ANDROGEN ACTION WITHIN THE VOMERONASAL ORGAN CONTRIBUTES TO THE SEXUAL DIFFERENTIATION OF THE BRAIN AND BEHAVIOUR

By © Yellow H. Martin

A Thesis submitted

to the School of Graduate Studies in partial fulfillment of the

requirements of the degree of

Master of Science, Department of Psychology, Faculty of Science

Memorial University of Newfoundland

June 2021

St. John's, Newfoundland and Labrador

Abstract

The vomeronasal organ (VNO), situated in the nasal cavity, is responsible for pheromone processing and plays a critical role in mediating socio-sexual behaviors in mice. Testosterone (T) masculinizes and defeminizes the brain and socio-sexual behavior during early development by acting directly on androgen receptors (AR) or indirectly via estrogen receptors (ER), following aromatization to estradiol. In Experiment 1, we asked whether androgens can act via the VNO in early development to affect the display of socio-sexual behaviors in adulthood by administering a microinjection of T locally to the VNO on the day of birth (PND1) in mice. In Experiment 2, we asked whether T acts on AR or ER by injecting the VNO with a vehicle, estradiol or the non-aromatizable androgen, dihydrotestosterone (DHT) on PND1. In Experiment 3, we took a complementary approach and assessed the necessity of androgen action by blocking AR and ER in the VNO with the aromatase inhibitor letrozole and AR antagonist flutamide on PND1. All three experiments utilized a behavioural battery including a buried food test, olfactory preference test, a resident intruder paradigm, and a test of sexual behavior. In Experiment 1, we found that a single microinjection of T on PND1 was sufficient to alter olfactory investigation and increase territorial aggression in males but did not affect female behavior. In Experiment 2, we found that a single microinjection of DHT on PND1 was sufficient to increase male territorial aggression with increases in boxing, biting, and attacking behavior towards males. In Experiment 3, we found that a single microinjection of letrozole on PND1 was sufficient to increase sexual behaviour with males displaying increased thrusts per mount towards a female intruder. FOS analysis, a marker of neural activity, after exposure to opposite sex odors did not yield any differences between groups in the nucleus accumbens, bed nucleus of the stria terminalis or the medial preoptic area. Our findings suggest that androgens may be acting both directly on AR and

indirectly through ER in critical periods of development within the VNO to affect adult socio-sexual behavior in male mice.

Keywords: Socio-sexual behaviour, vomeronasal organ, androgens, organizational period, pheromones

Declaration of Co-Authorship

I hereby declare that this thesis incorporates material that results from joint research supervised by Dr. Ashlyn Swift-Gallant. Specifically, Sarah Cross (honours student) aided in behavioural testing, dissections, and coding in Experiment 1; Stephanie Tuck (directed studies student) aided with FOS analysis and coding in Experiment 2; Katelyn Parsons (honours student) completed coding for Experiment 3; and Stephanie Salia aided in behavioural testing and dissections for all experiments. In all cases, primary contributions, experimental designs, data analysis, interpretation, and writing were performed by myself.

Acknowledgements

First and foremost, I would like to thank my supervisor Dr. Ashlyn Swift-Gallant. Ashlyn exposed me to new techniques, countless opportunities for advancement and learning, and a realm of study that excited and impassioned me. Further, Ashlyn consistently demonstrated patience, kindness, and respect, which provided me the room to nurture ideas and challenge myself. Her mentorship critically impacted my growth as both a person and researcher and it was an absolute pleasure working alongside her. Thank you, Ashlyn, for taking a chance on me.

I would also like to thank Dr. Christina Thorpe for her many years of mentorship, and support without which I would not be the scientist I am today. Further thanks extend to my committee members, Dr. Susan Walling and Dr. Carolyn Walsh for their time and expertise which allowed me to view my research through a different scope.

Thank you kindly to all the members of the Swift-Gallant lab who aided in testing, slicing, coding, and analysis. To Sarah and Katelyn, thank you for your time, ideas, questions, curiosity, and contributions that helped propel this project to completion. To Stephanie Salia, thank you for your selflessness, drive, work ethic and for motivating me over the past two years. You were an essential part of this process. I am truly thankful to have worked alongside you and to have gained a lifelong friend.

A special thank you extends to my friends and family for their endless encouragement. Some of the greatest lessons I have learned in life have come from you. To Anna, Luci, Michaila, Kyle, Sarah, Steph, and Courtney, thank you for being my rocks and letting me nerd out about science. To my dogs Lupin and Baloo, thanks for the cuddles, big belly laughs, and many kisses. Finally, to my mother Cynthia Tomblin, thank you for everything. Your dedication,

resiliency, and determination has always left me awestruck. You embody what it means to be a strong, intelligent woman, and I am extremely fortunate to have you as a role model.

Table of Contents

List of Figures

Page

List of Tables

Page

List of Abbreviations

ACo	Anterior Cortical Amygdala
ADT	Androst-4-ene-3,6,17-trione
ATD	1,4,6-androstatriene-3,17-dione
ANOVA	Analysis of Variance
AO	Anterior Olfactory Nucleus
AOB	Accessory Olfactory Bulb
AR	Androgen Receptor
BAOT	Bed Nucleus of the Accessory Olfactory Tract
BC/LA	Bulbocavernosus and Levator Ani Muscle
BORIS	Behavioural Observation Research Interactive Software
BNST	Bed Nucleus of the Stria Terminalis
BV	Blood Vessel
CP	Cribriform Plate
DAB	Diaminobenzidine
DHT	Dihydrotestosterone
E	Estrogen
Ent	Entorhinal Cortex
ER	Estrogen Receptor
ERα	Estrogen Receptor Alpha
ERβ	Estrogen Receptor Beta
FOS	Marker of Neuronal Activity
GL	Glomeruli

GG	Grueneberg Ganglion
IR	Immunoreactive
KO	Knock Out
LOT	Nucleus of the Lateral Olfactory Tract
MC	Mitral Cells
Me	Medial Amygdala
MePD	Posterodorsal Medial Amygdala
MOB	Main Olfactory Bulb
MOE	Main Olfactory Epithelium
MPA	Medial Preoptic Area
MPOA	Medial Preoptic Area
NAcc	Nucleus Accumbens Core
NAs	Nucleus Accumbens Shell
NGS	Normal Goat Serum
OB	Olfactory Bulb
OR	Olfactory Receptor
OSN	Olfactory Sensory Neuron
PBS	Phosphate-Buffered Saline
Pir	Piriform Cortex
PLCo	Posterolateral Cortical Amygdala
PM	Premammillary Nucleus
PMCo	Posteromedial Cortical Amygdala
PND	Post-Natal Day

SO	Septal Organ
T	Testosterone
Tfm	Testicular Feminization Mutant
TP	Testosterone Propionate
TRPC2	Transient Receptor Potential 2 Ion Channel
Tu	Olfactory Turbercle
VMH	Ventromedial Hypothalamus
VNO	Vomeronasal Organ
VR	Vomeronasal Receptor
VSN	Vomeronasal Sensory Neurons
VTA	Ventral Tegmental Area
VTT	Ventral Tenia Tecta
WT	Wild-Type

Androgen Action Within the Vomeronasal Organ Contributes to the Sexual Differentiation of the Brain and Behaviour

Communication is an essential element contributing to an animal's survival and reproductive success. Of the many forms of communication, rodents are particularly reliant on odor communication such as that communicated through pheromones (Baum & Bakker, 2013). Pheromones are chemical signals used as a means of communication between members of the same species that are detected through the sense of smell and act as signaling processes that elicit responses in conspecifics (Wilson & Bossert, 1963; Kikusui et al., 2018). Of noteworthiness is the role pheromone detection plays in mate choice, reproductive status/success, and partner fitness (Monfils & Agee, 2018).

