**The Effects of a Hyperandrogenic Prenatal Environment on Early and Later Life Social and Non-Social Behaviour**

by

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ABSTRACT

**The Effects of a Hyperandrogenic Prenatal Environment on Early and Later Life Social and Non-Social Behaviour**

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Gonadal sex hormones, such as testosterone (T) and estradiol (E2), are instrumental for sexual differentiation. During development, these hormones give rise to sexually dimorphic features and behaviour. Subsequently, hormones have activational effects on sexually dimorphic behaviors throughout adulthood. Most behavioral research has focused on activational effects, leaving the developmental effects less understood. In particular, the developmental effects of T on social behaviors, such as social approach and social recognition (SR), as well as non-SR memory (object recognition (OR), are little understood. In this study, we sought to elucidate how exposure to heightened levels of prenatal T interacts with later-life activational effects to enhance or impair social and non-social cognition. We treated pregnant CD1 mice with 10µg of testosterone propionate (TP) or the oil injection vehicle, on embryonic days 12, 14 and 16. Prior to puberty (PD 34-42), mice were tested in (A) SA: measuring innate preference for sociability; (B) SR: measuring the ability for an individual to discern a novel conspecific from previously encountered mouse; and (C) OR: measuring item declarative/recognition memory. Mice were then gonadectomised (GDX) or sham operated and given hormone replacement (12.5µg E for females or T for males). Mice were re-tested in SA, SR, and OR as adults. Overall, all mice showed an innate preference for the social stimulus (measured by SA). Pre-puberty we found that increased T exposure in-utero had an enhancing effect on males in OR and in both males and females in SR. In adulthood, we found that males, but not females, who received T replacement during adulthood and were exposed to heighted levels of prenatal TP performed worse in the OR test than T-replaced males who were not prenatally TP exposed. Prenatal control T replacement males outperformed prenatal control female E replacement in OR. SR, by contrast, was impaired in males. In GDX males that did not receive T replacement, prenatal TP exposure worsened performance compared to males that did not receive prenatal T. Overall, we found that exposure to a heighted levels of TP in-utero interacts with sex hormone manipulations in adulthood to produce sexually differentiated effects on OR and SR.

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## Declaration of Work

This thesis was written by me however, I was helped during the course of my studies by a graduate student, various undergraduate students, and multiple lab technicians. Colin Howes, University of Guelph, assisted with all paradigms and tissue collections. Emily Martin, University of Guelph, assisted with all paradigms, serum analysis, and SR and OR coding. Anthony Giuga, University of Guelph, assisted with all paradigms. Angela Tiessen, Ontario Veterinary College, assisted with OR data collection. Hailey Katzman, University of Guelph, assisted with OR data collection. Marian Castro and Caitlin O'Flynn, who both assisted with mice breeding and blood collection. Michael Marcotte, University of Guelph, assisted with serum hormone analysis. Gabriela F Mastromonaco, Toronto Zoo, ran the fecal testosterone analysis.

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## List of Abbreviations

AFP — α-fetoprotein
AMH — Anti-Müllerian hormone
AOS — Accessory olfaction system
ANOVA — Analysis of Variance
AR — Androgen Receptor
ASD — Autism Spectrum Disorder
DHEA — dehydroepiandrosterone
DHT — Dihydrotestosterone
E — Estrogen
E2 — Estradiol
EB — Estrogen Benzoate
ELISA — Enzyme-linked Immunosorbent Assay
$E_n$ — Embryonic Day
ER — Estrogen Receptor
ERα — Estrogen Receptor α
ERβ — Estrogen Receptor β
ERαKO — ERα Knockout
FGF9 — fibroblast growth factor 9
GnRH — gonadotropin-releasing hormone
GPER — G-protein coupled estrogen receptor
HVC — HVC (proper name) region of oscine songbirds
IR — Investigation Ratio
IRHAB — average IR of habituation session
IRTEST— IR of test session
LH — luteinizing hormone
MBH — Male Brain Hypothesis
MeA — medial amygdala
mPFC — medial prefrontal cortex
MWM — Morris Water Maze
NMDA receptor — N-methyl-D-aspartate receptor
OR — OR
OT — Oxytocin
PCR — Polymerase Chain Reaction
PD — Postnatal Day
RM-ANOVA — Repeated measures Analysis of Variance
SDN-POA — sexually dimorphic nucleus of the preoptic area
Sf1 — steroidogenic factor 1
SOX-9 — SRY-box 9
STaR — Steroidogenic Acute Regulatory Protein
STFP — Social Transmission of Food Preferences
T — Testosterone
TDF — testis determining factor
Tfm — testicular feminization mutation
TP — testosterone propionate

VmH — ventromedial hypothalamus

## Introduction

Exposure to gonadal sex hormones in utero, and immediately post-partum, initiates developmental processes leading to sex-differences in the structure of the brain and in behavior in adulthood. Understanding how prenatal and developmental exposure to gonadal sex hormones engender these sex differences is crucial for understanding how cognitive behaviours such as learning and memory and sociability develop in males and females. In the present study, we sought to elucidate how gonadal sex hormones, such as testosterone (T), interacts with embryonic development to produce early and later-life sex differences in social behaviour. Further, by manipulating the hormonal environment at puberty, via hormone deprivation/replacement, we sought to elucidate how later-life activational changes induced during adulthood interact with the organizational foundations set at the prenatal/prepubertal periods to produce distinct sexual dimorphisms in behaviour.

### ANDROGENS

The androgen class of hormones includes a variety of cholesterol-derived steroid hormones involved predominately in the development and maintenance of the male phenotype. Testosterone (17β-Hydroxy-4-Androsten-3-one) is the most abundant and studied androgen. The production of T, and other steroidal androgens can be derived via *de novo* synthesis from pregnenolone by a series of enzyme-mediated reactions (Figure 1). Pregnenolone, the immediate precursor of progesterone, is produced by side-chain cleavage of cholesterol inside mitochondria, a process regulated in the testes by pituitary luteinizing hormone, which controls the amount of steroidogenic acute regulatory protein (STaR; Clark, Wells, King, & Stocco, 1994; King et al., 1995) available to facilitate cholesterol transport to the inner mitochondrial membrane. Testosterone itself is the immediate precursor of estradiol (E2), the principal mammalian

estrogen (E), which is synthesized by the enzyme aromatase (aromatization). Estrogens are primarily produced in the ovaries and the placenta (during pregnancy). Other Es, such as estrone and estriol, can be synthesized from androstenedione via aromatization (Perel, Wilkins, & Killinger, 1980)

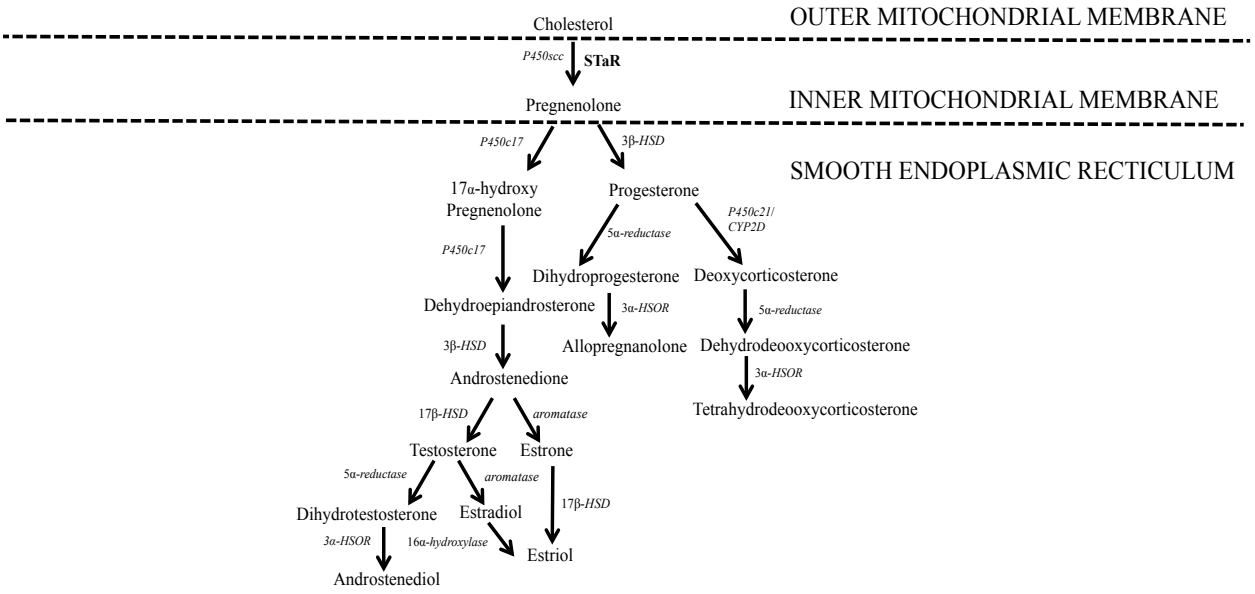



**Figure 1:** Androgens can be synthesized de novo from cholesterol or from other hormone precursors. The synthesis begins in the mitochondria and moves to the smooth endoplasmic reticulum for final processing.

Initially, it was thought that androgen synthesis was localized to peripheral tissues such as the testes and the adrenal cortex; however, Baulieu (1991) first showed that sex-hormone production could occur in neural tissue as well— coining the term "neurosteroids." STaR mRNA expression in the mouse brain was then later shown— supporting the notion that androgens and Es are actively synthesized from cholesterol in neural tissues (Furukawa, Miyatake, Ohnishi, & Ichikawa, 1998; Wehrenberg, Prange-Kiel, & Rune, 2001)

Androgens exert their behavioral and physiological effects via three main mechanisms of action that are often co-dependent (reviewed in (Heinlein & Chang, 2002)). Within a simplified context, peripheral and local T are bound by cytoplasmic androgen receptors (AR) which translocate to the nucleus to have direct effects on gene expression. Testosterone can also be converted into the potent non-aromatizable androgen dihydrotestosterone (DHT) via 5α-reductase. DHT then binds to cytoplasmic androgen receptors and goes on to have similar genomic effects as T by up-regulating synthesis of target proteins. Growing research suggests the presence of a membrane-bound AR (Yang et al., 2011) which may suggest rapid effects of androgens; however, this is beyond the scope of this thesis. Testosterone is also known to exhibit estrogenic effects via conversion to E2 via the enzyme aromatase. Estradiol binds to cytosolic estrogen receptors (ER) such as ERα, ERβ, or the membrane bound G-protein coupled estrogen receptor (GPER) to exhibit long-lasting genomic effects or rapid cell signalling cascades. The production of these hormones and their targets are important to understand as they play an instrumental role in sexual development and differentiation of the fetus' peripheral tissues and brain.

**SEXUAL DEVELOPMENT & DIFFERENTIATION**

Sexual development is a process that is initiated by chromosome genes, influenced by epigenetic factors (reviewed in Ratnu et al., 2017), and driven by gonadal sex hormones. Indeed, in mammals' females exhibit two X chromosomes while males exhibit a X and Y chromosome. The *Sry* gene— which is expressed on the Y chromosome— (Sinclair et al., 1990; Koopman, Gubbay, Vivian, Goodfellow, & Lovell-Badge, 1991) encodes testis determining factor (TDF) which actively drives the masculinization of the sexually undifferentiated primordial germ cells into testicular Leydig cells. Leydig cells are important for masculinization of the fetus as they

produce androgens such as T, androstenedione, and dehydroepiandrosterone (DHEA). In the absence of the Y-chromosome, and the *Sry* gene, X-linked and other autosomal chromosomes go on to produce the ovaries as well as other female related accessory organs. It is important to note that while the *Sry* gene is important for the development of the testes three other genes and their protein transcripts— SRY-box 9 (SOX-9; Koopman et al., 2001), steroidogenic factor 1 (Sf1), and fibroblast growth factor 9 (FGF9)— are instrumental in the development of the male accessory organs. Indeed, TDF acts as a transcription factor binding upstream of the SOX-9 promoter to up-regulate gene expression (Harley, Clarkson, & Argentaro, 2003). Next, SOX-9 interacts with FGF9 to produce a feed-forward loop, which in turn up-regulates SOX-9 protein expression and causes the proliferation of Sertoli cells (Moniot et al., 2009). Interestingly, in absence of the FGF9 gene a female phenotype ensues as the Sertoli cells do not develop (Moniot et al., 2009). Next, SOX-9 and Sf1 interact which allows for the production and secretion of Anti-Müllerian hormone (AMH) from the fetal Sertoli cells (Sekido & Lovell-Badge, 2008). It is thought that AMH acts by binding to AMH type II receptors on the Müllerian ducts (female accessory-organ precursor) to induce apoptosis of fetal Müllerian duct tissue (Baarends, 1994; Allard et al., 2000). Production of T and its conversion to DHT in target tissues, from fetal Leydig cells, promote the development of the Wolffian ducts, which produce the vas deferens, the epididymis, and the seminal vesicles which are male-specific (Renfree, Fenelon, Wijiyanti, Wilson, & Shaw, 2009).

Female sexual differentiation has been traditionally thought of as a "default pathway" wherein the simple absence of the Y-chromosome promotes the development of female-typical morphology. However, in recent years it has been shown that in females—and not in males— the presence of two X-chromosomes activates the *cis*-acting non-coding RNA *Xist,* which

transcriptionally silences one of the X-chromosomes (reviewed in Borensztein et al., 2017). This process prevents the overexpression of X-related genes in females and differentiates X-linked gene expression in males and females. In addition to the effects *Xist* has on sex-chromosomes, it has recently been suggested that *Xist* is able to act upon trans-elements to also silence autosomes (White, Willard, Van Dyke, & Wolff, 1998; Duthie et al., 1999; Keohane, Barlow, Waters, Bourn, & Turner, 1999; Hall, Clemson, Byron, Wydner, & Lawrence, 2002; Popova, Tada, Takagi, Brockdorff, & Nesterova, 2006; Tang et al., 2010). Further, it has been found that the X-chromosome in sexually dimorphic somatic cells expressed significantly more female-biased genes relative to autosomes, suggesting that sexually dimorphic areas are actively feminized (Reinius et al., 2012). The idea that a female-specific transcript can act upon sexually undifferentiated genes in sexually dimorphic regions lends to the idea of active feminization and repression of virilization.

## SEX DIFFERENCES IN BRAIN DEVELOPMENT

### Organizational

The term "organizational effects," first introduced by Phoenix et al, (1959), describes the effects hormones play to set the morphological and functional foundation of the brain during prenatal and early-life development. That is, sex hormones, such as T, act to engender a sexually dimorphic brain— which will produce observable sex-biased behaviour in early as well as later-life. While T is considered crucial for the development of male-specific behaviour, E2 mediates the development of most sexual dimorphisms in the rodent brain (reviewed in McCarthy, 2008). In-utero E2 acts on ER to masculinize and defeminize the brain. In male rodents, T produced by the developing testes crosses the blood-brain barrier, is converted to E2 in neural tissue, then masculinizes target tissue. In female rodents, this process is blunted by the presence of α-

fetoprotein (AFP); a glycoprotein produced by the fetal yolk sac and liver. AFP binds maternal and locally synthesized Es, preventing them from crossing the placenta— and inevitably the fetal blood-brain barrier— to prevent the masculinizing effects of E2 in the undifferentiated female brain (Bakker et al., 2006). It is interesting to note that AFP only has a strong affinity for E2 in rodents (Swartz & Soloff, 1974) making the translational research of this mechanism non-applicable to humans.

Increased androgenic exposure and therefore masculinizing effect in-utero are sometimes seen naturally. Indeed, in mice, a female fetus that develops in between two developing males sometimes show ambiguous genitalia and more male-biased behaviours later in life (Gandelman, vom Saal, & Reinisch, 1977; Ryan & Vandenbergh, 2002). In humans, females born with congenital adrenal hyperplasia, a genetic disorder which can cause increased exposure to androgens, show ambiguous genitalia as well as increased aggressive behaviour (Pasterski et al., 2007). This exposure to androgens may generate a masculine phenotype irrespective of genetic sex.

Many anatomical sex differences have been identified in the brain. The first instance of sex-specific brain morphology was noted by Raisman and Field (1973), who found that in female rats, non-amygdaloid synapses on dendritic spines in the preoptic area is higher than in males. Later, Gorski, Gordon, Shryne, and Southam (1978) first described the existence of the sexually dimorphic nucleus of the preoptic area (SDN-POA) in rats, and found the nuclei volume to be greater in males. Subsequent research on neuroanatomical sex differences showed that the thalamus and the third interstitial nucleus of the anterior hypothalamus is larger in males, having a greater cellular volume and more complex cells. (Hofman, & Swaab, 1989; Allen, Hines, Shryne, & Gorski, 1989; McCarthy, 2016). Later, the bed nuclei of the stria terminalis have also

been shown to be larger in males (Allen & Gorski, 1990)— containing a greater number of somatostatin-releasing neurons (Breedlove & Hampson, 2002). The posterodorsal sub-nucleus of the medial amygdala (MeA) was shown to have a greater number of excitatory synapse in males than females (Cooke & Woolley, 2005). These and other neuroanatomical sex differences are thought to arise due to T's anti-apoptotic effects on neural tissue (Davis, Popper & Gorski, 1996). While T is oftentimes cited as developmentally neuroprotective, it is not always clear whether it acts through its non-aromatized product or through one of its many metabolites. For example, E2 in regions such as the hippocampus in males and arcuate nucleus and amygdala in females organizationally promote synaptogenesis, cell proliferation and survival (Gillies & McArthur, 2010); in other regions, such as the ventromedial hypothalamus in males and preoptic area in females, it inhibits them (Gillies & McArthur, 2010). This biphasic mechanism of neuro-protection by sex hormones suggests that the effects of gonadal steroid hormones are multifaceted and that more research needs to be done to uncover how neural structures truly become sexually dimorphic at a cellular level and how these differences have implications for different brain functions and sex-specific behaviours.

Testosterone and its metabolites, as previously described, are instrumental for prenatal sexual differentiation and sexually differentiated morphology. Additionally, developmental exposure to T is also important for differences in early-life behaviour. Indeed, studies in non-human primates have shown that heighted levels of prenatal T promote male behavioral phenotype in juveniles (Hines, 1982), including higher frequencies of rough-and-tumble play and mounting behavior (Goy, R. W., & McEwen, B. S., 1980). Xu et al., 2014 showed that by indirectly increasing prenatal T with Letrozole (an aromatase inhibitor), rat neonates exhibit decreased ultrasonic vocalizations— suggesting a change in social interactions. While androgens

play a crucial role in the development of male characteristics, a secondary prenatal T surge occurs after birth that also has lasting developmental implications. Indeed, it has been shown that in male mice, 2 hours following birth, plasma T more than doubles (Corbier, Roffi, Rhoda, & Kerdelhué, 1984). However, after 4-6 hours plasma T rapidly declines in mice and remains relatively low until puberty (Motelica-Heino, Castanier, Corbier, Edwards, & Roffi, 1988). Females on the other hand, do not exhibit the postnatal T surge seen in males. This massive influx of T is produced by the gonads, and is prevented by castration at birth in mice (Rhoda, Corbier, & Roffi, 1983). This phenomenon is not well understood. It is postulated that this surge in T is caused by early male-specific activation of gonadotropin-releasing hormone (GnRH); which then activates luteinizing hormone (LH) which acts directly on Leydig cells to produce the surge of T (reviewed in Clarkson & Herbison, 2016). These pre- and early post-natal factors program sex differences in postnatal brain aromatization; hence, contributing to postnatal sexual differentiation (reviewed in Kaye & Kaye, 2013). Further, these organizational effects interact with the activational effects at puberty and in adulthood to produce sex-specific behaviour in adolescence and adulthood.

