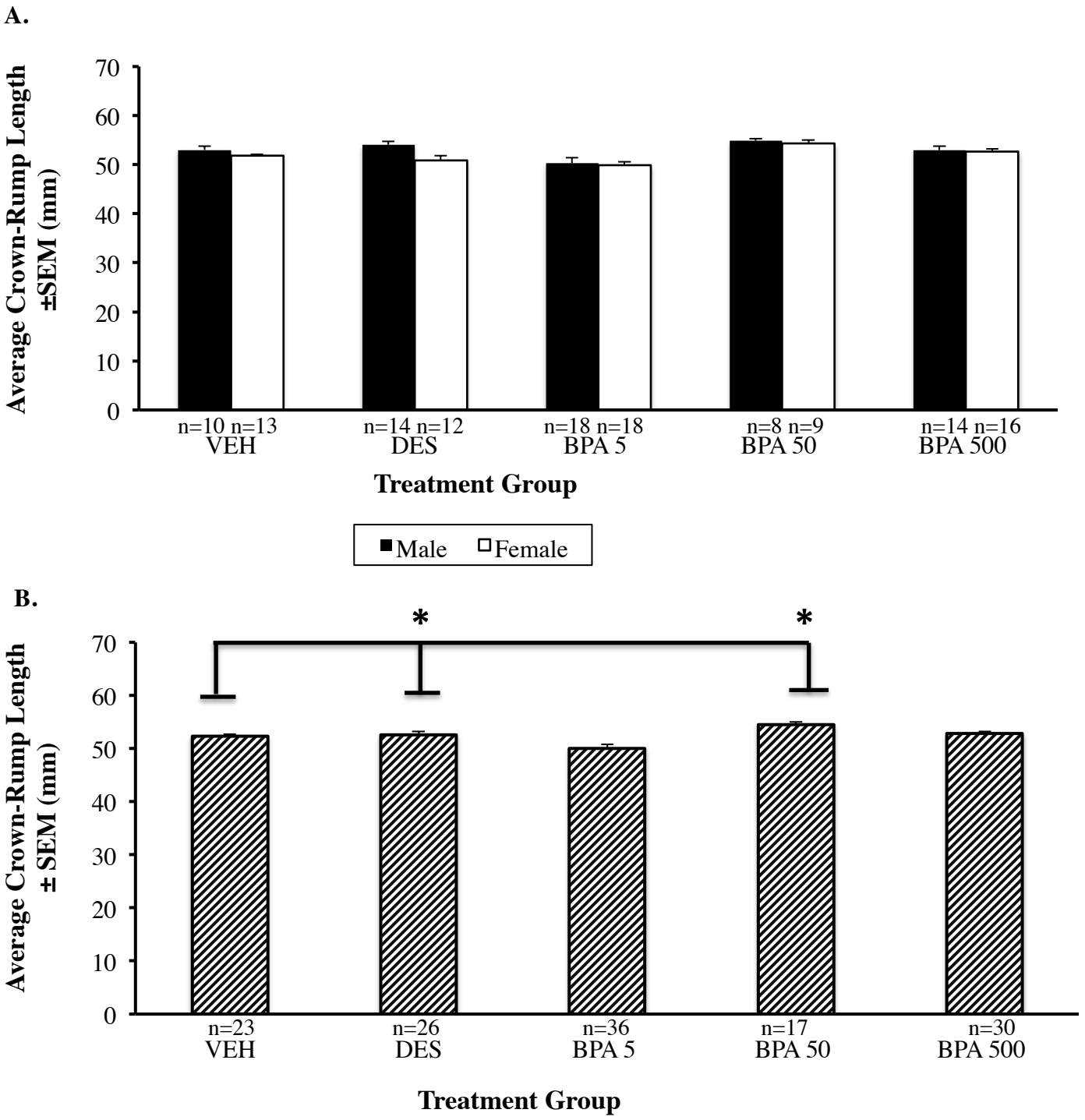


Figure 23. PND 5 Average Crown-Rump (CR) Lengths. Graphs represent the average CR lengths calculated from raw CR values. N values represent the number of animals that were measured and sacrificed on PND 5 for both graphs. Error bars represent the standard error of mean CR lengths for both graphs. Statistical analyses were conducted on square root corrected CR lengths: square root (CR/BW*)= CR** for both graphs. **A. PND 5 Average CR lengths per Sex per Treatment group.** Raw CR length values were separated by sex and averaged per treatment group. Sex did not significantly influence CR** lengths (1-way ANOVA, $F(1,122)= 2.520$, $p>0.05$). **B. PND 5 Average CR lengths per Treatment group**. Treatment significantly influenced CR** lengths (1-way ANOVA, $F(4,122)=3.760$, $p<0.05$), with DES and BPA 50 groups in particular having significantly longer CR** lengths versus the Vehicle pups (Dunnett's post-hoc, $p<0.05$). * indicates $p<0.05$.

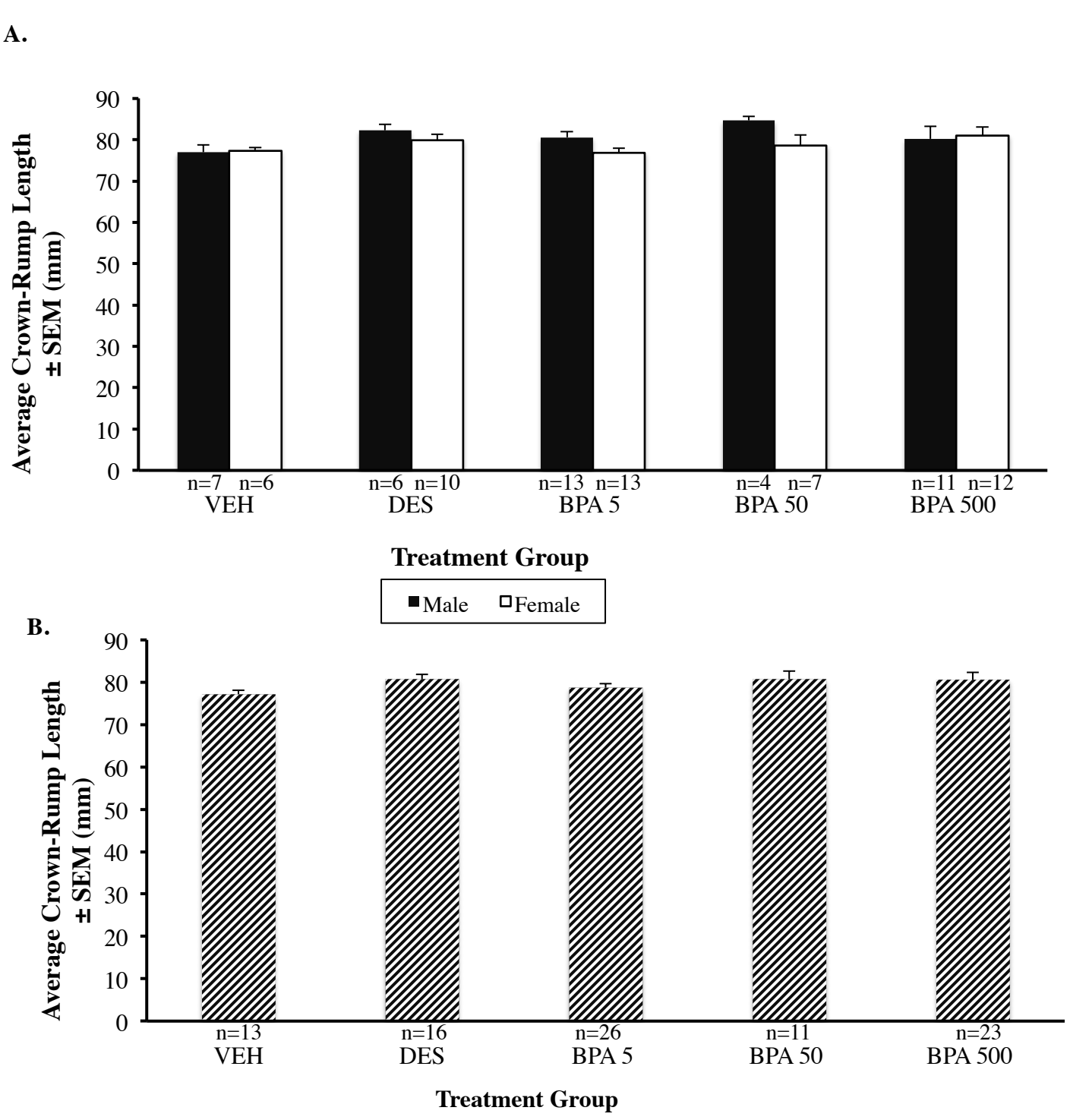
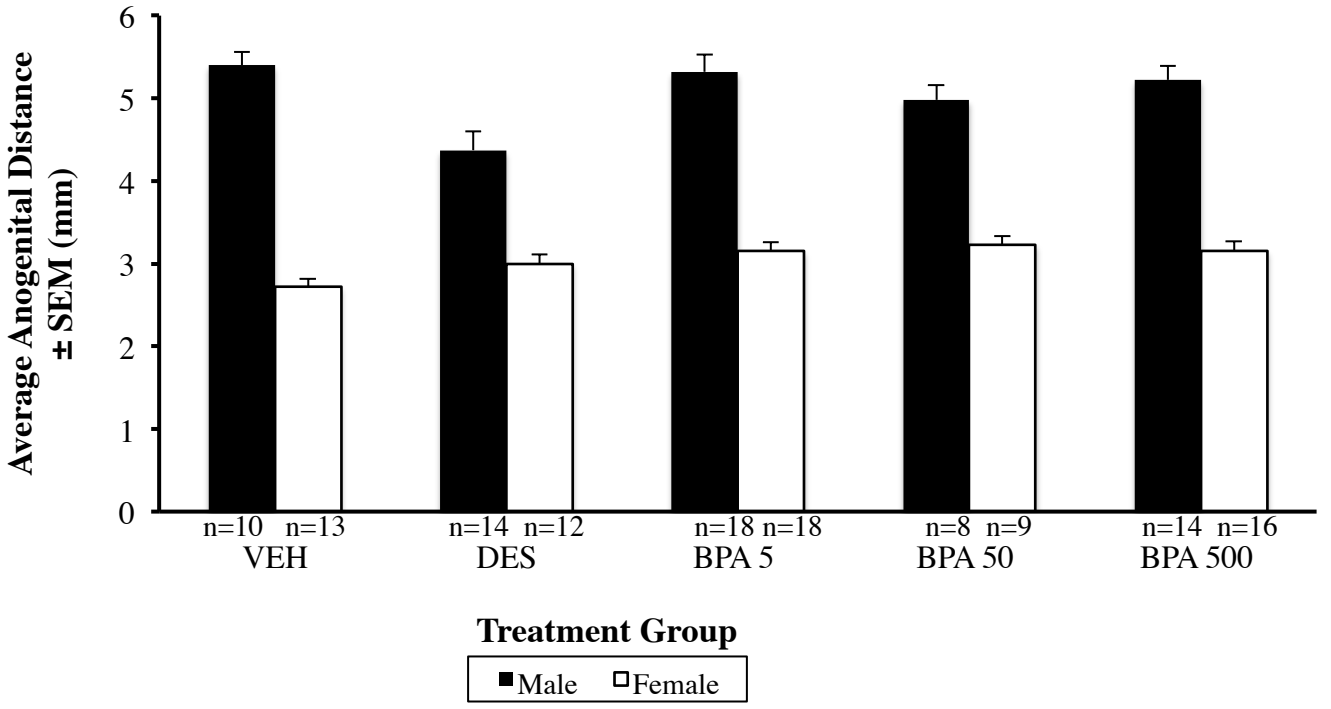