Olfactory Systems and Processing Centers

Pheromones are detected in rodents by chemosensory neurons located within the olfactory system. There exist two organs within the nasal cavity that contribute to olfaction in mice: the main olfactory epithelium (MOE) and the vomeronasal organ (VNO) (Dulac & Axel, 1998). While there is some overlap in the downstream neural targets, these organs largely perform separate sensory functions. The MOE is receptive to airborne odorants through the expression of olfactory receptors (ORs) whereas the VNO in comparison is reactant to pheromones, which are largely non-volatile and act upon vomeronasal receptors (VRs). When an odorant enters the nasal cavity, it can be received by the MOE, which then projects to the olfactory bulb (OB) and in turn to the olfactory cortex and additional higher order cognitive processing centers. Alternatively, signals detected by the VNO travel on axons to the accessory olfactory bulb (AOB), which then bypasses cortical processing areas heading straight through the limbic system to hypothalamic nuclei including both the core and shell of the nucleus accumbens

(NAcc, NAs), posterodorsal medial amygdala (MePD), bed nucleus of the stria terminalis (BNST), medial preoptic area (MPOA), ventral tegmental area (VTA), and the ventromedial hypothalamus (VMH) (for diagram of MOE and VNO projection pathways see Figure 1). Action upon this sexually dimorphic neural circuitry activates endocrine signalling, ultimately affecting change or stimulating social, sexual or aggressive behaviour. While the main olfactory system is capable of discriminating between male and female odors (Beny & Kimchi, 2016), the VNO is critical for communication and socio-sexual behaviours in rodents (Cross et al., 2021).

Socio-Sexual Behaviours in Mice

Socio-sexual behaviours include behaviours such as face/body/anogenital investigation that allow animals to discriminate between potential mates or adversaries, as well as include behaviours such as aggression and sexual behaviour that are critical for reproduction and survival. These socio-sexual interactions between conspecifics of the same and opposite sex have been shown to occur in a variety of mammalian species from rodents to beluga whales (Bailey & Zuk, 2009; Lilley, Ham, & Hill 2020). It is believed that socio-sexual behaviours exist to create hierarchies, establish dominance, engage in play or aggressive behaviours, to act on sexual excitement, and to engage in reproductive behaviour (Albonetti & Dessì-Fulgheri, 1990; Bailey & Zuk, 2009). Sexual behaviours displayed among conspecifics include investigative behaviours (e.g., anogenital or face/body investigation of an opposite or same sex partner), appetitive behaviours (e.g., ear wiggling), and consummatory behaviours (e.g., mounting, intromissions, lordosis) (McCarthy, 2012). Conversely, aggressive behaviour is considered an adaptive behaviour that occurs during instances of social conflict and is often observed as territorial and maternal aggression (Kwiatkowski et al., 2021). In instances of social aggression, both males and females can exhibit chasing, attacking, biting, boxing, and tumbling behaviours in response to an

intruder (Olivier & Young, 2002; Soma et al., 2008; Takahashi, & Miczek, 2013). Socio-sexual behaviours can vary widely between sexes due to the sexual differentiation of the brain and behaviour.

Sexual Differentiation of the Brain and Behaviour

Sexual differentiation of the mammalian brain refers to how biological processes shape features of brain regions and neural cells to differ between males and females that have influence over psychological and sex-typical behaviours (Kammel & Correa, 2019). Sexual differentiation is mediated mainly by sex hormones such as testosterone (T) or its metabolite estradiol (E) (Morris et al., 2004). This differentiation occurs during critical periods in development such as the perinatal and pubertal time windows. The perinatal period marks the initial androgen surge that triggers the commencement of sex development; in mice this occurs from embryonic day 18 until postnatal day (PND) 1 (Corbier et al., 1992; Meisel & Sachs, 1994; Romeo, 2003). Further sexual maturation occurs in the pubertal window with the development of reproductive physiology and behaviour along with completing the brain organization of the perinatal period (Romeo, 2003); in mice, the peripubertal rise in sex hormones begins between PND 28-30 (Romeo, 2003; Zuloaga et al., 2008). Perinatal effects can denote a long-term effect of hormonal action referred to as organizational effects. Phoenix et al., (1959) were the first to denote permanent effects of androgens during this early critical period – they found that female guinea pigs exposed to androgens in late gestation through the early neonatal period demonstrated masculinized sexual behaviour such as mounting and thrusting. Further findings of increased male-typical sexual behaviours and preferences have been observed in female mice from a single systemic dose of androgens on the day of birth (Bodo & Rissman, 2008a). Conversely, activational effects refer to transient hormonal effects causing a short-term change in behaviour

or physiological activity (Swift-Gallant & Monks, 2017). The sexual differentiation of the brain and behaviour often relies on both early organizational effects of hormones and transient activational effects of hormones in adulthood for the full display of sex-typical behaviour.

Androgenic Sexual Differentiation

It is well established that steroid hormones organize sexually dimorphic neural regions during development (Romeo, 2003). Steroid hormones hold influence over neuronal survival, neurite growth, neurogenesis, synaptogenesis, and neurotransmitter synthesis among other factors. Androgens, such as testosterone, masculinize and defeminize the brain and behaviour by acting directly on androgen receptors (AR) or indirectly via estrogen receptors (ERα and/or ERβ), following conversion to estradiol in the brain by the enzyme aromatase. Males produce higher quantities of testosterone, but a large portion is converted to estradiol, which ultimately is a critical modulator of the sexual differentiation of brain and behaviour (Zuloaga et al., 2008). Dihydrotestosterone (DHT) is catalyzed from testosterone and is considered more potent, acting as an agonist of the androgen receptor (AR) (Massa et al., 1972). Since testosterone or its metabolite DHT can act directly on AR, or testosterone can be converted to estradiol before acting on estrogen receptors (ER), it is often unclear whether testosterone is acting via estrogenic or androgenic mechanisms. As DHT is an androgen that does not easily convert into estradiol, it is often used and compared to the effects of estradiol to determine whether androgens are acting via AR or ER to mediate sexual differentiation of the brain and behaviour (Maseroli et al., 2020).