**Activational**

After puberty hormones act on target tissue to "activate" further changes at the cellular and behavioural level. These are often referred to as the activational effects of sex hormones. In males, puberty is marked by an increase in T and DHT secretion by the testes, which drives the development of male secondary sexual characteristics and promotes male biased behaviours, such as aggression (Archer, 1991), risk-taking behaviours (Vermeersch, T'sjoen, Kaufman & Vincke, 2008), and sexual drive (Singh & Kulathinal, 2005). Females on the other hand, experience an increase in E and progesterone, which drives the menstrual cycle and the

development of female-specific secondary sexual characteristics (Lee, Xemakis, Winer, & Matsenbaugh, 1976). Ahmed et al. (2008) showed that during puberty new neurons, arise in the hypothalamus, preoptic area, and the medial amygdala in rats. Further, Ahmed et al. (2008) suggest sex differences in pubertal addition of cells coincide with adult sexual dimorphisms. In general, the sex that gains more cells during puberty has a larger volume in adulthood. Additionally, removing gonadal hormones before puberty eliminates these sex differences, indicating that gonadal steroids direct the addition of new cells during puberty to maintain and accentuate sexual dimorphisms in the adult brain. The pioneering paper published by Phoenix, et al. (1959) showed that prenatal exposure to testosterone propionate (TP) caused female guinea pigs to exhibit "male-like" mounting behaviour later in life. These studies show that organizational effects can set the frameworks of behaviour and neural morphology that can interact with the activational effects of puberty and during adulthood to produce observable sex differences in physiology and behaviour.

## THE EFFECT OF ANDROGENS ON LEARNING AND MEMORY

### Sex Differences & Neurobiology

Brain regions involved in learning and memory processes, such as the hippocampus, amygdala, and hypothalamus show significant sex differences in morphology and functionality (Hajszan, MacLusky, & Leranth, 2008; Meitzen et al., 2012). For instance, studies in rats have demonstrated that E2 is associated with increased hippocampal N-methyl-D-aspartate receptor (NMDA receptor) sensitivity and dendritic spine density in females; and further, elevations in E2 due to the estrous cycle result in heightened hippocampal excitability (Scharfman, Mercurio, Goodman, Wilson, & MacLusky, 2003; Woolley & McEwen, 1992; Woolley, 1998). In addition, female rats show reduced hippocampal dendritic spine density after being

gonadectomized, which is recovered by exposure to T or E2. Letrozole can block the enhancing effects of T, which suggests that the improving effects of T on hippocampal spinogenesis may be dependant on E2 (Leranth, Hajszan, & MacLusky, 2004). A decrease in hippocampal dendritic spine density is also seen in males following orchiectomy; TP replacement recovers hippocampal spine density (Jacome et al., 2016; Leranth, Shanabrough, & Redmond, 2002). Similarly, treatment with an androgen antagonist and DHT increased hippocampal spine density in the CA1 subfield in male rats— while E2 has no effect. However, these effects of androgens on hippocampal spine density are also found in male rats who exhibit the Tfm (testicular feminization) mutation, which results in the synthesis of a non-functioning AR. Therefore, these effects of androgens may not be mediated by AR (MacLusky, Hajszan, Johansen, Jordan, & Leranth, 2006). Moreover, both male and female rats show increases in hippocampal spine density in the CA1 subfield following treatment with DHEA and flutamide, an androgen receptor antagonist. The response to DHEA in males is unaffected by treatment with Letrozole, suggesting that these changes are not mediated by conversion to E2 in males (MacLusky, Hajszan, & Leranth, 2004). Furthermore, the decrease in CA3 hippocampal spine density following gonadectomy is accompanied by mossy fiber expansion and increased apical dendritic length in male, but not female rats, which suggests a compensatory response to reductions in gonadal steroids among male, but not female, rats (Mendell et al., 2016). These findings demonstrate that, while gonadal hormones are involved in hippocampal plasticity and spinogenesis in both male and female rodents, the mechanisms involved are sexually dimorphic and still somewhat unclear. These differences in underlying morphology and physiology are likely to have implications for sex-differences in cognition.

**Sex Differences and Hormonal Regulation of Non-Social Cognition**

Males outperform females in spatial memory tests in both rodents and humans (Rizk-Jackson et al., 2006; Roof, 1993; Astur et al., 1998; Jonasson, 2005). Evidence from experiments with humans and rats indicates that T has organizational effects on the brain during development that enhance spatial learning and memory during later-life (Isgor & Sengelaub, 1998; Mueller et al., 2008; Roof & Havens, 1992; Williams et al., 1990). Indeed, male rats tend to outperform females on tasks involving the hippocampus such as the Morris Water Maze (MWM) Task and object recognition (OR) paradigms (Roof & Stein, 1999). Exogenous androgens have been shown to engender the development of a "male-like" hippocampus, improving performance and eliminating sex differences in spatial learning tasks when administered neonatally to female rats, whereas castration and treatment with anti-androgens impairs the acquisition of working memory tasks, such as maze performance, in male rats (Daniel, Winsauer, Moerschbaecher, 2003; Spritzer, 2011).

These findings suggest that gonadal steroids are able to facilitate later-life cognition through organizational effects during development (Joseph, Hess, & Birecree, 1978; Roof, 1993; Roof & Havens, 1992). Non-social cognition encapsulates a variety of behaviours, such as individual learning and spatial memory, one such tasks that exploits these cognitive abilities is Object Recognition.

*Object Recognition (OR)*

Object Recognition, developed by Ennaceur and Delacour (1988), exploits a rat's natural tendency to explore novel objects over familiar objects. This task can be configured to measure working memory, attention, anxiety, and preference for novelty in rodents (Goulart et al. 2010; Silvers et al. 2007). It has been shown that male mice are superior to females in localizing and recognizing objects (Frick & Gresack, 2003) and that E2 administration improves OR both with

long-term and rapid E2 manipulations (Fernandez & Frick, 2004; Vaucher et al., 2002; Walf, Rhodes & Frye, 2006; Phan et al., 2011). However, less is known about the effects of androgens on OR. Aubele et al. (2008) showed that castration blocked OR in male rats and that this impairment was recovered with TP replacement. This effect may be androgenic. Picot et al. (2016) showed that central nervous system specific AR knockout impaired in OR. Conflicting studies also exist, where castration of male mice did not affect OR (Benice & Raber, 2009); however, these differences may be due to differences species (mice vs rats) in cognitive load or memory demands (3-minute inter-stimulus interval vs 5-minute inter-stimulus interval) between the studies. While it seems that T and E play important roles in object recognition, it is unknown how they interact. A novel study by Taylor, Manzella, Huffman, Cabrera, and Hoffman (2017) suggest that when TP and E2 are highly bioavailable, T enhancement of spatial performance occurs and when E2 has low bioavailability, spatial memory is rescued in female mice, suggesting the importance of these two sex hormones in facilitating spatial memory. Despite what is known about OR, to our knowledge there are no studies that have looked at developmental effects on the engenderment of OR cognitive abilities specifically.

**Sex Differences and Hormonal Regulation of Social Behaviour & Social Cognition**

Social Cognition includes socially motivated behaviour such as social learning—the ability to learn from another—and Social recognition (SR)— the ability to learn about another (reviewed in Galef and Giraldeau, 2001; Choleris, Clipperton-Allen, Phan & Kavaliers, 2009). Mice are known to form social hierarchies and participate in a variety of social cognitive behaviours (Anderson, 1961). At the foundation of social behaviour is sociability— or simply, the propensity of interacting with others individually or in a group setting— (Moy et al., 2004) these behaviours are described below.

*Fundamental Sociability*

Sociability can be dichotomized: (1) behaviors that bring animals together, such as affiliative, parental, or copulatory behaviors and (2) behaviors that separate animals, such as aggressive behaviors (Caldwell, 2012). Some animal species are individualistic and intolerant of conspecifics, while other species are quite social and establish dominance hierarchies that allow group living (Caldwell, 2012). These differences in sociality may arise in relation to variation in circulating levels of gonadal hormones, such as T, and/or glucocorticoids, variation in neurotransmitter and hormone receptor distribution, expression and density (e.g., oxytocin, "OT" and vasopressin), and/or steroid-binding globulin proteins that may facilitate or inhibit steroids reaching target tissues (reviewed in Blumstein et al., 2010; Clipperton-Allen et al., 2011). Social interactions between animals may be understood through their tendencies to approach or avoid social stimuli, interest in investigating a conspecific, or inclination to engage in affiliative or agonistic behaviour in response to a conspecific (reviewed in Choleris, Clipperton-Allen, Phan, & Kavaliers, 2009; Ervin et al., 2015). Most laboratory rodents, including mice and rats, are social species (Latham & Mason, 2004); however, there is variability in sociality between individuals as well as between species (Young, 2009).

*Social Learning*

Through social learning animals are able to learn advantageous skills and techniques from one another that ultimately promote their fitness (Lindstrom, Selbing, Olsson, 2016). For instance, social learning and communication plays a role in the identification and localization of novel food sources; this socially based learning is important for circumnavigating potentially risky trial and error learning (Galef, 1996). Social learning has been documented across a variety of species. For instance, meerkats (*Suricata suricatta*) teach food-handling skills to their

offspring, as this species diet comprises venomous creatures, it is important to pass these skills on in order to promote survival (Thornton & McAuliffe, 2006). Other species like oscine songbirds, such as the zebra finch (*Taeniopygia guttata*), use social learning to acquire a sexually selected song, which is important for ensuring later reproductive succession of the individual (reviewed in (Bolhuis & Gahr, 2006)). Because singing is a typically sex-linked male trait, it is not surprising that gonadal sex hormones play a modulatory role in song learning process. For instance, in male zebra finch, the HVC, a nucleus correlated with song learning, has a greater cellular mass during breeding season when there is a T surge; this suggests that androgens drive the reproductive success of male songbirds and play a pivotal role in the learning and crystallization of birdsong (Sherry & Macdougall-Shackleton, 2014). The implications of hormonal induced social learning have also become apparent. In the Social Transmission of Food Preferences paradigm (STFP), a task where an observer animal socially acquires a food preference from a conspecific demonstrator, female mice perform better in proestrus, when endogenous E2 levels are highest (Choleris et al., 2011). Clipperton et al. (2008) found that ERα impairs performance, while ERβ prolongs the preference for a demonstrated food. This study gives insight into the activational effects of sex hormones however; to our knowledge only one study has assessed the developmental effects of sex hormones on the STFP paradigm, showing that neonatal administration of E has no effect on social learning in female rats (Berretti et al., 2014). In Howes et al., 2016 we sought to find an effect of prenatal TP treatment on early-life social learning set and how this organizational framework, set prenatally, interacts with the activational effects of sex hormones introduced at puberty and during adulthood. We found that during adolescence male and female mice may show an advantage for social learning compared to adult mice. Next, we found that prenatal treatment with T enhanced social learning in male

mice while castration blocked it in mice treated prenatally with T, and T replacement was not sufficient to recover it. We also found that ovariectomy reduced the duration social learning in female mice treated prenatally with sesame oil, and that social learning enhanced by E2 replacement. These results suggest that the gonadal steroid effects in adolescence and adulthood depend on organizing action of T and its metabolites among male mice, and our findings appear to reflect an improving effect of increased exposure to T on social learning in gonadally intact males. Further, these results show the importance of E2 on social learning in female mice.

*Social Recognition (SR)*

Social Recognition is vital for many social behaviors such as the establishment of social hierarchies, mate/kin selection, and the avoidance of potentially dangerous out-groups (reviewed in (Kavaliers et al., 2004)). The SR paradigm takes advatage of a mouse's propensity to investigate a novel stimulus; therefore, during this paradigm it is expected that the mouse will investgate the novel conspecifc more frequenly, and for a longer duration, than a previously encounted conspecific (see (Gabor, Phan, Clipperton-Allen, Kavaliers, & Choleris, 2012)). Much like social learning, hormones have shown to play a critical role in the ability for mice to distinguish between novel and familiar conspecifics. Social Recognition in female animals is dependent on GPER (Lymer et al., 2017) as well as ERα, ERβ, which have shown to interact with OT and vasopressin (Ferguson, Aldag, Insel, & Young, 2001; Choleris et al., 2006). It has been suggested that ERs may affect SR through regulation of OT, which is instrumental for successful SR in both male and female animals (reviewed in Gabor et al., 2012). In other tests of SR Spiteri et al. (2010) showed that by knocking down ERα in the MeA SR was impaired. In males ERαKO mice have been shown to exhibit impaired SR in trials involving the introduction of a novel ovariectomized female, other work has found intact SR when a sedated same-sex

conspecific was presented (Imwalle et al., 2002; Sánchez-Andrade & Kendrick, 2011). These studies suggest that ERα are crucial for SR behaviour. Despite the need for ERα in males and females to perform SR, sex differences have also been found in SR, where male rats, regardless of gonadectomy, were able to socially discriminate, whereas in only intact females this was the case (Karlsson, Haziri, Hansson, Kettunen, & Westberg, 2015). These studies tap into the activational and organizational nature of hormones effects on social cognition and but do not address how developmental manipulation of sex hormones may alter later-life SR abilities and how the activational effects seen during adulthood interact with the organizational framework laid out by exposure to/and manipulation of prenatal sex hormones.

It is suggested that androgen receptors are responsible for the SR of female but not male conspecifics (Tejada & Rissman, 2012); furthermore, it is suggested that the androgen receptor in males regulates genes related to OT and E, suggesting an interplay between the three hormone systems in SR (Karlsson, Studer, Kettunen, Westberg, 2016). To our knowledge there have been no studies that have assessed the effects of prenatal exposure of T on SR; however, Axelson, Smith, & Duarte (1999) showed that male mice exposed prenatally to an androgen antagonist do not exhibit deficits in SR following treatment with a vasopressin antagonist in adulthood, suggesting that androgens are required to set the organizational foundation for SR for later in life and that there is a male-specific dependency on vasopressin for SR. Though not specifically targeting androgens, Wolstenholme et al. (2013) showed that prenatal exposure to bisphenol A, a ubiquitous endocrine-disrupting chemical and xenoestrogen, to interfere with the habituation phase of SR in female mice, possibly impairing the ability to recognize a previously encountered conspecific potentially through an estrogenic mechanism. This is an important consideration as T

is converted to E and therefore, is important to understand how Es shape the formation of SR prenatally.

These studies suggest that androgens and Es are instrumental for the setting the foundation of SR behaviour during development. However, more studies still need to be conducted to understand how sex hormones organize SR behavior and how this organizational framework interacts with the activational effects of sex hormones at puberty and during adulthood.

## PRENATAL EXPOSURE TO ANDROGENS AS MODEL OF AUTISM

Over recent years the effects of heightened androgen exposure during development have been implicated in developmental disorders such as Autism Spectrum Disorder (ASD). ASD is a collection of pervasive neurodevelopmental disorders characterized by impairments in social interaction, communication, obsession with specific objects or subject matter, and stereotyped atypical behaviour (DSM-5, 2013). It is well-known that there exists a substantial sex bias that may hint at one possible underlying mechanism that give rise to ASD. Indeed, boys are diagnosed at a greater rate than girls at a ratio of 4:1 (Centre for Disease Control, 2014); furthermore, emerging evidence suggests this male bias may be due to the mechanisms that drive sexual development and differentiation. A compelling hypothesis, termed the "Extreme Male Brain Hypothesis," (EMBH), has begun to have a substantial impact on the field of ASD research. The hypothesis suggests that the phenotypic outcomes associated with ASD may be attributed to heightened prenatal androgen exposure in-utero. Baron-Cohen et al. (2002) were the first individuals to introduce this hypothesis, noticing that males and persons with ASD outperformed non-ASD females on the systemizing component of the empathizing and systemizing quotient but had low empathizing scores. Those scoring low on the empathizing

component typically expressed asociality or an inability to predict others behavioural responses. Furthermore, those who scored high on the systemizing component expressed, to a greater degree, the ability to analyze and predict outcomes with oftentimes, a lack of emotional affect or interpretation. This lead the researchers to believe that ASD may be an alteration in the mechanism that drives the development of the male sex.

Later research by Auyeung et al. (2012) showed that prenatal levels of T correlate with ASD symptoms. Baron-Cohen et al. (2014) then found the first direct evidence of elevated steroidogenic activity in ASD. Other studies have found similar features, for example, Henningsson et al. (2009) showed that male ASD individuals expressed generational under-transmission of the GGN polymorphism of the AR. Further research has shown that there is a dysregulation of ERβ and aromatase in ASD individuals (Crider et al., 2014) Additionally, there is evidence to suggest that the inheritance of the familial linked polymorphism of the AR may play a role in the development of ASD. These research findings converge on the idea that a hyper-androgenic prenatal environment and sex hormone dysregulation may be critical in the development of ASD phenotype. In the present study, we hope to use measures of fundamental sociability, social learning (see Howes et al., 2016), SR, as well as non-social measures (OR) to build an animal model of the EMBH so that it may be translated to humans for the development of potential therapeutics in the future.

## *Objectives*

Here we have attempted to provide evidence on how the organizational framework, set prenatally, can interact with later-life activational effects during adulthood to provide differential effects on social and non-social memory. Additionally, because of the growing support of the EMBH, and the involvement of elevated prenatal androgens, it is important to produce an animal

model of this theory and assess the social behaviour of our mice, which can be likened to humans.

Based on previous literature noted above, the prenatal hormonal environment affects outcomes for sex-typical social behaviour; therefore, we hypothesize that:

1. Exposure to heighted levels of prenatal T will affect social behaviour— particularly in males
2. The organizational behavioural framework se down by sex hormones during gestation and early-life will interact with activational effects post-puberty to produce different behavioural phenotypes
3. Females will show resilience to prenatal manipulation of T due to the protective nature of E

To test these hypotheses, we manipulated the hormonal milieu by increasing exposure to T in-utero via systemic administration of testosterone in pregnant dams. We then entered the offspring through an adolescent test battery that assessed both social (social transmission of food preferences, social approach/avoidance, SR and the resident intruder task) and non-social (OR, flavour recognition and dark-light anxiety test) behaviours. After this test phase, we gonadectomized the mice and gave hormone replacement via Silastic implants to establish a baseline for pubertal hormonal effects and to verify if the behavioural outcomes were a result of the activational effects of hormones interaction with the organizational framework set by our prenatal treatment. Soon thereafter, we submitted the mice through a second, similar, behavioural test battery and assessed the same behaviours. Upon completion of the behavioural paradigms we extracted the brains for future processing.

## Experimental Timeline

Table 1

| Age | Event | Experimental Day |
|---|---|---|
| Pre-conception | Mating pairs formed | 0 |
| E1 | Conception – indicated by vaginal plug | 1 |
| E12, E14, E16 | Prenatal treatment | 12 - 16 |
| PD 0 | Birth and cross-fostering | 18 - 21 |
| PD 20 | Weaning of pups | 38 - 41 |
| PD 34 - 42 | Behavioural test battery I | 52 - 60 |
| PD 43 | Gonadectomy or sham surgery | 61 - 64 |
| PD 58 | Hormone replacement (silastic implant) | 76 - 79 |
| PD 68 - 76 | Behavioural test battery II | 86 - 94 |
| PD 77 | Sacrifice and tissue extraction | 95 - 98 |

*Note*: 1. E1, E12, E14, and E16 indicate time point of pregnancy in days (embryonic day). PD 0 through PD 77 indicate the age of the subjects in days (postnatal day).

Male and female mating pairs at sexual maturity (2-3 months) were placed together for 12h -72h. Upon the presence of the vaginal plug (E1; Embryonic Day 1) the male was removed and the female was single-housed. At E12, E14, and E16 10 μg testosterone propionate was injected subcutaneously into the pregnant dam. At birth (PD0; Postnatal Day 0) litters were culled and cross-fostered to an untreated adoptive dam. At PD20 the adoptive dam was removed, the pups were weaned, and the offspring were triple-housed with same-sex siblings. During PD34 -PD42 the mice performed in the behavioural test battery I. The next day the mice were either gonadectomised or received sham surgery. On PD58 mice underwent hormone replacement surgery and received silastic implants filled with either: testosterone or cholesterol (males) or estrogen benzoate or sesame oil (females). Ten days following surgery, the mice performed in behavioural test battery II. On PD77 the mice were sacrificed for tissue collection.