Figure 24. PND 15 Average Crown-Rump (CR) Lengths. Graphs represent the average CR calculated from raw CR values. N values represent the number of animals that were measured and sacrificed on PND 15 for both graphs. Error bars represent the standard error of mean CR lengths for both graphs. Statistical analyses were conducted on corrected CR lengths: (CR/BW*)= CR* for both graphs. **A. PND 15 Average CR lengths per Sex per Treatment group.** Raw CR length values were separated by sex and averaged per treatment group. Sex was not found to influence CR* length (1-way ANOVA sex $F(1,79)= 0.558$, $p>0.05$). **B. PND 15 Average CR lengths per Treatment group.** Treatment significantly influenced CR* length (1-way ANOVA, $F(4,79)= 3.192$, $p<0.05$).

A.



B.

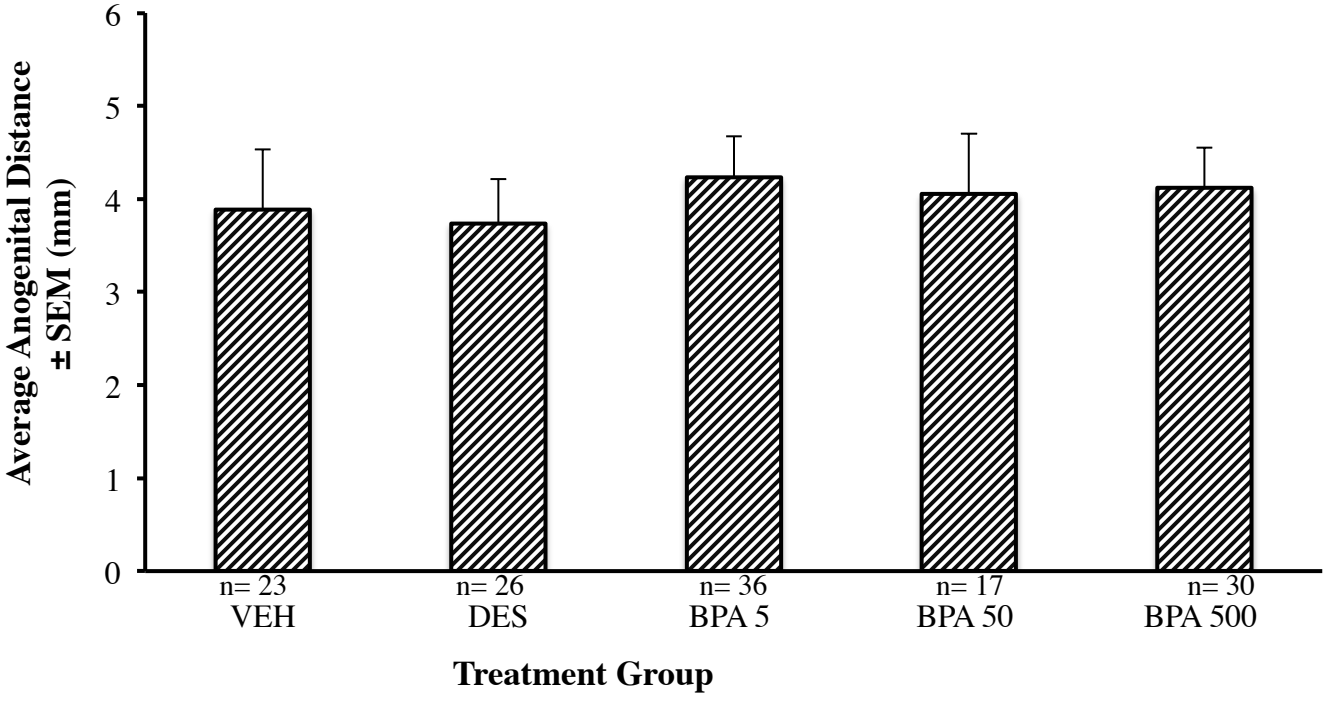


Figure 25. PND 5 Average Anogenital Distances (AGD). Graphs represent the average AGD calculated from raw AGD values. N values represent the number of animals that were measured and sacrificed on PND 5 for both graphs. Error bars represent the standard error of mean AGD for both graphs. Statistical analyses were conducted on square root of corrected AGD: (AGD/CR*)= AGD **. **A. PND 5 Average AGD per Sex per Treatment group.** Sex significantly influenced AGD** (1-way ANOVA, $F(1,122)= 56.439$, $p<0.05$). Overall, males had a longer AGD** than females, as anatomically expected. **B. PND 5 Average AGD per Treatment group.** Treatment did not influence AGD** (1-way ANOVA, $F(4,122)= 1.870$, $p>0.05$).

