ENDOCRINE DISRUPTING CHEMICALS ALTER STEROID TRANSPORT PROTEINS AND STEROID FREE AND BOUND FRACTIONS

By

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The effects of endocrine disrupting chemicals (EDCs) on the development of sexually dimorphic characteristics in vertebrates have been studied extensively for over sixty years. Throughout this time, studies have focused mainly on the disrupted steroidogenesis and steroid signaling pathways. Often, only freely suspended plasma steroids were reported, despite the fact that a large portion of plasma steroids are bound to circulating steroid transport proteins. When bound to transport proteins, steroids have reduced binding to steroid nuclear and membrane receptors and increased binding to transport protein receptors. Thus, steroid transport proteins regulate steroid activity. Sexually dimorphic characteristics of tissues require specific concentrations and milieus of steroids during development and disruption of these steroid milieus leads to abnormal differentiation. Therefore, it is likely that the developing organism also requires specific free and bound ratios depending on the sex. However, few studies have addressed the effects of EDCs on sexual dimorphism of steroid transport proteins and steroid fractions despite the impact that transport proteins have on steroid signaling, development, and physiology. To address this, I first summarize the current literature on the regulation of transport proteins, their interactions with steroid signaling, and how transport proteins are affected by

environmental contaminates. These findings are culminated into a new model of steroid signaling that includes transport protein mediated pathways. Second, using HPLC-MS/MS, I show that a normal sexual dimorphism exists in free and bound steroid ratios for the steroids progesterone, corticosterone, testosterone, and 17β-estradiol. Additionally, I found a normal sexual dimorphism in the ratio of the bound precursor progesterone, to its potent metabolites, with females having higher progesterone to metabolite ratios than those in males. When exposed to the model EDC, Vinclozolin, sexual dimorphism of free and bound steroids was lost, with males and females responding to disruption in different ways. The disruption of plasma steroid ratios was found in the absence of changes to liver steroid transport protein concentrations. The results of this study show that steroid free and bound ratios as well as ratios of precursor to metabolite steroids can be altered by EDCs. This study clearly shows that future work in determining effects of EDC should also determine free and bound fractions of steroids to better understand effects of contaminants.

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CHAPTER 1: EDCs ALTER STEROID TRANSPORT PROTEINS

Introduction

Since Berthold's seminal work in the 1800s (Tata, 2005), the evolution of the field of endocrinology has been fueled by great minds and has led to great advancement in understanding disease prevention and treatment (Medvei, 1993). However, we face a crisis in the form of ubiquitous environmental pollutants, called endocrine disrupting chemicals (EDCs) (Gore et al., 2015), that disrupt endocrine function and especially jeopardize embryonic development of humans and wildlife. The previous work of endocrinologists has described how environmental contaminants can affect steroidogenesis, steroid receptor function, and mechanisms of hormone feedback (Diamanti-Kandarakis et al., 2009; Gore et al., 2015), however, these are not the only components regulating steroid action.

For decades, we have known that circulating steroid transport proteins regulate the activity and permeability of steroids through cell membranes and that these proteins exist in across vertebrate taxa. More recent work has shown that some cells have receptors that can bind hormone bound steroid transport proteins and induce intracellular effects. There is a paucity of studies that determine the mechanisms through which EDCs can alter steroid transport protein concentrations and their receptor functions. This deficit in our understanding of how EDCs affect steroid binding protein synthesis and function is an important gap in our understanding of the mechanisms through which EDCs alter health. The goals of this review are three-fold. First, we will synthesize the current understanding of steroid transport protein concentration and function during development. Second, we will summarize the current knowledge and identify critical gaps in our understanding of how steroid transport proteins are altered by EDCs. Finally, we will present a model of endocrine disruption that incorporates transport proteins (Uriel et al., 1975).

Steroids drive cellular differentiation, proliferation, and function in a tissue specific manner across the life span in all vertebrate taxa (Arnold, 2009). The correct timing and concentration of secreted steroid hormones are, therefore, essential for the normal development of tissue structure and function. Alterations to sex steroid signaling at critical times, especially during embryonic development, can lead to a diverse array of abnormalities in differentiation, growth, and metabolism (Gore et al., 2015). In fact, environmental contaminants known as EDCs can antagonize or agonize native steroid function and induce a variety of health problems. For example, the anti-androgenic fungicide Vinclozolin alters genitalia and testes development (Christiansen et al., 2008), and the xenoestrogen bisphenol A (BPA) used in production of some plastics induces altered mammary duct development (Munoz-de-Toro et al., 2005), endometrial growth (Markey et al., 2005), and prostate enlargement (Gupta, 2000).

It is generally accepted that steroid signaling is dependent on steroid production, and nuclear receptor quantity and function (Mangelsdorf et al., 1995; Moras and Gronemeyer, 1998). Indeed, these mechanisms of steroid function have been the focus of much work detailing the effects of endocrine disrupting pollutants (Bergman et al., 2013). We know, for example, that the EDC atrazine can upregulate aromatase and increase conversion of androgens to estrogens (Sanderson et al., 2002; Sanderson et al., 2000), resulting in disrupted reproductive development (Hayes et al., 2002). Alternatively, vinclozolin exposure antagonizes the androgen receptor blocking its downstream effects (Kelce et al., 1997; Kelce et al., 1994), and during development decreases androgen receptor quantities (Amato et al., in revision).

Although these well-known and thoroughly studied mechanisms are important, steroid transport proteins and their cognate receptors play critical roles in endocrine regulation and function. There is a paucity of studies that determine the mechanisms through which endocrine

disrupting chemicals (EDCs) can alter steroid transport protein concentrations and their receptor functions.

Normal circulating sex hormone availability is regulated by steroid transport proteins. For example, in the fetus Alpha-1-fetoprotein (AFP), Sex Hormone Binding Globulin (SHBG), Albumin (ALB), and Corticosteroid Binding Globulin (CBG) are serum steroid transport proteins produced by the liver, that bind circulating steroids with high affinity in developing male and female organisms across vertebrate taxa (Abelev, 1971; Keisler et al., 1995). The existence of steroid proteins, their high affinity to circulating steroids, abundance during embryonic development, and importance for maintaining adult homeostasis has been known for decades (Abelev et al., 1963). Steroids bound to transport proteins are less lipophilic and have reduced permeability into cell membranes. This change in permeability has a profound effect on the activity of the cognate steroid as entry of steroids, through the plasma membrane, allows steroids to bind intracellular nuclear receptors. As seen in (*Fig 1A*) bound and activated steroid receptors can then enter the nucleus and bind to a specific sequence (hormone response elements (HRE) of the promoter that modulates transcription of a number of downstream factors important for sexual and metabolic development (Mangelsdorf et al., 1995).

Steroids in complex with circulating transport proteins are sequestered into the blood, where they form a circulating pool or "buffer" of inactive steroids that are more resistant to catabolism (Vermeulen et al., 1969). In this form they can be accessed as cellular needs arises and can be easily transported in plasma to distal target tissues. This buffer system is described as the "free hormone transport hypothesis" (Mendel, 1989). For instance, studies of glucocorticoids in sparrows have shown there are no seasonal changes in free plasma cortisol levels in response to handling stress, despite a greater concentration of total (free and bound) cortisol in the

breeding season. This was due to the fact that, CBG concentration proportionately varied with the total cortisol levels as the animals were handled, resulting in increased total cortisol, but no net change in free cortisol (Breuner and Orchinik, 2002). Stress (due to handling) during the breeding season leads to larger increases the pool of circulating (total) cortisol relative to the nonbreeding season without changing concentrations of free presumable active hormone.

In addition to promoting protection from degradation and transport to distant tissues, steroid-bound transport proteins can play a role in steroid function within target cells. Ligand complexed transport proteins have been shown to bind to specific membrane receptors on some steroid target cells (Hryb et al., 1990). When bound, these receptors elicit intracellular responses, via cyclic adenosine monophosphate (cAMP) activation of protein kinase A (PKA), that influence intracellular steroid function (Dagar and Bhattacharjee, 2015). This mechanism works directly via PKA or by facilitating steroid-nuclear receptor complex aggregation in the cytoplasm and/or nuclear receptor complex binding to an HRE (Heinlein and Chang, 2002; O'Malley et al., 1995; Rosner et al., 1999). For example, binding of 17β-estradiol-SHBG complex to the SHBG membrane receptor activates the “fast” cAMP-PKA pathway, and increases secretion of prostate specific antigen in human prostate explants, a model steroid dependent process (Nakhla et al., 1997). Prostate specific antigen secretion via the estradiol-SHBG-receptor complex was unaffected by estrogen receptor (ER) antagonists, while a PKA antagonism reduced prostate specific antigen (Rosner et al., 1999). Androgen-SHBG- receptor complex binding also induces cAMP/PKA changes and enhances androgen dependent responses of bound androgen nuclear receptor (AR) via phosphorylation of transcription factors (Heinlein and Chang, 2002). Overall, membrane receptors for AFP (Terentiev and Moldogazieva, 2013), SHBG (Heinlein and Chang, 2002), and CBG (Borski, 2000) have been found in several different vertebrate tissues and

depending on the complexed steroid, may agonize or antagonize intracellular steroid signaling (Esteban et al., 1991; Fortunati et al., 1996; Rosner et al., 1999; Villacampa et al., 1984). The fact that transport proteins have cellular receptors that alter cellular physiology when bound by steroid-transport protein complexes illustrates that both free and bound hormone fractions play important roles in endocrine system function, and that they could be targets of endocrine disruption.

Synthetic chemicals alter endocrine system function during development, and can lead to a variety of disorders at birth and later in life (Bergman et al., 2013). Such EDCs are widely produced and used, leaving populations of humans and wildlife exposed to detrimental alterations of hormone dependent development. EDCs can affect hormone function during steroid biosynthesis, metabolism, and receptor binding, often affecting multiple systems simultaneously (Diamanti-Kandarakis et al., 2009). However, the effects of EDCs on steroid transport proteins has been sparsely studied and currently presents a major gap in understanding the effects of EDCs on human and wildlife development, endocrine function, and health. Steroid transport proteins could be susceptible to alterations caused by EDC-induced changes in transport protein synthesis, and/or binding capacity (amount of ligand bound by given a concentration of protein) via competitive inhibition or allosteric modulation.

AFP

AFP is a fetal and neonatal serum binding glycoprotein found across diverse vertebrate taxa (Mizejewski, 2004) that binds circulating estrogens with high affinity (ka~0.8x10^8 M-1) (Savu et al., 1981) and several other circulating molecules at lower affinity such as bilirubin, fatty acids, retinoids, steroids, heavy metals, dyes, flavonoids, phytoestrogens, dioxins, xenoestrogens, and other organic pharmaceuticals (Andrews et al., 1982a; Hong et al., 2012; Mizejewski, 2004).

Notably, AFP has very low affinity for testosterone (Swartz and Soloff, 1974). It is worth noting that some studies specific to humans, show negligible binding to estrogens, while others show evidence of conditional binding where high concentrations of 17β-estradiol or pretreatment with estrogen metabolites increased 17β-estradiol-AFP complexing. The contexts under which AFP binds to estrogens needs to be studied in more detail. High estrogen environments are common in certain cancers. (Swartz and Soloff, 1974; Uriel et al., 1975; Vakharia and Mizejewski, 2000), so AFP may play an especially important role in these cases.

The ontogeny of AFP is best studied in mammals where it is present and functional throughout fetal sexual differentiation and neonatal periods. It is secreted primarily from visceral yolk sac and liver hepatocytes during mammalian fetal development (Andrews et al., 1982b; Janzen et al., 1982; Tilghman and Belayew, 1982) with the earliest recorded fetal mRNA expression in mice was found at embryonic day 9.5 (E9.5) (Gualdi et al., 1996) and by E26 days in humans. These dates coincide with development of primitive hepatocytes in the hepatic bud for their respective species (Jones et al., 2001). The highest concentrations of AFP are produced during mid-late fetal development with trace amounts of protein produced postnatally in humans (Ruoslahti et al., 1974) and rodents (Sell et al., 1974). AFP is the most dominant transport protein produced in the mammalian fetus and one of the most highly expressed proteins overall (Andrews et al., 1982a). The binding of AFP to estradiol during development protects the fetus from inappropriate exposure to maternal estrogens (De Mees et al., 2006) and possibly environmental estrogens (Mizejewski, 1995). Studies in rats have shown that it is necessary embryonically for the development of normal reproductive physiology and behavior (Bakker et al., 2006; Gabant et al., 2002; McEwen et al., 1975). For example, *afp*-null female rats are anovulatory (Gabant et al., 2002), and lack the female copulatory behavior of lordosis (response to male mounting attempts)

(Bakker et al., 2006), resulting in infertility. As its name implies, it is absent in healthy adults, and is only detectable in some estrogen receptor related breast and liver cancers (Sell et al., 1976; Sell et al., 1974) as well as some germ line cancers (Ruoslahti et al., 1974).

Normally, AFP is expressed in dividing hepatocytes in the developing fetus and expression slows as cells leave the proliferative state (Sell et al., 1974). Estrogen signaling plays a critical role in driving hepatocyte proliferation (Ciana et al., 2001) and liver growth (Uebi et al., 2015). For example, male rat livers treated with 17β-estradiol had increased liver proliferation and estrogen receptor alpha (ERα) protein expression (Francavilla et al., 1984), while livers exposed to tamoxifen, an ERα antagonist, slowed hepatocyte proliferation (Francavilla et al., 1989). Estrogens, then likely indirectly increase AFP via its proliferative actions. Indeed, it has been shown that estradiol or estriol treatment increases hepatocyte proliferation and serum AFP in a liver regeneration study using rats as a model (Alvaro et al., 2000). Though additional work is required to confirm that these findings also occur in the fetus, it can be surmised that estrogen and thus estrogen mimicking EDCs could indirectly increase AFP production by increasing the proliferative cells that produce it.