## Methods and Materials

### Animals & Housing

Male and female CD1 mice (*Mus musculus*) were purchased for breeding (2 months old; Charles River, Saint-Constant, QC, Canada). The CD1 strain was used for several reasons: 1) this strain is genetically outbred (Chia, Achilli, Festing, & Fisher, 2005); therefore, results will hold ecological validity and generalizability; 2) Outbred strains are known to be good mothers due to their ability to care for a large litter (Silver, 1995); therefore, CD1 are optimal for cross-fostering and successful adoption; and 3) tests for social and social cognitive behaviour are established in the CD1 strain; and have been used to investigate the role of steroid hormones.

Mice were housed on a reversed light/dark cycle (12:12h, lights on at 2000h) at 21±1°C. Breeding pairs were held in clear polyethylene cages (26cm x 16cm x 12cm) with corncob bedding, environmental enrichment (paper nesting material and paper cup), and rodent chow (14% Protein Rodent Maintenance Diet, Harlan Teklad, WI) and tap water *ad libitum*. Dams and litters were kept on a high protein diet (18% Protein Rodent Maintenance Diet, Harlan Teklad, WI) until after the litters were weaned on PD20. Prior to behavioural battery I, mice were tripled-housed in same sex groups of siblings in clear polyethylene cages (26cm x 16cm x 12cm) with corncob bedding, environmental enrichment (paper nesting material and paper cup), and rodent chow (14% Protein Rodent Chow) and tap water *ad libitum.* Following all surgeries mice were single-housed under the same conditions as above. All procedures were approved by the University of Guelph Institutional Animal Care and Use Committee, and were in accordance with the guidelines of the Canadian Council on Animal Care.

**Stimulus Animals**

Two of the behavioural paradigms described (SR and social approach/avoidance) require stimulus mice; therefore, additional mice were bred alongside the experimental mice. If non-experimental litter births did not match experimental births additional, age-matched, mice were

purchased from Charles River Laboratories (St. Constance, QC, Canada). During the first round of behavioural batteries the non-experimental stimuli were kept gonadally intact; they underwent gonadectomy surgery ~10 days prior to the second round of behavioural testing (PD68-76) to eliminate variation due to fluctuating gonadal hormones in adults and to assess organizational effects of sex hormones, without the interfering activational effect of endogenously produced sex hormones. Care was taken to ensure that an experimental animal did not encounter a stimulus animal more than once throughout the administration of the test batteries.

**Hormone Treatments**

Table 2

*Experimental Groups*

| **Prenatal Treatment**[1] | | | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|
| Sesame Oil | | | | | | Testosterone Propionate | | | | | |
| Male (n = 88) | | | Female (n = 87) | | | Male (n = 100) | | | Female (n = 90) | | |
| **Hormone Replacement**[2] | | | | | | | | | | | |
| Cholesterol (n = 27) | Testosterone (n = 30) | Sham (n = 31) | Sesame Oil (n = 24) | Estrogen Benzoate (n = 33) | Sham n = 30) | Cholesterol (n = 29) | Testosterone (n = 35) | Sham (n = 36) | Sesame Oil (n = 31) | Estrogen Benzoate (n = 28) | Sham (n = 31) |

*Note*: This table outlines the experimental treatment groups. 1. Pregnant dams were s.c. injected with sesame oil (vehicle) or testosterone propionate. Males and females are treated as different groups; therefore, for adolescence the experimental group total is four; 2. Males and females were split into 12 different groups based on prenatal treatment and hormone replacement. Males were implanted with either cholesterol or testosterone and females were implanted with either estrogen benzoate or sesame oil. One-third of males and females received sham surgery and no implant.

*Prenatal Treatment*

Pregnant dams received either a 0.05ml subcutaneous injection of 10µg testosterone propionate (Galenova, Saint-Hyacinthe, QC, Canada) suspended in a sesame oil vehicle or sesame oil (Sigma-Aldrich, Oakville, ON, Canada) as a control treatment on prenatal days 12, 14, and 16. These days coincide with embryonic sexual differentiation as well as the development of sexually dimorphic brain regions involved in social behaviour such as the amygdala, hippocampus, and hypothalamus (Finlay & Darlington, 1995; Ikeda, Tanaka, & Esaki,

2008; Shimogori et al., 2010). The injection sites were sealed with superglue to minimize leakage (All Purpose Krazy Glue, Elmer's Products, Inc., Toronto, ON).

*Hormone Replacement*

Half of the mice from the gonadectomized prenatal TP treatment and oil control groups were given hormone replacement via Silastic capsules at puberty (1.98 mm inner diameter, 3.18 mm outer diameter for females; 1.57 mm inner diameter, 3.18 mm outer diameter for males; Dow Corning Corporation, Midland, MI, USA) with 0.5 cm of Silastic medical adhesive (silicone type A, Dow Corning Corporation, Midland, MI, USA) at each end. The functional length of the capsule for the females was 2 cm while the male's was 1 cm. The diameter and the functional length are directly proportional to the flow at which the hormone is released from the capsule. Capsules of the size used here have all been previously shown to produce normal physiological levels of circulating hormones (see Myers, 1971; Clipperton-Allen, Cragg, Wood, Pfaff, & Choleris, 2010; Ribeiro, Pfaff, & Devidze, 2009). The lumen of the Silastic implants for females was filled with 0.07ml of 12.5uL EB in sesame oil (Sigma-Aldrich, Oakville, ON, Canada) or only sesame oil control (Sigma-Aldrich, Oakville, ON, Canada). The lumens for male capsules were filled with crystalline testosterone (Galenova, Saint-Hyacinthe, QC, Canada) or a cholesterol control (Sigma-Aldrich, Oakville, ON, Canada). All capsules were soaked overnight in 1% bovine serum albumin (Sigma-Aldrich, Oakville, ON, Canada), to avoid a spike in hormone release following insertion, and washed quickly in ethanol and physiological 0.9% NaCl saline solution prior to implantation.

**Experimental Apparatus**

The behavioural paradigms took place in the home-cage of the experimental mouse. All testing was conducted under red light between 0900h and 1900h, during the animals' dark active

phase. Behaviour was recorded using JVC Everio HD GZ-E300 digital camcorders. Perforated clear Plexiglas cage lids were used in place of stainless steel lids in order to allow for unobscured video recording from above.

## Procedures

### Animals and Breeding

Three-month-old experimentally naïve male and female CD1 mice were paired for breeding. The breeding pairs were formed at the beginning of the dark phase (0800h) and were monitored daily for the presence of a vaginal plug during the light phase (2000h). Once the plug had been identified, the male was removed and euthanized; this marked embryonic day 1 (E1). The cages were not changed until 8 days after the birth of the litter; this was done to prevent unintended stress on the pregnant dams/litters.

### Culling and Cross-fostering

On the day of birth, the litters were genotyped (described below) and culled to 4 males and 4 females on the day of birth. The culled pups were euthanized by lethal exposure to isoflourane and frozen at -80 ºC for analysis at a later time. Litters were then cross-fostered to a non-treated CD1 dam that had given birth in the past 48 hours. This was done to avoid differences in maternal behaviour resulting from treatment with testosterone propionate (Mann & Svare, 1983).

### Postnatal Development

The litters were weighed on PD1 and subsequently weighed every 4 days up until PD20. The litters were monitored daily for the appearance of fur, eye opening, and ear-opening to assess potential effects of the prenatal T treatment on outward morphology and development. Furthermore, we monitored female mice for vaginal opening daily post-weaning. Subsequent to

vaginal opening, the females were monitored daily for the onset of estrus via vaginal lavage (described below). Final body weights were taken of each mouse at the end of the study.

**Sample/Tissue Collection**

Twenty-four hours prior to the first maternal hormone injection and 24 hours after the last injection (E16), the cage was changed and 1 hour later fecal samples were collected from the pregnant dams. The samples were then stored at -80ºC for later assessment of fecal T concentration (described below).

On the day of pup delivery, the birth mother was sacrificed and had blood taken via cardiac puncture to assess T serum levels. To monitor circulating hormone levels of the offspring, blood was taken at three separate time points throughout the experiment: During gonadectomy surgery, during hormone replacement surgery, and during perfusion. The former two collections were taken via saphenous vein puncture while the latter was drawn via cardiac puncture. The blood was placed on ice then centrifuged at 12,000 rpm for 5 min to isolate the serum. The serum supernatant was aspirated off and stored at -80ºC for later hormone analysis.

On the day of birth, a toe was clipped, from each of the offspring, collected and stored at -80ºC for genotyping. Clotisol (Creative Science, Ballwin, MO, USA) was applied to the excision sites in order to stop any bleeding.

The day following the last behavioural test in adulthood, mice were deeply anesthetized with avertin, delivered through an i.p injection (at least 340 mg/kg), and perfused transcardiacally with 0.9% saline followed by 4% paraformaldehyde (PFA) (Sigma-Aldrich, Oakville, ON) in dd$H_2O$. Gonads and prostate glands were carefully dissected out and weighted. All brains were removed and stored at 4˚C in 4% PFA in Phosphate Buffered Saline (PBS) overnight and subsequently transferred to 30% sucrose solution to cryoprotect the tissue.

Approximately 24 hours later, they were removed from the sucrose solution and stored at -80˚C until immunohistochemical analysis.

**Hormone determination with Enzyme-linked Immunosorbent Assay (ELISA)**

The fecal samples were analyzed using an ELISA at the Toronto Zoo by Gabriela F Mastromonaco (Reproductive Biology Unit, Toronto, Ontario, Canada).

The ELISA was carried out by Martin & Marcotte (2017; unpublished data). Briefly, an extraction method was developed to eliminate the blanks seen in the ELISA assay with undiluted serum samples when the samples were hemolyzed. Each sample was weighed to determine the volume of serum and was diluted 5x the sample volume with diethyl ether (Sigma-Aldrich, Oakville, ON, Canada) with 0.1% ascorbic acid (Thermo Fisher Scientific, Mississauga, ON, Canada). Ascorbic acid was added to prevent peroxide formation in the ether, which would destroy the steroids. Each sample was vortexed for 2 minutes and left at room temperature for an additional 15 minutes. The samples were then placed in an ethanol dry-ice bath until the aqueous phase had frozen. The solvent phase was removed and left to evaporate overnight. The hormones were then resuspended in 0.1% human serum albumin (Sigma-Aldrich, Oakville, ON, Canada).

Total T concentration in maternal serum was measured using a Testosterone ELISA Assay (ab108666; Abcam, Boston, MA, USA). To correct for extraction efficiency, both the standards and the serum samples were extracted and dissolved in human serum albumin (Sigma-Aldrich, Oakville, ON, Canada) then assed in the ELISA. Briefly, 25 µL of testosterone standards (0.0 ,0.2 ,1.0 ,4.0 ,16.0 ng/mL), control, and samples were added to a 96-well plate. Next, 100 µL of Testosterone-Horseradish Peroxidase Conjugate was added to each well. The wells were then covered with foil and left to incubate for 1 hour at 37ºC. Next, the wells were washed 3x with 300 µL of 1x wash buffer. The liquid was thoroughly aspirated ensuring no

remaining substrate was left in the wells. Next, 100 µL of TMB substrate was pipetted into each well and incubated for 15 minutes at room temperature in the dark. Finally, 100 µL of the Stop Solution was added to each well; the microplates were shaken gently in order to thoroughly stop the colour development. The 96-well plate was read at against a 450nm wavelength on a Microplate Autoreader EL311 (Bio-Tek Instruments, Winooski, VT).

**Polymerase Chain Reaction (PCR) for Sex Determination**

The toe clipping tissue taken at day of birth was used for PCR to amplify the Y-linked *Sry* gene in male tissue for sex identification. The toe clippings were individually placed in 0.5mL PCR tubes (Polysciences, Pennsylvania, USA) and transferred to the molecular biology lab on dry ice. The toe clippings were lysed (180µL ATL buffer/20µL proteinase K; Qiagen, Limburg, Netherlands) at 52 ºC for 45 min and the DNA was isolated using a QIAamp DNA Mini Kit (Qiagen, Limburg, Netherlands; protocol included in kit). Two microliters of dissolved DNA were used for PCR in a 25µL reaction. Taq polymerase (0.25 U/µl, AmpliTaq Gold, Perkin Elmer, Norwalk, CT) diluted in a PCR buffer with 0.2mM each dNTP (Life Technologies/Gibco-BRL, Rockville, MD) 0.2µM SRY (317kb) primers forward: 8276–8295 5′-TGGGACTGGTGACAATTGTC-3′ and reverse: 8677–8658 5′-GAGTACAGGTGTGCAGCTCT-3′and 0.2µM beta-actin (220kb) specific primers forward: 5’-GGGACTC- CAAGCTTCAATCA-3’ and reverse: 5’-TGGAG- GAGGAAGAAAAGCAA-3’ (Life Technologies/Gibco-BRL, Rockville, MD) as a control (The SRY oligonucleotide and PCR protocol was adapted from Lambert et al., 2000). Samples were cycled under the following conditions: 95ºC for 5min. then 35 cycles at the same temperature for 35s, 50ºC for 1min. and 72ºC for 1min. Final elongation occurred for 5min. at 72ºC. The products were visualized using gel electrophoresis. The samples that showed bands at ~402 kd were genotyped as males.

**Surgeries**

Mice received 10mg/kg carprofen (50mg/kg Rimadyl, Pfizer Canada Inc., Kirkland, QC, Canada) subcutaneously (s.c.) 2h prior to surgery to act as an analgesic and an anti-inflammatory treatment. Immediately before the incision was made mice revived 0.5mL of hydrating physiological saline s.c. in the dorsal scruff and a local anesthetic (0.02mL of a 3.5ml buprivacaine (Hospira Inc., Montreal, QC, Canada), 1.5ml lidocaine (Alveda Pharmaceuticals, Toronto, ON, Canada) cocktail in 5.0mL saline) to the site of incision s.c. Next, the site of the incision was shaved and sterilized with soap, isopropyl alcohol, and tincture of Savlon. All surgeries were performed under anesthesia with isoflurane gas (Baxter Corporation, Inc., Mississauga, ON, Canada). Incisions were closed using 9mm EZ Clip surgical staples (Stoelting Co., Illinois, USA). Sham surgeries for the control groups followed the protocols as outlined below except that the gonads were pulled out of the body then placed back without excision.

Animals were continually monitored for 1h following surgery, and were single-housed and monitored daily for at least 7 days post-surgery. Animals that showed signs of infection or poor recovery were treated with warm saline and 50 mg/kg carprofen, and were euthanized if no improvement was observed; twenty (or ~2%) mice were euthanized.

*Ovariectomy Surgery*

At 42 days of age female mice underwent bilateral removal of the ovaries. The mice had their dorsal-lumbar portion shaved bare. A small 2-cm incision was made through the skin on the dorsal surface. Ovaries were removed via 1-cm bilateral incisions in the dorsal muscles. Ovaries were excised after clamping the fallopian tubes and cutting the fat tissue underneath.

*Castration Surgery*

At 42 days of age male mice underwent bilateral removal of the testes received a 1cm ventral-midline incision into the deep fascia of the scrotum to reveal the tunica of the testes. The tunica was pulled below the testes where the inferior portion of the spermatic cord was ligated before the testes were excised.

*Silastic Implantation Surgery*

At 58 days of age, mice received a 3cm dorsal-medial incision. The silastic implants were implanted subcutaneously on the left dorsolateral side.

**Estrus Stage Determination**

One week following ovariectomy surgery, female mice received vaginal lavages using a 50uL pipette tip loaded with distilled water to assess whether the ovariectomies were successful and to determine the stage of the estrus cycle in sham-operated females. The tip was inserted into the introitus of the vagina and flushed 5-8 times. The vaginal solution was placed on slides where they were assessed for the stages of the estrous cycle with a light microscope (described below). Briefly, proestrus, is characterized by a predominance of nucleated epithelial cells; estrus, by clustered cornified squamous epithelial; metestrus, leucocytes and few nucleated epithelial cells; and diestrus, by a predominance of leukocytes (Caligioni, 2009; McLean, Valenzuela, Fai, & Bennett, 2012). Hormone replacement and sesame oil replacement mice that appeared to be in the diestrus phase of the reproductive cycle were included in the statistical analyses (6 females were removed).

**Behavioural Test Battery**

All social and non-social behavioral tests (see Table 2; social approach-avoidance, OR, and SR— procedures described below/in this thesis) were repeated at two distinct time points and followed two distinct testing paths. The testing paths were designed to minimize cognitive

fatigue and to minimize priming effects (i.e., OR and social recognition are similar paradigms; therefore, the testing paths were kept separate).

Mice were triple-housed prior to the testing battery. The initial adolescent test battery occurred between postnatal days 34-42. Mice were retuned to triple-housing conditions following the first behavioural battery. One day later, prior to puberty, mice received gonadectomies and silastic implantations surgeries (see Table 2); mice were single-housed, to recover from surgery/to minimize the risk of their implants being removed. Next, a second test battery was administered— occurring between postnatal days 68-76 with adult mice. All tests were recorded during the dark phase of the light cycle under red light using a JVC Evario HD (GZ-E300). There were 12 test groups based on prenatal and adulthood treatments in males and females (see Table 2). During adolescence stimulus mice were gonadally intact while in adulthood they were gonadectomized. All items (perforated cylinders, objects, and Plexiglas lids) used in the paradigms were washed thoroughly between each use with odorless detergent (Sparkleen, Fisher Scientific), rinsed with baking soda and water to eliminate odor and thoroughly dried. The three main tests discussed in the thesis (SR, OR, and Social Approach-Avoidance Paradigms) were run in the home cage (with enrichment removed) of our experimental mice and was recorded for later behavioral scoring.

Table 3

*Order of Behavioural Testing*

| **Testing Day** | **Testing Path A** | **Testing Path B** |
|---|---|---|
| Day 1 | STFP | Either STFP or Flavour Recognition |
| Day 2 | Dark-Light | Dark-Light |
| Day 5 | OR* | Social Approach-Avoidance* |
| Day 8 | Social Reactivity | SR* |

*Note:* Each animal was tested in one of two sets of behavioural tests. ¾ of animals were tested in the social transmission of food preferences task (STFP), ¼ were tested in flavour recognition (discussed in Howes et al., 2016), while ½ of animals were tested in each of the other tasks (OR, Social Approach-Avoidance, and SR). Mice who engaged in a task during

adolescence did not engage in the same task in adulthood; with the exception of STFP, Dark-Light, and Social Reactivity). This was done to minimize priming effects. This table shows the order in which these tasks were performed. Asterisks indicate paradigms outlined in this thesis.

*Social Approach-Avoidance*

The experimental mouse was presented with a social and non-social stimulus introduced into the home-cage on opposing corners on the long side of the cage (see Figure 2).

During a 10min test session, experimental mice were exposed to a sex/age-matched stimulus mouse or a cotton ball dabbed with 0.5mL of 1% vanilla extract (McCormick® Pure Vanilla Extract, London, ON) in distilled water. Stimuli were presented in Plexiglas tubes (7-cm diameter, 12-cm high, with thirty-six 4-mm perforations to allow passage of olfactory cues as described in Choleris et al., 2006). The stimulus mice were previously habituated to the Plexiglas tubes.