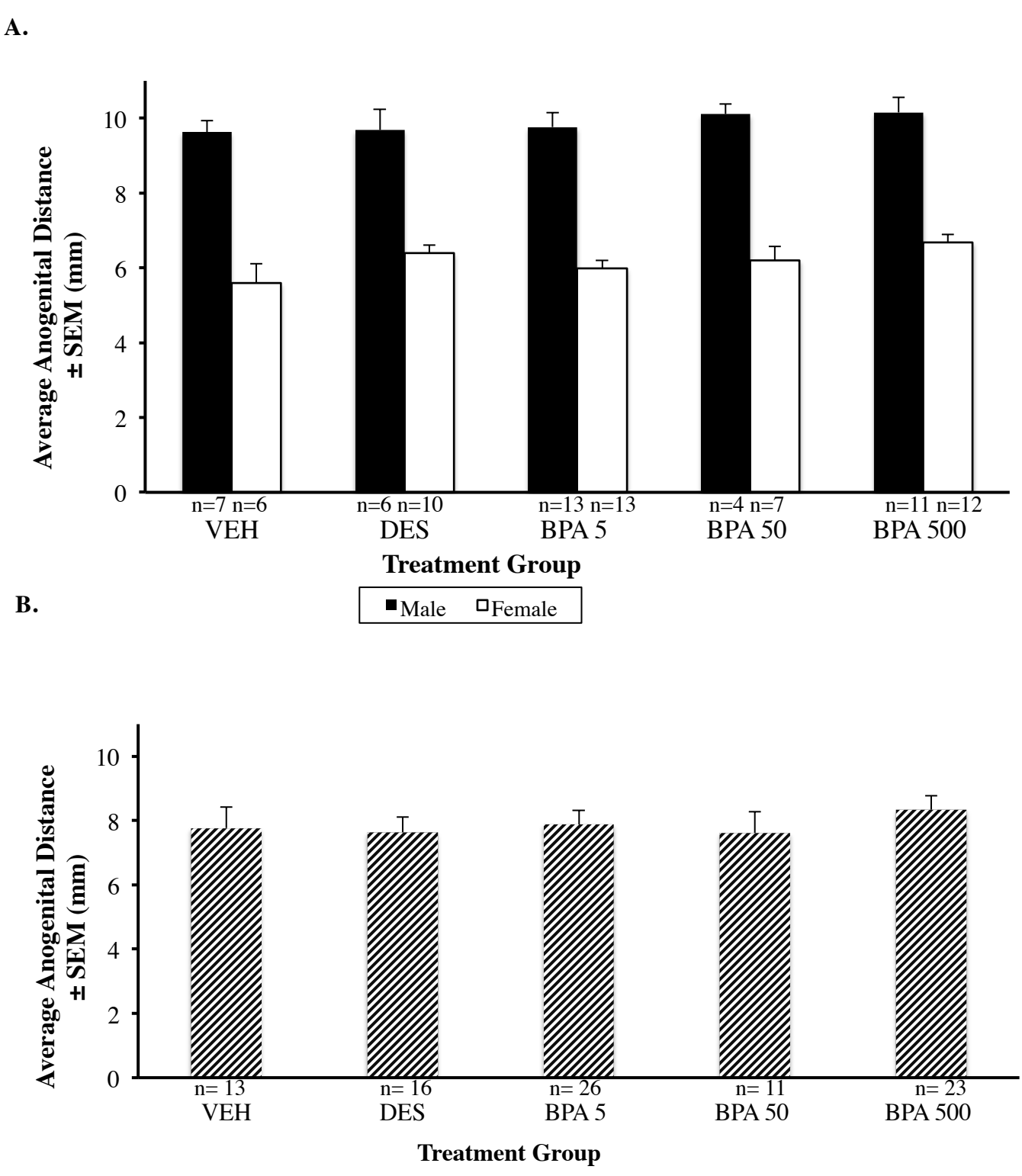


Figure 26. PND 15 Average Anogenital Distances (AGD). Graphs represent the average AGD calculated from raw AGD values. N values represent the number of animals that were measured and sacrificed on PND 15 for both graphs. . Error bars represent the standard error of mean AGD for both graphs. Statistical analyses were conducted on square root of corrected AGD: (AGD/CR*)= AGD ** for both graphs **A. PND 15 Average AGD per Sex per Treatment Group.** Sex significantly influenced AGD** (1-way ANOVA, $F(1,79)= 44.59$, $p<0.05$). Overall, males had a longer AGD than females, as anatomically expected. **B. PND 15 Average AGD per Treatment Group.** Treatment did not influence AGD (1-way ANOVA, $F(4,79)= 2.291$, $p>0.05$).

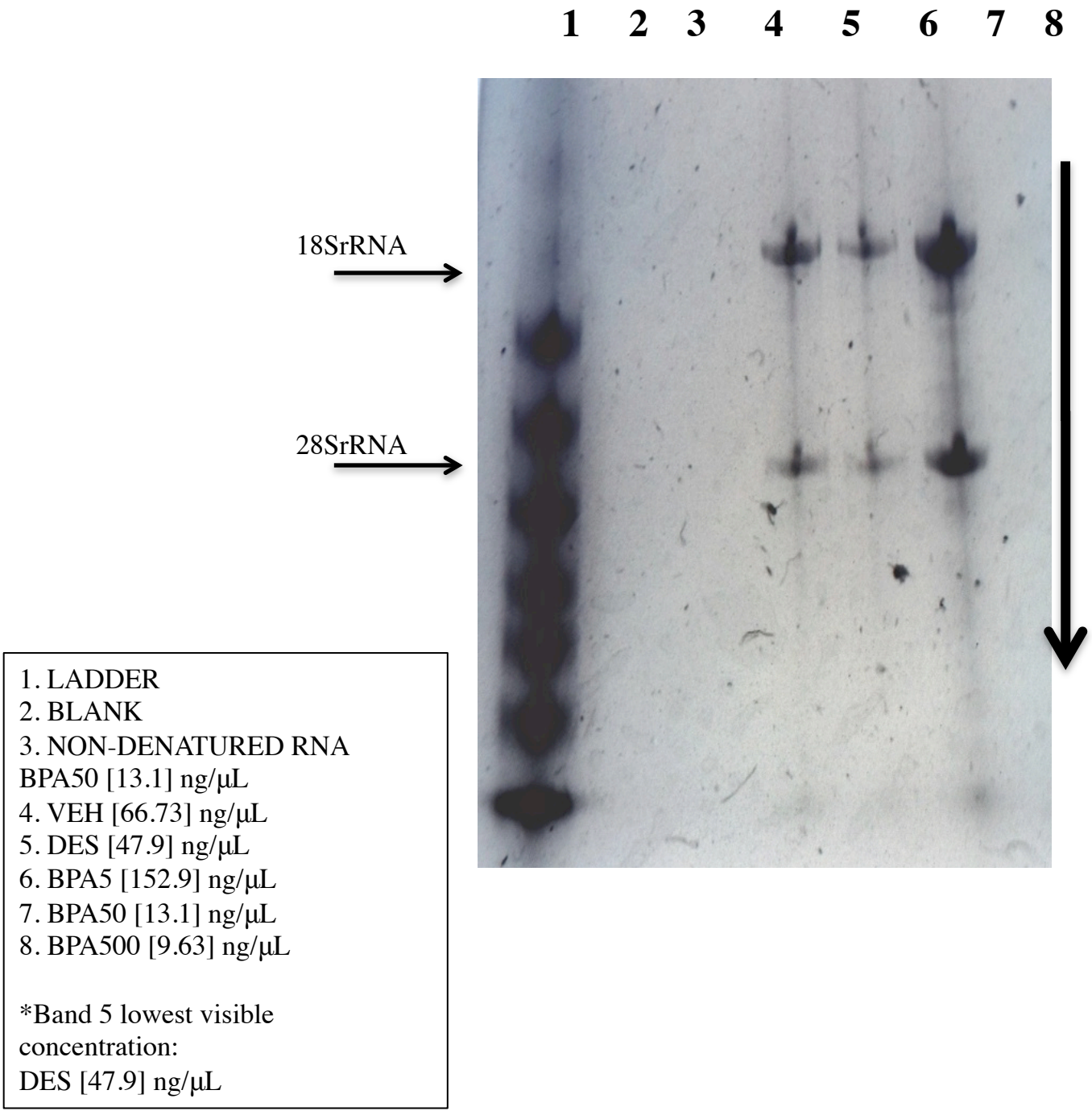
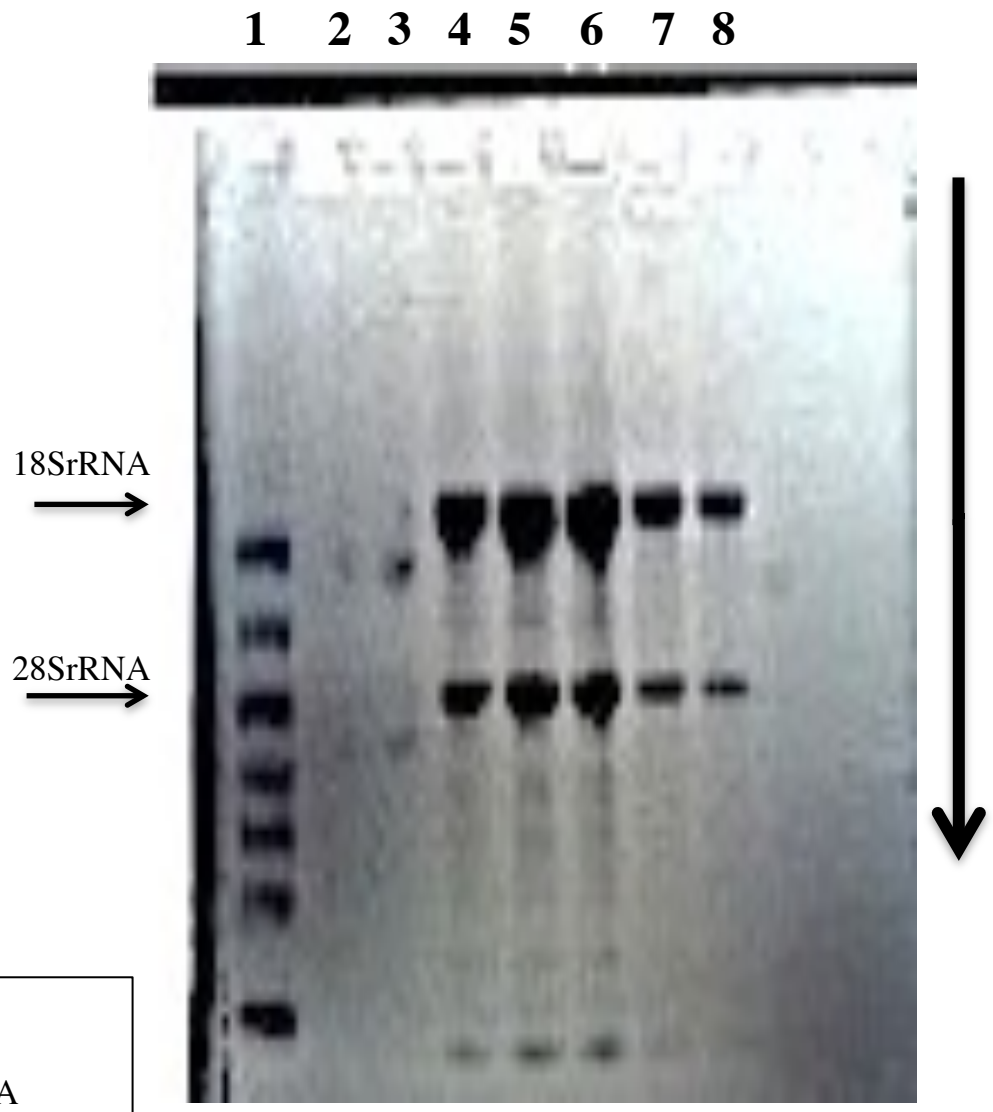
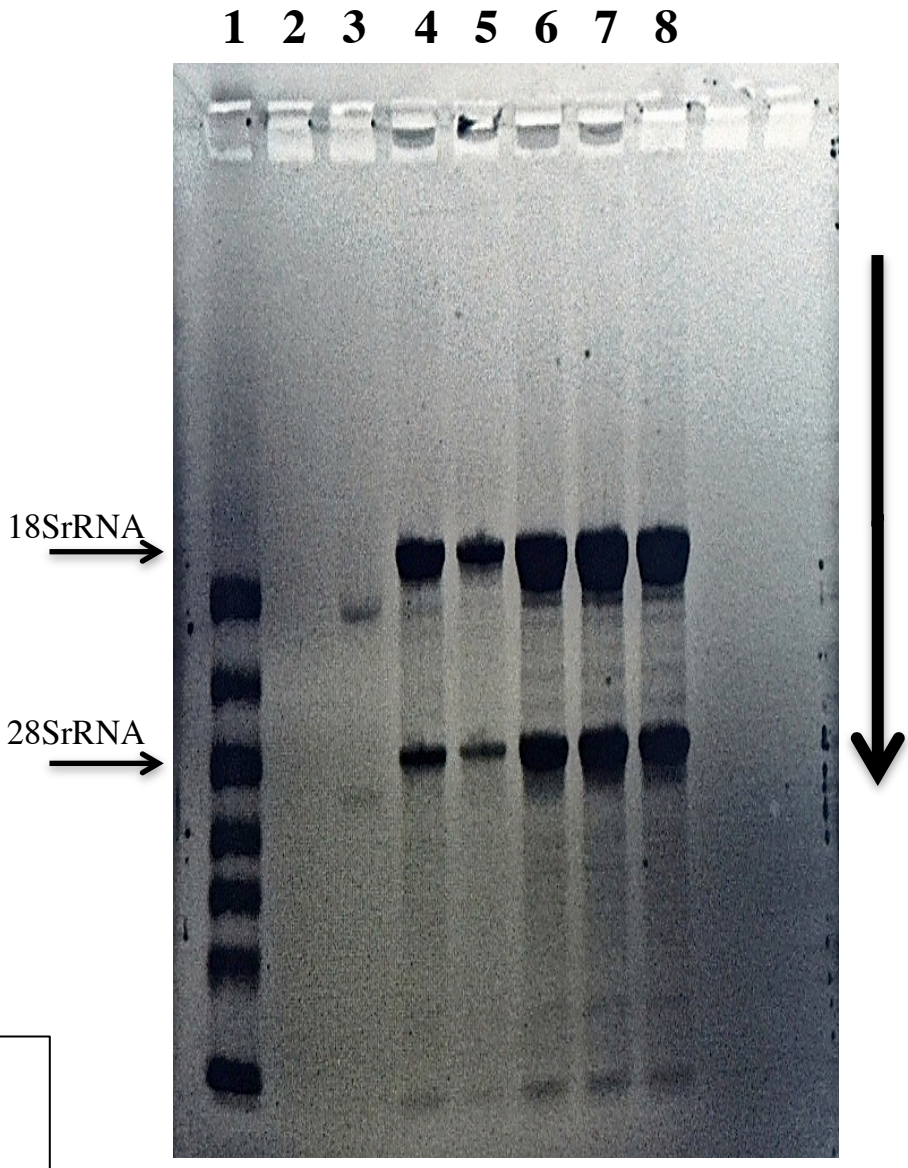