Sexual Differentiation of the VNO

As part of the accessory olfactory system, the VNO relays information to many sexually differentiated regions including the accessory olfactory bulb (Segovia & Guillamon, 1991), the bed nucleus of the stria terminalis (Segovia & Guillamon, 1988), the medial preoptic area, and

the ventromedial hypothalamus (Segovia & Guillamon, 1993). There is also evidence that the VNO itself is sexually differentiated in early neonatal development, and it is also steroid hormone dependent. In rodent early development, androgens mediate the sexual differentiation of the VNO by altering its structure (Segovia & Guillamon, 1982; Segovia et al., 1984). Specifically, the overall volume, neuroepithelial volume, and number of bipolar neurons within the VNO are larger in males than females. Androgenic manipulations during critical periods of development alter the structural organization of this organ; for example, testosterone propionate (TP) administration to a female on the day of birth leads to masculinization of the adult VNO. Sex differences in the functionality of the VNO have also been noted. Opposite-sex odours elicit activity in differing areas of the VNO in males and females, and these sex differences are steroid hormone dependent (Halem et al., 2001). Specifically, upon examination of apical and basal neurons within the VNO neuroepithelium after exposure to opposite-sex bedding, it was found that estradiol treated females display greater apical neuron immediate early-gene immunoactivity than estradiol treated males. Further, testosterone treated males display activity in both apical and basal neurons. With an abundance of evidence implicating the VNOs influence on both the brain and behaviour, and the sex hormone dependence of the sexual differentiation of the VNO structure and function, it is hypothesized that the VNO is a site of androgen action in mediating the sexual differentiation of socio-sexual behaviours.

Aromatization Inhibition on Socio-Sexual Behaviours

Aromatase is a key enzyme responsible for the biosynthesis of estrogens from androgens. Aromatase is widely distributed intracellularly in multiple tissues including the gonads, adipose tissues and the brain. Inhibition of aromatase has been found to have an impact on spatial memory (Zhao et al., 2018), and sexual and aggressive behaviours in rodents (Olvera-Hernández

et al., 2015). Studies have demonstrated reduced male preference for estrous females through use of the aromatase inhibitor 1,4,6-androstatriene-3,17-dione (ATD) (Brand et al., 1991). Further, castrated rats treated postnatally with aromatase inhibitor androst-4-ene-3,6,17-trione (ADT) demonstrated a decrease in masculine sexual behaviour in adulthood following testosterone treatment as well as an increase in female sexual behaviour, such as lordosis, with administration of estradiol (Booth, 1978). Both of the aforementioned studies had extenuating factors that may have contributed to the observed behavioural changes including castration, adult administration of androgens, and steroidal effects of ADT and ATD. There now exists a non-steroidal aromatase-inhibiting compound called letrozole, which allows researchers to remove the possibility of steroidal effects. Letrozole has ubiquitous effects in that it can affect both the brain and reproductive organs while its administration produces a systemic effect due to inhibiting the production of estradiol. Olvera-Hernández et al. (2015) found that when letrozole was administered prenatally, male rats displayed feminization of partner preference, arousal and sexual behaviour. This suggests that letrozole may be a better alternative in aromatase inhibition research and provides further evidence that the aromatization of testosterone to estradiol is required for male-typical socio-sexual behaviours.

Antagonistic Effects on Socio-Sexual Behaviours

Antagonists are a class of drugs that act as a blocker to dampen a response by binding to a receptor and preventing its activation. When androgen and estrogen receptors are blocked with antagonists during critical periods of development, sex hormones are unable to act upon their respective receptor to influence the sexual differentiation of the brain and behaviour. Gladue and Clemens (1978) clearly demonstrated the importance of steroid hormone receptors by administering the antiandrogen flutamide prenatally and observing the effects when estradiol

benzoate was introduced to adult rat's post gonadectomy. They found that both males and females who were exposed to flutamide displayed increased feminine sexual behaviour (i.e., lordosis) suggesting that prenatal androgens inhibit the development of female sexual behaviour (defeminization). Similarly, Vagell and McGinnis (1998) used the AR and ER antagonists hydroxyflutamide and RU 58668, respectively, to determine the necessity of AR and ER receptors for socio-sexual behaviour. The authors administered a dose of testosterone to gonadectomized male rats who had previously received either the AR or ER antagonist. Rats with inhibited ARs displayed no copulatory behaviour, partner preference or scent marking upon systemic administration of testosterone. Conversely, rats with inhibited ERs were capable of copulatory behavior and partner preference upon administration of testosterone, however scent marking, and vocalizations were impaired. Thus, the authors determined that gonadal steroid receptor activation was indeed necessary for a wide range of socio-sexual behaviours with individual receptors fulfilling differing socio-sexual behaviors. Further a study by Domínguez-Salazar et al. (2002) assessed whether the prenatal blockage of AR through the antagonist flutamide would affect socio-sexual behaviours. While these males, like control males, showed heightened neural activity (FOS immediate early gene) in response to female odor cues in the accessory olfactory system, flutamide did impair copulatory behaviours including intromissions and ejaculations. In addition to the effects of antiandrogens in early critical periods in development, blockage of AR in adulthood has also been found to inhibit male rat sexual behaviour, although such effects are time dependent (Cunningham et al., 2012). For example, male rats who received androgen antagonists with testosterone replacement immediately following castration showed only a marginal decrease in sexual behavior compared to those who received the antagonists with testosterone three weeks post castration. These findings suggest

that testosterone conversion to estradiol can maintain sexual behaviours immediately following castration; however, after weeks of little-to-no androgen exposure, estradiol is insufficient in restoring male sexual behaviour. Conversely, regardless of timing, antiandrogens do not seem to affect intermale aggression, supporting a role for testosterone action via ER for aggressive behaviours (Clark & Nowell, 1980).

Testicular Feminization Mutant Mouse Models

Testicular feminization mutation (Tfm) mice are deficient in functional ARs due to a naturally occurring mutation in the androgen receptor gene (Lyon & Hawkes, 1970). The deficiency in androgen signaling caused by Tfm results in a decrease in male-typical aggressive and socio-sexual behaviours (Zuloaga et al., 2008). For example, using this mutant, Ohno and Geller (1974) found that ARs directly influence aggressive behaviour in male mice, such that gonadally intact Tfm mice displayed a decrease in aggressive behaviour compared to their wildtype male counterparts. Conversely, Scordalakes and Rissman (2004) state that low levels of circulating sex hormones in Tfm rodents are the main reason that these mutants show decreased aggressive behaviour. They found support for this hypothesis by comparing castrated wild-type (WT) and Tfm male mice who were both given E as adults. Both groups displayed similar levels of aggression post supplementation, demonstrating that Tfm males likely show decreased aggression due to a decrease in circulating E, rather than non-functional AR. Conversely, the evidence suggests that AR has a direct role on sexual behavior, independent of circulating estradiol. Ohno and Geller (1974) were the first to report that Tfm male mice display impaired male-typical copulatory behaviour (i.e., mounts and thrusts). Bodo and Rissman (2007) explored male sexual behaviours further, and found that in contrast to wildtype males, Tfm males showed

no partner preference and preferred to explore male-soiled bedding, even with administration of E in adulthood, suggesting a role for AR in these behaviours.