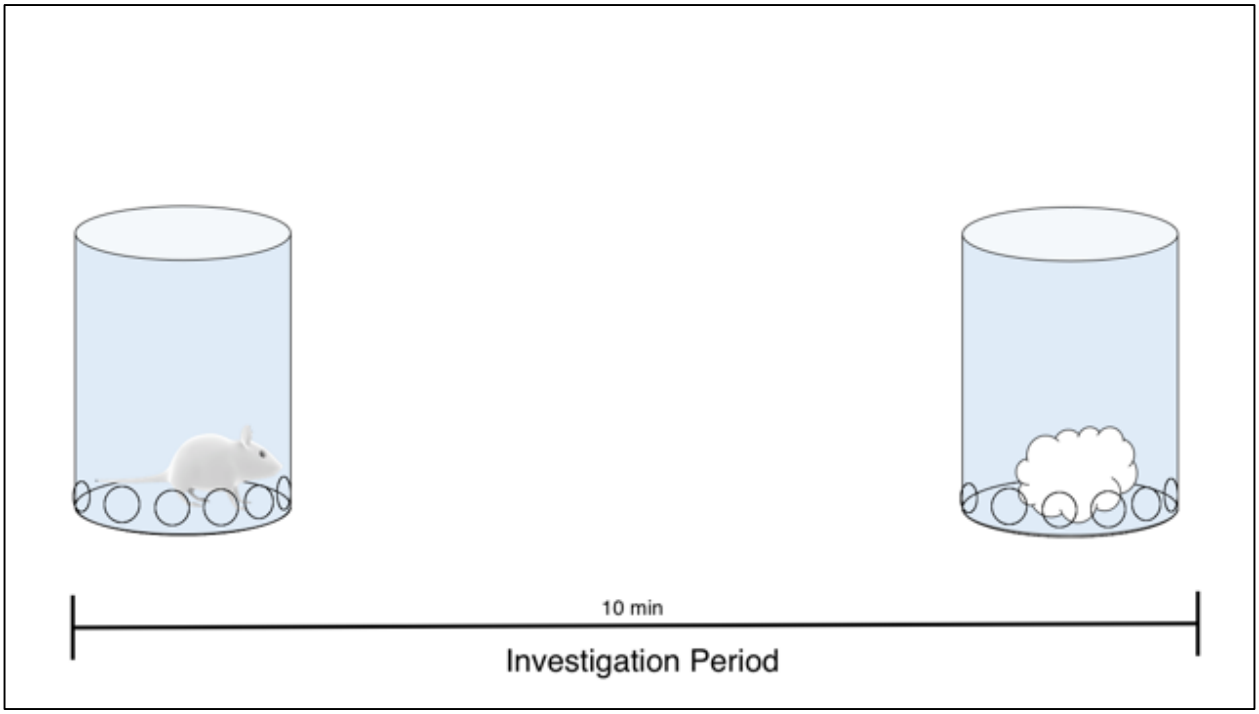



**Figure 2:** Schematic of Social Approach-Avoidance Paradigm. Two perforated Plexiglas tubes are placed in the test mouse home cage. A stimulus mouse is presented in one tube while a

vanilla scented cotton ball is presented in the other on opposite sides of the home cage. The test mouse is allowed to investigate the stimuli for 10 minutes.

*Object Recognition (OR)*

During habitation sessions (4-min x 3-repetitions) two identical objects were placed adjacent on the same wall in the home cage of the experimental mice and held in place using Velcro. During the inter-test intervals (3-min x 3-repetitions) the objects were removed. During the test phase (4 min) a novel object was introduced along with a familiar object from the habituation phase. The location of the object that was replaced at test was counterbalanced across experimental mice. The objects used were: stainless steel drainer catcher, glass cube, and a plastic hairclip. These objects have been previously tested to ensure the mice show no preferential bias (described in Phan et al., 2011).

*Social Recognition (SR)*

Two progressively familiar stimulus mice were introduced into the experimental mouse's home-cage for 3 four-minute habituation sessions inside perforated Plexiglas tubes described above. During the inter-test intervals (3-min x 3-repetitions), the tubes with stimulus mice were removed. During the test phase (4-minutes) a novel conspecific was introduced along with a familiar stimulus mouse from the habituation sessions. The location of stimulus mice that was replaced during the test phase was counterbalanced across experimental mice. Each stimulus mouse was presented in clean tubes each phase.

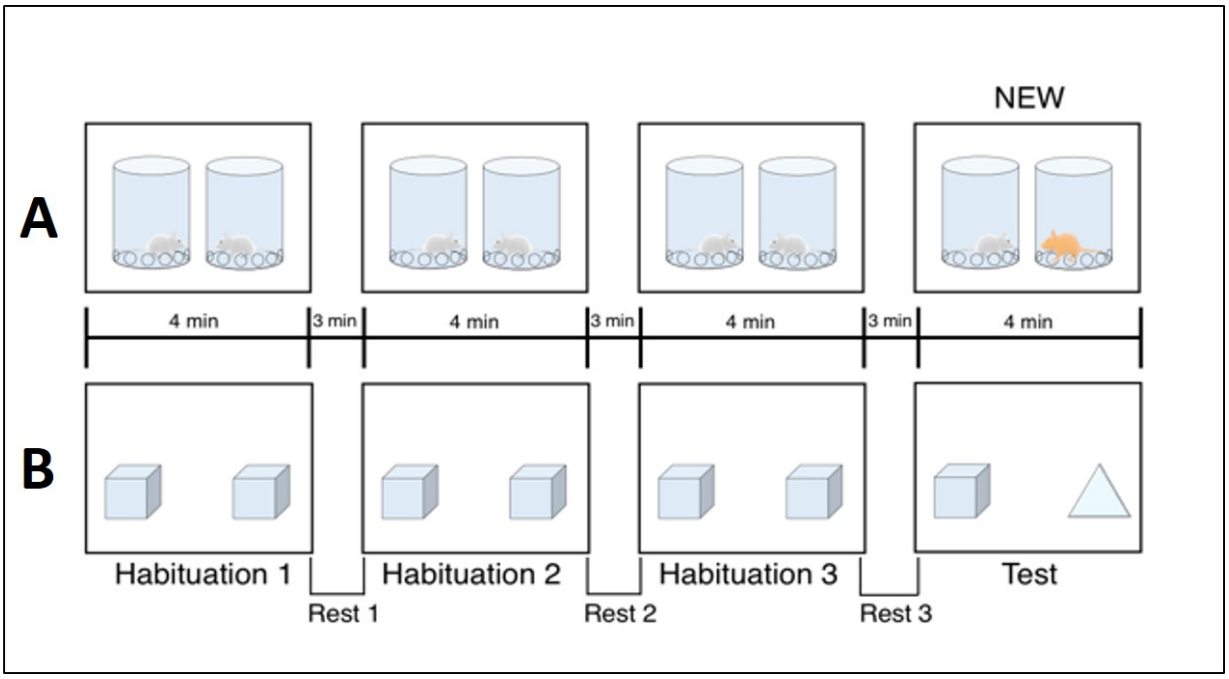



**Figure 3: A**: Schematic of SR. Stimulus mice are place in perforated Plexiglas tubes and placed in the home cage of the test mouse. **B**: Schematic of OR. Shapes are presented in the home cage of the test mouse and attached to the wall using Velcro. Habituations are 4-minute investigation periods where the same stimuli (mice/shapes) are presented. Rest is a 3-minute inter-stimulus interval period where no stimuli are presented to the test mouse. Test is a 4-minute investigation period where one of the previously encountered stimulus is reintroduced along with a novel stimulus.

**Behavioural Analysis**

Behavioural data (duration of behaviours) was collected from videos using JWatcher Video Analysis Software (JWatcher, UCLA) by trained observers blind to the treatment. The 12 behaviours analyzed are summarized in Table 4. Investigatory behaviour was defined as actively sniffing the stimulus (stimulus mouse/cotton ball through Plexiglas perforations or object).

*Social Approach-Avoidance*

A Social Investigation ratio ($IR_{Social}$) was calculated:

$$Social\ Investigation\ Ratio = \frac{Social\ Investigation}{Total\ Investigation}$$

where Social Investigation is defined as the investigation of the stimulus mouse and Total Investigation is defined as the sum of investigation of the non-social scented cotton and social stimulus.

*Object and Social Recognition*

Mice show a preference for novelty (Ennaceur & Delacour, 1988); Therefore, we used preferential investigation of the novel stimulus to determine whether mice are able to differentiate novel from familiar stimuli.

An novel stimulus investigation ratio (IR) was calculated:

$$Investigation\ Ratio = \frac{Novel\ Investigation}{Total\ Investigation}$$

where Novel Investigation is the investigation of the novel stimulus (or the stimulus to be replaced at test for the habituation sessions) and Total Investigation is the investigation of the familiar and novel stimulus. The IRs of the habituation sessions (IRHab) were averaged to account for any variation in investigatory behaviour throughout the habituation phase. The IR averaged IRHab was compared to the IR during the test phase IRTest. When IRTest > IRHab, a preference for the novel stimulus is present at test and stimulus recognition is suggested.

Total Investigation was also calculated across each habituation/test or a single test session for social-approach avoidance, OR, and SR:

$$Total\ Investigation = Novel\ Investigation + Familiar\ Investigation$$

To measure the motivation of the mice to investigate the stimuli. If no statistically significant differences between experimental groups occurred in total investigation, it was concluded that differences in social or object preferences were not secondary to changes in overall investigatory behaviour.

**Table 4:** ***List of Behaviours Analyzed During Paradigms***

| Behaviour | Description |
|---|---|
| Stretch Approach | Stretching towards stimulus with hind paws planted |
| Sniff Stimulus | Sniff/Investigation of stimulus |
| Bite Stimulus | Biting cylinder or objects |
| Dig | Moving of bedding backwards with forepaws |
| Bury | Moving of bedding forwards with forepaws |
| Horizontal Activity | Includes walk, explore, sniff that does not fall into any of the above categories |
| Vertical Activity | Rear and lean on wall, lid sniff, lid chew (two paws on the floor of the cage), single jump |
| Inactivity | Sit, laydown, sleep, freeze |
| Self Groom | Self groom and scratch |
| Stereotypies | Strange behaviours: spin-turns, repeated jumps, repeated lid chews (>3), head shakes, etc. |
| Non-social Investigation | Non-social sniffing of cylinder (above holes) |
| Sit on Object | Sitting/climbing on object |

*Note:* Mouse behaviours scored during social approach/avoidance, SR, olfactory discrimination, and OR tests (adapted from Lymer et al., 2017).

**Statistical Analysis**

*General Data Handling*

Mice with total investigation durations of less than 5sec during test (3% of animals) as well as outliers (>2SDs ± mean; 2% of animals) were excluded. Statistical analysis was conducted using SPSS (IBM SPSS Statistics for Macintosh, Version 22.0). Arcsin transformations were applied to investigation ratio data (SigmaStat version 3.5, Systat Software, Inc., IL). The optical density values from the ELISA were ran in duplicated then averaged and fitted to a four-parameter logistic curve using the LL4 function in the "Analysis of Dose-Response Curves Package" in R Statistical Software (R Foundation; Ritz & Streibig, 2005). Tukey-HSD was used as a post-hoc test when results were significant. Results were considered statistically significant when $p<0.05$.

*Social Approach Avoidance*

For between group effects ($IR_{social}$) was analyzed with a 3-way analysis of variance (ANOVA) with sex, prenatal treatment, and hormone replacement (in adulthood only) as the main factors and $IR_{social}$ as the dependent variable. When an interaction was significant the simple main effects were calculated using the marginal means. *A priori* binary mean comparisons were conducted between each group and their respective control using paired *t*-tests. A second analysis was conducted to assess within group effects. The $IR_{social}$ was compared to 0.5 (chance) using a one-sample t-test to discern if the behaviour was significantly greater than what would be expected by chance.

*Object and Social Recognition*

The investigation ratios were analyzed with a mixed-design repeated measures analysis of variance (mixed RM-ANOVA) with sex, prenatal treatment, and hormone replacement (in adulthood only) as the between subjects' factor and phase of paradigm (IRHab or IRTest) as the within subjects' factor. A *priori* binary mean comparisons were conducted only between IRHab

and IRTest of each group using paired *t*-tests, as correcting for multiple post-hocs would result in high type II error. A statistically significant difference between IRHab and IRTest suggests that the mice were able to recognize the familiar stimulus. Furthermore, a one-way ANOVA was conducted on sex, prenatal treatment, and hormone replacement with the IRTest being the dependent variable. This was used to assess treatment effects. The duration (in seconds) of other behaviours and total stimulus investigation (Table 4) were analyzed using a mixed RM-ANOVA with sex, prenatal treatment, or hormone replacement (adulthood only) as the between subjects' factor and phase of paradigm (habituation 1,2,3, test) as the repeated measure within subjects' factor. Simple main effects were also analyzed (see above) as well as mean comparisons of each treatment to control for Object and SR.

*Total Investigation*

Total investigation of the stimulus was analyzed using a RM-ANOVA to compare each phase (SR and OR only), prenatal treatment, and hormone replacement condition. *A priori* binary mean comparisons were conducted using paired *t*-tests.

*Other Behaviours*

The other behaviours described in Table 4 were analyzed identical to SR and OR. If the behaviours were dichotomous (stretching, biting, burying, digging, sitting on object, and non-social investigation)— approaching or avoiding a stimulus— an investigation ratio was calculated (as described in SR and OR analysis). If the behaviour was a non-dichotomous (stereotypies, horizontal/vertical locomotion, and grooming), the raw duration of the behaviour was used and followed the same statistical analysis described in total investigation of the social-approach avoidance paradigm.

*Serum Analysis*

An independent sample *t*-test was conducted to compare plasma testosterone levels in dams that received testosterone propionate to those of dams that received sesame oil.

## Results

### Summary of Results

Overall, it was verified that the prenatal TP treatment increased circulating levels of maternal T. This treatment had negligible effect on overall morphologic development, except for the accelerated onset of vaginal opening— a sign of sexual maturity (Mayer et al., 2010). The weight of the prostate glands in adults showed expected castration-induced reduction, which was partially restored by the crystalline T replacement. Similarly, female body weights showed the expected increase post-ovariectomy, which was fully reversed by the EB replacement.

During adolescence males were impaired in OR, which was recovered by the prenatal TP treatment. Finally, there was an enhancing effect of the prenatal TP treatment on SR in both adolescent males and females.

During adulthood, both males and females showed good OR without any major enhancing or impairing effects of treatments. Finally, there seemed to be an impairing effect of the prenatal TP treatment on SR in males that was recovered by crystalline T replacement. In females, ovariectomy significantly impaired SR. Statistically significant results—as well as non-significant results that lend to the overall interpretation of the data— are reported below.

### Developmental/Physiological Data

*Maternal Serum & Fecal Analysis*

The dams injected with the prenatal TP treatment had significantly higher plasma testosterone levels than the sesame oil control dams when the plasma was collected after the E16 TP injection (Martin & Marcotte, unpublished data; Fig.4, $Z = 81.5$, $p < 0.05$). No significant

difference was found between plasma testosterone levels at day of birth between dams treated with sesame oil and testosterone propionate (Fig. 4AB).

In analysis of fecal testosterone metabolites, a RM-ANOVA revealed a significant main effect of time ($F_{(1, 25)} = 12.14$, $p = 0.002$). Binary mean comparisons revealed a statistically significant difference in fecal T metabolites between the pre-injection and the post-injection for the mice prenatally exposed to the sesame oil control ($t_{(11)} = 2.72$, $p < 0.05$) and mice prenatally exposed to the TP treatment ($t_{(14)} = 2.28$ $p < 0.05$). However, there were no significant differences between the overall change in fecal T over the course of the prenatal treatment period between dams injected with sesame oil and dams injected with TP (Fig. 5, $t_{(25)} = 0.28$, $p = .78$).

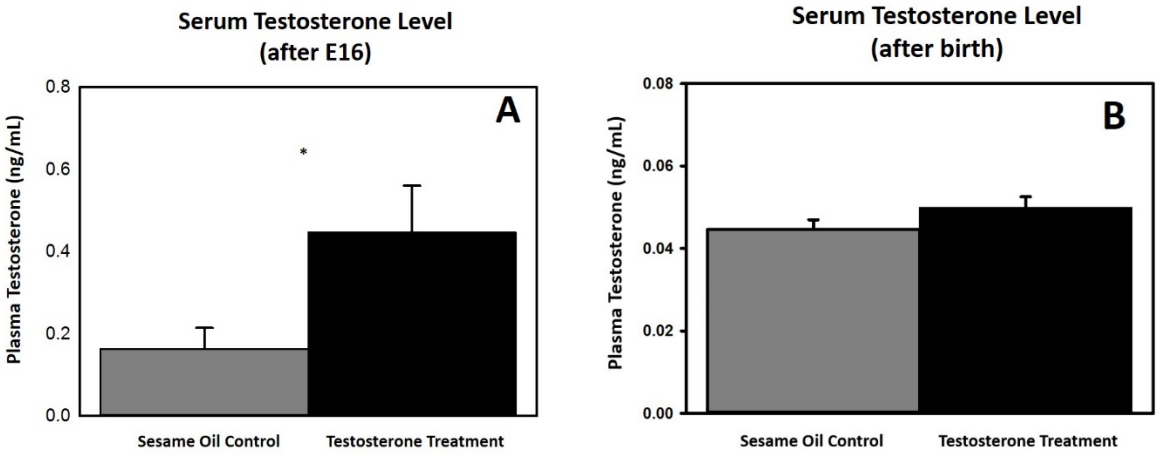



*Figure 4:* Measurements of endogenous testosterone in serum taken from damns. **A**: Testosterone in maternal serum taken after the E16 injection (control: n = 18, testosterone: n = 19). **B**: Testosterone in maternal serum taken after birth of litter (control: n = 18, testosterone: n = 21). Error bars ± SEM. *p<0.05.

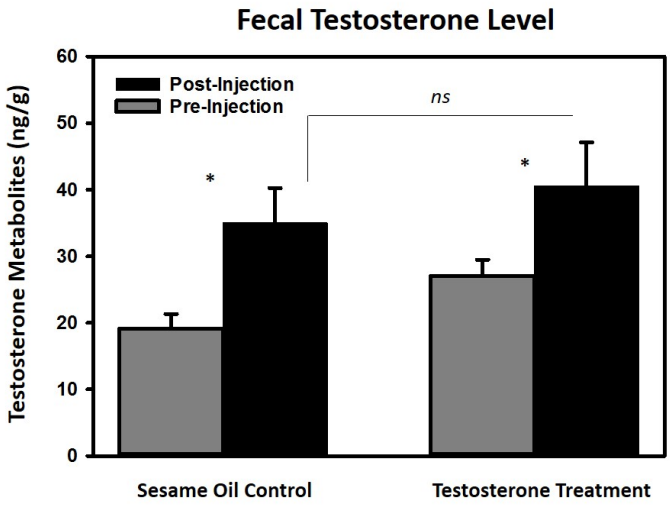



*Figure 5:* Testosterone metabolites in maternal fecal samples collected 24 h prior to first injection and 24 h following the final injection (control: n = 12, testosterone: n = 15). Error bars ± SEM. *p<0.05, *ns* non-significance.

*Development*

A mixed ANOVA showed a significant main effect of time on litter weight ($F_{(1,55)}$ = 900.18, $p < 0.01$); planned comparisons revealed no significant differences in litter weight between the sesame oil control and the TP exposed litters (Fig. 6) reflecting the fact that prenatal TP at the dose tested here did not affect postnatal pup growth.

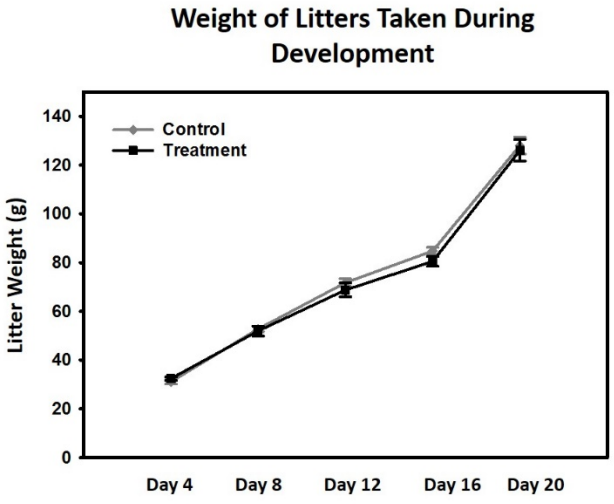


*Figure 6:* Weight of litter in grams. This represents the average total weight of a litter (culled to eight). Each weighing took place every 4 days following birth until weaning. Error bars ± SEM.