However, diluted plasma from adult mice fed estradiol or control chow did not display high-affinity binding of estradiol. This lack of binding was interpreted as evidence that estradiol does not increase AFP levels in adults or neonates (Sheehan and Branham, 1981). Adults typically do not have high levels of AFP, and AFP is unlikely to be regulated by estrogens in this life stage, as estrogens cycle in the adult female drastically throughout the reproductive cycle, but AFP levels do not (they remain low). In addition, the use of binding capacity as an index of AFP concentrations is indirect. In contrast to adults, neonatal rats have high AFP levels (up to 4 mg/ml), and AFP at this age has a high estradiol binding capacity (60 μM). Similar to the adult

mice binding capacities, the dissociation constants did not change with estradiol treatment in neonatal rats, and this lack of change was, again, interpreted as a failure of estradiol to alter AFP levels. Although AFP production still occurs in neonates, it is known to be in decline at this stage. The role of AFP to protect the fetus from inappropriate estradiol exposure changes at birth as the brain requires estradiol to undergo sexual differentiation (McCarthy, 2008). Therefore, the neonatal stage might be the physiological window within which estradiol regulation of AFP production is being programed to no longer occur. Currently, influence of endogenous estradiol on fetal hepatocyte proliferation/growth and subsequent production of AFP during the appropriate developmental time window when AFP production is greatest is still unknown and should be formally evaluated.

AFP and Endocrine Disruption

There is a staggering number of known estrogenic compounds currently produced or still prevalent in soil (Xu et al., 2008) and water ways (Kuch and Ballschmiter, 2001), and this number grows as more commercial and industrial chemicals are manufactured. A few studies have investigated the effects of xenoestrogens on AFP production and function. For example, embryoid bodies formed in culture by human embryonic stem cells exhibited an increase in AFP mRNA expression when exposed to estradiol, the estrogen receptor modulator raloxifene, and endocrine disrupting chemical bisphenol A (Kim et al., 2012).

In fish, the known EDCs, diethylstilbestrol (pharmaceutical estrogen (Giusti et al., 1995)) and 4-nonylphenoxyacetic acid (commercial/industrial estrogen, (Routledge and Sumpter, 1996)) showed significant competition with estradiol in binding to AFP, but only at concentrations about 100-fold greater than estradiol (Milligan et al., 1998). Therefore, in high concentrations, EDCs could prevent proper transport of estradiol to target tissues or away from undesired tissues

such as the prenatal female mouse brain (McCarthy, 2008), which would further exacerbate the direct effects of the EDCs. Alternatively, in lower concentrations, EDCs may not be effectively bound to AFP, and thus can freely circulate and enter sensitive tissues. Taken together, there are a number of environmentally available chemicals that might change AFP production and binding, particularly synthetic estrogen contaminants in waterways (Baronti et al., 2000). Future studies of estrogen and estrogen mimics should include AFP quantification and free/bound steroid fractions in their studies.

SHBG

SHBG is 50-70 kDa gylocoprotein, that binds sex steroids, primarily dihydrotestosterone, testosterone, and estradiol at high affinities. SHBG is produced primarily in embryonic liver hepatocytes and secondarily in the yolk sacs of all studied vertebrate taxa except birds (Hammond, 2011; Wingfield et al., 1984) its common occurrence in embryonic tissues, there are few studies that focus on how SHBG is regulated during fetal development. The work that has been conducted on the regulation of SHBG has focused on the adult life stage, showing evidence that SHBG is regulated by hepatic nuclear factor-4α (HNF-4α) (Jänne and Hammond, 1998), peroxisome proliferator activated receptor gamma (PPARγ) (Selva and Hammond, 2009a), thyroid hormones (Miguel-Queralt and Hammond, 2008), and the sex steroids (androgens and estrogens) (Anderson, 1974). Insulin may also have a potential role in SHBG regulation as increased insulin and insulin resistance have been strongly correlated with increased testosterone and SHGB in women, and with a weaker correlation in men (Haffner, 1996).

Hepatic nuclear factor-4α (HNF-4α), a member of the liver produced hepatic nuclear factor receptor family (a subset of the nuclear receptor superfamily), has been identified as a key transcription factor in upregulating SHBG expression in hepatocytes (Jänne and Hammond, 1998;

Winters et al., 2014). HNF-4α is highly conserved across taxa and drives transcription of genes necessary for many adult liver functions, including xenobiotic/toxicant metabolism as well as carbohydrate and lipid metabolism. Furthermore, HNF-4α is requisite in embryonic development to drive liver differentiation (Li et al., 2000), and to no surprise, genetic knock-out of this gene is lethal (Hayhurst et al., 2001). HNF-4α was shown to drive SHBG translation when it was introduced to a *SHBG* promoter-luciferase reporter gene construct (Jänne and Hammond, 1998).

Conversely, SHBG translation is repressed by PPARγ (Selva and Hammond, 2009a), a member of the PPAR nuclear receptor family, that is associated with uptake of lipids and increased lipogenesis (Lee et al., 2003). PPARγ has a response element on the *Shbg* promoter and binding of PPARγ agonists showed repression of SHBG production, while PPARγ antagonists showed stimulation of SHBG production in HepG2 cells lines (Selva and Hammond, 2009a).

SHBG production has been shown to be positively correlated with thyroid hormones as shown with thyroid hormone treatment in cell lines (Mercier-Bodard et al., 1991; ROSNER et al., 1984) and with thyroid pathologies (Anderson, 1974). Though a thyroid hormone response element is not found on the *shbg* promoter, thyroid hormones have been shown to indirectly drive SHBG mRNA and protein production by increasing HNF-4α production (Selva and Hammond, 2009b).

Estrogen increases hepatic SHBG production. For example in adult men administration of 20 pg of ethinylestradiol per day for 5 weeks increased in SHBG, as did the synthetic antiandrogen clomiphene (Anderson, 1974). Furthermore, ERα was required to increase SHBG production in human hepatoma cell lines when treated with mitotane, a known andrenocorticoid steroidogenesis inhibitor that decrease renal androgen production (Nader et al., 2006). This increase in SHBG was accompanied by an increase in testosterone but is likely independent of

and not driven by the increased testosterone as treatment with antiandrogens did not decrease SHBG concentration. In addition, ERα and β in hepatocarcinoma cells were found to down regulate PPARγ (Lin et al., 2013), which is expected to increase expression of SHBG by reducing PPARγ mediated HNF-α repression. Although this specific mechanism has not yet been directly tested, synthetic estrogens are known to strongly increase serum SHBG *in vivo* (Anderson, 1974) and estradiol and synthetic estrogens increased SHBG *in vitro.*

The direct role of androgens in SHBG regulation is not well understood as there are only a handful of studies that have addressed this relationship and with mixed results. For instance, some male patients diagnosed with hyperandrogenism, characterized by high circulating testosterone levels, had lower SHBG, whereas others had higher levels (Toscano et al., 1992). This variation might be related to the underlying mechanisms driving the hyperandrogenism. There is some evidence, in women, that androgens inhibit SHBG (Anderson, 1974). For example, women treated with Danazol (synthetic androgen) had decreased plasma SHBG (Gershagen et al., 1984) and increased levels of free testosterone (Forbes et al., 1986) relative to untreated women. It is clear that more studies are necessary to clarify the direct effects of androgens on SHBG especially in pregnant women where developing fetuses are potentially affected by the mothers' exposure to environmental and endogenous androgens.

While direct mechanisms are lacking, there are several studies that draw indirect links between androgen and SHBG via interactions between immune and hormone signaling systems (Simó et al., 2015). It is well known in both humans and wildlife studies that androgens regulate activity of immune cells, with both pro- and anti-inflammatory properties (Cohn, 1979; Klein, 2000; Olsen and Kovacs, 1996), which is reviewed in (Olsen and Kovacs, 1996). For instance, androgen is known to have a stimulatory effect on the proinflammatory tumor necrosis factor-α

(TNF-α) (Ashcroft and Mills, 2002) and an inhibitory effect on the adipocytokine, adiponectin (Nishizawa et al., 2002). Suppression of TNF-α has been associated with increased levels of serum SHBG (Sattar et al., 2007; Simó et al., 2012), and increases in anti-inflammatory adiponectin have been correlated with higher levels of SHBG (Crawford et al., 2015; Simó et al., 2015). Additionally, inhibition of adiponectin, which is involved in regulation of insulin resistance and fatty-acid oxidation (Yamauchi et al., 2002), by testosterone may indirectly reduce SHBG production by reducing HNF-α signaling (Crawford et al., 2015). However, to our knowledge, no direct application of a non-aromatizable androgen has been studied in the context of fetal hepatic and placental SHBG production.

SHBG and Endocrine Disruption

Given the known mechanisms of SHBG regulation shown with thyroid hormones, and estrogens, and the indirect effects of androgens, endocrine disruptors could change SHBG production and function. Direct studies of EDC-induced effects on SHBG are uncommon, but we know EDCs affect the hormones that are believed to regulate SHBG. Thyroid hormones drive production of SHBG (Selva and Hammond, 2009b) and SHBG could therefore be altered by chemicals that affect the regulation of thyroid signaling. There has been a tremendous body of work that implicates a number of contaminants as thyroid disruptors (most notably polychlorinated biphenyls, BPA, perchlorate, and dioxins) with large potentials for altered brain development (Zoeller and Crofton, 2000) and metabolism (Patrick, 2009). Thyroid disruption has been shown in a wide variety of taxa including mammals (Patrick, 2009), amphibians (Carr et al., 2003), fish (Noyes et al., 2011), and birds(Wada et al., 2009) among others.

Estrogens increase production of SHBG (Anderson, 1974). EDCs with estrogenic or antiestrogenic effects, therefore, have a significant potential for altering steroid transport proteins such as SHBG. Studies of oral contraceptives and menopause treatment utilizing ethinylestradiol (Pugeat et al., 2010) have shown increased SHBG. Conversely, cancer therapeutics like tamoxifen, which binds and antagonizes ER signaling (Shiau et al., 1998) in breast cancer cells (Van Kammen et al., 1975), also show increased SHBG, suggesting that disruption of SHBG is complex, and likely nonlinear. We know that estrogens have nonmonotonic dose response curves (Lagarde et al., 2015), so high and low estrogen signaling could lead to similar effects. This highlights a concern for endocrine disruption of SHBG, as synthetic estrogens from widely used pharmaceuticals (ethinylestradiol, esterified estrogens, etc), urinated estrogenic metabolites, and common commercial/industrial products (e.g BPA and bisphenol S) are prevalent in the human and natural environments (Laurenson et al., 2014; Peng et al., 2008), and low concentrations are physiologically relevant.

Currently, there are no studies that directly address possible endocrine disruption of HNF-4α (Lyche et al., 2011), but PPARγ, has shown to be altered by many EDCs, with detailed review in (Casals-Casas et al., 2008) and (Desvergne et al., 2009). Alterations in these transcription factors likely alter SHBG production and contribute to steroid related diseases, but this hypothesis and its implications on human and wildlife health need to be evaluated in detail.

Disruption of SHBG production likely alters steroid activity in exposed organisms, but EDC-induced alterations in SHBG binding ability could also be detrimental. Exploration of SHBG's active site affinity for possible pharmaceutical applications found that the structure is also permissible to non-steroidal ligands (Avvakumov et al., 2010). As with the synthetic estrogens mentioned above, a number of xenobiotics including EDCs can bind SHBG (Hong et al., 2015),

and some change the conformation of the protein to affect native steroid binding (Tollefson et al., 2001) and binding capacity (Van Kammen et al., 1975). For example, organochlorines and heavy metals were shown *in vitro* to facilitate testosterone and inhibit estrogen binding to SHBG of Green Turtles (*Chelonia* mydas) (Ikonomopoulou et al., 2009), and phytoestrogens can affect the binding effectiveness of SHBG to natural ligands (Martin et al., 1995). Interestingly, one study showed several known EDCs, including the heavy commercialized BPA, were able to bind human SHBG, though with significantly lower affinity than testosterone and estrogen. The effects of such low-level binding on physiology and health are unknown and should be investigated. The implications for this nonspecific binding have not been explored but might be complex and far reaching. For example, the SHBG membrane receptor is activated only when the SHBG-ligand complex binds to it (Hryb et al., 1990). This binding results in fast intracellular responses to alter intracellular steroid receptor responses (Rosner et al., 1999) and depicted in (*Fig 1C*) Direct binding of EDCs to SHBG that alter binding of SHBG to native ligands, could likely disrupt the SHBG receptor function, and induce mechanisms of endocrine disruption that have not been contemplated.

SHBG synthesis, circulating concentration, and binding function are at clear risk of alteration due to environmental chemicals, and should be studied in more detail. Furthermore, given that the normal function of SHBG is to control circulating bound and free fractions of estrogens and androgens, sex hormone concentrations are relevant endpoints to evaluate when studying the effects of EDCs on SHBG physiology. Importantly, we also have much to learn about the role that SHBG plays in regulating fetal hormone concentrations, and how those hormones feedback to regulate fetal transport proteins. Without this basic research, we cannot fully evaluate the effects that fetal exposure to EDCs on SHBG physiology.

Albumin

Albumin is a 65-70 kDA, globular, and non-glycosolated transport protein produced in both the fetal and adult liver across mammalian taxa (Huntley et al., 1977; Lampreave et al., 1982; Liao et al., 1980; Sellem et al., 1984; van den Akker et al., 2008; Viani et al., 1991). Albumin binds several circulating lipophilic molecules such as steroids, retinoids, and fatty acids at low affinity, but relatively high binding capacity (the total amount of ligand bound to amount of albumin). Albumin is first detectable in the mouse on E15 and by 130 days of human fetal development (Dancis et al., 1957). Albumin production and secretion from liver hepatocytes increases in the mouse from E15 until post-birth where it replaces AFP as the most abundant mammalian serum transport protein. Currently, there are few studies that specifically address whether or not steroids regulate hepatic synthesis of albumin, however, albumin has been shown to be increased by glucocorticoid exposure in rat hepatocyte culture (Bancroft et al., 1969; Nawa et al., 1986) and repressed by estrogens in reptiles (Selcer and Palmer, 1995), amphibians (Riegel et al., 1986), and fish (Flouriot et al., 1998).