Figure 27. PND5 Low Concentration Range Gel. Male samples with RNA concentrations in the range of [9.63-152.9] ng/uL. The 2:1 ratio of 28SrRNA: 18SrRNA is visible indicating RNA is intact.



1. LADDER
2. BLANK
3. NON-DENATURED RNA BPA500 [368.1] ng/µL
4. VEH [507.7] ng/µL
5. DES [867.3] ng/µL
6. BPA 5 [798.3] ng/µL
7. BPA 50 [579.5] ng/µL
8. BPA 500 [368.1] ng/µL

F**igure 28**. **PND 5 High Concentration Range Gel.** Male samples with RNA concentrations in the range of [368.1-867.3] ng/uL. The 2:1 ratio of 28SrRNA:18SrRNA is visible indicating RNA is intact.



1. LADDER
2. BLANK
3. NON-DENATURED RNA
 VEH [211.8] ng/µL
4. VEH [211.8] ng/µL
5. DES [206.1] ng/µL
6. BPA 5 [275.9] ng/µL
7. BPA 50 [476.6] ng/µL
8. BPA 500 [449.7] ng/µL

Figure 29. PND 15 High Concentration Range Gel. Male samples with RNA concentrations in the range of [206.1-476.6] ng/uL. The 2:1 ratio of 28SrRNA: 18SrRNA is visible indicating RNA is intact.

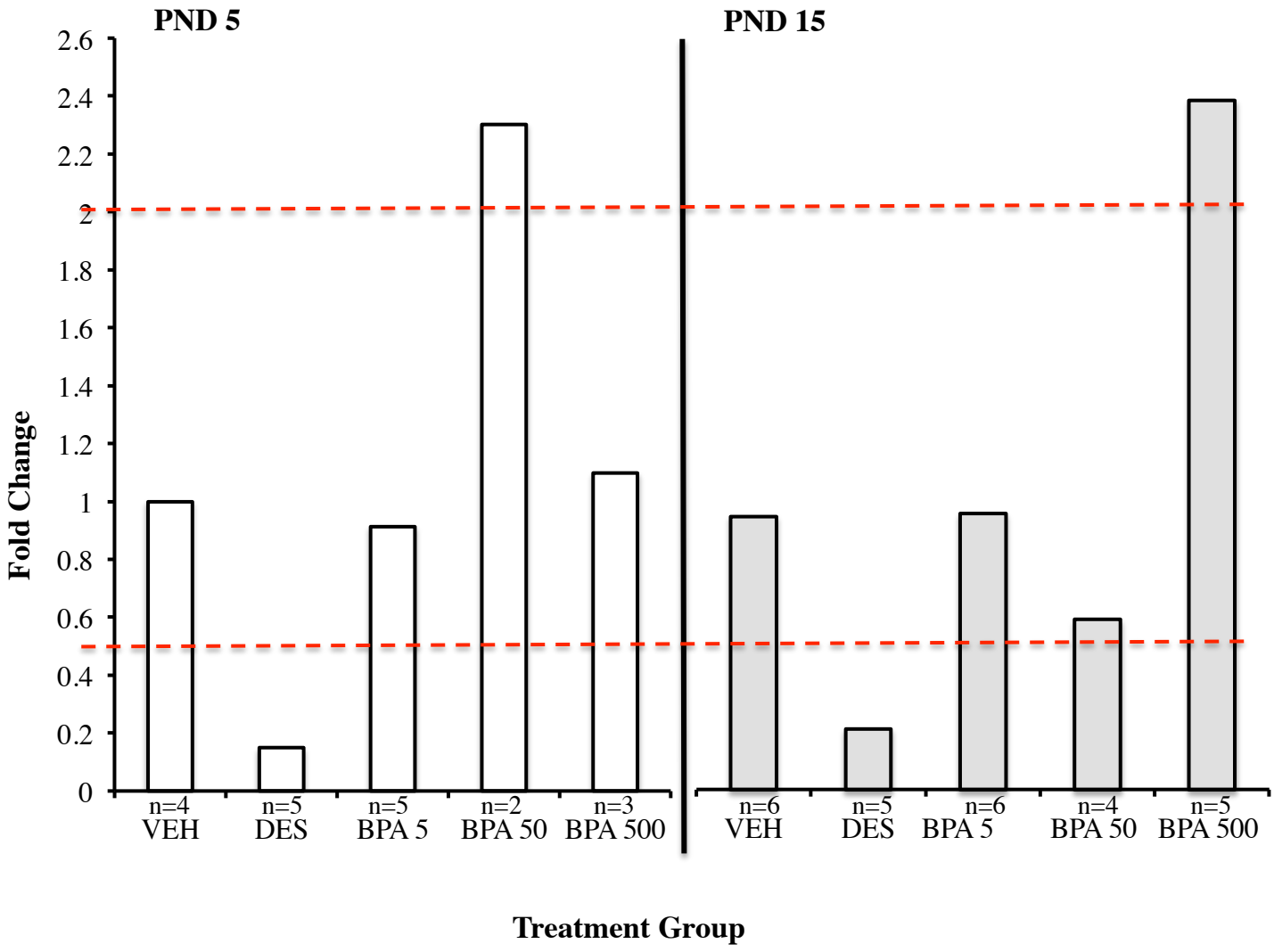
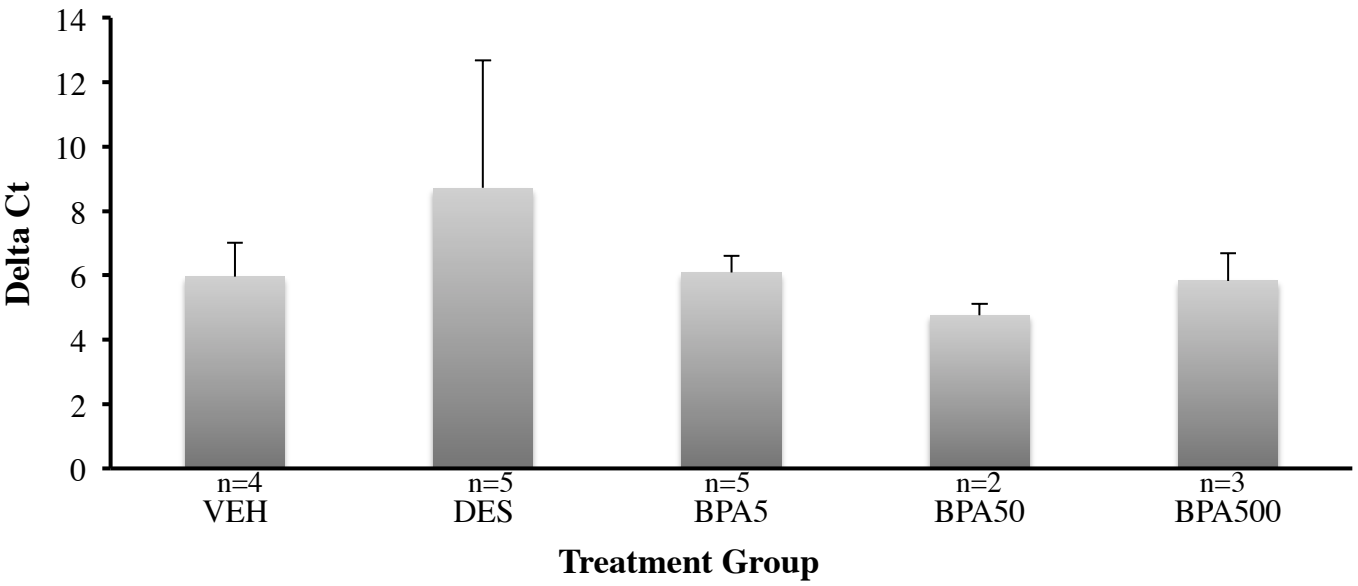