AR/ER Gene Knockouts

The role of androgens via AR and ER have also been studied using modern genetic techniques, such as gene knockout (KO). Ogawa et al. (2000) utilized this technique to knockout ERα, ERβ, and both together (αβERKO) in male mice to evaluate the role of each receptor for male socio-sexual and aggressive behaviours. Knockout of ERα eliminated ejaculation, reduced intromissions and reduced latency to aggress whereas male sexual behaviours in ERβ mice were unaffected and aggressive behaviours were minimally affected. In contrast, αβERKO male mice exhibited a reduction in aggressive behaviour (lunging, biting, attacking) and did not display any male sexual behaviours towards an estrus female. The authors concluded that socio-sexual and aggressive behaviours are determined by differing patterns of ER genes with the knockout of ERα decreasing sexual and aggressive behaviours, the knockout of ERβ increasing lordosis displays in response to estradiol, and αβERKO significantly reducing all male-typical behaviours studied. Kudwa et al. (2006) also found profound effects of ER KO on male-typical behaviours. They found that male-typical socio-sexual behaviours were decreased following ERα-KO, suggesting that ERα is involved in the masculinization of behaviour, whereas ERβ-KO increased the display of lordosis in males in response to estradiol, suggesting that ERβ is involved in defeminization of sexual behaviours. Other ERα-KO studies have shown that administration of testosterone in these males is insufficient to restore male-typical sexual behaviours, and preference for female odor cues is also decreased in these males (Rissman et al., 1999). Other studies on ERα KO mice show that ERα is also required for female-typical sexual behaviour such as receptivity and lordosis (Rissman et al., 1997).

Similar to ERKO models, ARKO models have demonstrated profound effects of AR on socio-sexual behaviours. Sato et al. (2004) produced a global ARKO model by utilizing a null AR mutation within the Cre-loxP system in mice. Male mice with the AR-null mutation demonstrated a complete ablation of both male-typical sexual and aggressive behaviours. While sexual behaviours were not rescuable upon administration of DHT to the AR-null mutated males, a partial restoration of aggressive behaviours occurred; this suggests that lower circulating androgens in this model may partially account for the decrease in male-typical aggressive behaviour. To examine the masculinization of the brain during the perinatal stage of development, AR-null mutated female mice were treated perinatally with DHT (Sato et al., 2004). These females displayed sensitivities in the induction of male-typical behaviours in adulthood in response to DHT and 17β-estradiol, but this brain masculinization was completely abolished by activation of ARs. Similar behavioural deficits have been observed in neural ARKO models such as that of Raskin et al. (2009) who used Cre-loxP to produce animals lacking AR expression selectively in the nervous system. In their model, mutant males were lacking in sexual motivation and performance and displayed less aggression. Juntti and colleagues (2010) also demonstrated behavioural deficiencies in their neural ARKO model. By using a specific deletion of AR within the male mouse nervous system, they observed that mutant males were capable of producing masculine sexual and territorial displays, however they contained deficits including reduced initiation of mating and reduced attacking in the presence of an intruder. Both Raskin et al and Juntti et al reported no differences in circulating androgens among neural ARKO males. Collectively, these studies demonstrate that the masculinization of the brain and behaviour requires AR function (often as a precursor to estrogen) and that the expression of male-typical behaviours is mediated by both AR and ER.

Surgical/Genetic Ablation of the Vomeronasal Organ

Previous research has demonstrated through multiple methods, such as surgical and genetic ablation, the importance of the VNO in influencing aggressive and sexual behaviours. Stowers and colleagues (2002) demonstrated that the transient receptor potential 2 (*TRPC2)* ion channel is required for the sensory activation of VNO neurons, and that KO of *TRPC2* disrupts the function of this organ. *TRPC2* channels are localized to the microvilli within the VNO, which are actin-based structures that specialize in the detection of chemical signals and are therefore believed to be a site of pheromone transduction. As *TRPC2* is essential in the functioning of the VNO and its expression is nearly VNO exclusive, with very low levels in other tissues (e.g., MOE, testes, brain, heart, spleen, liver, and erythrocytes; Wissenbach et al., 1998; Liman et al., 1999; Hofmann et al., 2000), KO of TRPC2 has been useful in determining the function of the VNO for socio-sexual behaviours (Liman & Dulac, 2007). For example, male mice with *TRPC2*-KO failed to exhibit male-male aggression, and instead showed sexual behaviours like mounting and thrusting towards male intruders introduced to their home cage. These males also continued to show sexual behaviours towards females. Kimchi et al. (2007) found that *TRPC2*-KO female mice showed a decrease in female-typical behaviours (i.e., lordosis), and an increase in male-typical behaviours such as mounting and thrusting towards both male and female conspecifics. There were $TRPC2^{-/-}$ females who were sexually receptive and litter producing, but these mice displayed decreased nesting behaviour and maternal aggression towards an intruder. Multiple studies have found that surgical ablation of the VNO changes male and female behaviours and preferences, however the extent of those changes is debated among researchers with some reporting effects similar to $TRPC2^{-/-}$ (Kimchi et al., 2007) while others report no changes in socio-sexual behaviour with adult ablation of the VNO (Martel & Baum, 2009). The latter

argument was supported by Pankevich et al. (2004) who demonstrated that male mice with their VNO ablated are capable of discriminating between sexes, however they experience a diminished preference for female odors. However, one important difference between *TRPC2*$^{-/-}$ and surgical VNO ablation is timing - *TRPC2*$^{-/-}$ results in lifelong disruption of the VNO, while mice with adult VNO surgical ablation retained a functional VNO throughout critical neonatal and pubertal periods in development. Cross and colleagues (2021) recently demonstrated that the pubertal period is a critical time for VNO functioning. While adult ablation resulted in minor deficits in olfactory preference, mice with peripubertal VNO ablations had decreased sexual odor preferences and neural activity in response to opposite sex odors, along with decreases in male territorial aggression. This suggests that behavioural manipulations within the VNO are sensitive to critical developmental periods for the sexual differentiation of neural circuitry and behaviour. Nevertheless, the evidence is strong that both male and female socio-sexual behaviour is dependent upon a functional VNO.

Study Overview

Given the role of both the VNO and sex hormones for the display of sex-typical socio-sexual behaviours, the current study explored whether the VNO is a site of androgen action in the mediation of socio-sexual behaviours during the organizational critical period of development. To examine this question, our first experiment assessed whether a microinjection of T administered locally to the VNO during the early critical period in development (post-natal day 1 [PND1]) was sufficient to affect socio-sexual behaviour. A single injection on PND1 was chosen, as previous research has indicated that a single systemic dose of androgen on the day of birth is sufficient to masculinize socio-sexual behaviours in female mice (Bodo & Rissman, 2008a). We hypothesized that a single microinjection would be sufficient to increase socio-

sexual behaviours in a sex-dependent manner. Specifically, we hypothesized that males would show increases in male-typical behaviours such as territorial aggression in response to a male intruder and sexual behaviour towards estrus females. We also predicted that female socio-sexual behaviours may be more male-typical as a result of the increase in circulating androgens in the VNO during the neonatal period. To examine the organizational effects of testosterone in the VNO, mice were compared on their preference for male or female soiled bedding and by analyzing their behavioural responses (e.g., sexual and aggressive behaviours) to same and opposite sex intruders introduced to their home cage.