As a high capacity binder of lipophilic molecules, disruption of album by EDCs could cause significant changes in the availability of endogenous steroid hormones. For instance, if albumin protein concentrations are increased or decreased or binding affinity to steroids is altered the amount of free native steroid (unbound) and steroid transport to target cells would be affected, potentially for all steroids. Alternatively, changes in albumin concentrations or structure could affect its binding to disruptive xenobiotics in the bloodstream and remove them from activity (Baker, 2002).

Albumin and Endocrine Disruption

Currently there are only a few studies that explore the effects of exogenous chemicals on albumin production or function. Some of these studies show that EDCs bind directly to albumin. For instance, the xenoestrogens alkylphenols 4-tert-octylphenol, 4-nonlphenol , and several polyphenols bind to albumin (Soares et al., 2007; Xie et al., 2013). Albumin has also been shown to bind to synthetic and naturally produced estrogens such as diethylstilbestrol (Savu et al., 1981; SHEEHAN and YOUNG, 1979) and the phytoestrogen genistein (Bian et al., 2004). Fewer studies have attempted to characterize changes in the conformation of albumin, though structural changes of albumin can be induced by perfluoroalkyl acids (Qin et al., 2010) and naphthols (Wu et al., 2007), which can potentially alter the normal binding of albumin to native and external steroid molecules. Therefore, albumin can directly bind to an EDCs at its active site, and/or undergo conformational changes due to EDC binding at allosteric sites. Given its extreme abundance and critical role in endocrine regulation, more work should focus on determining how common EDCs affect albumin production, circulating concentrations, and binding capacity.

CBG

CBG, also referred to as transcortin, is a monomeric glycoprotein that is approximately 50-60 kDa and produced in the fetal and adult liver in all studied vertebrate taxa including amphibians, reptiles, and birds and mammals. Generally, across taxa CBG has high affinity to corticosteroids, slightly lower to androgens, and a weak affinity to estrogens though the strength to a particular steroid class may change depending on the species (Malisch and Breuner, 2010). For example, CBG in birds, who lack the strong (Brien, 1981)androgen binding SHBG, has a higher affinity to androgens than seen in other taxa (Wingfield et al., 1984).

CBG is known to bind Serum concentrations (0.002 – 0.378 µg/mL) and binding capacity (0.023 – 0.432 µg/mL at 4°) ranging across adult species (Seal and Doe, 1965). Blood CBG concentrations closely mimic blood corticosteroid levels through development, with large steady increases of both in the late fetal stages until a plateau at the end of the neonate period (Henning, 1978) .CBG concentrations and binding capacities have been shown to be naturally dimorphic in some species such as the rat, with females having significantly higher concentrations (Van Baelen et al., 1977) and capacity to bind corticosterone than males (Gala and Westphal, 1965). Dimorphism in CBG was not seen in humans (Brien, 1981). Testosterone appears to be a key driver of CBG as gonadectomy eliminated with males having CBG levels near females, which was rescued to the lower normal male levels with testosterone treatment (Van Baelen et al., 1977). The binding capacity of CBG to corticosterone decreased when female rats were exposed to 17β-estradiol and testosterone while males had increased levels. Both males and females showed decreased CBG capacity when exposed to progesterone (Gala and Westphal, 1965). CBG synthesis has been shown to be influenced by corticosteroids and estrogens (Rosner, 1990). For instance, corticosteroids were found to inhibit CGB protein concentrations while estrogens caused an increase (Feldman et al., 1979). Diseases that increase cortisol levels, such as Cushing's syndrome and Addison's disease, result in lower concentrations of CBG and lower binding capacity to corticosteroids. (Frairia et al., 1988). However, increases in circulating CBG occurred with treatment of estrogens in adult humans (Seal and Doe, 1962), and during pregnancy (Daughaday et al., 1959), when concentrations of endogenous estrogens are known to be naturally high.

CBG and Endocrine Disruption

Evidence has shown that there are environmentally pervasive corticosteroids (Hinson and Raven, 2006), estrogens (Kuch and Ballschmiter, 2001), antiandrogens (Kelce et al., 1994), and progestins (Chatterjee et al., 2008) with that act as endocrine disruptors. As noted in the previous section, all the above classes of steroids can affect either CBG concentrations or binding capacity. As with the previous transport proteins, the direct effects of EDC exposure on CBG has been poorly investigated, though the few studies that follow do suggest CBG concentration and function can be affected by pharmaceuticals taken medically or unintentionally via water contamination. Humans treated with the synthetic corticosteroid, cortisone acetate, had increased CBG binding capacity (Schlechte and Hamilton, 1987). Treatment with exogenous glucocorticoids have been shown to increase production of CBG in rat liver (Feldman et al., 1979) and circulating levels in human plasma (Schlechte and Hamilton, 1987). However, the anticorticosteroid ketoconazole (and antiandrogenic EDC (Taxvig et al., 2008)) increased CBG concentrations in patients with Cushing's syndrome (Frairia et al., 1988).

Synthetic estrogens used in contraceptives increase the bound fraction of cortisol in plasma (Bulbrook and Hayward, 1969). Estrogens also affect the percent of CBG bound cortisol. As an example, when treated with the pharmaceutical estrogens, diethylstilbestrol and ethinyl estradiol, women with breast cancer and men with prostate cancer had increased bound cortisol in circulation relative to before treatment (Sandberg and Slaunwhite, 1959). CBG binding capacity is also increased by exposure to a mixture of synthetic estrogens (conjugated estrogens, mestranol, norgestrel, and norethindrone) (Moore et al., 1978). Interestingly, women taking oral contraceptives have increased CBG, and higher serum total cortisol after stress tests (Kuch and Ballschmiter, 2001), suggesting that pharmaceutical and EDC exposure could affect stress response via altered CBG. Contraceptives have been detected in public drinking waters (Sun et al.,

2009) and natural bodies of water (Waste, surface, ground, stream, river, and lake) (Liu et al., 2011) putting human and animal populations dependent on the fresh water at risk of altering CBG. This is particularly important for aquatic species such as fish that have multiple modes of EDC exposure: osmoregulation and respiration through the gills, exposed food sources, and through egg yolk deposition (Mills and Chichester, 2005). Disruption to CBG represents a critical threat to stress and metabolic maintenance in all vertebrate taxa, as glucocorticoids, the chief ligand of CBG, represent a conserved response to stress in all of these organisms. Altered glucocorticoids levels have been shown to effect immunocompetence in mice (Malisch et al., 2009) and early nest abandonment in European starlings (Love et al., 2004), and overall parental care (Wingfield et al., 1998). Effects of EDC induced alterations of CBG may be most crucial during the reproductive seasons where glucocorticoids have shown to fluctuate in many taxa of birds, reptiles, fishes, amphibians (Breuner and Orchinik, 2002; Romero, 2002), and mammals (Romero et al., 2008).

Conclusion

Over the past century we have advanced from identifying the sources of hormones and their chemical structure to understanding their conserved nature, evolution, feedback regulation, and that each one can induce a multiplicity of effects (Lösel and Wehling, 2003). Our first 100 years of studying hormones cumulated into our understanding that all free circulating steroid hormones cross the lipid membranes of cells and interact with nuclear receptors to induce their effects (Tata, 2005). In the late 20th century, however, a new mechanism of steroid action was introduced (Norman et al., 2004), and it is now well accepted that free steroid hormones can bind to steroid receptors at the cell membrane to induce much quicker non-genomic responses than their classic genomic mechanisms (Schwartz et al., 2016). Already, comparative models of rapid

steroid membrane receptor signaling have been developed to study auditory learning in fish (Remage-Healey and Bass, 2004) and birds (Remage-Healey et al., 2011). With more research it is likely that rapid, non-genomic signaling will be found in neural development of other taxa as well.

This rich and important body of work was accomplished without deep consideration of the role that steroid transport proteins were playing. Most studies suggested that steroid hormones were only functional when they were liberated from their blood-solubilizing proteins (Mendel, 1989), and could bind to cell membrane receptors, or cross the cell membrane and bind to nuclear receptors. Therefore, transport proteins were believed to decrease the concentration of free and functioning steroid hormones available to affect cell physiology. Relatively recent work, however, has shown that transport binding proteins play more interesting roles in driving steroid hormone action. Researchers have found that bound steroids paly a direct role in cellular physiology as their binding proteins have cell membrane receptors that can illicit cellular responses when bound by the steroid hormone-binding protein-hormone complex (Heinlein and Chang, 2002; Leonhardt et al., 2003; Li et al., 2002; O'Malley et al., 1995; Rosner et al., 1991).

The roots of the studies that investigate the effects of endocrine disruption on binding-protein biology stretch back decades, some as early as the 1970s (Van Kammen et al., 1975; Williams et al., 1978). This work does show evidence of altered steroid transport protein synthesis, binding capacity and affinity, and altered circulating hormone concentrations across multiple taxa. Here, we suggest that the effects of EDCs on steroid transport proteins likely presents an important and drastically understudied form of endocrine disruption and requires increased study regardless of taxa.

Furthermore, the existence of steroid transport specific receptors suggests an additional pathway of steroid signaling that has received little attention. As the evidence presented in the review has shown, steroid signaling and its disruption is not limited to the "classic" genomic signaling pathway, or even membrane steroid receptors (Lösel and Wehling, 2003), but also transport protein receptors. All three signaling pathways have the potential to be disrupted. EDC exposure can agonize or antagonize intracellular steroid nuclear receptors directly altering how they bind to the HRE and changing transcription of steroid dependent transcripts (*Fig 1.1A and 1.1D*). Steroid membrane receptors can also be agonized or antagonized by EDC binding, altering activation of Ca^{2+} channels and MAPK/ERK signaling and affecting both recruitment of transcription factors and direct phosphorylation of steroid nuclear receptors (*Fig 1.1B and 11.E*). Lastly, EDCs can alter the ability of steroid transport proteins to bind their ligands or their membrane bound receptors. This disruption can again be agonistic or antagonistic, resulting in changes to Ca^{2+} channels and cAMP mediated PKA activation. Changes to PKA cascades induced by hormone-transport protein complex binding to membrane receptors affect phosphorylation and recruitment of steroid nuclear receptor cofactors and downstream gene expression (Menazza and Murphy, 2016; Pietri et al., 2016) (*Fig 1.1C and 1.1F*). Here we argue that these mechanisms (*Fig 1.1A-C*) of steroid function are not mutually exclusive but are integrated across cells, tissues, and body systems and that dysfunction in any of these mechanisms likely feedback to alter other aspects of the endocrine system. Therefore, each of these mechanisms must be considered as potential targets for endocrine disrupting chemicals. This model expands upon the brilliant work that endocrinologists have done since the field's conception in the mid-1800s and provides a synthetic picture of steroid signaling and facilitates the generation of new hypotheses to test novel mechanisms of endocrine disruption. Before we can understand the full effects of EDCs on wildlife

and human health we must fully characterize how these pervasive chemical pollutants affect steroid transport protein production and function.

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CHAPTER 2: FETAL STEROID FREE AND BOUND FRACTIONS ARE DIMORPHIC AND ARE ALTERED BY THE MODEL ANTIANDROGEN EDC, VINCLOZOLIN, IN A SEX SPECIFIC WAY

Introduction

Sexually dimorphic tissue development and function requires tight regulation of steroid concentrations. Aberrant steroid signaling during critical windows of development, within which the fetus is especially sensitive to hormone signaling, results in a number of endocrine-related diseases at birth or that manifest in adulthood. It is well established that perturbations in ligand-receptor binding and steroids concentrations can be detrimental especially to the fetus (Colborn et al., 1993). What is less well understood is the role steroid transport proteins serve to control proper signaling and mitigate improper signals (i.e. from environmental contaminants) (Uriel et al., 1972), and maintain normal dimorphism. Steroid transport proteins bind circulating steroids during critical stages of sexual differentiation and segregate steroids into two distinct and functionally different fractions: bound and unbound steroids which interact with cells in fundamentally different ways.

Steroid transport proteins regulate sex hormone availability and diffusion through the lipid bilayer. In the fetus, Alpha-1-fetoprotein (AFP), Sex Hormone Binding Globulin (SHBG), Albumin (ALB), and Corticosteroid Binding Globulin (CBG) are plasma transport proteins produced by the liver, that bind circulating steroids with high affinity in developing male and female organisms across vertebrate taxa (Abelev, 1971; Keisler et al., 1995). Steroids bound to

transport proteins are less lipophilic and have reduced permeability through cell membranes and binding to nuclear receptors is therefore reduced. Consequently, steroids that are bound to circulating binding proteins are sequestered in the blood, where they form a circulating reserve or "buffer" of steroids that are more resistant to catabolism (Vermeulen et al., 1969), and can be easily transported in plasma to distal target tissues (Mendel, 1992), so they can be accessed as cellular needs arises.