Other developmental milestones such as ear opening, eye opening, appearance of first fur, vaginal opening, and first estrus were measured and are summarized in Table 5 and they confirm that postnatal development was generally not affected by prenatal TP, apart from a half day acceleration in vaginal opening.

***Table 5: Summary of other developmental landmarks***

| | Sample Size | | Day (Mean) | | Significance | t-value |
|---|---|---|---|---|---|---|
| Developmental Landmarks | Control | Treatment | Control | Treatment | | |
| Ear opening | 12 | 13 | 4.58 | 4.53 | *ns* | 0.17 |
| Eye opening | 12 | 13 | 12.75 | 12.8 | *ns* | 0.16 |
| First fur | 12 | 13 | 6.83 | 6.53 | *ns* | 0.46 |
| [1]Vaginal opening | 40 | 39 | 30.30 | 29.56 | $p < 0.05$ | 0.49 |
| First estrus | 49 | 50 | 36.53 | 36.11 | *ns* | 0.44 |

1. Vaginal opening occurred significantly earlier in females exposed to heighted levels of testosterone prenatally compared to those exposed to the sesame oil vehicle.

*Gonad & Prostate Weights*

There were no significant differences in adult gonad weights in either sex between mice prenatally exposed to TP or a sesame oil control among male or female groups (Fig. 7). Conversely, a mixed ANOVA revealed a main effect of the adult treatment condition on prostate weight (Fig. 7; $F_{(2,175)} = 23.95$, $p<.001$). Post-hocs revealed that prostates weighed significantly more in the gonadally intact males than in the castrated mice ($p<.001$) and than the castrated mice that received the crystalline testosterone hormone replacement ($p<.004$). Post hoc analysis

found that in castrated mice prostate weights were heavier in those that had received the crystalline testosterone replacement compared to cholesterol replacement controls ($p$<.001). Further, T replacement in adulthood increased prostate weight among castrated males treated prenatally with the sesame oil control ($t_{(45)}$ = 3.14, $p$<.001) and prenatal TP ($t_{(51)}$ = 2.56, $p$<.01) relative to the adulthood cholesterol controls. Thus, prostate weights showed the expected response to castration and confirmed the partial effectiveness of our T replacement protocol. It is interesting to note that both replacement groups prostate size did not significantly differ from each other.

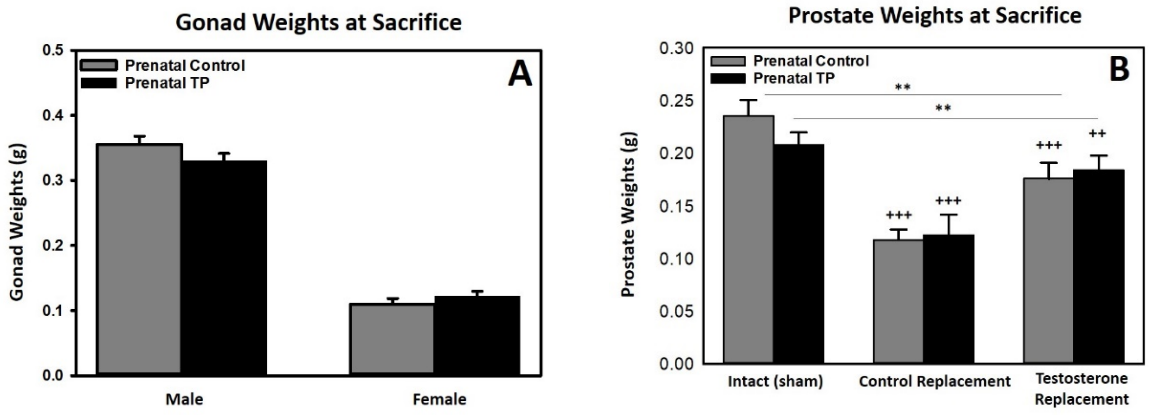



*Figure 7:* Gonad and prostate weights taken at time of sacrifice. (**A**) Testes and ovaries taken from intact (sham) mice. (**B**) Prostates taken from all male groups (accessory tubules removed). + indicates a significant difference between adult treatment groups within the same sex and prenatal treatment group, either between intact and GDX mice, or between GDX and GDX and GDX with adulthood T replacement mice. Error bars ± SEM. Adult treatment ++p<0.01, +++p<0.001, **p<0.01 (between groups).

*Adult Body Weight*

Overall, males had significantly greater body weights than female mice (Figure 8; $F_{(1, 343)}$ = 424.38, $p$ < .001). A 3-Way ANOVA revealed a main effect of sex (Figure 8; $F_{(1, 343)}$ = 424.38, $p$ < .001), adult treatment ($F_{(2, 343)}$ = 7.23. $p$ = .001) on adult body weight, and a significant interaction between sex and adult replacement/sham surgery ($F_{(2, 343)}$ = 13.86. $p$ < .001). Simple main effects revealed an effect of adult implant intervention/sham on body weight in female

mice ($F_{(2, 167)} = 24.70$. $p < .001$) and pairwise comparisons revealed that gonadally intact female mice had lower body weights than both ovariectomized female mice ($p < .001$), and ovariectomized female mice that received EB replacement ($p < .001$), while ovariectomized mice that received EB replacement had lower body weights than ovariectomized mice that received sesame oil replacement ($p = .006$). Planned comparisons indicated that ovariectomized mice were significantly heavier than intact mice among female mice treated prenatally with the sesame oil control ($t_{(48)=} 6.51$, $p < .001$) and prenatal TP ($t_{(52)} = 3.37$, $p = .001$). Further, in the mice treated prenatally with the sesame oil control and later ovariectomized, body weight was significantly lower in mice that received the EB replacement compared to those that received control oil replacement ($t_{(46)} = 3.66$, $p < .001$).

In mice treated prenatally with sesame oil and later left gonadally intact, males had greater body weights than females ($t_{(56)} = 13.86$, $p < .001$). In mice treated prenatally with sesame oil and later gonadectomized, males had greater body weights than ovariectomized females ($t_{(47)} = 6.10$, $p < .001$), and males that received testosterone replacement were heavier than females that received EB replacement ($t_{(45)} = 10.42$, $p < .001$) Likewise, in mice exposed prenatally to the TP treatment, gonadally intact males had greater body weights than intact females ($t_{(62)} = 10.24$, $p < .001$), castrated male mice had greater body weights than ovariectomized female mice ($t_{(49.10)} = 5.31$, $p < .001$), and castrated mice that received the crystalline testosterone replacement had greater body weights than ovariectomized mice that received EB replacement ($t_{(52)} = 6.87$, $p < .001$).

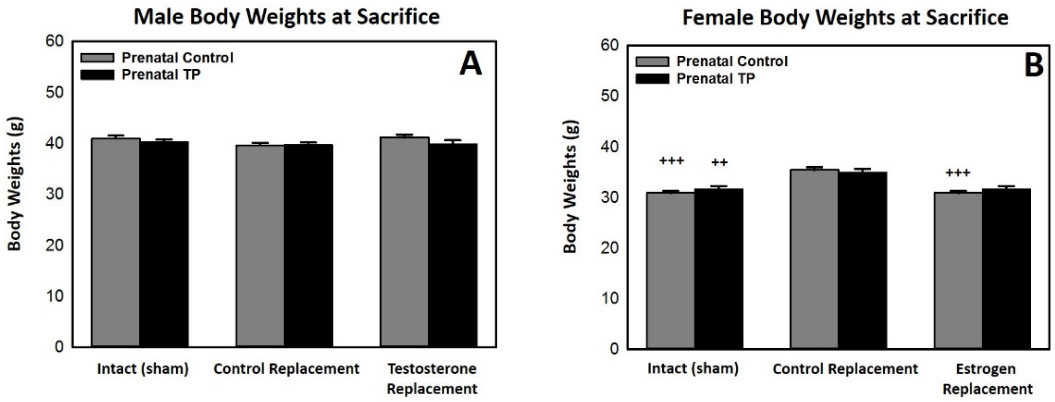



*Figure 8:* Adult body weights taken at sacrifice. **A**: Males; **B**: Females. Error bars ± SEM. Adult Treatment ++$p<0.01$, +++$p<0.001$.

Overall, we were unable to detect a difference in either fecal or plasma T between dams treated prenatally with sesame oil and dams treated prenatally with TP. We found no differences in litter weight between mice treated prenatally with testosterone and sesame oil during the first 20 days of life, or in adulthood at perfusion. Gonadectomy increased adult body weight in female mice, though this change was reversed in mice that received EB replacement. Treatment with prenatal T resulted in earlier vaginal opening in female animals, but did not change onset of estrus. In male mice, prostate weight was decreased in gonadectomized animals—this change was reversed in mice that received T replacement.

**Social Approach-Avoidance Paradigm**

*Adolescence*

The univariate ANOVA revealed no effects of sex or prenatal treatment on the social approach-avoidance paradigm (Figure 9). *a priori* mean comparisons indicated that all prenatal treatment groups investigated the social stimuli significantly greater than would be expected by chance (Figure 9; in males (control: $t_{(9)} = 4.87$, $p<0.05$; treatment: $t_{(9)} = 8.14$, $p<0.001$) and females (control: $t_{(9)} = 4.265$, $p<0.01$; treatment: $t_{(9)} = 8.28$, $p<0.001$). Thus, social approach was not impaired by prenatal TP treatment.

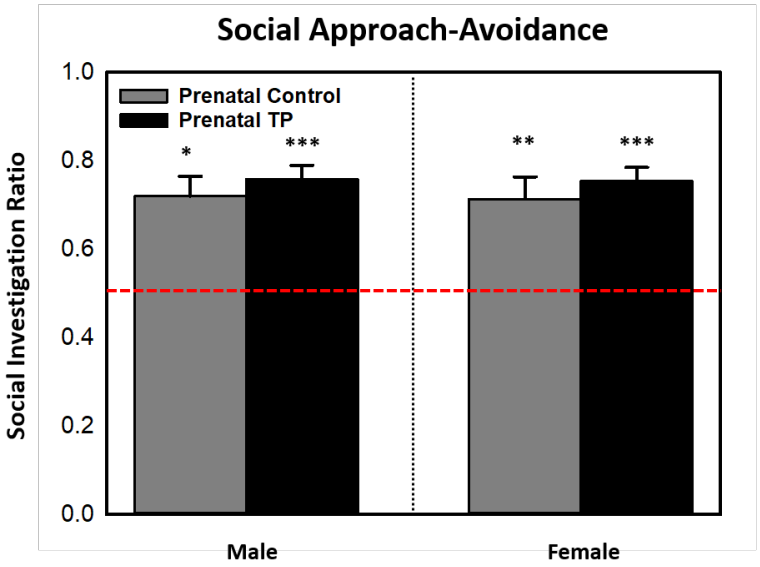



*Figure 9:* Social Approach-Avoidance paradigm done during adolescence. Grey bars represent prenatal exposure to the sesame oil control while black bars represent prenatal exposure to the TP treatment. **Left Panel**: Males (control: n = 10, testosterone: n = 10). **Right Panel:** Females (control: n = 10, testosterone: n = 10). Redline marks chance (0.5). *p<0.05, **p<0.01, ***p<0.001 (significantly different than would be expected by chance). Error bars ± SEM.

A RM-ANOVA revealed no significant effect of sex or prenatal treatment on total investigation (Fig. 10) indicating that males and females, regardless of treatment, did not differentially investigate the social and non-social stimuli.

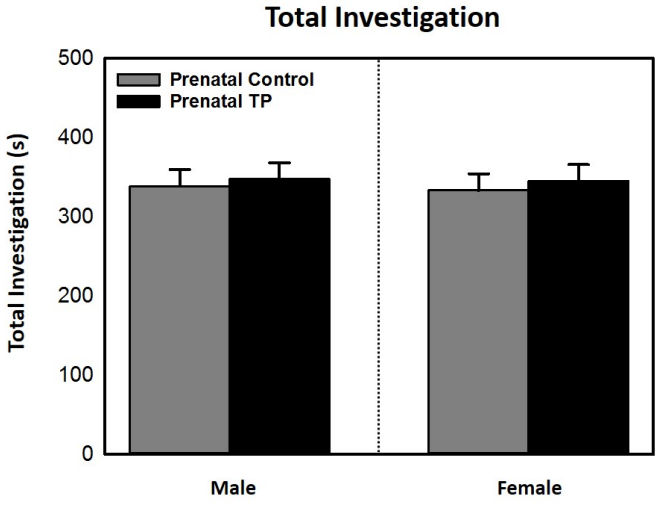


*Figure 10:* Total investigation during the Social Approach-Avoidance paradigm at adolescence. Grey bars represent prenatal exposure to the sesame oil control while black bars represent prenatal exposure to the TP treatment. **Left Panel**: Males (control: n = 10, testosterone: n = 10). **Right Panel:** Females (control: n = 10, testosterone: n = 10). Error bars ± SEM.

*Adulthood*

A univariate ANOVA revealed no significant main effects or interactions; however, a main effect of prenatal treatment trended towards significance (Figure 11; $F_{1,125} = 3.148$, $p = 0.08$). A one-sample t-test indicated that each group investigated the social stimuli significantly more than would be expected by chance (Figure N; prenatal control: males (sham: $t_{(9)} = 24.10$, $p < 0.001$, cholesterol replacement: $t_{(11)} = 6.63$, $p < 0.001$, T replacement: $t_{(9)} = 21.70$, $p < 0.001$) and females (sham: $t_{(9)} = 35.99$, $p < 0.001$, sesame oil replacement: $t_{(9)} = 5.53$, $p < 0.01$, EB replacement: $t_{(9)} = 19.30$, $p < 0.01$), as well as prenatal TP exposed males (sham: $t_{(9)} = 8.43$, $p < 0.01$, cholesterol replacement: $t_{11)} = 5.98$, $p < 0.01$, T replacement: $t_{(9)} = 8.10$, $p < 0.001$ ) and females (sham: $t_{(9)} = 8.94$, $p < 0.001$ , sesame oil replacement $t_{(11)} = 12.38$, $p < 0.001$:, EB replacement: $t_{(9)} = 14.96$, $p < 0.001$).

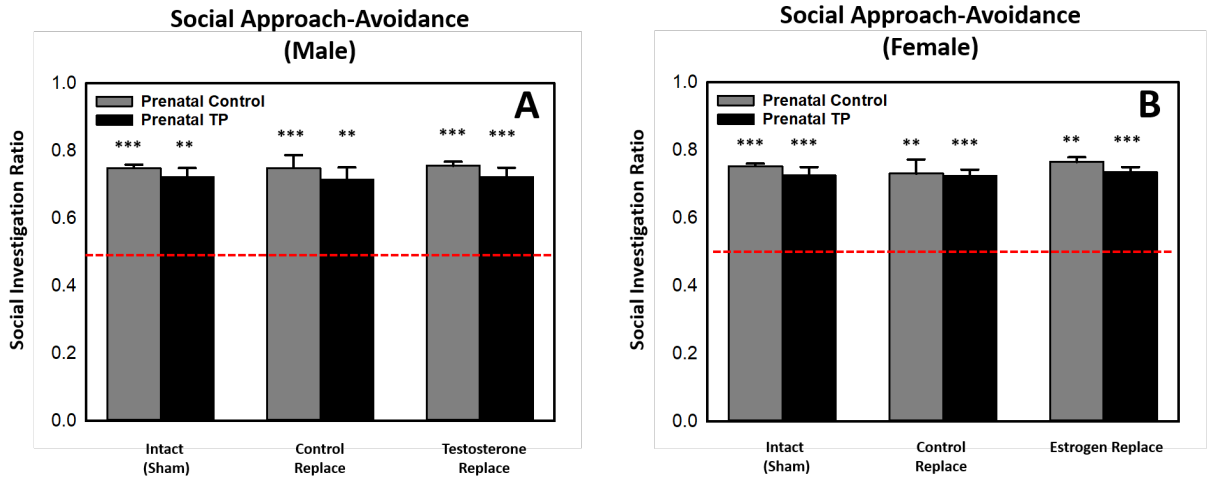



*Figure 11:* Social Approach-Avoidance paradigm done during adulthood. Grey bars represent prenatal exposure to the sesame oil control while black bars represent prenatal exposure to the TP treatment. **A:** Males prenatally exposed to sesame oil (control), (sham: n = 10, control: 12,

replacement: n = 10**)** and males prenatally exposed to testosterone (treatment) (sham: n = 10, control: n = 11, replacement: n = 10**). B:** Females prenatally exposed to sesame oil (control), (sham: n = 10, control: 9, replacement: n = 10) and Females prenatally exposed to testosterone (treatment) (sham: n = 10, control: n = 12, replacement: n = 10). Redline marks chance (0.5). *p<0.05, **p<0.01, ***p<0.001 (significantly different than would be expected by chance). Error bars ± SEM.

A univariate ANOVA indicated no significant effects on total investigation of sex, prenatal treatment, or adulthood replacement/sham surgery interventions (Fig. 12).

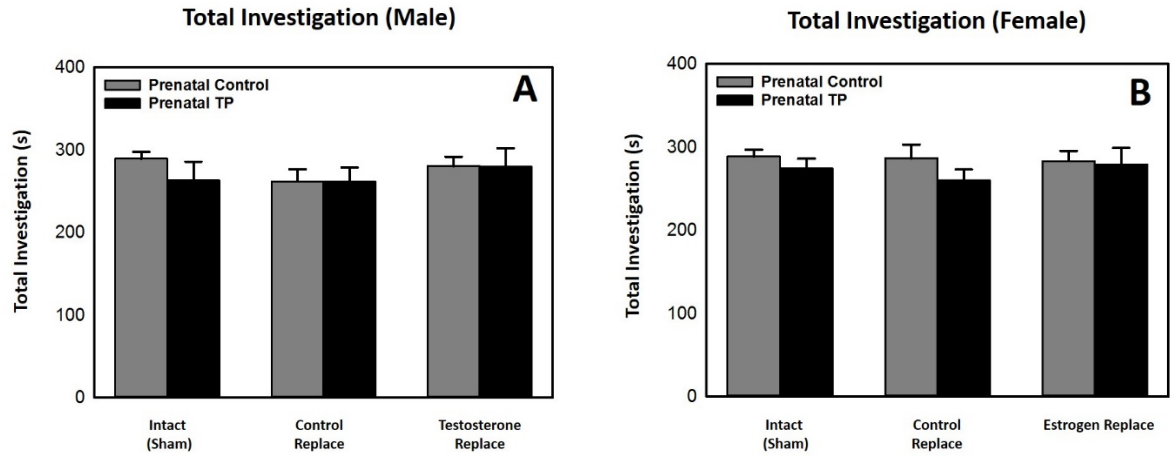



*Figure 12:* Total Investigation collected during the Social Approach-Avoidance paradigm done during adulthood. Grey bars represent prenatal exposure to the sesame oil control while black bars represent prenatal exposure to the TP treatment. **A:** Males prenatally exposed to sesame oil (control), (sham: n = 10, control: 12, replacement: n = 10**)** and males prenatally exposed to testosterone (treatment) (sham: n = 10, control: n = 11, replacement: n = 10**). B:** Females prenatally exposed to sesame oil (control), (sham: n = 10, control: 9, replacement: n = 10) and Females prenatally exposed to testosterone (treatment) (sham: n = 10, control: n = 12, replacement: n = 10). Error bars ± SEM.

**Social Recognition (SR)**

*Adolescence*

There was an enhancing effect of prenatal TP on SR in juveniles of both sexes (Fig. 13). A RM-ANOVA revealed a main effect of phase ($F_{1,44} = 29.65$, $p<0.001$), prenatal treatment ($F_{1,44} = 8.19$, $p = 0.006$), and a significant phase by prenatal treatment interaction ($F_{1,44} = 14.54$, $p<0.001$). A one-way ANOVA revealed a significant effect of prenatal treatment where mice prenatally exposed to TP had a greater IRTest than the prenatal sesame oil control counterparts.

Analysis of the simple main effects revealed that males who were prenatally exposed to TP had a significantly greater IRTest than the prenatal sesame oil control males ($p = 0.001$).