Our second experiment assessed whether androgens act via AR and/or ER within the VNO to mediate socio-sexual behaviours. We examined this question by administering either estradiol or the non-aromatizational androgen DHT within the first 24 hours of birth (PND1) during the perinatal critical period of sexual differentiation in mice. As in Experiment 1, the mice were evaluated in an odor preference, resident intruder, and sexual behaviour paradigm. As sexual and aggressive behaviour seems to be mediated by sex hormones via both AR and ER in neonates, it is hypothesized that both manipulations of AR and ER in the neonatal period will affect behaviour, specifically increasing male-typical behaviours.

Finally, we investigated a complementary approach by blocking AR and ER in the VNO through the use of the aromatase inhibitor letrozole and AR antagonist flutamide on PND1 and assessed mice in adulthood on the same behavioural paradigms as Experiments 1 and 2. Here, we hypothesized that by blocking AR/ERs, mice would display a decrease in male-typical behaviours in adulthood. As a control, microinjections were also administered systemically in Experiment 3. VNO microinjections were compared with the systemic administrated to deduce the effects of the antagonists acting locally in to the VNO. Specifically, it was hypothesized that

the same microdose administered systemically should have a more dilute effect than when administered locally, if there is indeed a role of sex hormones within the VNO.

Immunohistochemistry was also performed for the analysis of the immediate early gene FOS, a marker of neural activity, in response to opposite sex odour in all three experiments. I hypothesize that hormone action via the VNO may affect sexual odour preferences and that a corresponding alteration in neural activity in response to sexual odour cues in the accessory olfactory pathway may be observed. If the opposite-sex odour preferences are increased due to our manipulation, we expect to see higher levels of FOS expression in regions involved with sexual preferences/behaviours (e.g., nucleus accumbens, bed nucleus of the stria terminalis, and medial preoptic area). Similarly, if decreases are observed in response to opposite-sex odours, then a corresponding decrease is expected in FOS expression within the brain regions along the accessory olfactory pathway, as previous research has demonstrated (e.g., Swift-Gallant et al., 2016).

2.0 Materials and Methods

2.1 Experimental Animals

Wild-type C57BL/6 breeding mice were obtained from Charles River Laboratories in Quebec, Canada. Mice were paired and bred with opposite-sex conspecifics to produce experimental animals. Cages were monitored daily to monitor the stages of pregnancy and to determine PND1 for new litters. Pilot work was conducted prior to experimentation to optimize the microinjection technique and confirm localization of solutions utilized (unpublished findings). Specifically, 4ul Nissl dye injected with a 32-gauge custom 45-degree beveled Hamilton syringe into the VNO of PND1 neonates was found to stain the VNO, with little-to-no stain found in the MOE or olfactory bulbs. All experimental animals received microinjections on

PND1 with doses determined by academic literature (i.e., $1/10^{th}$ of the dose typically administered systemically to see changes in socio-sexual behaviour); testosterone: 10 μg in 0.004 ml (systemic dose = 100 μg in 0.025 ml; Edwards & Burge, 1971), estradiol: 8 μg in 0.004 ml (systemic dose = 50 μg in 0.05 ml; Bateman & Patisaul, 2008; Edwards & Herndon, 1970), DHT: 10 μg in 0.004 ml ($1/10^{th}$ systemic dose) (systemic dose = 100 μg in 0.1 ml; Bodo & Rissman, 2008a), letrozole: 0.004 μg in 0.004 ml (systemic dose = 0.001 μg/ml; Olvera-Hernández et al., 2015), and flutamide: 10 μg in 0.004 ml (systemic dose: 100 μg/ml; Penatti et al., 2009; Robinson et al., 2012; Huber et al., 2018). All experimental mice were individually housed in a temperature-controlled room (23 °C) in transparent plastic cages connected to an oxygen ventilation system. The colony room was maintained a 12-hour light-dark cycle, with the light cycle beginning at 0700. All mice were given *ad libitum* access to food and water, with the exception of an 18-24-hour food deprivation of experimental mice prior to the Buried Food Test. Mice were weaned 21 days after birth (PND21) and were housed in groups of 2-4. They were subsequently singly housed approximately 45-60 days after birth (PND 45-60). Mice were between 74 and 79 days of age at time of behaviour testing, with dissection occurring on day PND80 (Refer to Figure 2 for experimental timeline and summary).

In Experiment 1, 45 experimental animals (n = 21 males, n = 24 females) were produced. Experimental animals received either a microinjection of testosterone (10 μg in 4 μL sesame oil) (n = 12 male, n = 12 female) or a sesame oil vehicle (4 μL) (n = 9 males, n = 12 females) in the VNO on PND1.

In Experiment 2, 66 experimental animals (n = 28 males, n = 38 females) were produced from breeding pairs. Experimental mice received a microinjection of either DHT (10 μg in 4 μL

sesame oil; *n* = 9 male, *n* = 14 female), estradiol (8 µg in 4 µL sesame oil; *n* = 9 male, *n* = 16 female) or oil vehicle (4 µL sesame oil; *n* = 10 male, *n* = 8 female) on PND1 in the VNO.

In Experiment 3, breeding pairs produced 72 male experimental animals. Experimental mice received either a microinjection of letrozole (0.004 µg in 4 µL sesame oil; *n* = 12), flutamide (10 µg in 4 µL sesame oil; *n* = 12), or an oil vehicle (4 µL sesame oil; *n* = 12) on PND1 in the VNO, or a microinjection of letrozole (*n* = 12), flutamide (*n* = 12), or an oil vehicle (*n* = 12) on PND1 subcutaneously in the same doses as the VNO groups. Female experimental animals were excluded from Experiment 3 due to a lack of observed socio-sexual behaviours in the previous two experiments.

All procedures were conducted in accordance with the Canadian Council on Animal Care Guidelines and were approved by Memorial University of Newfoundland's Committee of Animal Care.

2.2 Stimulus Animals

Stimulus C57BL/6 mice were of reproductive age, between two and five months at the time of surgery/experimentation. At approximately two months old, stimulus mice were gonadectomized and were given a minimum of two weeks recovery time before exposure to experimental animals. During surgery, female stimulus mice were implanted with a capsule containing 17β-estradiol in sesame oil (50mg in 0.025µL sesame oil; 1.98 mm id/ 3.17 mm od (Swift-Gallant et al., 2020) placed near the upper spine, as described in Swift-Gallant et al., 2020. Stimulus mice were housed in groups of 2-3 mice.

Prior to testing, sexually experienced male urine was collected from breeding mice for the Resident Intruder Test. To obtain the urine, mice were first restrained and held over a tray, and then received gentle pressure on their abdomen causing urination. Samples from multiple

males (*n* = 5-6) were pooled, collected into 0.5mL aliquots and stored at -20°C until testing; urine reached room temperature on testing day, and then was swabbed on the rear of stimulus males before each trial. Stimulus females were injected with 500mg progesterone (in 0.1 mL corn oil) to induce estrus 2-5 hours prior to the Sexual Behaviour Test.

Soiled bedding was collected from sexually experienced male mice and hormonally primed (500mg progesterone in 0.1 mL corn oil) estrous-induced females prior to behavioural testing. Specifically, mice were transferred to clean cages and maintained with the same bedding for 36-48 hours at which point the soiled bedding was collected and stored at -20°C until use; soiled bedding reached room temperature before use in the Olfactory Preference test and terminal exposure (Swift-Gallant et al., 2016b).