Classically, the bound fraction of steroids has been considered "inactive" in its ability to drive steroid signaling in the classic steroid genomic signaling pathway, where only the fraction of steroids that is unbound to transport proteins are "active" and can permeate the lipophilic cell membrane to activate gene transcription. This long-standing theory is known as the "free hormone transport hypothesis" (Mendel, 1989). However, in addition to protecting steroids from degradation and aiding in transport to distant target tissues, recent evidence shows that binding proteins bound to steroid can play a role in non-genomic steroid signaling. Indeed, steroid-complexed binding proteins have been shown to bind to binding protein specific membrane receptors on diverse target cells such as human endometrial tissue (Avvakumov et al., 1986), prostate (Hryb et al., 1986), hippocampus (Herbert et al., 2005) breast cell line (Porto et al., 1992b), as well as rat testes (Porto et al., 1992a), and hippocampal cell lines (Caldwell et al., 2000). Steroid bound transport proteins bound to their receptors activate phosphorylation cascades that modify intracellular steroid function and the binding of steroid nuclear receptors to their promoters (Porto et al., 1992a). Thus, bound steroids, in addition to free, play an important role in steroid signaling.

We know that synthetic endocrine disrupting chemicals (EDCs) can affect all aspects of hormone function: including steroid biosynthesis, metabolism, receptor binding, or often affecting multiple systems simultaneously (Diamanti-Kandarakis et al., 2009). However, the effects of EDCs on steroid transport protein concentrations, and their interactions with their cognate steroids have been sparsely studied. The vast majority of EDC studies do not distinguish free or bound fractions of steroids, generally reporting only on free or total concentrations. Limited data suggests that EDCs can affect transport protein synthesis (Pugeat et al., 2010), and disrupt their natural binding capacity via competitive inhibition at high concentrations (Milligan et al., 1998). These changes could affect free and bound steroid concentrations resulting in changes for both genomic and non-genomic steroid signaling. In adulthood, gonadal and adrenal steroids are known to bind receptors in the hypothalamus and pituitary in a negative feedback loop, reducing secretion of the gonadotropins and corticotropins (respectively) and thus limiting further secretion of gonadal and adrenal steroids. No evidence currently shows how changes in steroid transport proteins directly affects negative feedback, however it is likely that free steroids can bind to brain nuclear receptors whereas bound steroids cannot. Disruption of binding protein concentrations or binding of would lead to alterations of the free and bound fractions of steroids, which may affect the ratio of genomic to non-genomic signaling and have impacts on steroid feedback. Additionally, steroids exist as a part of the cholesterol metabolic pathway, where changes in the fractions of upstream precursor-molecules like progesterone, can alter available substrate for downstream steroids such as glucocorticoids and androgens or estrogens steroids.

Ultimately, the lack of knowledge of how EDCs affect steroid transport proteins and their interactions with steroids limits the interpretations that can be made in EDC studies and presents

a major gap in understanding the sex-dependent risks EDCs pose to human and wildlife development, endocrine function, and health (Mogus and McCoy, 2018, in review). Here we test the hypothesis that exposure to the model EDC and antiandrogen, Vinclozolin, during sexual differentiation alters murine concentrations of free and bound fetal plasma progesterone, corticosterone, testosterone, and 17β-estradiol, and that these changes affect the ratios of precursor steroids to their metabolized products (e.g. Progesterone/Testosterone), and that the nature of the changes are sex dependent. Then we test the hypothesis that fetal mouse liver steroid binding protein concentrations (alpha-1-fetoprotein, albumin, and corticosteroid binding globulin) are altered by Vinclozolin and in a sex dependent way.

Materials and Methods

Animal Studies

All studies were carried out under an approved protocol established by East Carolina University (ECU) Institutional Animal Care and Use Committee (AUP D-297 and AUP D-297a). Eight-week-old CD-1 mice were purchased (Charles River Breeding Laboratories Raleigh, NC) and acclimated for at least 7 days to 70-72 F on a 12 h light-dark cycle with free access to food and water (Purina ISOCHOW).

Vinclozolin Exposure

CD-1 mice were time mated by placing males and virgin females together in the evening, and females were checked for vaginal plugs each subsequent morning. A vaginal plug indicated that a mating had occurred that night and the female was identified as embryonic day (E) 0.5 at noon the day the plug was identified. Pregnant dams (N=5) were treated with 125 mg/kg Vinclozolin

(cat# 45705, Sigma-Aldrich, St. Louis, MO), dissolved in Tocopherol striped corn oil (cat# 901415, Millipore, Burlington, MA), or Tocopherol striped corn oil alone (Solvent control) once a day from E 13.5-16.5. All doses were given between 11am- 1pm via gavage. This dosing regimen is known to induce endocrine disruption and induce hypospadias (feminized male genitalia) 100% of the time in mice (Amato and McCoy, 2016). Masculinization of the genitalia is androgen dependent (Miyagawa et al., 2009), so using this regimen assures disruption of androgen signaling.

Sample Collection

To determine steroid concentrations within fetal plasma, pregnant dams were dissected 1 hr. after their fourth and final treatment dose on day E16.5, and embryos were removed. Embryonic age was confirmed by Theiler stage criteria (Stage TS24) (Theiler, 2013). The carotid and thoracic arteries of each embryo was cut, and blood was collected using a heparinized 20-gauge x (0.9mmx25mm) needle (cat# 305175, BD, Franklin Lakes, NJ) attached to a 1cc syringe (cat# 9625, BD, Franklin Lakes, NJ). The syringe tip was filed down and dulled to prevent puncture and to reduce clogging (de Lurdes Pinto et al., 2008). Collected blood was centrifuged for one minute without prior clotting, and plasma supernatant was collected via a pipetter. Plasma samples were immediately stored at -20˚C. The carcasses were dissected in cold 1x PBS (cat# BP665-1, Fischer Scientific, Fairlawn, NJ) and sex was determined based on gonad phenotype, which is not altered using this dosing regimen. Fetal livers were collected, weighed, and snap frozen in liquid nitrogen. Frozen livers were stored in a -80˚C freezer until processing for proteomics. Livers were transported over dry ice to the Center for Human and Health and Environment at North Carolina State University for protein extraction and proteomic analysis.

Fetal Plasma Metabolomics

Plasma sample preparation

Plasma was thawed and pooled based on sex within each dam (n=5). Depending on volume collected, between 20-50µL and were transferred to 16x125mm borosilicate glass tubes (cat# 47729-578, VWR, Radnor, PA) and then spiked with a 20µL of mixture of deuterated steroid standards, consisting of: corticosterone-D8 (0.25µg/mL; cat # Q3460-020, Steroloids Newport, RI), 17β-estradiol-D5 (0.5µg/mL; cat# E0950-000, Steroloids, Newport, RI), progesterone-D9 (0.005 µg/mL, cat# Q2600-014, Steroloids, Newport, RI)), and testosterone-D3 (2.5µg/mL, cat# T-046, Cerilliant, Round Rock, TX) diluted in Optima Grade Acetonitrile (cat# A955-1, Fischer Scientific, Fairbanks, NJ). A remaining volume of Optima Grade Water (cat# W6-1, Fischer Scientific, Fairlawn, NJ) was added to each sample to bring the total volume up to 500µL of solution.

An external calibration curve was prepared using mixtures of non-deuterated standards of corticosterone (cat# C-117, Cerilliant, Round Rock, TX), 17β-estradiol (cat# E-060, Cerilliant, Round Rock, TX), progesterone (cat# P-069, Cerilliant, Round Rock, TX), and testosterone (cat# T-037, Cerilliant, Round Rock, TX). The non-deuterated steroids were mixed and serially diluted in Optima Grade Acetonitrile (cat# A955-1, Fischer Scientific, Fairbanks, NJ) to make a standard external calibration curve with the range: 0.0005, 0.005, 0.05, 0.5, and 5.0 µg/mL of steroid. Twenty µL of each mixed external steroid concentration was added to 480 µL of water in individual 16x125 mm borosilicate glass tubes. Solvent blank tubes were prepared by adding 500 µL of optima grade water. All samples were then extracted.

Unbound steroid extractions

To extract unbound steroids, calibration curves, and blanks three mL of methyl-tert-butyl-ether (cat# 306975, Fischer Scientific, Fairbanks, NJ) was added to each tube. Samples equilibrated for one min, vortexed at 700rpm for five minutes, settled for two min, and vortexed for two min. With organic and aqueous phases clearly separated, sample tubes were placed in a methanol (cat# 34860, Fischer Scientific, Fairlawn, NJ)/dry ice slurry until the aqueous phase was frozen. The organic phase was poured off and collected in 12x75mm borosilicate glass collection tubes (cat# 14-961-26, Fischer Scientific, Fairlawn, NJ), and dried via a constant stream of N_2 gas at room temp. The aqueous phase was thawed, and the extraction process was repeated for a total of five extraction cycles, each time pouring off only the organic phase into the same 12x75mm collection tubes and drying with N_2 gas. After the fifth extraction cycle, the 12x125mm aqueous phase tubes were stored in -20°C until use in the bound steroid extraction.

Bound steroids extraction

Bound steroids were extracted from the frozen aqueous phase generated in the procedure above using the same protocol but with the following additional steps. First, 16x125mm tubes with aqueous phase precipitate were spiked with an additional 20µL of deuterated standard mix. This was necessary as the first series of extractions removed all the spiked standards (Fig. *2.1*). A new standard tube was added with 20µL of deuterated standard spiked into 480µL of water was added as a reference that that we could measure the amount of residual standard left from the free steroid extraction. A new external standard series was also added. To release the bound steroids from their binding proteins, all tubes were boiled at 100°C for two minutes to denature

proteins and release bound steroid (Grabski and Novagen, 2001). All tubes were then extracted as in the above unbound steroid extraction.

Extracted steroid resuspension

Dried organic extract from bound and unbound extractions (in 12x75mm) tubes were resuspended for liquid chromatography/mass spectrometry (LCMS) analysis in 65µL of 70% optima grade acetonitrile. The solutions were then vortexed for five minutes to collect residual extract on the sides of the tubes, and centrifuged for five minutes at 10,000rpm and 4˚C. The solutions were then transferred to autosampler vials and stored at -20˚C until LCMS analysis.

Liquid chromatography/mass spectrometry

Extracted steroids were identified using a Sciex Exion LC 100 Liquid chromatography system coupled with Sciex API 3200 triple quadrupole mass spectrometer. A Phenomenex Kinetex 2.6µm C18 100 Å, 100x3 mm column (cat# 00D-4462-Y0, Phenomenex, Torrance, CA, USA) was used for positive and negative ionization analysis.

Positive ionization

Testosterone, progesterone, corticosterone, and two estradiol transitions were optimized using positive electrospray ionization. The LC method for these targets included the following mobile phases: Solvent A = 5% acetonitrile with 0.1% formic acid (cat# F0507, Fischer Scientific, Fairlawn, NJ) and Solvent B = 100% acetonitrile. LC gradient consisted of 1min 100% A/0% B, 1-7min increased to 5% A/95% B, 7-9min 5% A/95% B, 9-10min decreased to 100% A/0% B, 10 min to 15 min 100% A/0% C. Flow rate was 0.3 mL/min at a 15µL on column injection. MS

instrument parameters for positive mode are shown in Table *2.1*. Transitions for steroids are listed in Table *2.2*.

LC-MS/MS Analysis

Plasma steroid molecule peaks detected by LC-MSMS were analyzed using Analyst software version 1.6.3. Peak integrations were analyzed with Mutliquant version 3.0.3 using external standard curves to determine sample concentrations. Internal (deuterated) standards were used to confirm observed concentration trends and values of the external curves.

Statistical Analysis

To control for technical differences between runs, plasma steroids were extracted and analyzed via LC-MS/MS as blocks, with a block consisting of a complete experimental replicate (one representative sample of each treatment group). Steroid concentrations were log transformed to contend for zero inflation (many concentrations were close to 0.0µg/mL) and analyzed with normal distributions using linear mixed models with dam as a random effect. Plasma steroid concentrations have inherent variation as their secretion is pulsatile and fluctuates through time and some biologically important changes may not be identified as statistically significant if threshold p-value is less than 5% ($P<0.05$). To contend for potentially important trends, we define significant statistical difference between groups as ($P<0.05$) and marginally significant statistical difference as ($P<0.10$).

Fetal Liver Proteomics

Liver sample preparation

Fetal livers were thawed and 25mg collected from liver samples. Excess blood was washed off liver with cold PBS. The 25mg samples were made by independently cutting the livers into several small pieces and randomly selecting up to 25mg of liver pieces to assure random sampling of the liver tissue. 2 volumes of cold lysis buffer, made of 1% sodium deoxycholate surfactant (SDC, Cat# S3014, Sigma-Aldrich, St. Louis, MO) /50mM ammonium bicarbonate (Cat# 09830, Sigma-Aldrich, St. Louis, MO), were added to each 25mg of liver sample. Liver solutions were fully homogenized with a probe blender for five to ten seconds (depending on sample) and then sonicated for 20 seconds in two intervals with one minute in an ice bath between intervals. Sample solutions were then centrifuged at 16,000xg for ten minutes at 4°C. Supernatant was removed via pipetter and pellets saved for proteomic analysis preparation. SDS-PAGE and BCA assays were conducted to access quality of protein yield.

50mM ammonium bicarbonate with 1% SDC was added to each pellet. Then MDL-Dithiothreitol (DTT, Cat# 43815, Sigma-Aldrich, St. Louis, MO) was added to each pellet solution to reach a concentration of 5mM and incubated for 60°C for 30 minutes. 15mM Iodoacetamide (IAM, Cat# I6125, Sigma-Aldrich, St. Louis, MO) was added to each sample solution and then incubated for 20 minutes in the dark (IAM is light sensitive). Protein clean-up preparation was completed using FASP (Filter Aided Sample Preparation) (Wisniewski et al., 2009). Vivacon 500 30kD spin filters (Cat# 50-311-816; Fischer Scientific, Fairlawn, NJ) were prepped by adding 20µL of 1% SDC to passivate the spin filters. Sample solutions were then transferred to the spin filters, avoiding the protein pellet, and then centrifuged at 14,000xg for 15 minutes to dry the pellet. Then 200µL of 8M Urea (Cat# U5378, Sigma-Aldrich, St. Louis, MO) in 50 mM ammonium bicarbonate and centrifuged at 12,000xg for 15 minutes to dry, then

repeated the process a second time with fresh urea. Next, 200µL of 50mM ammonium bicarbonate (no urea) was added to each sample and then centrifuged at 14,000xg for 15 minutes to, repeating the process a second time.