Planned binary-mean comparisons revealed the IRTest to be significantly higher than at the IRHab for males ($t_{(22)} = 3.24$, $p = 0.004$) and females ($t_{(22)} = 7.82$, $p < 0.001$) treated prenatally with TP but not in prenatal sesame oil control groups.

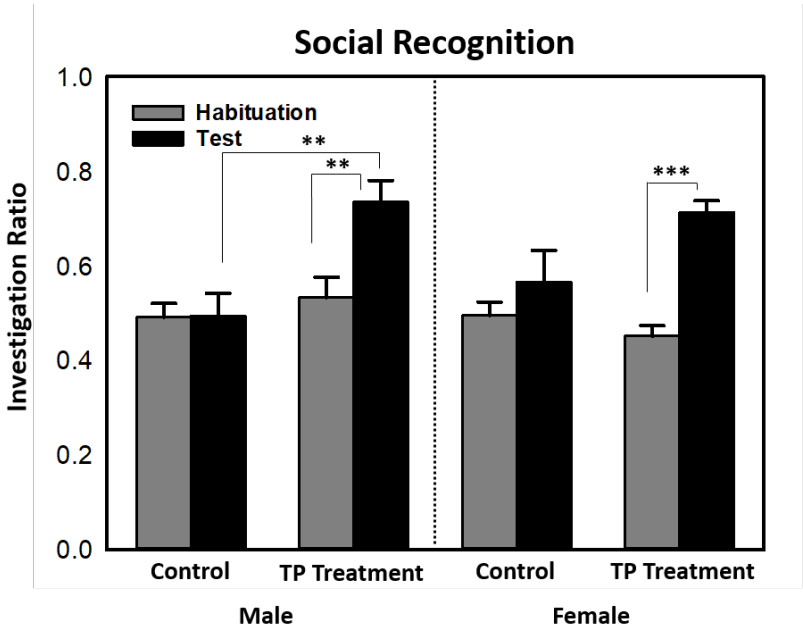



*Figure 13:* SR paradigm done during adolescence. Grey bars represent habituation session while black bars represent the testing phase of the paradigm. **Left Panel**: Males prenatally exposed to sesame oil (control) or testosterone (treatment) (control: n = 12, testosterone: n = 12). **Right Panel:** Females prenatally exposed to sesame oil (control) or testosterone (treatment) (control: n = 12, testosterone: n = 12). Error bars ± SEM. *p<0.05, **p<0.01, ***p<0.001.

A RM-ANOVA on total investigation revealed a main effect of phase ($F_{1,43} = 9.99$, $p = 0.003$) and sex ($F_{1,43} = 5.23$, $p = 0.03$). A one-way ANOVA indicated a significant effect of sex at H2 (Figure 14*;* $F_{1,46} = 8.17$, $p = 0.006$).

Pairwise comparisons indicated that prenatal sesame oil control males had significantly higher total investigation than prenatal sesame oil control females at H2 ($p = 0.04$).

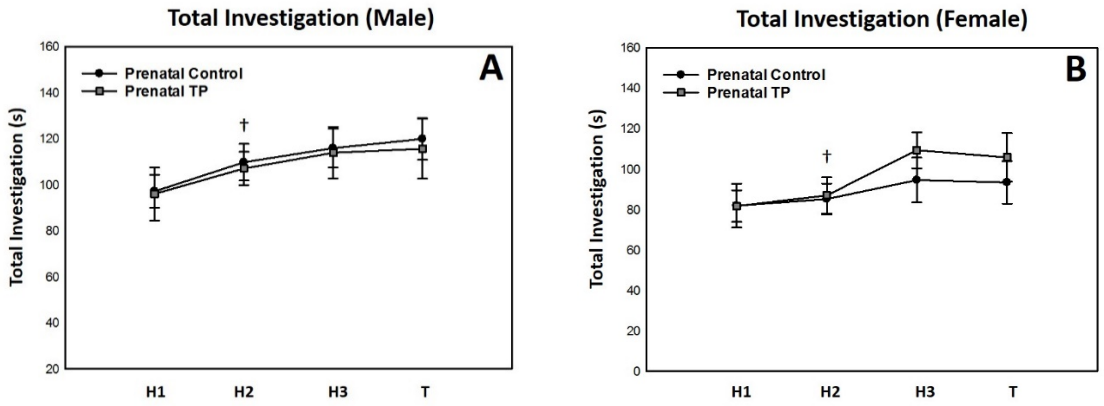



*Figure 14:* Total Investigation during SR paradigm conducted during adolescence. H1, H2, and, H3 represent the habituation sessions of the paradigm while T represents the testing phase. **A**: Males prenatally exposed to sesame oil (control) or testosterone (treatment) (control: n = 12, testosterone: n = 12). **B:** Females prenatally exposed to sesame oil (control) or testosterone (treatment) (control: n = 12, testosterone: n = 12). Error bars ± SEM. † indicates a sex difference.

*Adulthood*

Overall, there was an impairing effect of prenatal TP on SR in adult males (Fig. 15A) but not females Fig. 15B); which could be rescued by crystalline T replacement in adulthood. In females, ovariectomy impaired SR regardless of prenatal treatment and this was not rescued by adulthood E replacement. A RM-ANOVA revealed a main effect of phase ($F_{1,109} = 36.38$, $p<0.001$). Pairwise comparisons revealed that in males who received cholesterol replacements, those that were prenatally exposed to TP had a significantly lower IRTest than prenatal oil control males ($p = 0.048$). *A priori* binary mean comparisons revealed significant difference between IRHab and IRTest in the prenatal sesame oil control group exposed females (sham: $t_{(22)} = 2.20$, $p = 0.019$, sesame oil replacement: $p = ns$, EB replacement: $p = ns$ ) and males (sham: $t_{(20)} = 2.87$, $p = 0.01$, cholesterol replacement: $t_{(18)} = 3.67$, $p = 0.002$, T replacement: $p = ns$). As well as prenatal testosterone exposed females (sham: $t_{(26)} = 3.33$, $p = 0.003$, sesame oil replacement: $p = ns$ , EB replacement: $p = ns$) and males (sham: $p = ns$, cholesterol replacement: $p = 0.15$ *(ns)*, T replacement: $t_{(24)} = 3.30$, $p = 0.003$), showing that in males prenatal control

intact, prenatal control replacement, as well as males prenatal T replacement show good social learning. While in females, only the intact groups regardless of prenatal treatment show significant SR.

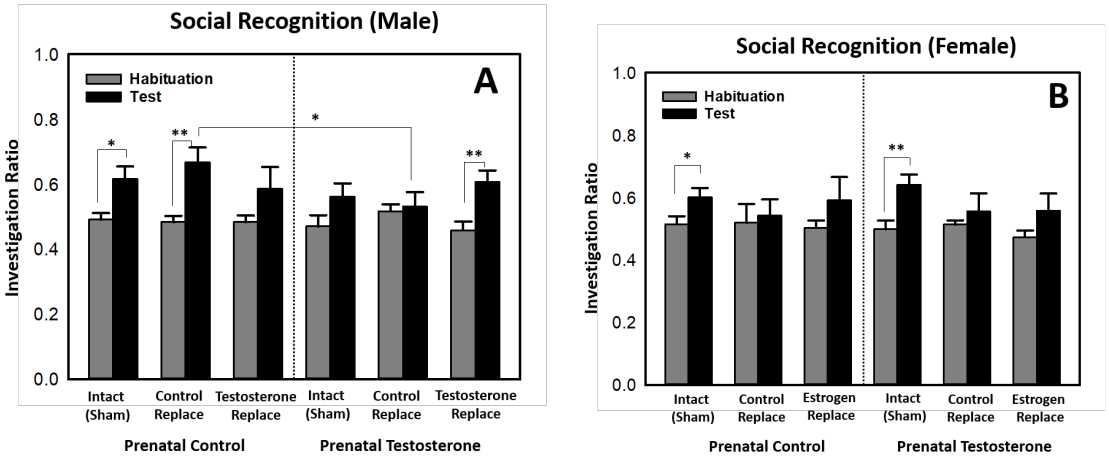



*Figure 15:* SR paradigm conducted during to adulthood. Grey bars represent habituation session while black bars represent the testing phase of the paradigm. **A (left panel)**: Males prenatally exposed to sesame oil (control), (sham: n = 11, control: 10, replacement: n = 12). **A (right panel)**: males prenatally exposed to testosterone (treatment) (sham: n = 12, control: n = 10, replacement: n = 13). **B (left panel)**: Females prenatally exposed to sesame oil (control), (sham: n = 12, control: 5, replacement: n = 8). **B (right panel):** females prenatally exposed to testosterone (treatment) (sham: n = 12, control: n = 6, replacement: n = 10). Error bars ± SEM. *p<0.05, **p<0.01.

A RM-ANOVA on total investigation revealed a prenatal exposure by phase effect $F_{1,57}$= 4.25, $p$ = 0.025. A one-way ANOVA on prenatal treatment as the main factor revealed a significant effect of prenatal treatment on the test phase ($F_{1,122}$= 4.10, $p$ = 0.045). Analysis of simple main effects revealed that prenatal sesame oil control mice had significantly higher total investigation at Test compared to the TP exposed mice in-utero (Figure 16*, C;* $p$ = 0.024).

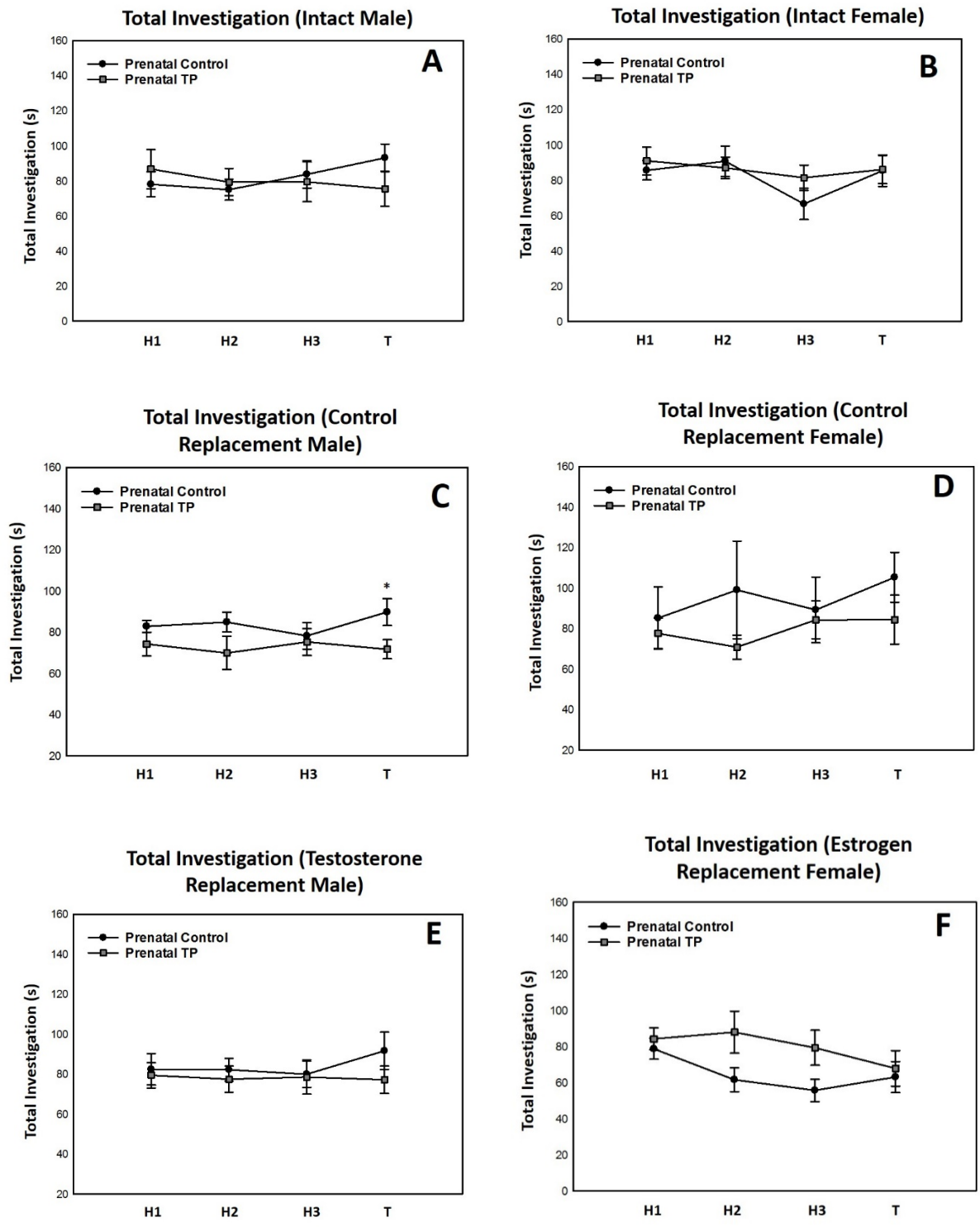



*Figure 16:* Total Investigation during SR paradigm conducted during adulthood. H1, H2, and, H3 represent the habituation sessions of the paradigm while T represents the testing phase. **A**: Males left gonadally intact after puberty (control: n = 11, testosterone: n = 12). **B:** Females left gonadally intact after puberty (control: n = 12, testosterone: n = 12). **C**: Males who were gonadectomized and received cholesterol control replacement at puberty (control: n = 10,

testosterone: n = 10). **D:** Females who were gonadectomized and received sesame oil control replacement at puberty (control: n = 5, testosterone: n = 6). **E**: Males who were gonadectomized and received testosterone hormone replacement at puberty (control: n = 12, testosterone: n = 13). **F:** Females who were gonadectomized and received estrogen replacement at puberty (control: n = 8, testosterone: n = 10). Error bars ± SEM. *p<0.01.

**Object Recognition (OR)**

*Adolescence*

Juvenile males, but not females, were impaired in OR and this impairment was reversed by the prenatal TP treatment (Fig. 17). Sex differences and TP effects cannot be explained by effects on total investigation of the objects (Fig. 18). The mixed RM-ANOVA revealed a main effect of paradigm phase ($F_{1,44} = 39.14$, $p<0.001$), prenatal treatment ($F_{1,44} = 4.71$, $p = .035$), and a significant interaction between phase of the paradigm and sex ($F_{1,44} = 5.58$, $p = .023$). A one-way ANOVA indicated a significant difference in IRHab phases between control and TP prenatal treatment groups ($F_{1,46} = 6.24$, $p = .016$). Binary mean comparisons of the simple main effects indicated that sesame oil control females had a higher IRTest than sesame oil control males ($p = .034$) and that females prenatally exposed to TP had a greater IRHab than prenatal sesame oil exposed females ($p = .034$). Furthermore, prenatal TP exposed males had greater IRTest than prenatal sesame oil exposed males ($p = .044$). Planned binary-mean comparisons revealed the IRTest to be significantly higher than at the IRHab for males treated prenatally with TP ($t_{(11)} = 3.48$, $p<0.01$), females prenatally exposed the sesame-oil control ($t_{(11)} = 4.04$, $p<0.01$), and females exposed prenatally to the TP treatment ($t_{(11)} = 3.57$, $p<0.01$) but not in males parentally exposed to sesame oil.

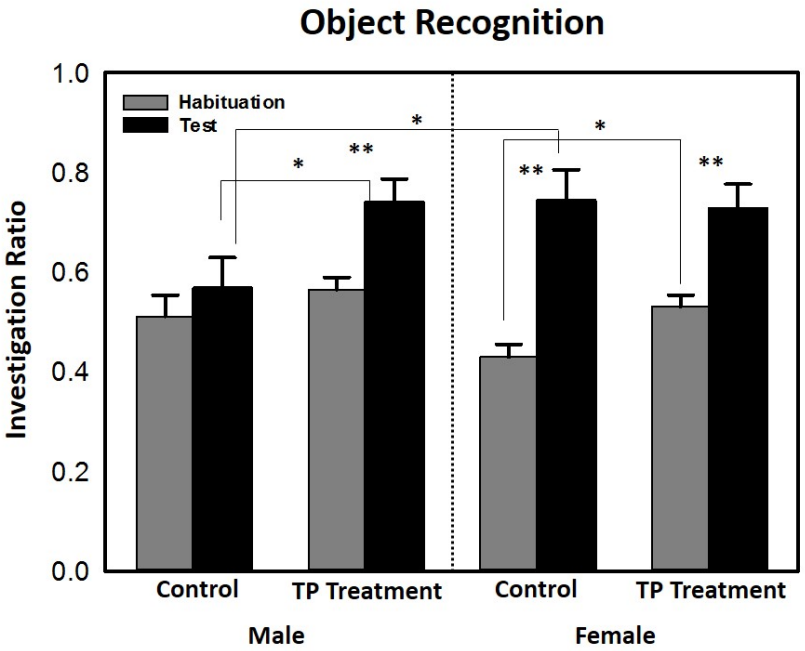



*Figure 17:* OR paradigm done during adolescence. Grey bars represent habituation session while black bars represent the testing phase of the paradigm. **Left Panel**: Males prenatally exposed to sesame oil (control) or testosterone (treatment) (control: n = 12, testosterone: n = 12). **Right Panel:** Females prenatally exposed to sesame oil (control) or testosterone (treatment) (control: n = 12, testosterone: n = 12). Error bars ± SEM. *p<0.05, **p<0.01.

A mixed RM-ANOVA on Total investigation of adolescent mice (Fig. 18*)* revealed a significant effect of phase ($F_{1,44} = 5.32$, $p = .026$), sex ($F_{1,44} = 17.59$, $p < 0.001$), and prenatal treatment ($F_{1,44} = 17.61$, $p < 0.001$). There was also a significant interaction between sex and prenatal treatment ($F_{1,44} = 15.60$, $p < 0.001$). A one-way ANOVA revelated a main effect of prenatal treatment between prenatal control and treatment groups during the test phase ($F_{1,47} = 9.90$, $p = 0.003$). Additionally, a one-way ANOVA revealed a main effect of sex during H1 ($F_{1,47} = 7.33$, $p = 0.01$), H2 ($F_{1,47} = 5.15$, $p = 0.03$), and Test ($F_{1,47} = 6.40$, $p = 0.02$). Post hoc analysis of simple main effects showed that sesame oil control males had significantly greater overall investigation during H1 ($p < 0.01$), H2 ($p < 0.04$), H3 ($p < 0.01$) and Test ($p < 0.05$) than sesame oil control females. This was not the case for TP treated males and females ($p = ns$). Post hoc

analysis revealed that TP treated females had greater total investigation than sesame oil control females during H1 ($p = 0.01$), H2 ($p = 0.07$), H3 ($p < 0.01$) and Test ($p = 0.02$).

Planned comparisons revealed that TP exposed females had higher total investigation than sesame oil exposed females within each phase of the paradigm (Figure 18; H1: $t_{(22)} = 3.60$, $p < 0.01$; H: $t_{(22)} = 3.11$, $p < 0.01$; H3: $t_{(22)} = 4.76$, $p < 0.001$; Test: $t_{(22)} = 2.90$, $p < 0.01$).