Figure 30. GR Fold Changes Calculated by ΔΔCt Method. qPCR was used to amplify GR, normalized to GAPDH reference gene. Fold changes are presented for both PND 5 and PND 15 male pups. N values represent the number of male adrenal gland samples used for the calculation in each treatment group, per timepoint. Downregulated fold changes (<0.5) were observed in the DES group in both PND 5 (fold change= 0.1488) and PND 15 (fold change= 0.2223) pups. Upregulated fold changes (>2.0) were observed in the PND 5 BPA 50 pups (fold change=2.302) and the PND 15 BPA 500 pups (fold change= 2.522).

A.



B.

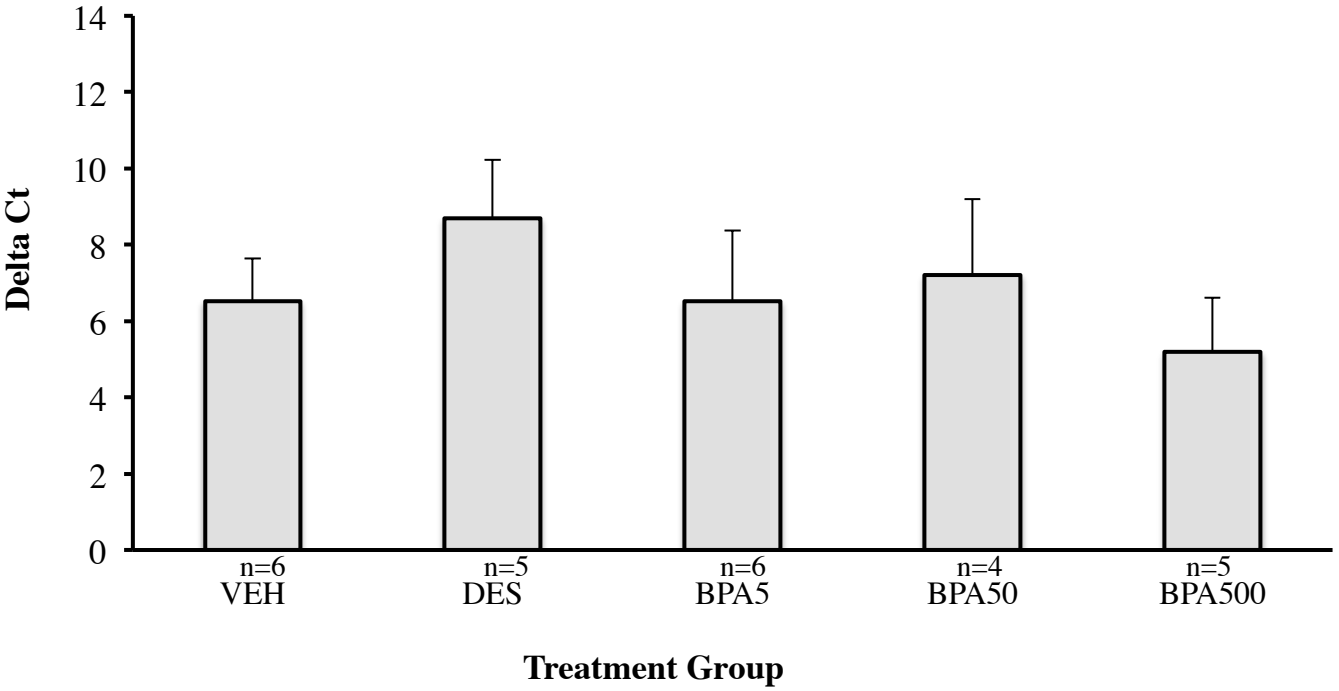


Figure 31. GR Average Delta Ct values. Average delta Ct values Ct^{GR}-(GAPDH) are presented for both **A.** PND 5 and **B.** PND 15 samples for GR gene expression normalized to GAPDH. N values represent the number of male adrenal gland samples used for the calculation in each treatment group, per timepoint. The GR ΔCt values for PND 5 pups (Kruskal-Wallis test: H(4,N=19)=8.274739, p=0.0820) and PND 15 pups (Kruskal-Wallis test: H(4, N=26)=2.716524, p= 0.6063) were not significantly affected by Treatment.

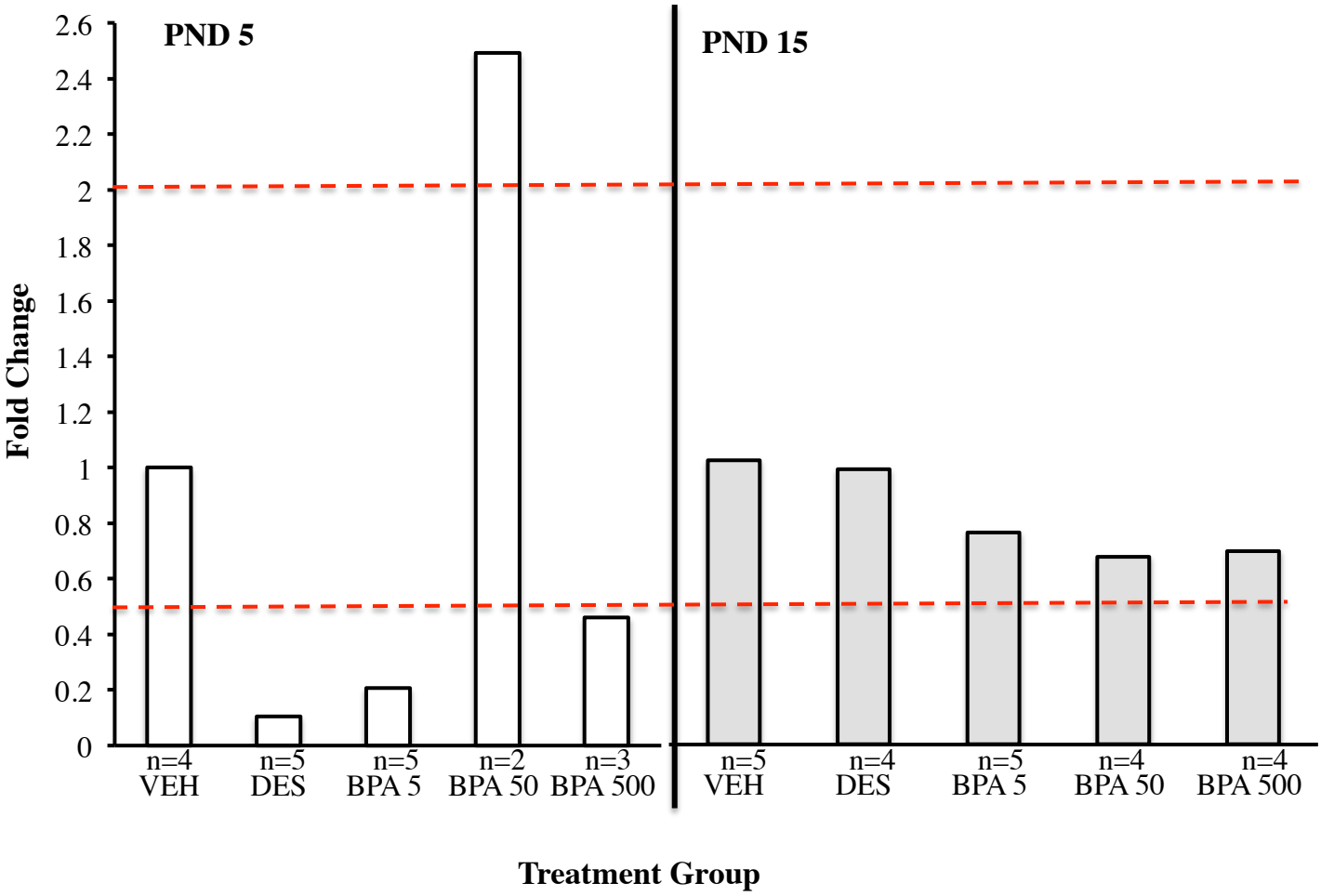
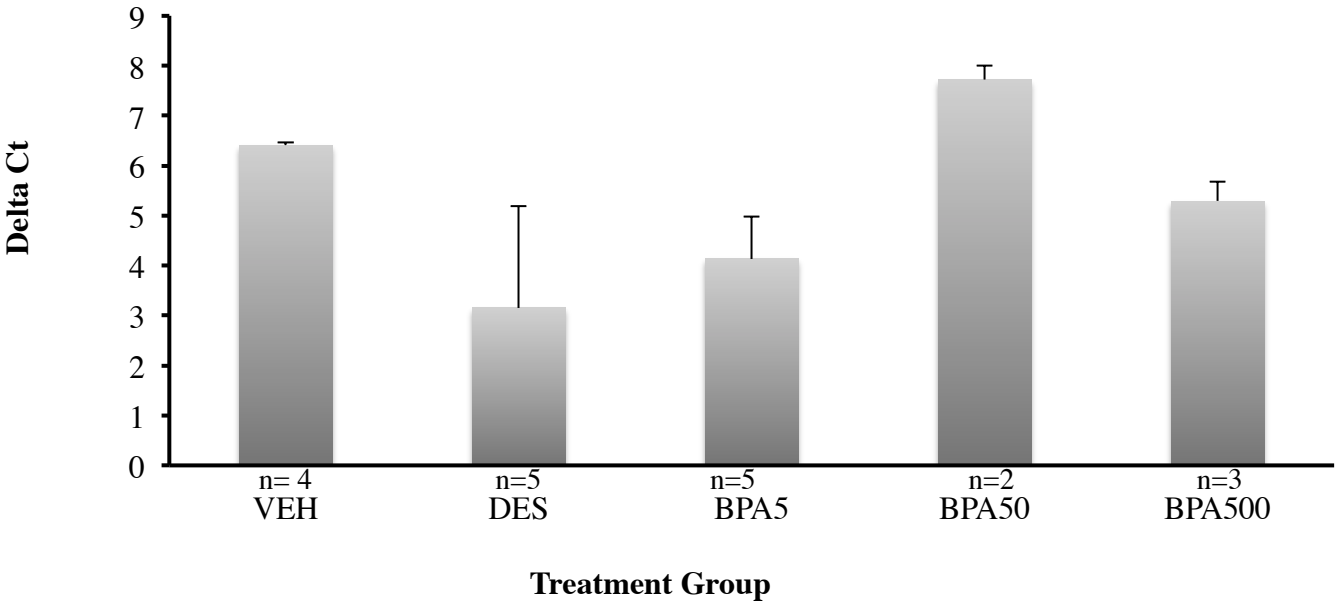