70µL solution of trypsin (Cat# V5111, Promega, Madison,WI) with 0.01% acetic acid was added to each sample in 50mM ammonium bicarbonate buffer. The solutions were digested for four hours at 37°C. Sample solutions were acidified (to pH of 2) with 4µL of 6M hydrochloric acid and then centrifuged at 12,000xg for 15 minutes. Filters were then washed with 20–30 µL (enough o reach 100µL total volume) and centrifuged at 12,000xg for ten minutes or until dry. Samples were kept in their microcentrifuge tubes at -20°C.

Liquid chromatography/mass spectrometry

Cleaned and digested protein peptides were identified using liquid chromatography tandem mass spectrometry (LC-MS/MS). Peptides (1µg) were loaded onto a 35cm C18 column (reverse phase ReproSilPur 120C-18-AQ 3µm particles, (Cat# H354, New Objective, Woburn, MA) and eluted using a 240-minute gradient with an Easy-nLC 1000 coupled to a Q-Exactive Plus orbitrap mass spectrometer. After a 2µL volume injection, sample peptide separation was achieved on the LC using a gradient of mobile phase A (98% water, 2% acetonitrile, and 0.1% formic acid) and mobile phase B (100% acetonitrile, 0.1% formic acid). The 270min method included an LC gradient with a linear increase from 2% B to 40% B across minutes 2 to 242. This was followed by an increase and wash at 80% B from 242–254 minutes, followed by equilibration of the column at 0% B (*Table 2.3*).

Tandem mass spectrometry was processed in top 12 data-dependent acquisition mode. MS1 and MS2 scans were performed at a resolving power of 70k and 17.5k. A dynamic exclusion window of 20 seconds was used to avoid redundant interrogation of abundant species. Bovine serum albumin was run on column after every three injections to confirm LC-MS/MS reproducibility.

Raw data were processed using MaxQuant linked to the mouse Uniprot protein sequence database. Multiple comparisons in large data sets such as the one used here have innate false discovery errors. To contend for this, the threshold false discovery rate (FDR) was set at 1% for both the peptide and protein levels. The “match between runs” program feature was used to increase peptide identification via molecular mass and retention time across runs.

Significant proteins by Fisher's exact test ($p<0.05$) were then verified by integrating the intensity under the extracted ion chromatogram from a single unique peptide for each protein across all runs. A two-sample t-test ($p<0.05$) based on integrated peak intensities discovered differences in 145 proteins for control versus vinclozolin males, 139 proteins for control versus Vinclozolin females, and 167 proteins for corn oil males versus females, and 104 proteins for Vinclozolin males versus females. Proteins were then quantified using the label-free MaxLFQ (Cox et al., 2014) program in MaxQuant. Protein group filtering, transformation, imputation, and T-test were analyzed using Perseus (Tyanova et al., 2016).

The differential proteins were validated using a triple quadrupole mass spectrometer operating in selected reaction monitoring (SRM) mode. Comparison of peptide abundance across treatments was performed using a 1-way ANOVA analysis followed by Least Significant Difference test of

significant findings. Relative protein quantitation was performed using label free methods (integration of area under the curve) using Skyline and the MSstats package.

Results

Vinclozolin eliminates sexual dimorphism

Progesterone

For free progesterone concentrations no significant effects were found for the for the main effects of sex ($\chi2 = 0.0006$, df = 5, p = 0.9798), or treatment ($\chi2 = 0.3433$, df = 4, p = 0.5579), or for their interaction ($\chi2 = 1.1048$, df = 6, p = 0.2932). For bound progesterone, no significant effects were found for the main effects of sex ($\chi2 = 2.2196$, df = 5, p = 0.1363) or treatment ($\chi2 = 0.2885$, df = 4, p = 0.5912), however, a significant interaction between sex and treatment was identified (*Fig. 2.2A*, $\chi2 = 5.1121$, df = 6, p=0.02376). Bound progesterone was found to be dimorphic between the sexes and was 1.54 times higher in female (x = 9.6406) relative to male (x = 6.2780) corn oil controls. Vinclozolin treated animals showed a reduced dimorphism in bound progesterone (effect size = -33.00% difference). Vinclozolin treatment slightly decreased bound progesterone in females (x = 9.2336) and increased bound progesterone in males (x = 8.9844) for a 1.03 difference between sexes. However, no significant effects were found for the for the main effects of sex ($\chi2 = 0.1998$, df = 5, p = 0.6549), or treatment ($\chi2 = 0.3554$, df = 4, p = 0.5511), or for their interaction ($\chi2 = 2.3119$, df = 6, p = 0.1284) for total progesterone concentrations.

Significant effects were found for the main effect of sex (*Fig. 2.2B*, $\chi2 = 4.7638$, df = 5, effect size =5.1960 p = 0.02901), while significant effects were not found for treatment ($\chi2 = 0.0.1330$,

df = 4, p = 0.7154) for the ratio free to bound progesterone. However, a significant interaction between sex and treatment was identified for the ratio of free to bound progesterone (*Fig. 2.2B*, $\chi 2$ =10.168, df = 6, p=0.001429). Corn oil control females and males showed a dimorphism in their free to bound ratio, with females (x = -1.9032) having a lower ratio of free relative to bound progesterone than males (x = 3.2924). Vinclozolin treatment increased the free/bound ratios in females (x = 0.0579, effect size = 3887% increase) and reduced the ratio in males (x = -0.0316, effects size 96.0% decrease), decreasing the dimorphism.

17β-estradiol

For free 17β-estradiol, no significant effects were found for the main effects of sex ($\chi 2$=1.8505, df=5, p=0.1737), or treatment ($\chi 2$=0.1488, df=4, p=0.6996), or the interaction between sex and treatment ($\chi 2$=0.2507, df=6, p=0.6166) for free 17β-estradiol. No significant effects were found for the main effects of sex ($\chi 2$=0.5713, df=5, p=0.4497), or treatment ($\chi 2$=0.1281, df=4, p=0.7205), or their interaction ($\chi 2$=2.4537, df=6, p=0.1172) for bound 17β-estradiol. No significant effects were found for the main effects of sex ($\chi 2$=1.8926, df=5, p=0.1689), or treatment ($\chi 2$=0.1540, df=5, p=0.6947), or between the interaction of sex and treatment ($\chi 2$=1.8464, df=6, p=0.1742) for total 17β-estradiol.

No significant effects were found for the main effect of sex ($\chi 2$=0.1554, df=5, p=0.6934) or treatment ($\chi 2$=0.4893, df=4, p=0.4842) for the free-to-bound ratio of 17β-estradiol. However, a significant interaction was detected between sex and treatment (*Fig. 2.3*, $\chi 2$ =7.0586, df = 6, p=0.007889) for the free to bound ratio of 17β-estradiol. Corn oil control females and males showed a dimorphism in their free to bound ratio of 17β-estradiol, with females (x = 0.1117)

having a less free relative bound 17β-estradiol than males (1.4600). Vinclozolin treatment increased the free/bound ratios in females (x = 0.3686, effect size = 329.99% increase) and reduced the ratio in males (x = 0.8687, effect size = 40.50% decrease), decreasing the dimorphism.

Corticosterone

There were no significant effects observed for the main effects of sex ($\chi2$=0.6766, df =5, p=0.4108) or treatment ($\chi2$=0.0524, df= 4, p=0.8189) for free corticosterone. However, a marginally significant effect was found between the interaction of sex and treatment ($\chi2$=3.4541, df=6, p=0.06309) for free corticosterone concentrations. For bound corticosterone, a marginally significant effect was seen for the main effect of sex ($\chi2$= 2.9998, df=5, p=0.08327), though not for treatment ($\chi2$=1.8187, df=6, p=0.1775). No significant effect was found between the interaction of sex and treatment ($\chi2$=0.1530, df=4, p=0.6956) for bound corticosterone. No significant difference was found between the main effect of sex ($\chi2$=0.6708, df=5, p=0.4128), or treatment ($\chi2$=0.0277, df=4, p=0.8678) for total corticosterone. A marginally significant effect was found between the interaction of sex and treatment ($\chi2$=2.7876, df =6, p=0.0950) for total corticosterone. No significant effect was found for the main effect of sex ($\chi2$=2.5555, df=5, p=0.1099), or treatment ($\chi2$=2.3783, df=4, p=0.1230), or between the interaction of sex and treatment ($\chi2$=0.0053, df=6, p=0.9422) for the ratio of free/bound corticosterone.

Testosterone ratios unchanged by Vinclozolin exposure

No significant effect was found for the main effects of sex ($\chi2$=05404, df=5, p=0.4622), or treatment ($\chi2$=0.0091, df=5, p=0.9241), or the interaction between sex and treatment ($\chi2$=0.8407,

df=6, p=0.3592) for free testosterone. No significant effect was found for the main effects of sex ($\chi2$=0.0472, df=5, p=0.8279), or treatment ($\chi2$=0.3100, df=5, p=0.577), or the interaction between sex and treatment ($\chi2$=0.0041, df=6, p=0.9490) for bound testosterone. No significant effect was found for the main effects of sex ($\chi2$=0.8957, df=5, p=0.3439), or treatment ($\chi2$=0.0055, df=5, p=0.9409), or between the interaction of sex and treatment ($\chi2$=0.9084, df=6, p=0.3405), for total testosterone. No significant effect was found for the main effects of sex ($\chi2$=0.1291, df=5, p=0.7194), or treatment ($\chi2$=0.0714, df=5, p=0.7892), or the interaction between sex and treatment ($\chi2$=0.0134, df=6, p=0.9079) for the ratio of free/bound testosterone.

Ratios of bound steroid precursor, Progesterone, to downstream targets lose sexual dimorphism when exposed to Vinclozolin.

Progesterone / Testosterone

No significant effect was found for the main effects of sex ($\chi2$=0.0189, df=5, p=0.8907), or treatment ($\chi2$=1.0234, df=4, p=0.3117), or the interaction between sex and treatment ($\chi2$=1.4261, df=6, p=0.2324) for the ratio free progesterone to free testosterone. A significant effect was found for sex (*Fig.3A*, $\chi2$=7.8258, df=5, p=0.0052) in bound progesterone to bound testosterone, but not for treatment ($\chi2$=0.0.6227, df=4, p=0.4300). A significant interaction was detected between sex and treatment (*Fig. 2.4A*, $\chi2$ =10.500, df = 6, p=0.0012) for the ratio of bound progesterone to bound testosterone. Corn oil control females and males showed a dimorphism in their ratio of bound progesterone to bound testosterone, with females (x = 1.4724) having a higher ratio of bound progesterone to bound testosterone than males (x = -2.4779). Vinclozolin treatment decreased the bound progesterone to bound testosterone ratios in females (x = 0.7497, effect size = 49.07% decrease) and increased the ratio in males (x = -1.1905 effect size =

308.04% increase), decreasing the dimorphism. No significant effect was found for sex ($\chi2$=1.0416, df=5, p=0.3075), treatment ($\chi2$=1.5549, df=4, p=0.2124) or the interaction between sex and treatment ($\chi2$=1.068, df=6, p=0.2356) for the ratio of total progesterone to total testosterone.

Progesterone / 17β-estradiol

No significant effect was found for the main effects of sex ($\chi2$=0.1473, df=5, p=0.7011), or treatment ($\chi2$=1.5723, df=4, p=0.2099), or the interaction between sex and treatment ($\chi2$=0.6267, df=6, p=0.4286) for the ratio free progesterone to free estradiol. A significant effect was found for the main effect of sex (*Fig. 2.4B*, $\chi2$=0.6.8336, df=5, p=0.0089), but not treatment ($\chi2$ =2.2759, df = 4, p=0.1314) in bound progesterone to bound 17β-estradiol. A significant interaction was detected between sex and treatment (*Fig. 2.4B*, $\chi2$ =8.5782, df = 6, p=0.0034) for the ratio of bound progesterone to bound 17β-estradiol. Corn oil control females and males showed a dimorphism in their ratio of bound progesterone to bound estradiol, with females (x = 0.4344) having a higher ratio of bound progesterone to bound 17β-estradiol than males (-4.3784). Vinclozolin treatment decreased the progesterone to estradiol ratios in females (x = -0.8010, effect size = 164.84%) and increased the ratio in males (x = 0.3224, effect size = 9,314.04% increase), decreasing the dimorphism.

No significant effect was found for the main effect of sex ($\chi2$ =1.0416, df =5, p=0.3075) in total progesterone to total 17β-estradiol, but a significant effect was found for treatment ($\chi2$ =3.3505, df = 4, p=0.0672) and for the interaction between sex and treatment ($\chi2$ =4.1102, df = 6, p=0.04263) for the ratio of total progesterone to total 17β-estradiol. Corn oil control females and

males showed a dimorphism in their ratio of total progesterone to total 17β-estradiol, with females (x = 0.0558) having a higher ratio of progesterone to 17β-estradiol than males (-1.7947). Vinclozolin treatment decreased the progesterone to 17β-estradiol ratios in females (x = -0.0842, effect size = 249.96% decrease) and increased the ratio in males (x = 0.0614, effect size = 3,022.96% increase), decreasing the dimorphism.