2.3 Buried Food Test

Prior to testing, experimental mice received one Froot Loop™ daily for three days for familiarization. Mice were then food-deprived for 18-24h prior to testing. For testing, large (45 x 25 x 21 cm) cages cleaned with 10% ethanol were placed on a table with 3 cm of bedding (Illendula et al., 2020). Mice were allowed to habituate for five minutes before returning to a holding cage. Experimenters then placed a Froot Loop™ 1cm deep in the bedding of the testing cage before reintroducing the mouse. Experimenters remained at least 1m away from the cage and watched the mouse for up to 15 minutes to record their latency to unearth the Froot Loop™ with a stopwatch. A Froot Loop™ was considered found when it was visible and had been pulled from the bedding. Cages were cleaned and bedding was replaced between each animal.

2.4 Olfactory Preference Test

Mice were placed within a large rodent cage (45 x 25 x 21 cm) with clean bedding along the bottom and three ramekins spaced equidistance apart (Cross et al., 2021). Mice were allowed

to habituate for five minutes during which time each ramekin contained clean bedding. Following habituation, mice were placed in a holding cage while experimenters replaced the ramekins with three new ramekins containing soiled male bedding, soiled female bedding, and clean bedding in a predetermined randomized order. Experimental mice were then placed back into the cages and were recorded with a Cannon video camera for ten minutes during which time experimenters left the room. Upon completion, the mice were removed from the testing apparatus and returned to their home cages. Each cage and ramekin were cleaned between each trial with 10% ethanol to remove any remaining odors.

2.5 Resident Intruder Test and Sexual Behaviour Test

During the Resident Intruder Test, mice were exposed to a male stimulus intruder whereas in the Sexual Behaviour Test, mice were exposed to a female stimulus intruder, within the home cage of the experimental mouse (Cross et al., 2021). Their cages were placed on a table with all enrichment items removed (nesting material, food, water, plastic house). As described previously, female intruders were hormonally primed prior to placement within the home cage of the experimental mouse, and male intruders were swabbed with sexually experienced male urine immediately prior to being placed in the experimental mouse's home cage. Interactions were recorded with a Cannon video camera for a 15-minute duration during which time the experimenters left the room.

2.6 Behaviour Coding

Videos from the Olfactory Preference Test, the Resident Intruder Test, and Sexual Behaviour Test were analyzed using Behavioural Observation Research Interactive Software (BORIS, version 7.9.7, retrieved from: http://www.boris.unito.it/pages/download.html). Experimenters were blind to the sex and treatment condition of mice during behavioural coding.

For the Olfactory Preference Test, time spent investigating bedding types in the three ramekins was recorded. An animal was considered to be investigating bedding if their nose was positioned downward within the ramekin. Following scoring of all videos, the randomized bedding assignment records were used to identify preference for soiled bedding. For each animal a female preference score (time in female bedding minus time in male bedding) was calculated.

Four blind investigators coded videos for the Resident Intruder Testing and Sexual Behaviour Test. Sexual behaviours were coded as number of mounts (climbing the rear of stimulus animal for copulation), thrusts (projecting the pelvic region towards a conspecific), intromissions (insertion of the copulatory organ into a conspecific during coitus) and lordosis (receptivity/inward curvature of the spine). Aggressive behaviours included duration and frequency of chasing (running after/swiftly following a conspecific), boxing (conspecifics pushing/hitting while standing on their hind legs), tumbling (somersaulting/rolling motion between two conspecifics), attacking (a forceful aggressive action/pounce towards a conspecific) and frequency of biting (rapid motion/snapping at conspecific with mouth/teeth). Further, duration and frequency of anogenital investigation (investigation of a conspecific from the tail to hind legs), face/body investigation (investigation of a conspecific from hind legs forward) and grooming (brushing, cleaning, or scratching) were analyzed.

2.7 Dissection and Immunohistochemistry

Experimental mice were dissected the day after the final behavioural test. Ninety minutes prior to perfusion, experimental animals were exposed to opposite sex soiled bedding for a duration of ten minutes within their home cage. All experimental mice were then weighed and overdosed with Avertin. This was followed by an intracardial perfusion with phosphate buffered saline (PBS) followed by 4% paraformaldehyde in phosphate buffer. The gonads,

bulbocavernosus and levator ani muscle (BC/LA) and seminal vesicles were dissected and weighed. The brains were dissected and post-fixed in 4% paraformaldehyde for 2 hours then transferred to 20% sucrose and stored in the fridge until sectioning. Brains were sectioned on a sliding freezing stage microtome at 30 μm sections into 4 series and stored in cryoprotectant at -20°C until histological processing.

One series of tissues from eight animals per group per experiment was stained for FOS (a marker of neuronal activity). Sections were placed in plated wells and rinsed with PBS between each step. Sections were blocked with a 10 min incubation in 0.3% H_2O_2 in PBS. This was followed by a 1 hr incubation in 10% normal goat serum (NGS, Vector S1000) in PBS with 0.3% gelatin and 0.1% Triton X-100 (PBS-GT) at room temperature. All sections (aside from controls) were incubated overnight (18-24 hours) in rabbit anti-FOS 1° antibody (Ab, Cell Signaling #2250; concentration 1:10,000) in PBS-GT with 1% NGS at 4°C. Next, sections were incubated in biotinylated goat-anti rabbit 2° Ab (Vector, BA1000, 1:500) in PBS-GT with 1% NGS in 1.5 mL tubes with 1 mL of solution for one hr. Sections were subsequently incubated in ABC solution (ABC Elite Kit, PK-6100, from Vector) for 1 hr, then visualized with diaminobenzidine (DAB) (49 mL 0.05 Tris buffer, 1 mL 1.25% DAB, 100 μL 3% H_2O_2, 120 μl 8% $NiCl_2$) for 2-3 min. After the last rinse, sections were mounted on subbed slides and left to dry overnight. Slides were then dehydrated 2x2 min in dH_20, 2x2 min in 70% EtOH, 5 min in 95% EtOH and 2x5 min in 100% EtOH. Slides were then cleared using xylene (2x5 min), and cover-slipped using Permount.

2.8 FOS Analysis

Bilateral images were captured on a brightfield microscope at 40x magnification for two sections of the medial preoptic area (MPOA), the bed nucleus of the stria terminalis (BNST), the

nucleus accumbens core (NAcc), and the nucleus accumbens shell (NAs). FOS counts for each image were collected using ImageJ software (version 1.53a, retrieved from: https://imagej.nih.gov/ij/download.html). Manual cell counts were performed using the cell counter function in ImageJ to count the number of immunoreactive (ir) cells (see Figure 3 for example of FOS-ir staining).