Figure 32. 18SrRNA Fold Changes Calculated by ΔΔCt Method. qPCR was used to amplify 18SrRNA, normalized to GAPDH reference gene. N values represent the number of male adrenal gland samples used for the calculation in each treatment group, per timepoint. Fold changes are presented for both PND 5 (left) and PND 15 (right) male pups. In PND 5 animals, downregulated fold changes (<0.05) were observed in DES, BPA 5 and BPA 500 groups and upregulated fold change (>2.0) was observed in the BPA 50 group. No 18SrRNA gene expression fold changes were observed in the PND 15 animals.

A.



B.

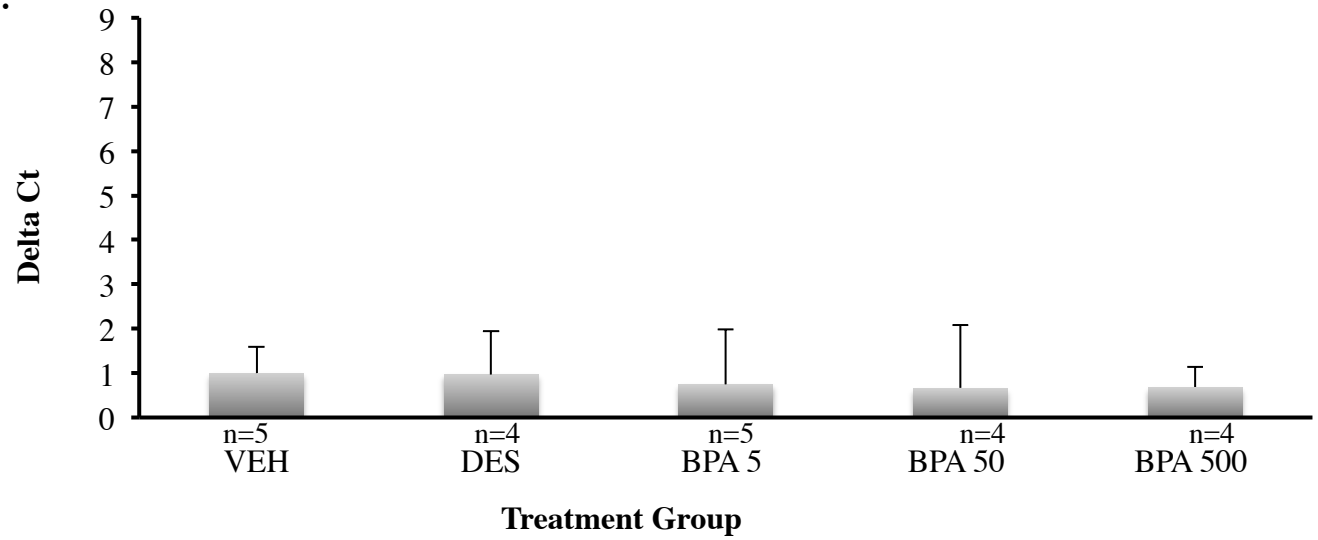


Figure 33. 18SrRNA Average Delta Ct values. Average delta Ct values Ct18SrRNA-(GAPDH) are presented for both A. PND 5 and B. PND 15 samples for 18SrRNA gene expression normalized to GAPDH. N values represent the number of male adrenal gland samples used for the calculation in each treatment group, per timepoint. Absolute values are shown above, as results for all treatment groups for both graphs were negative values. PND 5 ΔCt values were significantly affected by Treatment (Kruskal-Wallis: $H(4, N=19)= 9.75$, $p=0.0449$). At PND 15, the ΔCt values for 18SrRNA were not significantly affected by Treatment (Kruskal-Wallis: $H(4, N=22)= 0.5158103$, $p= 0.9719$).

Table 1. Determination of pregnancy staging based on days of gestational dosing.

The determination of each dams' stage of pregnancy at day 1 of dosing was retrospectively estimated based on the date of parturition (see text for details). Values were obtained by subtracting the number of days of gestational dosing the dam received from the standard 21-23 days of rat gestation.

Days of Gestational Dosing	Onset of Dosing (in days)
6	15-17
7	14-16
8	13-15
9	12-14
10	11-13
11	10-12
12	9-11
13	8-10
14	7-9
15	6-8

Table 2. RNA concentration and absorbance reading ranges.

RNA concentration and absorbance reading ranges. RNA concentrations for both **A)** PND 5 and **B)** PND 15 male adrenal gland samples were tested in triplicate by spectrophotometer. Samples were selected from the GD 9-11 window.

A.

PND 5	**Concentration Range**	**260/280 Range**	**260/230 Range**
VEH	66.73-211.8	2.07-2.10,2.75	0.20-1.40
DES	47.9-206.1	1.90-2.06	0.22-0.85
BPA5	152.9-375.9	2.12-2.14	0.46-1.26
BPA50	211.2-476.6	2.11-2.12	0.56-2.16
BPA500	211.9-449.7	2.12-2.14	0.96-1.46

B.

PND 15	**Concentration Range**	**260/280 Range**	**260/230 Range**
VEH	186.4-527.7	2.08-2.12	1.62-2.12
DES	47, 260-867.3	1.65-2.17	0.11, 0.86-2.00
BPA5	199.2-798.3	0.22, 1.90-2.11	0.6, 1.11-1.99
BPA50	308.8-579.5	2.09-2.13	0.91, 1.65-2.00
BPA500	92.8-368.1	2.08-2.13	0.25-0.62

Table 3. Reference and Target Gene Primer Sequences.

Primer sequences were obtained using the NCBI BLAST database. 18SrRNA and GAPDH sequences were selected from the literature, and verified using NCBI BLAST. GR primers were custom-generated from NCBI Blast.