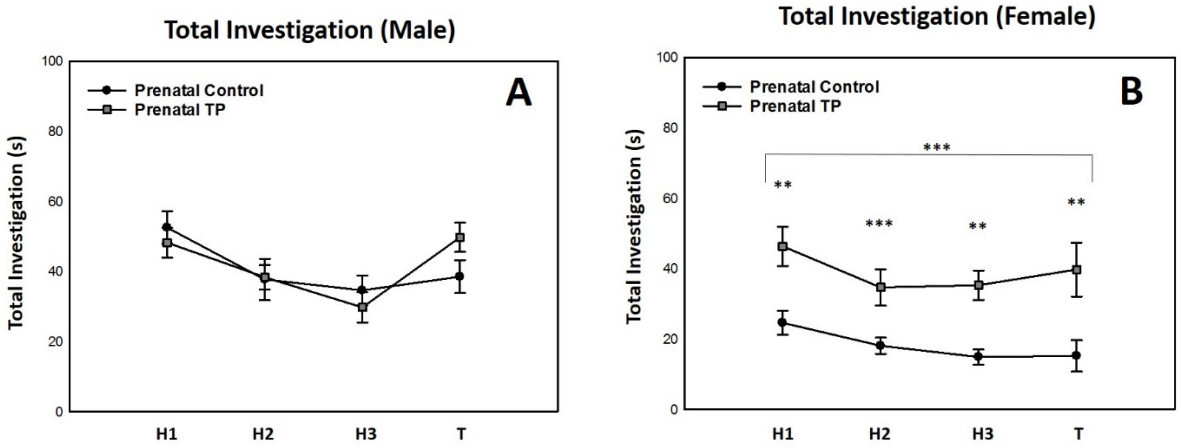



*Figure 18:* Total Investigation during OR paradigm conducted during adolescence. H1, H2, and, H3 represent the habituation sessions of the paradigm while T represents the testing phase. **Panel A**: Males prenatally exposed to sesame oil (control) or testosterone (treatment) (control: n = 12, testosterone: n = 12). **Panel B:** Females prenatally exposed to sesame oil (control) or testosterone (treatment) (control: n = 12, testosterone: n = 12). Significance line represents an overall difference between prenatal TP and prenatal sesame oil control groups. * indicates a significant difference between each group total investigation within each time bin. Error bars ± SEM. *p<0.05, **p<0.01, ***p<0.001.

Analyses conducted on other behaviours (See Table 4) showed an increase in horizontal locomotion in the female prenatal TP group (Fig. 19) compared to female prenatal control; with no overall sex differences. Therefore, the effects on horizontal locomotion and total object investigation suggest prenatal TP enhancement of active behaviors in females but not in males.

A mixed RM-ANOVA revealed a main effect of phase ($F_{1,42} = 45.58$, $p < 0.001$) and prenatal treatment ($F_{1,42} = 4.06$, $p = 0.05$). A one-way ANOVA on total horizontal movement in

females revealed that TP treated females exhibited significantly greater bouts of horizontal activity compared to sesame oil exposed females ($F_{1,94} = 17.25$, $p < 0.001$).

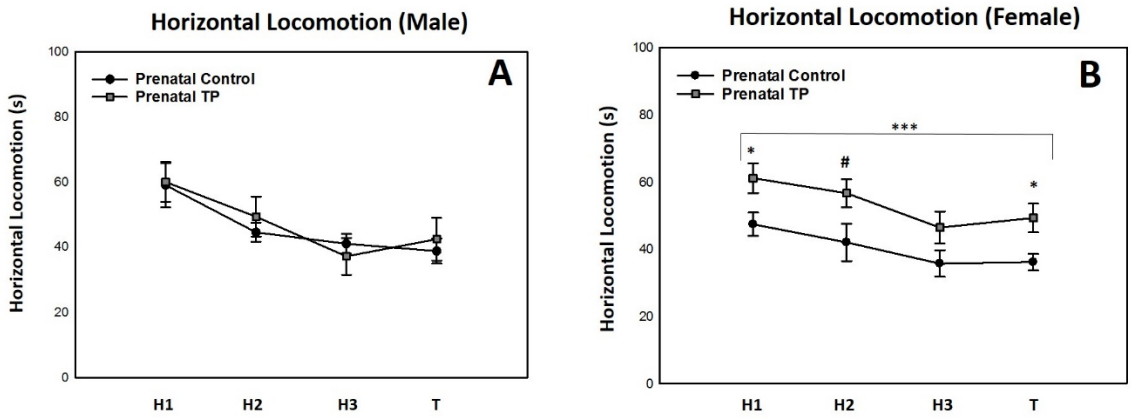



*Figure 19:* Horizontal locomotion during OR paradigm in adolescent mice ***A:*** Males prenatally exposed to sesame oil (control) or TP (treatment) (control: n = 12, testosterone: n = 12). ***B:*** Females prenatally exposed to sesame oil (control) or TP (treatment) (control: n = 12, testosterone: n = 12). Significance line represents an overall difference between prenatal TP and prenatal sesame oil control groups. * indicates a significant difference between each group horizontal locomotion within each time bin. Error bars ± SEM. *p<0.05, **p<0.01, ***p<0.001.

*Adulthood*

Overall, mice of all groups showed good OR, with no major impairments nor improvements by prenatal or adulthood hormonal manipulations. A mixed RM-ANOVA revealed a main effect of paradigm phase ($F_{1,140} = 181.71$, $p<0.001$) and sex ($F_{1,140} = 5.25$, $p<0.05$), as well as a phase by prenatal treatment interaction ($F_{1,140} = 4.23$, $p<0.05$) and a sex by prenatal treatment by adult treatment interaction ($F_{2,140} = 3.35$, $p<0.05$). A one-way ANOVA conducted with sex as the main factor revealed a significant difference between males and females during IRHab ($F_{1,150} = 9.69$, $p= 0.002$) where males had a higher investigation ratio than the females overall.

Analysis of the simple main effects revealed that prenatal sesame oil control males (Fig. 20A) that received crystalline testosterone replacements in adulthood, had greater IRHab ($p =$

0.02) and IRTest ($p = 0.04$) than the female prenatal sesame oil control who received the EB replacement (Fig. 20B). Further analysis indicated that male prenatal sesame oil control, who received crystalline testosterone replacements, had significantly higher IRTest ($p = 0.018$) compared to the prenatal TP males, who received crystalline testosterone replacements after puberty. In females that were left gonadally intact (sham) those who were exposed to prenatal TP in-utero, had a significantly higher IRHab than those who were prenatally exposed to the sesame oil control in-utero ($p = 0.01$).

*A priori* planned comparisons revealed a significant difference between habituation and test in the prenatal sesame oil control group exposed males (sham: $t_{(11)} = 5.47$, $p < 0.001$, cholesterol replacement: $t_{(11)} = 3.29$, $p < 0.001$, T replacement: $t_{(11)} = 4.76$, $p < 0.001$) and females (sham: $t_{(10)} = 5.80$, $p < 0.001$, sesame oil replacement: $t_{(10)} = 5.47$, $p < 0.01$, EB replacement: $t_{(16)} = 3.82$, $p < 0.01$), as well as prenatal TP exposed males (sham: $t_{(12)} = 3.30$, $p < 0.01$, cholesterol replacement: $t_{(14)} = 3.03$, $p < 0.01$, T replacement: $t_{(14)} = 5.59$, $p < 0.001$ ) and females (sham: $t_{(16)} = 3.91$, $p = 0.001$ , sesame oil replacement $t_{(6)} = 2.83$, $p = 0.03$:, EB replacement: $t_{(9)} = 3.38$, $p = 0.008$), suggesting all groups have in tact OR.

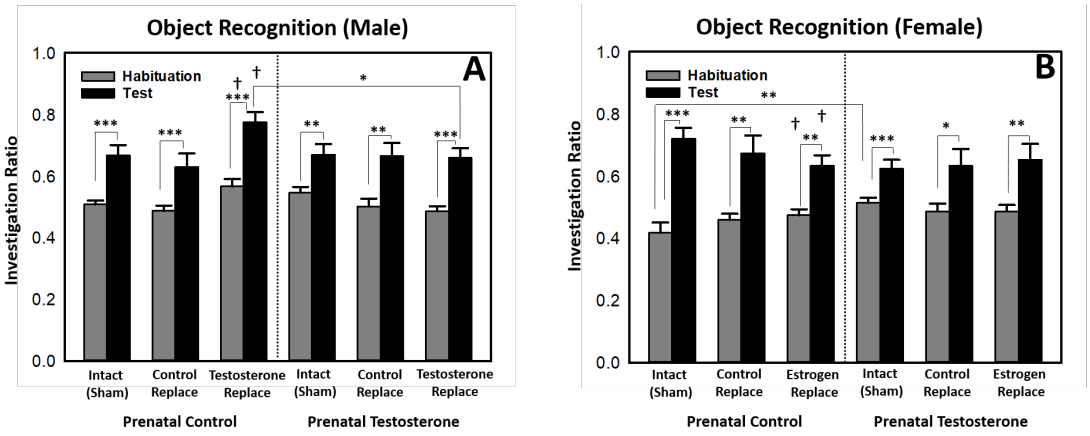
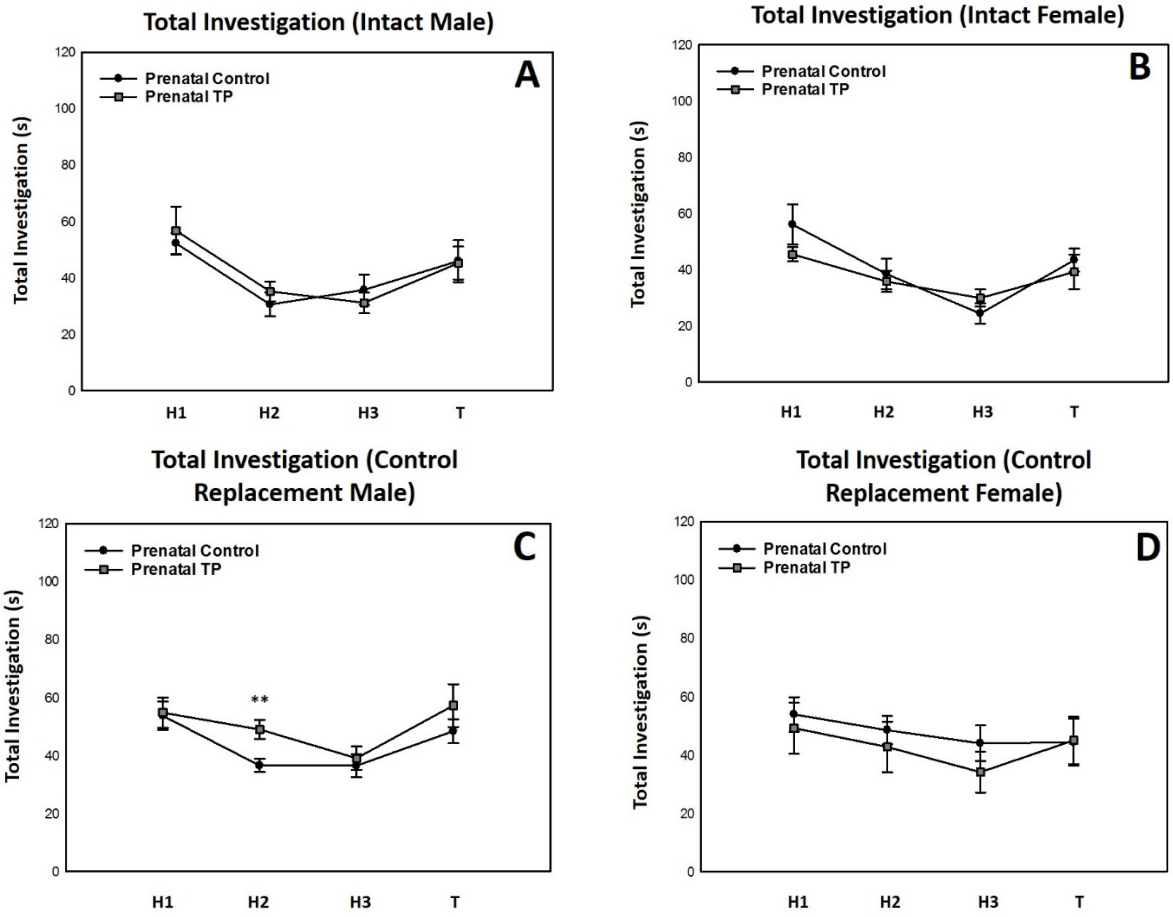



*Figure 20:* OR paradigm done during to adulthood. Grey bars represent habituation session while black bars represent the testing phase of the paradigm. **A (left panel)**: Males prenatally exposed to sesame oil (control), (sham: n = 13, control: 12, replacement: n = 14**). A (right panel):** males prenatally exposed to testosterone (treatment) (sham: n = 14, control: n = 15, replacement: n =

16**). B (left panel)**: Females prenatally exposed to sesame oil (control), (sham: n = 12, control: 11, replacement: n = 14). **B (right panel):** Females prenatally exposed to testosterone (treatment) (sham: n = 12, control: n = 8, replacement: n = 10). Error bars ± SEM. *p<0.05, **p<0.01, ***p<0.001. † indicates a significant sex difference (p<0.05).

A RM-ANOVA on total investigation revealed a main effect of phase ($F_{1,118} = 9.92$, $p$= 0.002). A one-way ANOVA revealed an effect of adult treatment on H2 and H3 (see Figures 21, A-F). Post-hoc tests revealed that female and male controls (sesame oil or cholesterol replacements respectively) had greater total investigation than gonadally intact (shams) mice during H2 ($p$ = 0.017) and H3 ($p$ = 0.031). *A priori* planned comparisons revealed significant difference between male cholesterol replacement controls at H2, where prenatal TP mice with cholesterol implants had a greater total investigation than males prenatally exposed to sesame oil $t_{(22)} = 3.15$, $p$ = 0.005 (Fig. 17).



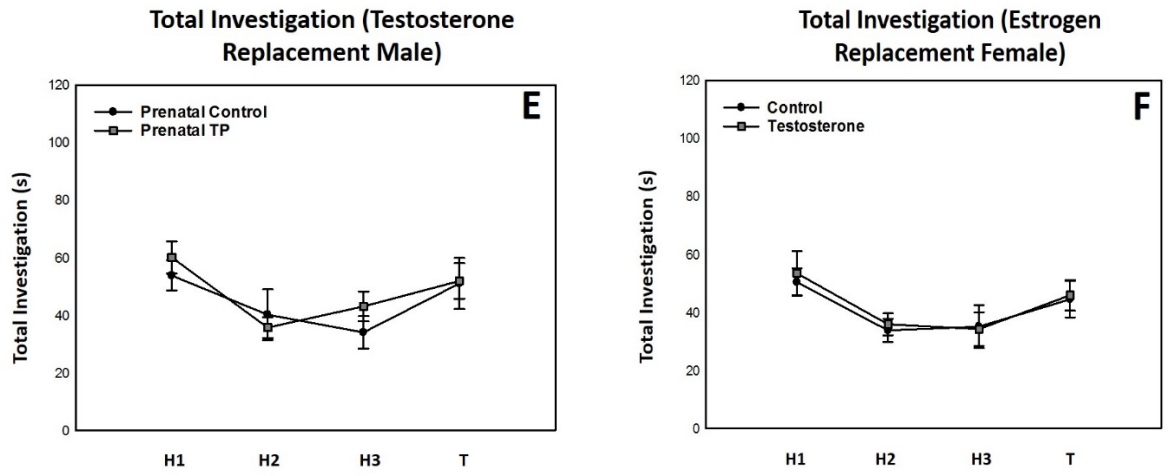



*Figure 21:* Total Investigation during OR paradigm conducted during adulthood. H1, H2, and, H3 represent the habituation sessions of the paradigm while T represents the testing phase. **A**: Males left gonadally intact after puberty (control: n = 13, testosterone: n = 14). **B:** Females Males left gonadally intact after puberty (control: n = 12, testosterone: n = 12). **C**: Males who were gonadectomized and received cholesterol control replacement at puberty (control: n = 12, testosterone: n = 15). **D:** Females who were gonadectomized and received sesame oil control replacement at puberty (control: n = 11, testosterone: n = 8). **E**: Males who were gonadectomized and received testosterone hormone replacement at puberty (control: n = 14, testosterone: n = 16). **F:** Females who were gonadectomized and received estrogen replacement at puberty (control: n = 14, testosterone: n = 10). Error bars ± SEM.

## Discussion

### Summary

Overall, it was verified that the prenatal TP treatment increased circulating levels of maternal T. This treatment had negligible effect on overall morphologic development except for an accelerating effect on the onset of vaginal opening— a sign of sexual maturity (Mayer et al., 2010).

Notably, we found that males and females exhibit a marked sex difference in OR capabilities and are similar in SR during adolescence. Further, we found that typically the prenatal TP treatment rescued or enhanced social or non-social recognition. In adulthood, we found that prenatal treatment with TP inhibited SR and interacted with activational hormone effects of sex hormones during adulthood to influence SR and OR to either impair or rescue

these behaviours. Fundamental sociability does not seem to be affected by the prenatal treatment or hormone replacement in adulthood, suggesting manipulation of the prenatal milieu with low doses of TP may not affect this behaviour.

**Developmental and Physiological Measures**

We were unable to detect a difference in the maternal plasma T taken after the delivery between dams injected with the sesame oil control or the TP treatment. Howes et al. (2016) hypothesized that the prenatal TP injection induced an increase in maternal T after the last injection on E16. This increase was no longer significant on day of delivery indicating that the elevation of T was metabolized and transient in nature. Martin and Marcotte (2017, unpublished data) later found that if the maternal serum was collected after the final injection (at E16) the serum T levels of the TP groups was greater than in sesame oil control dams. Taken together, these results suggest a transient rise in serum T after injection, which then tapers off and returns almost to baseline on the day of birth. While the data collected from the fecal samples (Figure 5) did not show a rise in T as a result of our treatment, the results may show that the handing of the dams was stressful, as there was a significant increase of fecal T in the control group and exposure to stressors during pregnancy has shown to be correlated with heighted levels of circulating T (vom Saal et al., 1990; Schradin, 2008). An elevation in prenatal stress could have serious implications towards the current study, as this would mean that we had no true control group. Indeed, gonadal sex hormones have been shown to modulate activity of the hypothalamic-pituitary-adrenal axis, which interacts with the hypothalamic-pituitary-gonadal axis is involved in regulating the stress response, and is closely associated with anxiety in humans (reviewed in Abelson, Khan, Liberzon, & Young, 2007; Pariante & Lightman, 2008).

The prenatal TP treatment had no effect on the growth of the litters (measured by weight, appearance of fur/opening of eyes/ears). Further, there were no observable morphological alterations to the female genitalia, as some studies show that prenatal exposure to exogenous TP can masculinize female genitalia (Wolf, Hotchkiss, Ostby, LeBlanc, Gray, 2002; Hotchkiss, Lambright, Ostby, Parks-Saldutti, Vandenbergh, Gray, 2007). This is most likely because serum T levels in this study were well within a physiological range (0.133 ng/ml - 0.4462 ng/ml) rather than supra-physiological does ranging from 0.1 to 10mg, resulting in maternal T levels of 0.5mg/ml to 1.0mg/ml—like in the above cited studies. Further, there was an effect on onset of vaginal opening, where TP exposed females showed a sooner onset than their control counterparts. This suggests that prenatal exposure to heightened levels of T accelerates onset of puberty; this is in line with previous literature that shows low levels of androgen exposure during late gestation in mice accelerates female puberty onset (Witham et al., 2012). Even though this this finding was statistically significant we should point out that the difference in puberty onset between the prenatal treatment and control groups was less than a day; therefore, it is unlikely this has major implications for the interpretation of the behavioural results.

Castration reduced prostate weight in adults, which was only partially recovered by T replacement via hormone implant; as, the weight of the prostate was not returned to that of intact males. Previous literature shows the prostate to be a bioassay of T levels and has shown to fluctuate in weight in reference to circulating T (Holmäng, Mårin, Lindstedt, & Hedelin, 1993; Shao, Kong, & Cunningham, 1994). This suggests that our T implants were not successful in bringing physiological T back to baseline in our castrated males, which may be why some male replacements groups were unable to optimally perform in some of the behavioural paradigms, as discussed below.

Ovariectomy also significantly increased body weight during adulthood, which was significantly decreased to the level of gonadally intact females by the EB replacement. This suggests our EB replacements were functional, as previous literature that shows Es play a role in the maintenance and regulation of weight (Blaustein, Gentry, Roy, & Wade, 1976). Overall there was no effect of our TP treatment on gonad weight, adult body weight, or prostate weight.

Taken together, the developmental data suggests that our TP treatment was within physiological range and it did not disrupt gross anatomical development, which adds to the translational aspect of our research towards an EMBH ASD model, which will be discussed later.