2.9 Statistical Analysis

Statistical analysis was conducted using Jamovi (version 1.2.27, retrieved from: https://www.jamovi.org/download.html). For Experiment 1, 2 x 2 sex (male, female) by treatment (control, testosterone) between-subjects factorial analysis of variances (ANOVAs) were used on the main variables: the female preference score, sexual behaviours (i.e., mounting, thrusting and intromissions), aggressive behaviours (i.e., frequency of chasing, tumbling, and a combined aggressive behaviour measure consisting of frequency of biting, attacking and boxing; these 3 behaviours were combined due to the low frequency of such behaviours), the gonad weight, and the number of FOS-ir cells in the MPOA, BNST, NAcc, and NAs. Multiple 2 x 3 sex (male, female) by treatment (Experiment 2: control, DHT, estradiol; Experiment 3: control, letrozole, flutamide) between-subjects factorial ANOVAs were used on the same dependent variables as Experiment 1. For Experiment 3, first, subcutaneously injected mice were compared to injected vehicle controls to assess whether microinjections administered systemically had any effect on somatic or behavioural measures. Then, VNO-injected groups (letrozole and flutamide) were compared to vehicle controls to assess the role of local injections on socio-sexual behaviours. Post hoc Tukey analyses were conducted for significant main effects and Cohen's *d* was used to measure effect sizes for significant post hoc tests. Effects were considered statistically significant at $p \leq 0.05$.

3.0 Results

3.1 Somatic Measures

In Experiment 1, a main effect of sex by treatment was found for overall body weight $F(1, 41) = 3.89$, $p = 0.05$. Post hoc analysis showed that males treated with testosterone had a significantly higher overall body weight compared to both females treated with testosterone ($t(41) = 9.739$, $p < .001$, $d = 3.976$) or given an oil vehicle ($t(41) = 0.421$, $p < .001$, $d = 3.846$), as seen in Figure 4. There were no significant differences noted between seminal vesicle weight, gonad weight or BC/LA weight between vehicle and testosterone treated males.

Experiment 2 found no significant differences between any groups in overall body weight, seminal vesicle weight, gonad weight, and BC/LA weight.

In Experiment 3, a main effect of treatment was established for overall body weight, $F(4, 67) = 4.12$, $p = .005$. Post hoc analysis revealed that subjects subcutaneously injected with letrozole weighed significantly less than those subcutaneously injected with flutamide ($t(67) = -3.202$, $p = .017$, $d = -1.307$) and oil ($t(67) = 3.257$, $p = .015$, $d = 1.152$; Figure 6). There were no significant differences in body weight found between VNO injected and control males. When assessing seminal vesicle weight, a marginal effect of treatment was found in VNO injected groups, $F(4, 67) = 2.40$, $p = .058$, with post hoc comparisons indicating those injected with flutamide in the VNO showing a decreased seminal vesicle weight than those injected with letrozole in the VNO ($t(67) = 2.871$, $p = .042$, $d = 1.1722$; Figure 6), however both treatment groups did not differ from controls. There were no significant differences found in testes weight. Additionally, there were no significant differences found for gonad weight or BC/LA weight.

3.2 Buried Food Test Confirms Olfactory System is Intact

The Buried Food test was performed as a control measure in all three experiments to test the function of the main olfactory system and to ensure no damage resulted from PND1 microinjections. In Experiment 1 there were no significant differences found between groups on latency to recover the buried Froot Loop™, ($p > .05$), indicating that the olfactory system was intact and was not affected by the VNO manipulation. In Experiment 2, there was a significant effect of treatment on latency to find the buried food, $F(2, 57) = 4.008$, $p = 0.024$. Post hoc analysis indicated that those injected with DHT spent significantly more time searching for the buried food than controls, $t(57) = 2.627$, $p = .026$, $d = -.884$ (Figure 7). In Experiment 3 there were no significant differences between any groups (controls, VNO-injected, and SUBQ-injected) in their latency to retrieve the buried food ($p > .05$), demonstrating that the olfactory system remained intact after the manipulation.

3.3 Testosterone Administration via the VNO Influences Socio-Sexual Behaviours in Male Mice

Experiment 1 sought to determine whether androgens such as testosterone were acting within the VNO to affect change during the organizational period of development. Animals were administered a microdose of testosterone or an oil vehicle on the first day of birth within the VNO to assess androgenic effects on socio-sexual behaviours and neural activity in response to opposite-sex odours.

3.3.1 Males Demonstrate Preferences for Female Odours

The odour preferences of animals were examined during the olfactory preference test and a marker of neural activity in response to opposite-sex odours (i.e., FOS-ir) was assessed in a

terminal odour exposure test to determine if androgen manipulations within the VNO altered sexual preferences.

The expected sex effect was found on the female preference score from the olfactory preference test, $F(1, 41) = 27.96$, $p < .001$. Post hoc analyses revealed that males had a higher preference for female bedding than female mice, $t(41) = 5.29$, $p = .001$, $d = .550$ (Figure 8), as expected. No main effects of treatment or interaction of treatment with sex was found for Experiment 1. FOS analysis measuring markers of neural activity/FOS expression indicated no changes/alterations in sexual odour preference as measured by treatment, sex, or sex by treatment interactions in any brain regions analyzed (MPOA, BNST, NAcc, and NAs) (p's > .05), further demonstrating opposite-sex preferences among all treatment groups.

3.3.2 Androgen VNO Manipulations Increase the Frequency and Duration of Social Behaviours in Male Mice

In assessing a multitude of socio-sexual behaviours, mice were exposed to a male intruder in the resident intruder paradigm, and a female intruder in the test of sexual behaviour. In Experiment 1, a significant main effect of treatment ($F(1,41) = 4.87$, $p = .035$), and sex ($F(1,41) = 13.08$, $p < .001$) was found on anogenital investigation duration (Figure 9). Post hoc analyses revealed that mice treated with testosterone via the VNO spent significantly less time investigating the anogenital region of a male intruder compared to the control group, $t(41) = -2.19$, $p = .035$, $d = -.657$. Further post hoc analyses revealed that males spent significantly more time investigating the male intruder compared to females, $t(41) = 3.62$, $p = .001$, $d = 1.09$.

Experiment 1 also revealed an effect of treatment on inter-male aggression. Specifically, a main effect of both treatment ($F(1,41) = 6.58$, $p = .014$), sex ($F(1,41) = 12.13$, $p < .001$) and a sex by treatment interaction ($F(1,41) = 6.58$, $p = .014$), on the frequency of tumbling in the

presence of a male intruder. Tukey's post hoc analyses revealed males treated via the VNO with testosterone displayed a greater frequency of tumbling towards a male intruder when compared to control males, $t(41) = 3.497$, $p = .006$, $d = 1.542$ (Figure 10). As expected, significant main effects of sex were found on chasing and observed aggressive behaviours, with males showing increased aggression when compared to females, $p < .05$.

Experiment 1 identified a main effect of sex $F(1,41) = 4.18$, $p = .047$) and treatment ($F(1,41) = 4.35$, $p = .043$) for the frequency of grooming in response to a female stimulus mouse. Post hoc analyses revealed that males groomed the stimulus mouse significantly less than females ($t(41) = 2.04$, $p = .047$, $d = -.614$), and VNO-testosterone treated mice groomed the stimulus animal less than controls ($t(41) = 2.09$, $p = .043$, $d = -.627$) (Figure 11). In addition, Experiment 1 revealed the expected main effect of sex for duration of mounting, number of thrusts, as well as number of intromissions in response to a female intruder. Males displayed more sexual behaviour than females on all aforementioned measures, $p < .05$, however there were no treatment effects on sexual behaviours in response to a female intruder.