Gene	Primer Sequence	Melt Temp
GR	FWD:AATGGGTACTCAAGCCCTGG	60°C
	REV:GCACCCCGTAATGACATCCT	
18SrRNA	FWD: CTTAGTTGGTGGAGCGATTTGTCTG	55°C
	REV:GTTATTGCTCAATCTCGGGTGGC	
GAPDH	FWD:CTGAGGAGTCCCCATCCCAA	55°C
	REV:GGTATTCGAGAGAAGGGAGGGC	

Table 4. Calculation steps for Ct and Delta Ct values.

i) Average Ct values per biological sample were calculated by taking the average of the triplicate Ct values per sample per treatment group. **ii)** The delta Ct (ΔCt) value per sample was calculated by taking the average Ct value per sample per treatment group and subtracting from the average Ct value of the reference gene (per treatment group). **iii)** The delta Ct (ΔCt) value per treatment group was calculated by subtracting the average Ct value per experimental group (done separately for both target genes 18SrRNA and GR) from the average Ct value of the reference gene (GAPDH) per experimental group. This was done to determine the difference between the average Ct value of each target gene and the reference gene.

i) Ct value calculation per sample
e.g. Ct^{GR} (VEH Sample 1) $Ct^{GR}_{\text{sample 1 average}} = (Ct_1^{GR}(VEH) + Ct_2^{GR}(VEH) + Ct_3^{GR}(VEH))/3$
ii) Delta Ct value calculation per sample
$\Delta Ct_1 = Ct^{GR}$ (e.g. VEH sample 1) – Ct^{GAPDH} (VEH avg) (per biological replicate)
$\Delta Ct_2 = Ct^{18SrRNA}$ (e.g. VEH sample 1) – Ct^{GAPDH} (VEH avg) (per biological replicate)
ii) Delta Ct value calculation per treatment group
Ct^{GR} (eg. VEH avg) – Ct^{GAPDH} (e.g. VEH avg) = ΔCt^{GR} (VEH group)
Ct^{GR} (eg. DES avg) – Ct^{GAPDH} (e.g. DES avg) = ΔCt^{GR} (DES group)
Ct^{GR} (eg. BPA 5 avg) – Ct^{GAPDH} (e.g. BPA 5 avg) = ΔCt^{GR} (BPA 5 group)
Ct^{GR} (eg. BPA 50 avg) – Ct^{GAPDH} (e.g. BPA 50 avg) = ΔCt^{GR} (BPA 50 group)
Ct^{GR} (eg. BPA 500 avg) – Ct^{GAPDH} (e.g. BPA 500 avg) = ΔCt^{GR} (BPA 500 group)

avg= average

Table 5. Calculation steps for Delta Delta Ct and Fold Change.

i) Once an average ΔCt value was obtained per treatment group, the ΔΔCt values were then calculated. The ΔΔCt is the difference between the average ΔCt of each treatment group and that of the Vehicle control group: (ΔCt^{GR} (treatment group) - ΔCt^{GR} (VEH)) for each target gene normalized to the reference gene GAPDH. **ii)** The final step is the fold change calculation, which would indicate whether gene expression of 18SrRNA and GR was up or down regulated in certain treatment groups compared to the GAPDH reference gene.

i) Delta Delta Ct (ΔΔCt)
VEH
ΔCt^{GR} (DES) - ΔCt^{GR} (VEH) = $\Delta\Delta Ct^{GR}$ (DES) value
ΔCt^{GR}(BPA 5) - ΔCt^{GR}(VEH) = $\Delta\Delta Ct^{GR}$ (BPA 5) value
ΔCt^{GR} (BPA 50) - ΔCt^{GR}(VEH) =$\Delta\Delta Ct^{GR}$ (BPA 50) value
ΔCt^{GR}(BPA 500) – ΔCt^{GR}(VEH) =$\Delta\Delta Ct^{GR}$ (BPA 500) value
ii) Fold Change= $2^{(-\Delta\Delta Ct)}$

avg= average

Table 6. Bodyweight Progression of PND 5 Pups.

Statistical analysis of average daily bodyweight (BW) progressions for animals sacrificed at PND 5 in the GD 9-11 exposure window are presented. The factors of Treatment and Time significantly affected BW progression (2-way ANOVA, $F(8,174)= 3.602$, $p=0.001$).

Between-subjects analysis	Treatment and Sex (3-way ANCOVA, $F(4,87)=0.623$, $p>0.05$)	Not significant
Individual Factor Analysis	Treatment (1-way ANOVA, $F(4,87)=1.203$, $p>0.05$)	Not significant
	Sex (1-way ANOVA, $F(1,87)= 2.147$, $p>0.05$)	Not significant
Within subjects	Treatment, Sex and Time (3-way ANCOVA, $F(8,174)=0.799$, $p>0.05$	Not significant
2-factor analysis	Sex and Time (2-way ANOVA, $F(2,174)= 1.718$, $p>0.05$)	Not significant
	Treatment and Time (2-way ANOVA, $F(8,174)= 3.602$, $p=0.001$).	Significant

$p<0.05$ is significant

Table 7. Bodyweight Progression of PND 15 pups.

Statistical analysis of average daily bodyweight (BW) progressions for animals sacrificed at PND 15 in the GD 9-11 exposure window are presented. The factor of time significantly affected BW progression (1-way ANOVA, $F(3,149)=3.127$, $p=0.025$).

Between-subjects analysis	Treatment and Sex (3-way ANCOVA, $F(4,47)=0.424$, $p>0.05$)	Not significant
Individual factor analysis	Treatment (1-way ANOVA, $F(4,47)=1.525$, $p>0.05$) Sex (1-way ANOVA, $F(1,47)=0.025$, $p>0.05$)	Not significant
Within-subjects analysis	Treatment, Sex and Time (3-way ANCOVA, $F(13,149)=0.746$, $p>0.05$)	Not significant
2-factor analysis	Sex and Time (2-way ANOVA, $F(3,149)=0.824$, $p>0.05$ Treatment and Time (2-way ANOVA, $F(13,149)=1.116$, $p>0.05$)	Not significant Not significant
	Time (1-way ANOVA, $F(3,149)=3.127$, $p=0.025$)	Significant

$p<0.05$ is significant

Table 8. Probabilistic Dietary Exposure to BPA for the General Population.

Adapted from Health Canada's Updated Assessment of Bisphenol A (BPA) Exposure from Food Sources (2012): Table 1. Probabilistic dietary exposure to BPA for the general population. The reproduction is a copy of the version available at http://www.hc-sc.gc.ca/fn-an/securit/packag-emball/bpa/bpa_hra-ers-2012-09-eng.php#t1

Age of Subpopulation	Male Mean µg/kg bw/day	Female Mean µg/kg bw/day
9-13 years	0.050	0.067
14-18 years	0.038	0.039
19-30 years	0.046	0.042
31-50 years	0.056	0.046
51-70 years	0.049	0.040
71 or more	0.052	0.061
All ages	0.055	0.054
Both	0.055	

µg/kg bw/day:
microgram per kilogram of body weight per day

Table 9. Probabilistic Dietary Exposure to BPA for Infants of Different Age Groups.

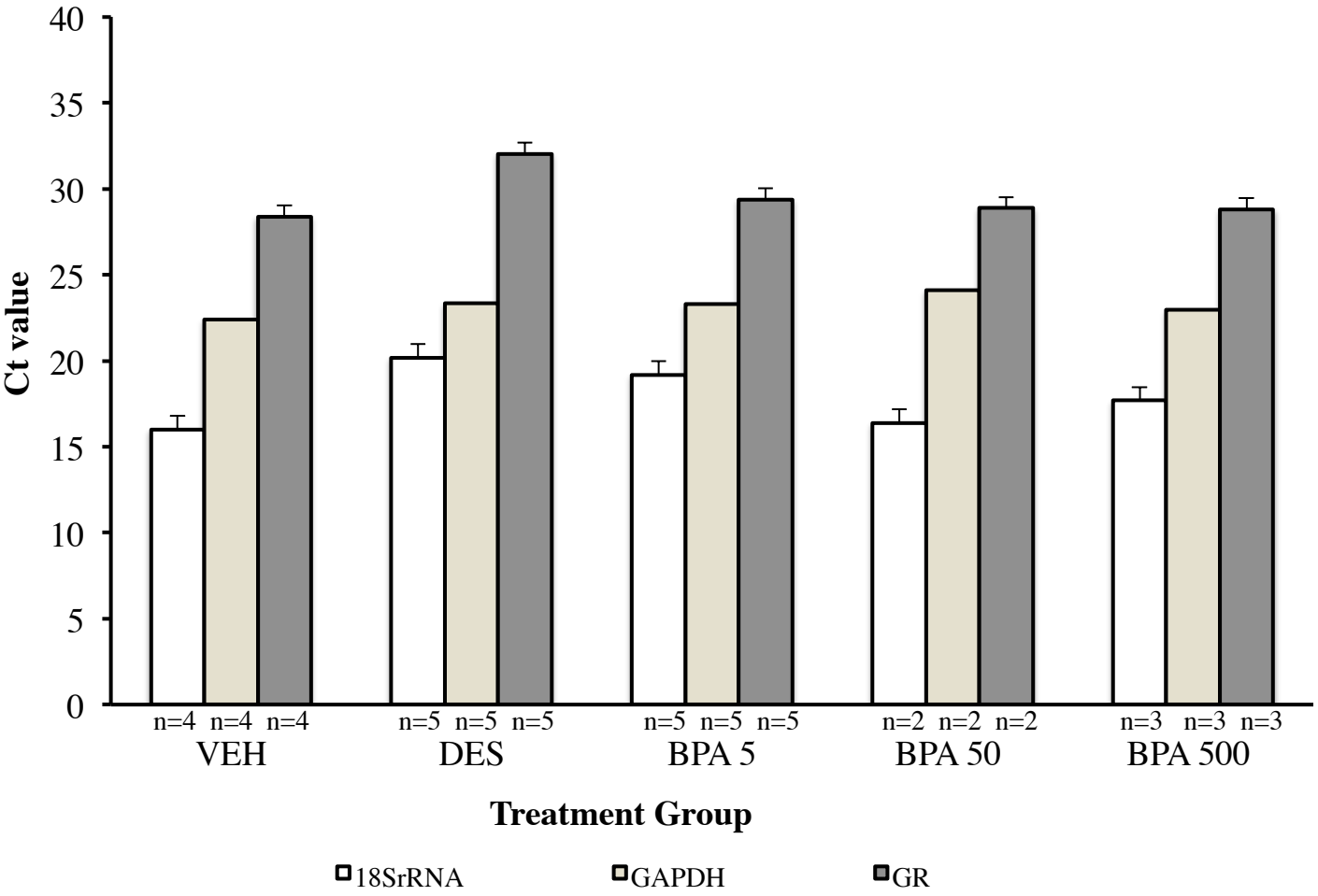
(Adapted from Health Canada's Updated Assessment of Bisphenol A (BPA) Exposure from Food Sources (2012). Exposure from Food Sources (2012): Table 2. Probabilistic dietary exposure to BPA for infants of different age groups. . The reproduction is a copy of the version available at http://www.hc-sc.gc.ca/fn-an/securit/packag-emball/bpa/bpa_hra-ers-2012-09-eng.php#t2