Progesterone/Corticosterone

No significant effect was found for the main effect of sex ($\chi2$=0.5548, df=5, p=0.4564) in ratio of free progesterone to free corticosterone, thought a significant effect was found for treatment ($\chi2$=3.9664, df=4, p=0.04642). No significant effect was found for the interaction between sex and treatment ($\chi2$=0.2371, df=6, p=0.6263) for the ratio free progesterone to free corticosterone. A significant effect was found for the main effect of sex (*Fig. 2.4C*, $\chi2$ =5.0594, df = 5, p=0.0245) for the ratio of bound progesterone to bound corticosterone, though no significant effect was found for treatment ($\chi2$ =0.0585, df = 4, p=0.8089). A significant interaction was detected between sex and treatment (*Fig. 2.4C*, $\chi2$ =8.5364, df = 6, p=0.0035) for the ratio of bound progesterone to bound corticosterone. Corn oil control females and males showed a dimorphism in their ratio of bound progesterone to bound corticosterone, with females (x = 0.0760) having a higher ratio of bound progesterone to bound corticosterone than males (-3.8444). Vinclozolin treatment decreased the bound progesterone to bound corticosterone ratios in females (x = -0.2211, effect size = 388.64% decrease) and increased the ratio in males (x = -1.1948, effect size = 321.76% decrease), decreasing the dimorphism.

Testosterone/ 17β-estradiol

No significant effect was found for the main effects of sex ($\chi2$=0.4832, df=4, p=0.4870), or treatment ($\chi2$=2.0455, df=6, p=0.1527), or the interaction between sex and treatment ($\chi2$=1.9422, df=5, p=0.1634) for the ratio of free testosterone to free estradiol. No significant effect was found for the main effects of sex ($\chi2$=0.0252, df=5, p=0.8740), or treatment ($\chi2$=0.0024, df=4, p=0.9610) for the ratio of bound testosterone to bound 17β-estradiol. A marginally significant interaction was detected between sex and treatment (*Fig. 2.5*, $\chi2$ =2.9142, df = 6, p=0.0878) for the ratio of bound testosterone to bound 17β-estradiol. Corn oil control females and males showed a dimorphism in their ratio of bound testosterone to bound 17β-estradiol, with females (x = -1.6603) having a lower ratio of bound testosterone to bound 17β-estradiol than males (-0.1588). Vinclozolin treatment increased the bound testosterone to bound 17β-estradiol ratios in females (x = -1.9076, effect size = 114.89% decrease). Vinclozolin decreased the mean bound testosterone to bound 17β-estradiol ratios in males (x = -1.0306, effect size = 648.99% decrease). This results in reduced dimorphism as Vinclozolin males are indistinguishable from females in regard to bound testosterone to 17β-estradiol ratios. No significant effect was found for the main effects of sex ($\chi2$=1.0416, df=5, p=0.3075), or treatment ($\chi2$=0.0088, df=4, p=0.9252), or the interaction between sex and treatment ($\chi2$=0.7157, df=6, p=0.3976) for the ratio total testosterone to total 17β-estradiol.

Fetal Liver Steroid Transport Protein Proteomics

Fetal liver proteomes were analyzed to detect normal dimorphism and changes in steroid transport proteins concentrations due to Vinclozolin exposure.

No sexual dimorphism in steroid binding proteins within liver

No significant difference was found for alpha-1-fetoprotein between male and female controls (p=0.28226). Fold change from male to female was found to be (-0.114244). No significant difference was found for corticosteroid binding globulin between males and female controls (p=0.913782). Fold change from male to female was (0.0274777). Albumin was also shown to have no difference between male and female controls (p=0.670666). Fold change from male to female controls was found to be (0.115814).

No significant difference was found for alpha-1-fetoprotein between male and female Vinclozolin (p=0.308734). Fold change from male to female was found to be (-0.176465). No significant difference was found for corticosteroid binding globulin between males and female Vinclozolin (p=0.786258). Fold change from male to female was (-0.09691672). Albumin was also shown to have no difference between male and female controls (p=0.762455). Fold change from male to female Vinclozolin was found to be (-0.0726662).

Vinclozolin exposure does not change fetal liver steroid binding protein concentrations.

No significant difference was found for alpha-1-fetoprotein between male control and male Vinclozolin (p=0.586694). Fold change from male control to male Vinclozolin was found to be (-0.0641747). No significant difference was found for corticosteroid binding globulin between male control and male Vinclozolin (p=0.876136). Fold change from male corn oil to male Vinclozolin was (-0.0477915). Albumin was also shown to have no difference between male control and male Vinclozolin (p=0.769335). Fold change from male control to male Vinclozolin was found to be (-0.0807247).

No significant difference was found for alpha-1-fetoprotein between female control and female Vinclozolin (p=0.427344). Fold change from female control to female Vinclozolin was found to be 0.126296. No significant difference was found for corticosteroid binding globulin between female control and female Vinclozolin (p=0.806646). Fold change from female to female was (0.0766034). Albumin was also shown to have no difference between female control and male Vinclozolin (p=0.649299). Fold change from female control to female Vinclozolin was found to be 0.107785.

Discussion

EDCs have been shown to alter free or total steroid concentrations, however, few studies have addressed how fractions of steroids change due to EDC exposure (free, bound, total steroid, or free/bound ratios) and if these changes alter normal dimorphism. Here we show that fetal plasma steroid fractions are dimorphic, and that the dimorphism is reduced by Vinclozolin (*Table 2.4*). Females show a higher concentration of progesterone, a higher ratio of bound progesterone to free progesterone, and a higher ratio of bound progesterone than bound metabolites (testosterone, 17β-estradiol, and corticosterone) and total progesterone to total 17β-estradiol. The opposite is the case for males, where males have more free progesterone to bound progesterone and a higher ratio of bound metabolites to bound progesterone. These data show a consistent dimorphism in the steroid fraction that males and females circulate, with males having more progesterone that can be easily metabolized to more potent steroid nuclear receptor signaling molecules and greater effect of steroid feedback mechanisms by binding inhibitive nuclear receptors in the hypothalamus and pituitary. Conversely, females have more progesterone bound to proteins and

unable to be converted to downstream steroids, less stimulation of negative feedback, and a higher rate of binding steroid binding protein receptors and driving non-genomic steroid signaling.

Vinclozolin treatment reduced the ratio of bound progesterone to free progesterone for females as well as reduced the bound progesterone to bound steroid metabolite ratio. The exact opposite effect was seen in males where free progesterone to bound progesterone concentrations were increased as wells as bound progesterone to bound metabolites increased. Vinclozolin shifts females to a higher ratio of rate of progesterone metabolism, a higher ratio of progesterone to its downstream metabolites, and potentially increases negative feedback mechanisms in females while doing the opposite in males. The only ratio that differed from this pattern was for bound testosterone to 17β-estradiol (metabolite of testosterone), where both control males and females had more bound 17β-estradiol than bound testosterone. Vinclozolin increased the bound testosterone to 17β-estradiol ratio in males while decreasing the ratio in females. In all cases, the effect of Vinclozolin reduced natural dimorphism by driving masculinizing female and feminizing male ratios.

Progesterone, an upstream steroid precursor to corticosterone, testosterone, and estradiol, was shown to be dimorphic in the bound fraction (*Fig. 2.1A*), with corn oil females having higher bound concentrations than corn oil males (*Fig. 2.1A*) and lower free to bound ratios than males (*Fig. 2.1B*). When exposed to Vinclozolin, remarkably males and females lose their dimorphism (*Fig. 2.1A&B*). It is known that Vinclozolin exposure demasculinizes male genitalia, while anatomical differences in Vinclozolin females are less extreme. This data shows that Vinclozolin

exposed females pups have reduced feminization in progesterone ratios and males have reduced masculinization of progesterone ratios. These results reveal two critical breakthroughs in our understanding of steroid dependent development. Steroid fractions, not just free concentrations of steroids, are normally dimorphic with females showing higher bound steroids and higher ratios of less potent precursor molecules to more potent metabolites than males. Strikingly, Vinclozolin eliminates this dimorphism. Given that Vinclozolin is an androgen receptor antagonist, it is interesting that we did not find statistically significant changes in testosterone free, bound, total, or ratios as we would expect that Vinclozolin would reduce inhibition of brain negative feedback and propagate testosterone production at the gonadal level. This suggests that binding of testosterone to transport proteins may be the chief change with Vinclozolin exposure rather than direct effects on brain feedback control. Other EDCs may have similar effects and future work with EDCs need to consider fractions as these effects would be missed if we only measured free concentrations.

Furthermore, bound progesterone alone was altered as well as ratios of bound progesterone to its downstream metabolites corticosterone, testosterone, and 17β-estradiol. The importance of progesterone as a key steroid precursor means that its relationship to its downstream metabolites is important. These data show that the ratio of bound progesterone to bound corticosterone (*Fig. 2.4A*), bound progesterone to bound testosterone (*Fig.2.4B*), and bound progesterone to bound 17β-estradiol (*Fig. 2.4C*) in corn oil females is higher than those ratios in males. Females naturally show a higher ratio of a bound precursor unable to directly bind transcriptional mechanisms, but capable of binding transport protein membrane receptors. When exposed to Vinclozolin, these differences in bound ratios are statistically lost with females decreasing their

bound progesterone to bound metabolite (*Fig. 2.4A-C*) ratios, whereas males increasing their bound progesterone relative to bound metabolites. Lastly, the bound ratio of bound testosterone to bound 17β-estradiol is altered by Vinclozolin (*Fig. 2.5*) with males losing their higher normal bound testosterone to bound 17β-estradiol ratio after treatment. These downstream changes combined with upstream progesterone changes illustrate the potential for complete shifts in steroidogenesis across the steroid pathway.

This study shows that the bound steroid fractions, were altered by Vinclozolin whereas the free fractions were not. With a higher fraction of bound progesterone females likely have a higher contribution of progesterone-corticosteroid binding globulin complexing to the corticosteroid binding globulin receptor resulting in increased non-genomic signaling compared to males. Females have higher bound progesterone and a higher ratio of bound to free progesterone (*Fig. 2.1B*). This suggests that males have a higher content of progesterone that can be more readily taken up into cells and metabolized to downstream steroids or more potent ligands to steroid receptors (corticosterone, testosterone, 17β-estradiol, etc.) compared to females. As an example, we can consider free to bound ratios of 17β-estradiol (*Fig. 2.2*), a potent estrogen and downstream metabolite of progesterone. Corn oil males have a higher ratio of free to bound 17β-estradiol than corn oil females and a higher potential for genomic steroid signaling in tissues where ER is expressed.

It has recently become more apparent that steroid signaling is beautifully complex and this study suggests that measuring single concentrations of free steroids as an endpoint in both directly manipulated and correlative studies can miss significant biologically important results. Our work

shows that ratios of steroids, not just single free steroids, are dimorphic and exposure to EDCs, such as Vinclozolin, can affect both male and female sexual differentiation. Having multiple steroids and fractions measured simultaneously provides a wider and more relevant scope of steroid signaling. This is especially true considering that steroids are not independently transcribed but synthesized in an interconnected metabolic pathway with concentrations of one steroid being dependent on another.

Proteomic analysis of steroid transport proteins shows that alterations in free and bound fractions occur in the absence of changes in liver steroid transport protein concentrations. Our results did not show a significant difference between corn oil and Vinclozolin treatment for liver albumin, alpha-1-fetoprotein, or corticosteroid binding globulin, nor were there significant differences in these proteins between sexes. This is important as this suggests that abundance of transport proteins isn't be directly affected by Vinclozolin, but rather the interaction between protein and the steroid is affected. Vinclozolin, or its metabolites, may competitively bind transport proteins, alter their conformation via allosteric binding, or affect protein structure by a posttranslational mechanism. Currently, none of these routes have been studied and further work will be necessary to distinguish which of the mechanisms may be altered.

There are potential caveats to our finding concerning the lack of significant changes in transport protein concentration. First, it has been previously noted that AFP production in the fetal liver may be related to fetal hepatocyte cell division, with increases in proliferation correlating with increases in AFP mRNA synthesis (Sell et al., 1974). Despite evidence that estrogens increase the rate of hepatocyte proliferation in adult rat liver regeneration (Francavilla et al., 1984;

Francavilla et al., 1986; Francavilla et al., 1989), it may be possible that AFP is not be directly linked to steroid nuclear receptor activation, but rather cell cycle mechanisms. Albumin, which is a close evolutionary ancestor of AFP and shares conservation in most structure (Pñeiro et al., 1982), was also shown to have a positive correlation between mRNA concentration and proliferation in mature hepatocytes (Hannah et al., 1980) and may have similar independence to steroid receptor mechanisms as AFP. It is possible that CBG and other transport proteins may have a similar cell division-based regulation during fetal development rather than steroidal regulated proliferation.

Second given the sheer abundance of these proteins, it is possible that change in protein concentration, though small, may be biologically important. Third and final, is that change (or lack thereof) in concentration of transport proteins in the liver tissue may not be representative of concentrations in the plasma, where transport proteins functionally regulate steroids. Due to the low volume of plasma available in our pups, circulating transport protein concentrations were impossible to retrieve. Furthermore, capturing liver tissue and interstitial fluid protein concentrations captures newly produced, circulating, and degrading transport proteins in the liver. Further study of protein expression may provide a more direct measurement of EDC effects on transport protein synthesis.

Conclusions

Free and bound fractions of fetal steroids are normally dimorphic with females having higher bound to free ratios and higher precursor steroid progesterone to metabolites ratios than males. Vinclozolin, the model antiandrogen EDC, alters fractions of free and bound steroids during fetal

development and changes to ratio of progesterone to its metabolites and in a sex-dependent way. Decreasing the ratio in females and increasing the ratio in females. These EDC driven alterations are sex-dependent and lead to a reduced dimorphic profile of steroids and steroid fractions that influence reduced dimorphic phenotypes seen in many common congenital diseases (i.g hypospadias (Amato and McCoy, 2016)). Most evidence of endocrine disruption focuses on single steroid to single phenotype changes without regard to their free or bound fraction or relation to other steroid metabolites. This perspective places individual steroids into solitary vacuums and neglects the complex interactions of steroids that drive dimorphic development. This study shows that steroid fractions, particularly the oft ignored bound fraction, and ratios to each other are naturally dimorphic in the fetus and thus are relevant to sex-dependent development, can be altered by EDCs, and steroid fractions must be considered when evaluating the developmental effects of EDCs to better understand the complete role that EDCs play in developmental disease.