**Social Approach-Avoidance**

*Adolescence and Adulthood*

Overall, all mice showed a preference for the social stimulus; this makes sense as social approach behaviour is arguably the "most basic behavioral component of all social interactions providing a means through which specific relationships between individuals can be established, maintained and strengthened" (Panksepp & Lahvis, 2007). As social-approach avoidance behaviour is a measure of fundamental sociability, the fact that we did not see an impairing or enhancing effect on this behaviour suggests the effects of prenatal TP seen in SR, social learning, and in aggression can not be attributed to changes in social approach. To our knowledge there has been only one study that looked at how heighted levels of androgens and estrogens in-utero affect the innate sociability of rodents. Xu et al, 2014 found that prenatal exposure to Letrozole— thus exposure to elevated levels of T and low levels of Es— in-utero, impairs social approach behaviour in adolescence. Our model differs however, as Xu et al. (2014) used rats and blocked T aromatization; whereas, we used a mouse model and did not control for the differential effects that the aromatization of T to E in-utero may implicate on behaviour. Further,

the dosing regimen of Letrozole was chronically delivered at 1μg/ml for 3 weeks which resulted in serum T levels well above a physiological range—averaging 40 ng/dl. Therefore, this suggests that T, and not E, drives the development of fundamental sociability in-utero. However, this is speculative as more studies need to be done to understand how T and E differentially affect the development of fundamental sociability.

**Social Recognition**

*Adolescence*

Overall, prenatal exposure to heighted levels of TP allows for SR to occur in both sexes. Mice that were not exposed to the prenatal T treatment could not perform SR. Further, prenatal T had an improving effect on SR in male and female mice, significantly increasing investigation during the test phase relative to test. Interestingly, this paradigm has been used successfully in adult male and female mice (reviewed in this thesis, Ferguson, Young, & Insel, 2002); thus, our study suggests this SR paradigm may not be optimal for testing SR in juveniles. To our knowledge there have been no studies assessing SR in juveniles; therefore, it is difficult to put our results into context with previous literature. It is possible, that the enhancing effect of prenatal TP may be due to an early onset of puberty, as the onset of vaginal opening was earlier in TP treated females. Furthermore, these effects can not be explained by differences in total investigation as there were no overall differences between control and treatment groups as well as no sex differences. Our findings suggest that juveniles are unable to socially differentiate from conspecifics—or perhaps the paradigm may be too difficult for mice in this age-group. Nonetheless, the results suggest that gonadal hormones may activate SR in adolescent male and female mice.

*Adulthood*

Overall, we found there to be an impairing effect of prenatal testosterone on SR in adult males but not females, which is rescued by T replacement in adulthood. Both gonadally intact and cholesterol replacement groups prenatally exposed to the sesame oil control show good SR; this is consistent with previous literature showing male can socially recognize regardless of the presence of gonads (Karlsson, Haziri, Hansson, Kettunen, & Westberg, 2015). At this time, we are not able to discern the levels of serum T provided by the T implant in our male mice; therefore, we can not know if the T levels in the T replacement mice returned serum T to within a physiological range. However, the prostate weights suggest that T levels were not recovered to that of in-tact males during adulthood. Subsequent analysis must be done to conclude if a low level of serum T is the cause of this inability to rescue SR in the replacement males or if the organizational effects are permanent and can be fixed later.

In the present study, prenatal exposure to heighted T seems to impair later-life SR. The pubertal T replacement seems to interact with the organizational frame-work set by the prenatal TP treatment to rescue SR capabilities in males. This finding is novel as no other studies have shown such an interaction in SR before. In adult females, both gonadally intact groups showed good SR, which is consistent with previous literature (Lymer et al., 2017; Gabor et al., 2014, Phan et al., 2012). Furthermore, gonadectomized control groups were impaired in SR, which is also consistent with previous literature (Karlsson, Haziri, Hansson, Kettunen, & Westberg, 2015); however, it is striking is that the hormone EB replacement did not rescue SR and that prenatal TP had no effects in females. This is interesting as long-term E replacement in female rats has shown to increase SR without affecting overall investigation (Tang et al., 2005). Furthermore, it has been shown that impairments caused by ovariectomy can be recovered by the delivery of Es in rats (Hlinak, 1993; Spiteri, & Ågmo, 2009). The inconsistencies between our

studies and others may simply be the dosage of EB and species used, as Hlinak, 1993 administered 100µg and to female rats Spiteri, & Ågmo, 2009 used a combination of EB and progesterone in rats as well. Other differences, such as variations in the design of SR protocols may help explain the inconsistencies in our findings, as Hlinak, 1993 SR paradigm was drastically different and used juvenile conspecifics, and Spiteri, & Ågmo has a 15-minute inter-stimulus interval. Much like in adolescence, these effects can not be explained by differences in total investigation as there were no overall differences between control and treatment groups as well as no sex differences. Taken together, it is likely that the inconsistencies between the current study and previous literature, on the impairing effects of ovariectomy and improvement with E replacement on SR, are a result of differences in species, dosage, and paradigms used in each study.

The fact that there is an impairing effect of prenatal TP on SR in adulthood suggests that the interpretation of accelerated puberty by prenatal TP in juveniles is plausible. Indeed, because heighted prenatal TP impaired SR in adulthood it is likely that during adolescence this prenatal exposure is just enough to bring SR to the level of an unexposed intact adult; however, it may interact with the influx of gonadal sex hormones introduced at puberty to have an impairing effect.

**Object Recognition**

*Adolescence*

The results suggest that male adolescent mice, and not female adolescents, are unable to differentiate between a novel or a previously encountered object and that prenatal exposure to heighted levels of T enhances object recognition. However, unlike our study, Cryenne and Brown (2011) showed no sex differences in investigation of a novel object; in adolescent rats. To

our knowledge, no studies have been done testing mice in OR in early adolescence. Therefore, it is difficult to make conclusions based on the available literature. Further, the prenatal TP treatment seems to have an enhancing effect on OR, as TP treated males showed significant OR. It has been shown that suppressing gonadal hormones in adolescent male rats reduced preference for novel objects while suppressing gonadal hormones in adolescent females did not affect response to the OR (Cryenne and Brown, 2011). Therefore, our results suggest that androgens and Es play a critical role in the development of OR capability.

The results also showed that males tend to investigate the objects more than females but exposure to prenatal TP eliminates this sex difference by increasing object investigation of females to that of males. Exposure to T has not shown to cause hyperactivity in a rodent model, many studies of humans have shown that prenatal exposure to T causes hyperactivity in females (Martel, Gobrogge, Breedlove, & Nigg 2008; Fink et al., 2007; Williams, Greenhalgh, & Manning, 2003). It is likely that the prenatal TP masculinized the investigatory behaviour in females, as our findings are consistent with research by Karlsson et al. (2015) who found males tend to investigate a previously encountered object at a much greater rate than females. While both female groups in our study could conduct OR, the prenatal TP females performed significantly more behaviours (horizontal locomotion) and still performed optimal in the paradigm— this would suggest that they were less focused and hyperactive than the control females, but were still able to perform OR at the same level.

*Adulthood*

Overall, all mice groups— regardless of sex, prenatal treatment, or hormone replacement showed that ability to differentiate between a novel or previously encountered objects. This is interesting because Aubele et al. (2008) found that male rats, who were castrated, were not able

to discriminate between novel or previously encountered objects. However, this paradigm had a significantly longer inter-stimulus interval spanning hours to days, whereas our inter-stimulus intervals were only 3 minutes. Furthermore, ovariectomy has been shown to cause significant deficit in OR in mice starting two weeks after surgery (Wallece et al., 2006). Furthermore, these deficits were associated with a reduction in pyramidal neuron dendritic spine density in hippocampal CA1 and the medial prefrontal cortex suggesting a role for morphological alterations in these observed memory deficits in OR; however, again the inter-stimulus interval used in OR lasted hours, which may help explain the lack of deficits we see in our OR paradigm. It is likely that the differences between our results and the literature may be attributed to the design of our OR paradigm and the nature of long-term object recognition, seen in the above studies, and working-memory recognition in our current study. These differences have implications for the acquisition and retention phases of object recognition, as alteration the habituation phases has implications for learning, while alteration the inter-stimulus interval has implications for working memory. Future research needs to be conducted to elucidate the effects of prenatal exposure to T on the short-term and long-term retention and acquisition phases of OR and other non-social memory tasks.

The prenatal control males who received the T replacement have better OR than the prenatal TP T replacement group. This suggests that exposure to prenatal TP may have desensitized the mouse to later-life androgens (i.e. T implant) as exogenous T in adulthood has been shown to suppress intratesticular T production (Crosnoe, Grober, Ohl, & Kim, 2013). However, this result must be interpreted with caution, as the prenatal TP exposure T replacement males still exhibited good OR. There was also an interesting sex difference where prenatal control males who received TP replacement outperformed female prenatal control groups who

received EB replacements showed that was not seen in the prenatal testosterone group. These findings suggest that both males and females perform similarly in OR, but T replacement enhances male performance in comparison to that of females who receive EB replacement, and that heighted prenatal exposure to T eliminates the difference in the replacement groups.

**Summary and Implications**

Overall, we found that exposure to a heighted levels of TP in-utero has direct effects and interacted with sex hormone manipulations in adulthood to produce sexually differentiated effects on OR and SR, as well as social learning, social interaction, agonistic behaviour, and anxiety-like behaviour (discussed in Howes, 2016).

We have shown that adolescent mice are unable to perform SR within the context of our paradigm and that heighted exposure to TP in-utero rescues, and in the case of males, enhances SR capabilities. This same enhancing effect is seen in adolescent males for OR., while there seems to be no effect in females. Our findings imply that prenatal exposure to TP enhances memory in adolescent males, regardless of the social or non-social context. While prenatal TP exposure enhances non-social recognition memory in adolescent males and shows no effect in females. These results are difficult to interpret as no studies have looked at the effects of prenatal exposure of T on OR and SR in adolescent mice. The sex difference may arise from the fact that females outperform males in OR when the novel object is highly similar to the previously encountered ones (Bettis & Jacobs, 2012)). This factor may have played a role, as the objects were tested in previous studies (see methods) and shown to exhibit no preferential bias. The way our TP treatment induced OR in males however is currently unknown.

In adulthood, we see an interaction of prenatal TP treatment with the hormone replacement manipulation in adulthood non-social memory, but not with endogenous hormones

(intact and sham controls). Prenatal control males who received T replacement in adulthood had greater OR capabilities than males who received the prenatal TP treatment and received the T replacement. This was not seen in SR, where the prenatal control T-replacement males were not able to perform SR, while the prenatal TP group who received T replacements could. Therefore, the prenatal TP interacted with the T replacement to impair OR but improve SR in males. Despite this, male's exposure to prenatal TP showed no SR without T replacement. This indicates that prenatal TP treatment in males had negative implications for male SR. All males and females performed OR well in adulthood; however, this was not the case in SR. Prenatal control T-replacement males and prenatal control, control replacement females showed poor SR but not OR. Further, prenatal TP intact and control replacement males and prenatal TP control replacement and EB replacement females showed poor SR but not OR. Taken together, heighted levels of prenatal TP have differential effects on social and non-social memory where SR memory is negatively implicated in males but not females in adulthood.

Howes (2016) found that male mice are less sociable than females, do not pay as much attention to social stimuli, and allocate more time to non-social activity than females. Further, exposure to heightened prenatal TP appears to shift male mice further towards a non-social behavioural phenotype, suggesting that these behaviours are further masculinized in male mice, but not in females. This was consistent in SR, as intact and sham males, who were exposed to prenatal TP, were not able to conduct SR in adulthood. comparing SR of adulthood prenatal TP females who are intact are able to perform SR, unlike their male intact counterparts— giving evidence for a female advantage in SR if exposed to prenatal TP. However, "hyper-masculinizing" the male by way of T replacement in the prenatal TP group, allows for SR; therefore, SR may be unique in terms of androgen responsiveness, as adult males are impaired by

organizational exposure to TP and the activational T replacement recuses it. This result is a fantastic interaction between organizational and activational effects of sex hormones effects on SR, as per hypothesis # 2 (see introduction). Alternatively, the prostate results suggest that T levels are lower in the T replacement groups and that the mice may be hypoandrogenic; therefore, it is possible that optimal levels of T are required to perform SR in TP treated males. Subsequently, Howes (2016) found that heightened exposure to T in early development elevates anxiety-like behaviour in male mice. This adds to a body of literature showing that effects of prenatal stress on later life anxiety behaviour tend to be sexually dimorphic showing that female mice are resilient to this effect (Bowman et al., 2004; Hellemans et al., 2010, 2008; Kinsley & Svare, 1987; Mueller & Bale, 2008; Souza et al., 2013; Weinberg, 1988; Weinstock, 2002; Wilson & Terry, 2013). Howes (2016) also suggests that that male mice are more sensitive than female mice to changes in gonadal steroid milieu during critical periods for masculinization and defeminisation. This statement holds true in adolescence, where there is an enhancing effect in both SR and OR in males and during adulthood where, the prenatal TP treatment interacts with the activational effects of the T replacement to rescue SR; this interaction is not seen in females.

As previously described, these results have serious implications for ASD. While our results showed fundamental sociability was unaffected, OR recognition memory was enhanced in males by prenatal TP. This is interesting as individuals with ASD show an advantage for visuospatial processing and an obsession with objects (DeRamus et al., 2014). While adolescent findings for SR were counter-intuitive to the ASD model (prenatal TP recovered SR capabilities) adulthood findings suggest that mice treated with the prenatal TP treatment had an overall reduction in social investigation during the test phase of SR. This lends to the idea that prenatal T in utero decreases overall SR and that our model may be effective. Further, there seems to be a

female protective advantage to the effects of prenatal TP, as it did not affect gonadally intact females. This has been noted in the literature as a "female protective effect" against ASD (Robinson, Lichtenstein, Anckarsäter, Happé, & Ronald, 2013). Furthermore, anxiety disorders are often comorbid with ASD, which shows a strong male bias (White et al., 2009). Therefore, it is possible that the suspected role of heightened levels of prenatal sex steroids in increasing vulnerability to ASD may also extend to increased susceptibility to comorbid anxiety disorders, especially among males.

Anxiety has been associated with decreased sociability in rats, while fear has been shown to potentiate aggressive behaviour in both rats and humans, suggesting that some of the changes in SR, OR, sociability and agonistic behaviour (discussed in Howes 2016) we observed in male mice treated prenatally with TP may have been related to increased anxiety (Marsee, Weems, & Taylor, 2007; Neumann et al., 2010). In addition, anxiety has been shown to impair recognition memory, associative memory, and spatial learning in rodents, while anxiolytic treatment has been shown to enhance social learning, suggesting that heightened anxiety may have contributed to impairments in social learning and SR by prenatal T in males (Choleris et al., 1998; Darcet et al., 2014; Harrison et al., 2009).

Taken together our results support our hypotheses, as we see that heighted prenatal TP in-utero promotes a sensitivity for later-life activational effects, which manifests in sex-specific differential behavioural outcomes. Furthermore, we saw "male-like" social behaviour in females in Howes (2016). We saw an interaction between prenatal TP and T replacement in males SR. Finally, we saw that male mice will show a disinterest in social interactions with conspecifics in Howes (2016). Therefore, to suggest our results translate to an animal model of the EMBH of

ASD is reasonable. This suggests that exposure to heighted levels of T during development can impair social behaviour, especially in males.

**Limitations**

This study was limited as it was a longitudinal study that was potentially prone to fluctuations in factors such as research staff, season, as well as overlapping cohorts of mice sharing the same colony room. Furthermore, the study was prone to stress as the mice underwent two separate testing batteries, had blood collected, and underwent two surgeries. The fecal data is indicative of a handling effect on the dams, where the prenatal control dams showed an increase in fecal T alongside the TP treated dams; therefore, it is likely stress played a role in increasing T— corticosterone should be measured to confirm this postulation. Blood collection and dual-surgery (gonadectomy and hormone replacement) are also inherently stressful procedures; this may have implications in the anxiety results from (Howes et al., 2016) and may have interacted with the results in this thesis as well.

Initially, we were unable to detect significant differences in T in maternal serum or fecal samples, possibly due to the timing of the analysis, as the elevation of T seems to be transient. However, this was rectified by Martin & Marcotte (unpublished data), who analyzed serum from a separate cohort 6 hours after the final TP injection (see methods section).

From the physiological data, it is evident that female mice exposed to prenatal TP have a half day earlier vaginal opening than their control counterparts. This suggests females entered puberty early and thus, may introduce significant variation in circulating E levels. Further, Howes et al. (2016) showed that females and males went through estrus and exhibited aggressive behaviours prior to gonadectomy, it is likely that hormonal surges of androgens and Es during puberty may have started producing secondary activational changes. Thus, it is likely we missed

the window of puberty and that some mice may have been undergoing activational changes before they received the implants— making it unclear if or results are driven primarily by our lone hormone replacement intervention, or if it was an interaction between a natural hormone surge and our intervention.

**Future Directions**

The brain tissue harvested at the end of this study will be used to analyze sexually dimorphic brain regions involved in social cognitive behaviours, such as SR, social learning, and aggressive behaviours, as well as non-social brain areas involved in OR. We will use these brain regions to correlate underlying neurobiological changes in hormone receptor and nonapeptide receptor expression (OT and vasopressin) to the behavioural outcomes we saw in this study; furthermore, we will be able to analyze how the organizational manipulation of androgenic hormones interact with the activational effects of puberty and adulthood to create distinct morphological differences in brain structures between sexes and different treatment groups. Specifically, we will look at hormone receptor expression within the medial amygdala (MeA), the ventromedial hypothalamus (VmH), the hippocampus, and the medial prefrontal cortex (mPFC) to assess the effect of our treatment. The effects of E and the MeA's involvement in SR behaviour has been well documented. Indeed, Spiteri et al. (2010) showed that by inhibiting ERα in the MeA impaired SR. Martin and Marcotte (2016; unpublished data) suggest that heighted exposure to prenatal T in-utero decreases ERα expression in the posterodorsal MeA in adult female mice. Additional receptors (AR, OT, Vasopressin, GPER) will be investigated and the analysis of ERα expression will be repeated in males. The VMH is associated with aggressive behaviour and highly expresses hormone receptors such as AR and ERs; this suggests that the VMH may be involved in the sex differences of agonistic behaviour in mice (Fuxjager et al.,

2010; Lin et al., 2011; Merchenthaler, Lane, Numan, & Dellovade, 2004) by studying the morphology of this structure we may be able to explain our results further. The hippocampus has been shown to undergo plastic changes in response to naturally occurring estrogenic fluctuations (Scharfman, Mercurio, Goodman, Wilson, & MacLusky, 2003; Woolley & McEwen, 1992; Woolley, 1998) and can be recovered by T following orchidotomy in males by not in females (Jacome et al., 2016; Leranth, Shanabrough, & Redmond, 2002). The mPFC is involved in social cognitive processes. Indeed, it has been shown that in humans, lesions to the mPFC severely impairs social cognition. Further, studies have shown reduced activity in the mPFC of ASD individuals (Castelli et al*,* 2002; Kana et al*,* 2009; Nieminen-von Wendt et al*,* 2003). Additionally, in female mice disruption of OT interneurons in the mPFC severely inhibits social interest in male mice during their sexually reproductive phase (Nakajima, Görlich, & Heintz, 2014). Therefore, it is important to evaluate the mPFC as well as hormone and nonapeptide receptor expression in this area, as well as other social/sexually dimorphic brain regions, like the hippocampus, MeA, and the VMH as discussed.

This study has only shown the effects of heighted prenatal T on social behaviour; however, T can act on AR's and ER's (via aromatization) therefore, the molecular mechanism driving these behavioural effects in early and later-life are not known. A follow-up study using DHT, an androgen that is not converted into any potent estrogens, should be conducted to assess whether these developmental effects are mediated by AR or ER binding. There is a very small body of literature on this research so many more studies will be generated based on this model.

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