Age Group	Mean µg/kg bw/day
0-1 month	0.083
2-3 months	0.143
4-7 months	0.164
8-12 months	0.092
13-18 months	0.110

*Males and females are both included in each age group of infants

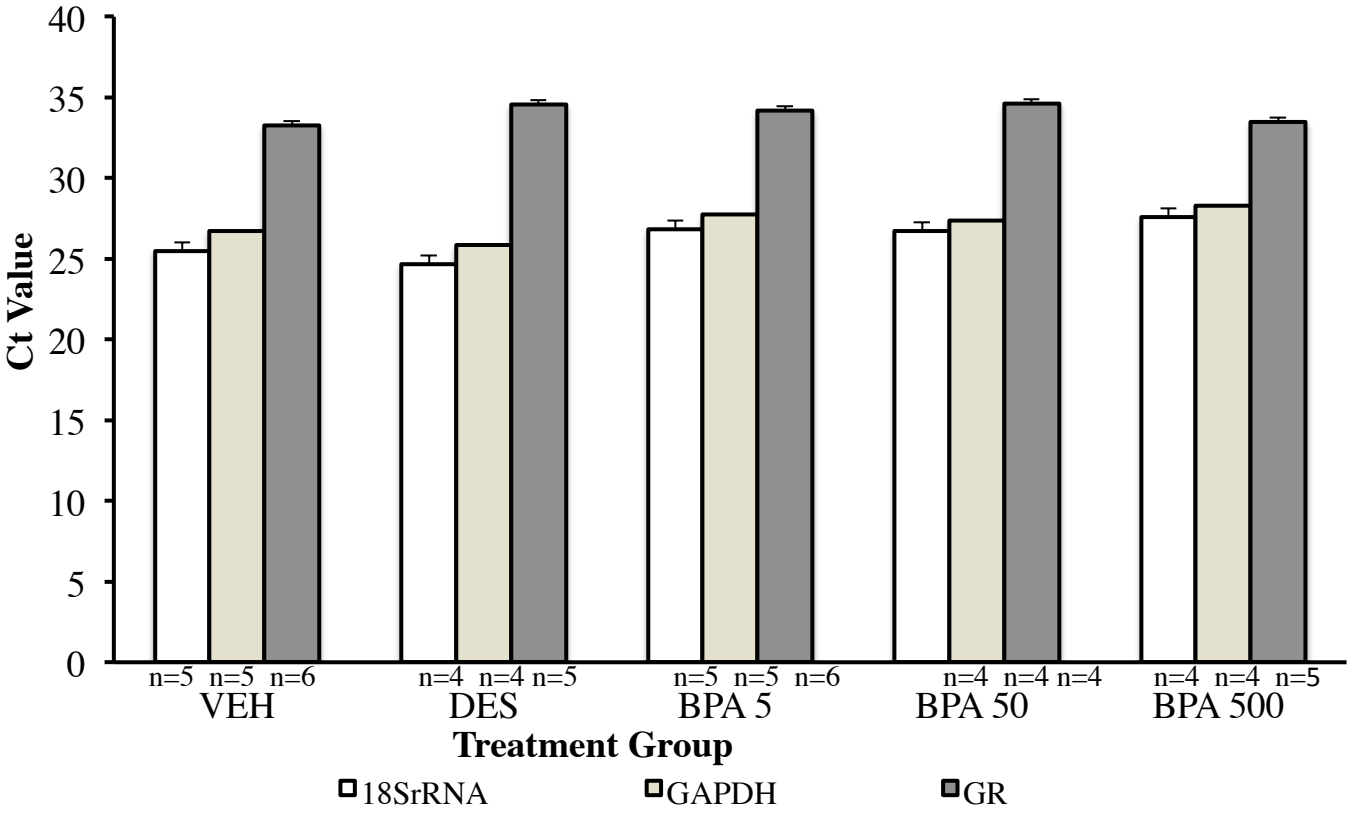
µg/kg bw/day ;
microgram per kilogram of body weight per day

APPENDIX 1.1



PND 5 Raw Ct values. Ct values obtained for every sample were averaged to obtain one value per gene per exposure group. Error bars represent standard error of raw Ct values.

APPENDIX 1.2



PND 15 Raw Ct values. Ct values obtained for every sample were averaged to obtain one value per gene per exposure group. Error bars represent the standard error of raw Ct values.

APPENDIX 2.1

18SrRNA VEH	DES	BPA 5	BPA 50	BPA 500
15.85	21.75	19.35	16.66	17.08
16.14	16.04	16.99	16.12	17.51
15.94	17.35	20.43		18.44
16.04	18.3	17.57		
	27.41	21.48		
GAPDH VEH	**DES**	**BPA 5**	**BPA 50**	**BPA 500**
23.03	26.22	24.38	24.28	23.70
21.69	22.12	23.11	23.96	22.29
22.22	21.99	22.99		22.91
22.66	22.11	22.98		
	24.18	23.02		
GR VEH	**DES**	**BPA 5**	**BPA 50**	**BPA 500**
27.86	29.30	28.86	28.62	28.48
27.83	29.06	28.84	29.13	28.12
29.93	35.60	29.91		29.78
27.83	29.14	29.56		
	37.06	29.77		

Average Ct values for PND 5 pups. The average of the Ct values for each PND 5 male sample (taken in triplicate) per gene is presented. These values were then used for the ΔCt statistical analysis and for the fold change (ΔΔCt) calculations and graphs.

APPENDIX 2.2

18SrRNA VEH	DES	BPA 5	BPA 50	BPA 500
25.73	22.23	24.46	24.51	28
26.02	26.52	24.55	25.85	26.85
26.88	25.95	25.56	25.68	26.85
25.48	23.92	29.7	30.91	28.67
23.37		29.92		
GAPDH VEH	**DES**	**BPA 5**	**BPA 50**	**BPA 500**
27.23	24.36	26.6	26.33	29.81
28.29	28.17	26.42	27.17	28.22
27.68	26.46	26.51	26.19	28.22
26.78	24.43	29.88	29.86	26.86
23.73		29.29		
GR VEH	**DES**	**BPA 5**	**BPA 50**	**BPA 500**
29.61	36.81	31.20	28.86	29.30
31.88	34.77	29.04	36.51	34.19
36.1	38.14	37.26	37.795	37.73
34	33.76	37.79	35.25	31.66
36.42	29.29	39.59		34.47
31.62		30.21		

Average Ct values for PND 15 pups. The average of the Ct values for each PND 15 male sample (taken in triplicate) per gene is presented. These values were then used for the ΔCt statistical analysis and for the fold change (ΔΔCt) calculations and graphs.

APPENDIX 3.1

PND 5 18SrRNA	Efficiency	R^2
VEH	88.3	0.998
DES	108.9	0.976
BPA 5	109.9	0.988
BPA 50	106.6	0.995
BPA 500	106.7	0.994
PND 5 GAPDH	**Efficiency**	**R^2**
VEH	109.0	0.949
DES	116.8	0.970
BPA 5	108.7	0.998
BPA 50	94.8	0.958
BPA 500	107.0	0.979
PND 5 GR	**Efficiency**	**R^2**
VEH	108.6	0.981
DES	114.2	0.925
BPA 5	113.5	0.981
BPA 50	109.4	0.947
BPA 500	100.3	0.981

PND 5 sample Efficiency and Correlation Coefficient values generated by qPCR runs. Efficiency (E) and correlation coefficient (R^2) values generated from standard curves with PND 5 male samples. Values were obtained using a representative sample from each exposure group for each gene.

APPENDIX 3.2

PND 15 18SrRNA	Efficiency	R^2
VEH	103.7	0.990
DES	109.4	0.981
BPA 5	98.2	0.979
BPA 50	95.2	0.99
BPA 500	114.8	0.986
PND 15 GAPDH	**Efficiency**	**R^2**
VEH	98.1	0.993
DES	86.4	0.993
BPA 5	111.1	0.989
BPA 50	107.0	0.984
BPA 500	96.5	0.988
PND 15 GR	**Efficiency**	**R^2**
VEH	97.1	0.989
DES	101.9	0.989
BPA 5	95.2	0.993
BPA 50	87.9	0.929
BPA 500	109.2	0.985

PND 15 sample Efficiency and Correlation Coefficient values generated by qPCR runs. Efficiency (E) and correlation coefficient (R^2) values were generated from standard curves with PND 15 male samples. Values were obtained using a representative sample from each exposure group for each gene.

APPENDIX 4.0

Health Canada / Santé Canada

January 17th, 2017

Ref: HC2016-0467

Ms. Julia Hajjar
Student
Master's Program – Health Sciences
University of Ottawa
Ottawa, ON

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