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CHAPTER 3: THESIS CONCLUCIONS AND IMPACTS

Years of research have shown the importance that steroid hormones contribute to sexual differentiation and healthy dimorphisms. Paralleled to these endeavors, are decades worth of studies that show roles that steroid transport proteins play in regulating steroid activity by separating steroids into free and bound fractions. Despite the wide range of academic attention that both steroids and their transports have garnered, few studies have sought to synthesis these fields of work into a more descriptive, albeit complex, model of steroid function and disruption *(Fig 1.1)*. Furthermore, understanding of this natural complexity represents the very solution for combating the ever-present threat of endocrine disruption. Here, presented in this Thesis, I have addressed four major aspects in the disruption of steroid signaling and the roles that steroid transport proteins play in this disruption.

First, presented in chapter I, I reviewed the past and current literature of the major steroid transport proteins, their regulation, and exposure to common environmental contaminants, and show that steroid transport proteins have the clear potential to be altered by a diverse array of endocrine disruptors, whether in concentration or function. These alterations were found to occur at the level of transport protein expression, the interface of transport protein and its cognate steroids, by modulating the structure of the protein, and/or by interfering with transport protein binding their receptors.

Second, using LCMSMS analysis of fetal plasma, I show that there are natural dimorphisms in fractions of free and bound steroids, with females generally having a higher fraction of bound steroid that males and that females have a higher concentration of bound precursors compared to

their male counterparts. This work is critical in showing that dimorphisms do exist in steroid fraction between male and females during early stages of sexual differentiation, and these differences in free and bound steroids may drive the mechanisms that divert males and females from a bipotential state. Most studies measuring steroid concentrations as an endpoint in endocrine disruption report only free steroids, though on occasion sum have provided total steroid fractions. This study provides the evidence to justify measuring free and bound steroids for future study as the dimorphism in steroid physiology shown here would have been impossible without consideration of steroid fractions. Future work should aim to manipulate concentrations of free and bound fractions of steroids to measure phenotypic endpoints dimorphism both anatomically and physiologically. One example, would be to directly alter free progesterone by increasing or decreasing its concentration. Doing so will likely alter available substrate for creating downstream steroid metabolites and alter their concentrations and may have considerable impacts on dimorphic development.

The third major contribution of this thesis, was in showing that a model EDC and antiandrogen can indeed alter steroid fractions. As noted in the review found in chapter one, there has been evidence of changes in transport proteins due to therapeutic and synthetic steroids, however studies of antiandrogen effects were lacking. What is notable in this study is that we found changes in steroid fractions directly in the form of altered fraction ratios with antiandrogen exposure and that these ratios where not based on changes of transport protein concentration of the liver. This suggests some form of disruption in steroid binding to the transport protein. Whether Vinclozolin is competitively binding transport proteins, displacing native steroids from the transport binding pocket, or modulating the transport allosterically is unknown. Future work

should further explore the binding kinetics and protein interactions of these transports and Vinclozolin.

The last major finding from this research, is that male and female fetuses responded to Vinclozolin differently, in terms of their steroid fractions. Males exposed to Vinclozolin exhibited a feminization of fraction while, females exhibited the opposite effect with a non-statistically significant, but clear and recurring trend toward masculinization. These changes show the importance in measuring steroid fractions as many common human reproductive diseases such as hypospadias and poly cystic ovarian syndrome as the results of reduced dimorphism in steroid physiology. EDC driven effects of altered steroid fractions may be culprits for the large increased diagnoses seen in such diseases. Interestingly, no dimorphism in the post translational modification, general moiety, or concentrations of transport proteins has been shown, though little study currently exists. Further biochemical analysis of male and female protein and maintenance of the protein post production would help solve this gap in knowledge.

It is has become apparent over the past several decades that humanity's successes in production have led to a demise in the efficacy of our world's biological development. With an ever-increasing list of disruptive chemicals that alter the physiology and anatomy of both humans and wildlife, describing the process by which EDCs affect development is paramount. In culmination of its works and words, this study has moved our knowledge of steroid transport physiology and disruption one step forward, with the hope that humans and wildlife will be one step closer to a healthier future.

Table 2.1. LC-MS Method Parameters for Positive Mode

Parameter	Unit	Parameter	Unit
LC flow pressure range	0-3000 psi	Ion spray Voltage	5500 V
Total low time	15 min	Source Temperature	500 °C
Injection Speed	15.0 µL/s	GS1	20 psi
Column temp	35 -65 °C	GS2	10 psi
Injection volume	15 µL	Declustering Potential	40 V
Curtain Temperature	25 °C	Collision Energy	50 eV

Table 2.2: Steroid and Deuterated Standard Transitions for Positive Mode

Molecular ID	Molecular Ion (Da)	Fragment (Da)	Collision Energy (V)
17β-estradiol	255.2	159.2	50
17β-estradiol	255.2	255.2	50
Testosterone	289.2	97.2	50
Testosterone	289.2	109.2	50
Testosterone-D3	292.2	97.2	50
Testosterone-D3	292.2	109.2	50
Progesterone	315.1	109.1	50
Progesterone-D9	324.445	100	50
Progesterone-D9	324.445	113	50
Corticosterone	347.1	121	50
Corticosterone-D8	355.403	100	50
Corticosterone-D8	355.403	125.1	50

Table 2.3: LC Method for Proteomics

Time (s)	duration (s)	Flow (nl/min)	% solvent B
2	2	300	0
242	240	300	40
244	2	300	80
254	10	300	80
255	1	300	0
256	1	300	0
270	14	300	0

Table 2.4. Summary of Steroid Fraction Dimorphism and Response to Vinclozolin

Steroid Fractions	Corn Oil Male	Corn Oil Female	Vinclozolin Male	Vinclozolin Female
Bound Progesterone	Lower	Higher	Increase	Decrease
Free/Bound Progesterone	Higher	Lower	Decrease	Increase
Free/Bound 17β-estradiol	Higher	Lower	Increase	Decrease
Bound Progesterone/Bound Corticosterone	Lower	Higher	Increase	Decrease
Bound Progesterone/Bound Testosterone	Lower	Higher	Increase	Decrease
Bound Progesterone/Bound 17β-estradiol	Lower	Higher	Increase	Decrease
Total Progesterone/Total 17β-estradiol	Lower	Higher	Increase	Decrease
Bound Testosterone/Bound 17β-estradiol	Higher	Lower	Decrease	Decrease

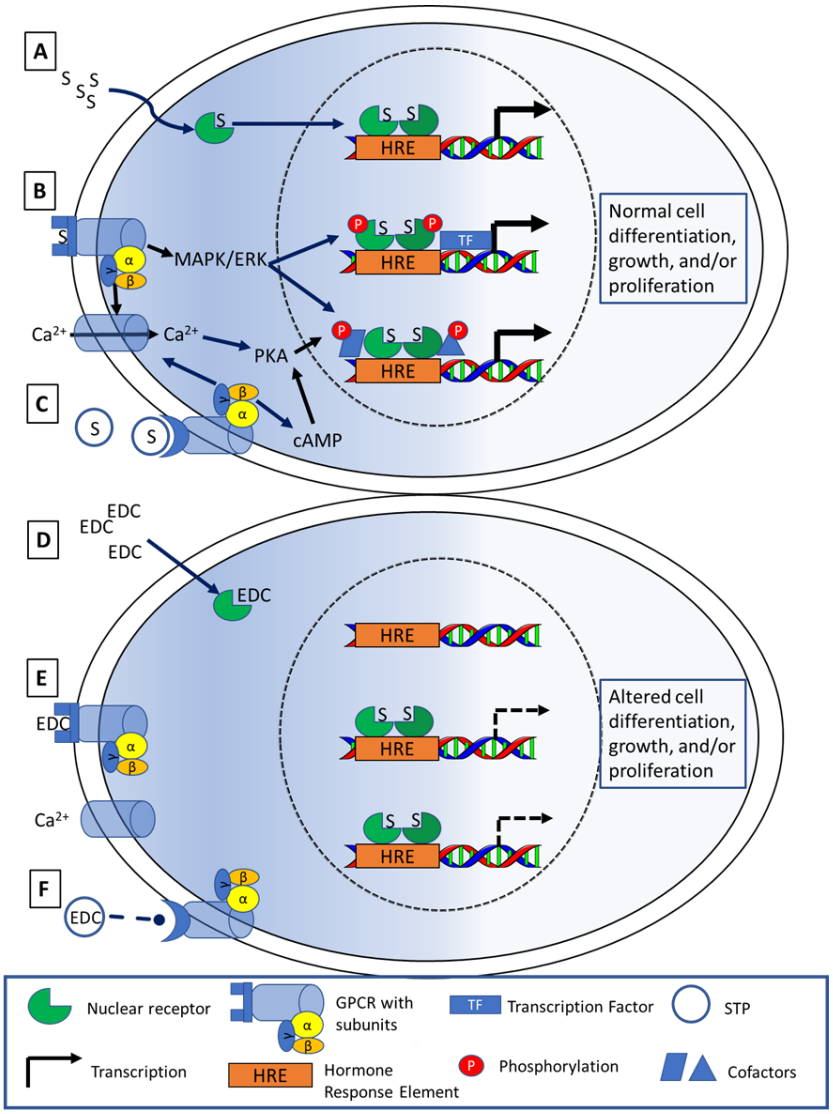

Fig 1.1 Steroid signaling and routes of EDC disruption. *On the top:* A) Classic "genomic" signaling. An unbound steroid molecule (S) permeates the cell membrane, where it binds intracellular nuclear receptor. The nuclear receptor forms a homodimer and is relocated to the nucleus. The homodimer complex binds to the hormone response element (HRE), driving transcription of steroid dependent genes leading to cell differentiation, growth, and/or proliferation. B) Steroids can also bind the G protein-coupled receptor driving MAPK/ERK pathways that activates transcription factors (TF) which alters steroid dependent transcription and/or directly phosphorylates (P) steroid receptors, enhancing steroid receptor binding to the HRE and facilitating steroid dependent transcription. GPCR steroid membrane can also modulate Ca^{2+} influx into the cell and activate the PKA cascade. PKA phosphorylates cofactors that increase steroid nuclear receptor binding to the HRE and steroid dependent transcription. C) Steroids bound to STPs may interact with specific STP receptors. Activation can result in Ca^{2+}influx and/or direct cAMP phosphorylation. cAMP can activate PKA, which in turn can phosphorylate cofactors that enhance nuclear receptor binding. *On the bottom:* Endocrine disrupting chemicals (EDC) alter pathways of steroid signaling and effect cell development. Shown here are antagonistic effects of EDCs, such as Vinclozolin, that antagonize hormone nuclear receptors. However, EDCs may be antagonistic or agonistic and may have different effects depending on the target cells' receptor types. D) EDCs can bind nuclear receptor agonistically preventing it from dimerizing and binding the HRE. This may result in loss of normal cell differentiation, growth, and/or proliferation. E) EDCs may bind steroid membrane receptors causing decreases to normal MAP, ERK, and PKA mediated nuclear receptor and transcription factor phosphorylation. F) EDCs bound to steroid binding proteins do not activate steroid transport protein receptor functions. Phosphorylation cascades are not activated and phosphorylation of cofactors is reduced. Incorporating steroid transport protein function into models of endocrine disruption will facilitate research efforts that aim to understand the health implications of exposure.

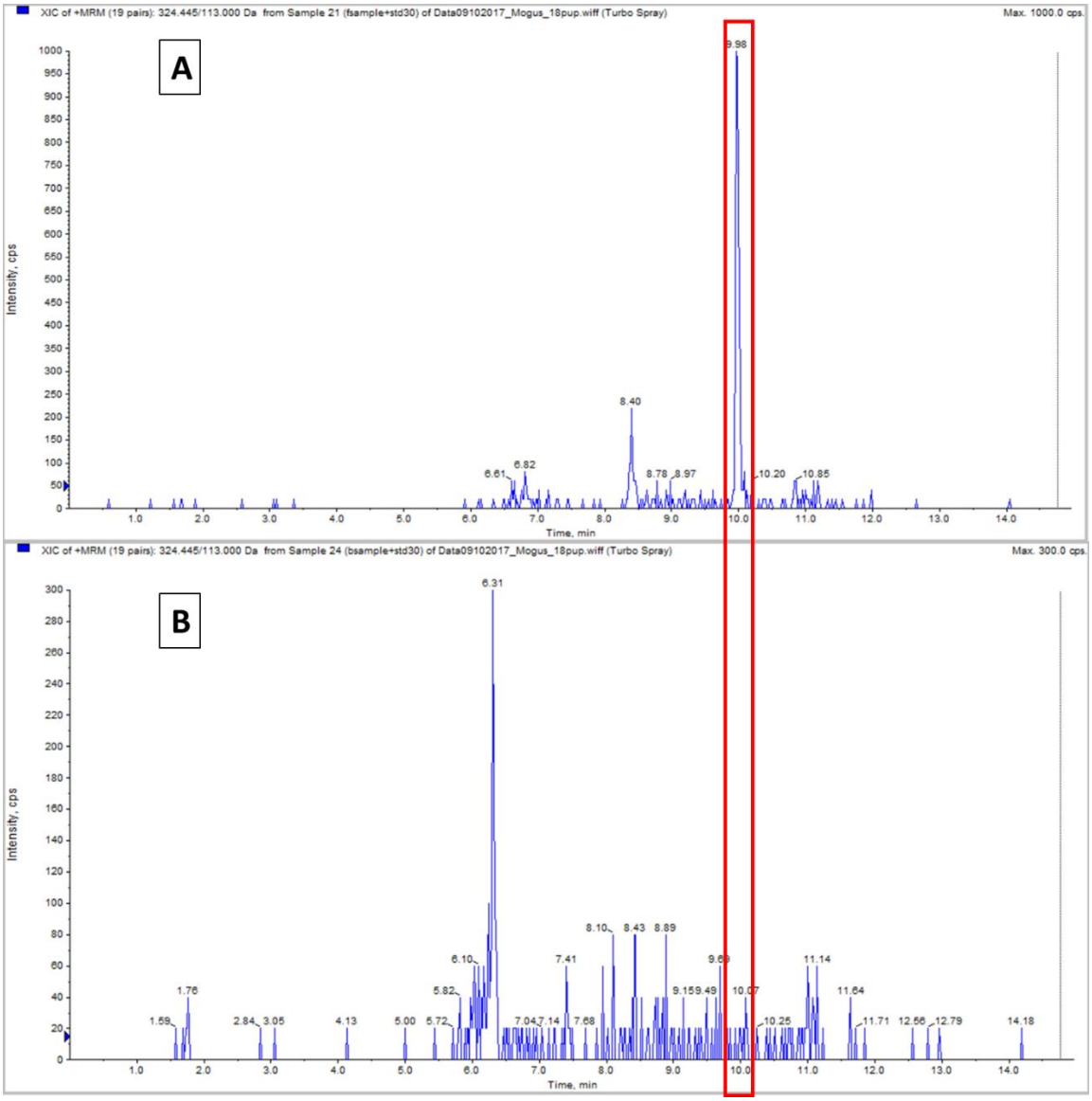

Figure 2.1: No detection of free steroid carryover into bound extraction. X-axis is molecule retention time in minutes, Y-axis is signal intensity represented as molecules signal counts per second (cps). (A) Chromatogram of free fraction corn oil female plasma injected with progesterone-D8 (Fragment peaks of 324.445/113.000 Da, expected retention time of 9.98 min) external standard. Peak reading shows relative intensity of for progesterone-D8 as 970 cps. Note that the y-axis scale spans 0 cps to 1000 cps. (B) Chromatogram of bound fraction in the same corn oil female plasma after free steroids and spiked porgesterone-D8 were extracted. No peak is seen at the expected retention time of 9.98 min for progesterone-D8 (324.445/113.000 Da), showing that there is no carryover of free steroid into bound steroid analysis. Note that the y-axis scale spans from 0 cps to 300

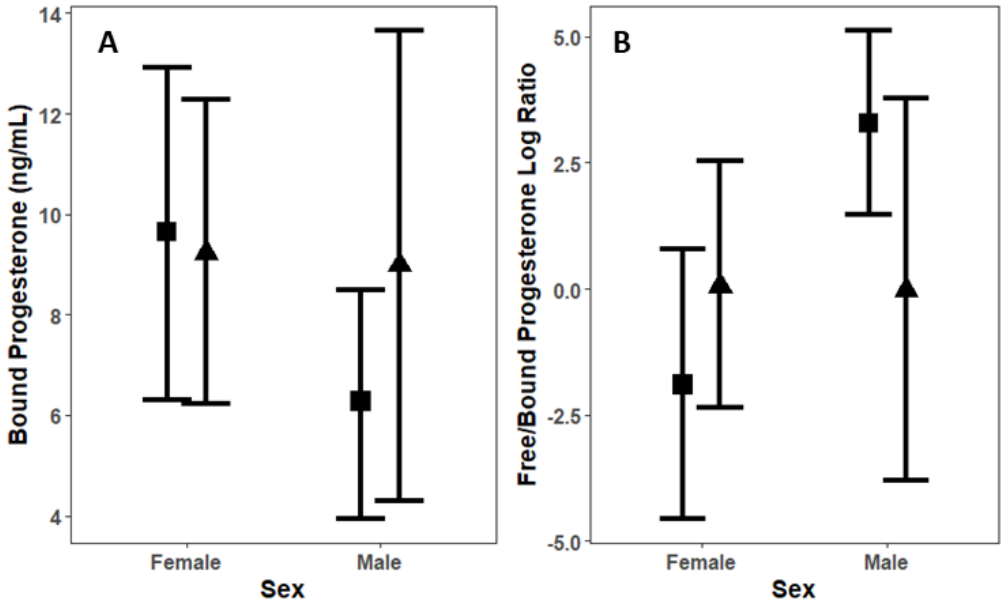

Figure 2.2: Progesterone fractions in males and females when exposed to Vinclozolin. (A) Log concentration of bound progesterone. (B) Log ratio of free-to-bound progesterone. Squares are corn oil control and triangles are Vinclozolin. A log ratio value of 0 shows 1:1 ratio. All positive values have a higher free than bound progesterone, all negative values show higher bound than free progesterone.

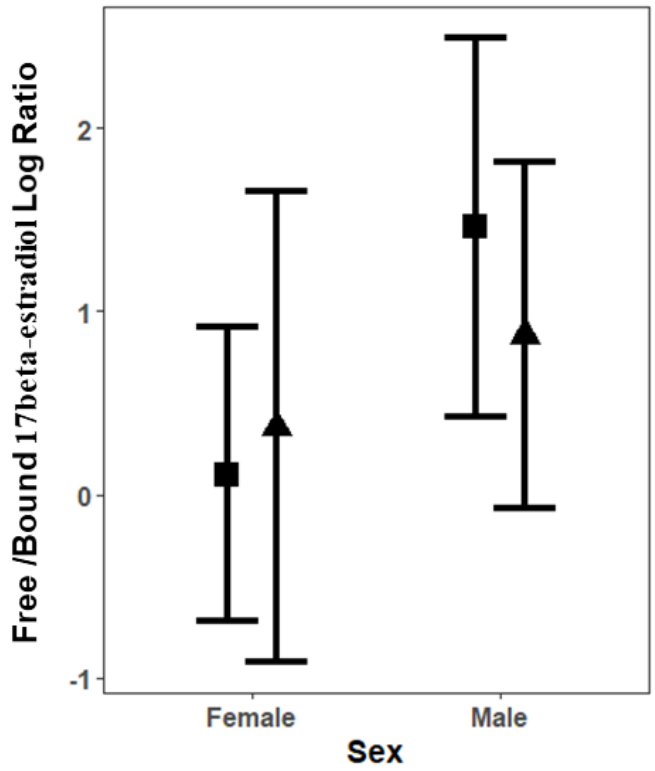

Figure 2.3: 17β-estradiol ratio in males and females when exposed to Vinclozolin. Log ratio of free-to-bound 17β-estradiol concentrations. Squares are corn oil control and triangles are Vinclozolin. A log ratio value of 0 shows 1:1 ratio. All positive values have a higher free than bound 17β-estradiol, all negative values show higher bound than free 17β-estradiol.

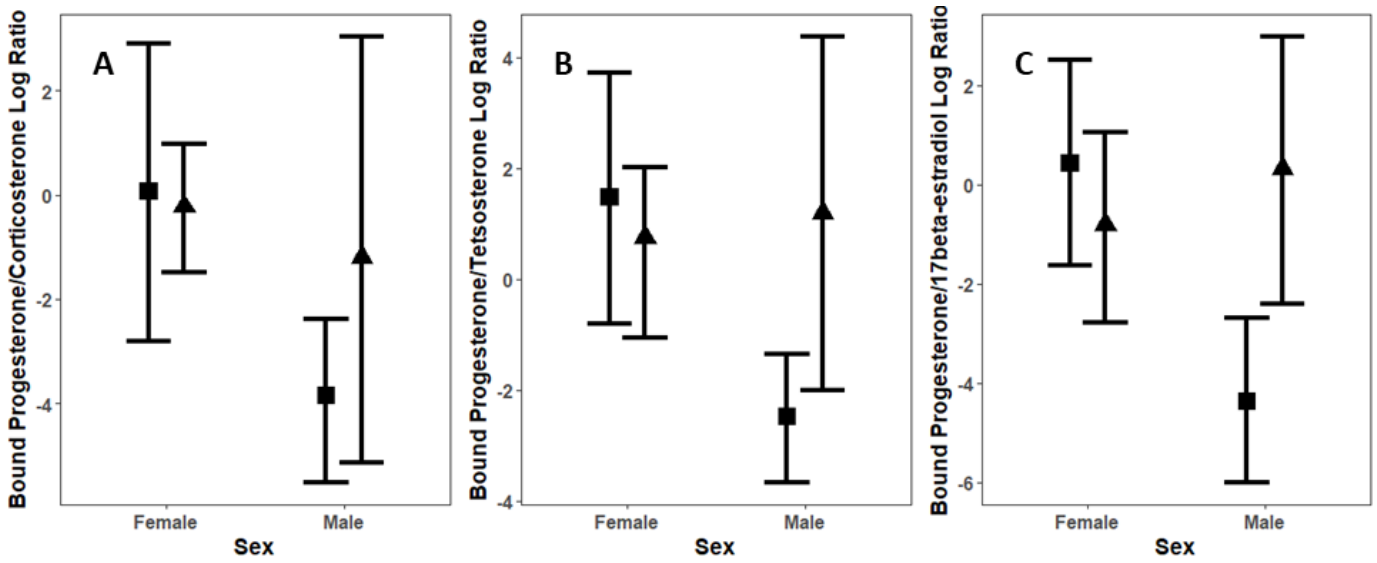

Figure 2.4: Bound progesterone to bound steroid metabolite ratios in males and females when exposed to Vinclozolin. (A) Log ratio of bound progesterone/corticosterone. B) Log ratio of bound progesterone/testosterone. (C) Log ratio of bound progesterone/17β-estradiol. Squares are corn oil control and triangles are Vinclozolin. A log ratio value of 0 shows 1:1 ratio. All positive values have a higher bound progesterone than bound metabolite, all negative values show higher bound metabolite that bound progesterone.

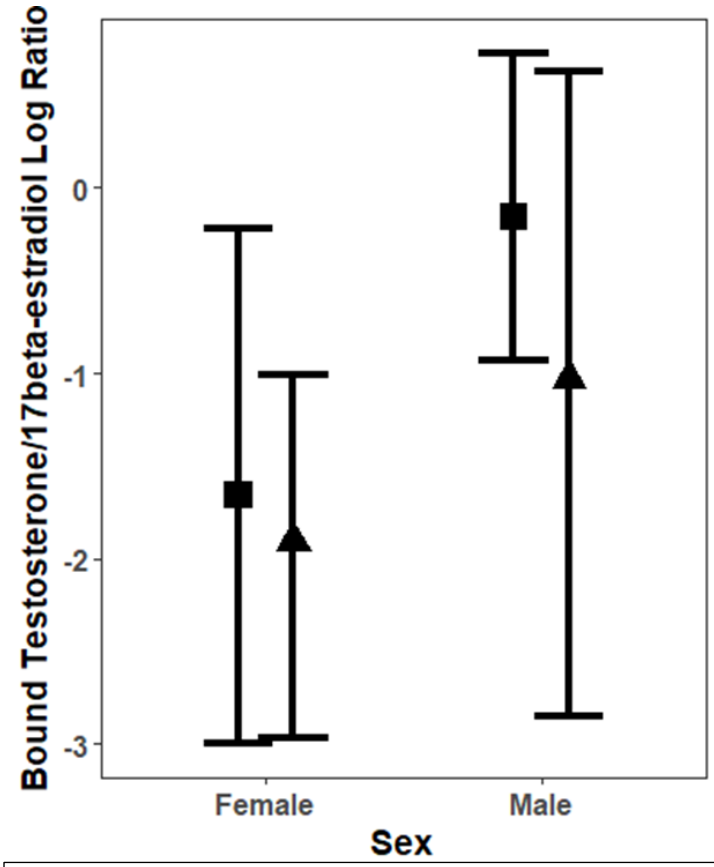

Figure 2.5: Bound testosterone to bound 17β-estradiol log ratio in males and females when exposed to Vinclozolin. Squares are corn oil control and triangles are Vinclozolin. A log ratio value of 0 shows 1:1 ratio. All positive values have a higher bound testosterone than bound 17β-estradiol, all negative values show higher bound 17β-estradiol than bound testosterone.

APPENDIX A: IACUC APPROVAL LETTER AUP #D297a

East Carolina University

mal Care and
U..e Commitee
212 Ed Warren Life
Sciences Building
East Carolina University
Greenville, NC 27834

252-744-2436 office
252-744-2355 fax

October 20, 2016

Krista McCoy, Ph.D.
Department of Biology
Howell Science Complex
East Carolina University

Dear Dr. McCoy:

Your Animal Use Protocol entitled, "Developing a Nutritive Therapy for Reducing the Severity and Incidence of Hypospadias" (AUP #D297a) was reviewed by this institution's Animal Care and Use Committee on October 20, 2016. The following action was taken by the Committee:

"Approved as submitted"

Please contact Aaron Hinkle at 744-2997 prior to hazard use

A copy is enclosed for your laboratory files. Please be reminded that all animal procedures must be conducted as described in the approved Animal Use Protocol. Modifications of these procedures cannot be performed without prior approval of the ACUC. The Animal Welfare Act and Public Health Service Guidelines require the ACUC to suspend activities not in accordance with approved procedures and report such activities to the responsible University Official (Vice Chancellor for Health Sciences or Vice Chancellor for Academic Affairs) and appropriate federal Agencies. **Please ensure that all personnel associated with this protocol have access to this approved copy of the AUP and are familiar with its contents.**

Sincerely yours,

Susan McRae, Ph.D.
Chair, Animal Care and Use Committee

SM/jd

Enclosure