3.3.3 Androgen Administration via the VNO Decreases Male-Male Copulatory Behaviour

Experiment 1 revealed a significant main effect of treatment ($F(1,41) = 8.14$, $p = 0.048$) and a significant sex by treatment interaction ($F(1,41) = 8.14$, $p = 0.048$) for mounting duration in the presence of a male stimulus mouse. Tukey's post hoc analyses indicated that testosterone treated males had a decreased average mounting duration when compared to control males, $t(41) = 2.783$, $p = .039$, $d = -1.227$ (Figure 12). In addition, a significant main effect of treatment ($F(1,41) = 5.52$, $p = 0.024$) as well as a sex by treatment interaction ($F(1,41) = 5.52$, $p = 0.024$) was revealed on the average number of thrusts performed in the presence of a male stimulus. Post hoc testing showed that testosterone treated males thrusted the male stimulus less than

control males, $t(41) = 3.202$, $p = .014$, $d = -1.412$ (Figure 13). Additional analyses demonstrated main effects of sex on number of mounts, duration of mounting, and number of thrusts with males displaying increased sexual behaviour in comparison to females, $p < .05$.

3.4 Androgens Act on ARs Within the VNO to Affect Socio-Sexual Behaviours

As Experiment 1 indicated a role for androgens within the VNO in the neonatal period on socio-sexual behaviours in male mice, Experiment 2 sought to identifying what receptors androgens were primarily working upon. To test whether androgens were acting directly on AR or indirectly on ER following aromatization of T to E, animals were given microinjections of an oil vehicle, E, or the less aromatizable androgen DHT on PND1 locally to the VNO.

3.4.1 Female Odours are Preferred by Males

As in Experiment 1, the sexual odour preferences of the experimental animals in Experiment 2 were assessed via the olfactory preference test and FOS expression analysis. As expected, a main effect of sex on female preference score was also found during the olfactory preference test for Experiment 2, $F(1,60) = 7.419$, $p = .008$. Post hoc analyses indicated that males had a higher female preference score than female mice, $t(60) = 2.72$, $p = .008$, $d = .692$. No main effects of treatment or interaction of treatment with sex was found for Experiment 2. FOS analysis measuring markers of neural activity/FOS expression also indicated no changes/alterations in sexual odour preference as measured by treatment, sex, or sex by treatment interactions (p's > .05).

3.4.2 Males Treated Locally in the VNO with DHT Displayed Increases in Territorial Aggression

In examining socio-sexual behavioural responses to opposite-sex intruders, Experiment 2 results indicated a significant sex by treatment interaction on the frequency of aggressive

behaviours *(F*(2, 60) = 3.97, $p < .024$). Post hoc analyses showed that males exposed to DHT aggressed (boxed, bit, and attacked) significantly more in response to a male intruder compared to control males, $t(60) = 3.066$, $p = .036$, $d = -1.409$ (Figure 14). No treatment or treatment by sex effects were found in frequency of chasing or tumbling towards a male intruder. Experiment 2 also revealed the expected main effects of sex on frequency of anogenital investigation and tumbling towards male intruders, $p < .05$.

Experiment 2 revealed a main effect of sex for grooming duration, with females displaying more self-grooming than males, $F(1,60) = 35.09$, $p < .001$. No other significant effects of sex, treatment, or sex by treatment interactions were found.

3.5 Male Sexual Behaviour is Affected by the VNO Treatment with the Aromatase Inhibitor Letrozole

Experiment 3 took a complementary approach to the previous two experiments by examining the necessity of ARs and ERs within the VNO during the organizational period of development. This was accomplished by providing a microinjection of an oil vehicle, the aromatase inhibitor letrozole, or flutamide, an androgen antagonist to the VNO on PND1. Further, the same microinjections were provided subcutaneously on PND1 to ensure all observable changes were due to local, rather than global action of these antagonists.

3.5.1 Males Display a Preference for Opposite-Sex Odours

As with the previous two experiments, experimental animals from both the VNO-injected and subcutaneously injected groups were subject to olfactory preference testing and FOS-ir expression analysis. Sex differences were not assessed in Experiment 3 due to the exclusion of female experimental animals. Olfactory preference testing demonstrated no significant differences between control and treatments groups for female preference score, $p > .05$; all males

displayed had a positive female preference score indicating a preference for opposite-sex odours. Consistent with the findings from the previous experiments, FOS analyses indicated no alterations in this marker of neural activity in response to opposite-sex odours by treatment, sex, or sex by treatment interactions (p's > .05).

3.5.2 Letrozole Injected in the VNO of Males Increased Sexual Behaviour Towards Female Stimulus Mice

In Experiment 3, male experimental mice were exposed to both a male stimulus, and a female stimulus mouse to examine their socio-sexual behavioural responses. A significant main effect of treatment was found on thrusts per mount in VNO injected animals, $F(2, 45) = 3.55$, $p = .037$. Post hoc tests showed that letrozole treated subjects performed a significantly greater number of thrusts per mount compared to control animals when exposed to a female stimulus animal, $t(45) = 2.586$, $p = .034$, $d = -.914$ (Figure 15). No other significant differences were noted in subcutaneous or VNO injected animals in response to a female intruder. In Experiment 3, males injected in the VNO with letrozole or flutamide did not differ from control males in any socio-sexual behaviours towards a male intruder.

3.5.3 Subcutaneously Treated Groups do not Differ from Controls in Socio-Sexual Behaviours

In Experiment 3, animals injected systemically with letrozole and flutamide were compared to controls to assess whether microdoses administered systemically would affect behaviour. Results indicated a main effect of treatment ($F(2, 45) = 3.56$, $p = .037$) on grooming duration in response to a male intruder. Tukey's post hoc test indicated that flutamide treated subjects showed an increase in grooming duration in the presence of a male intruder compared to letrozole treated mice $t(45) = 2.457$, $p = .046$, $d = -1.003$, however neither treatment group

differed from controls ($p > .05$) (Figure 16). No other significant differences were noted in response to a male or female intruder in subcutaneously treated mice. Thus, while there were differences between subcutaneous letrozole and flutamide groups in grooming behaviour, neither differed from control animals, suggesting that these microdoses administered systemically were insufficient to affect socio-sexual behaviours.

4.0 Discussion

It is well established that androgens act during early developmental periods to sexually differentiate the brain and behaviour (Toran-Allerand, 1976; Sata et al., 2004; Bodo & Rissman, 2007; Bodo, 2008). This organizational period associated with an androgenic surge produces long term changes in the displays of socio-sexual behaviours lasting well into adulthood. Further, it is understood that the pheromonal processing center in rodents known as the VNO is critical for the display of socio-sexual behaviours in response to conspecifics. Through the commonalities and influence on socio-sexual behaviour occurring during the organizational period of development and within the VNO, we sought to examine the interplay via androgenic manipulations. Thus, the current study aimed to extend our understanding of endocrine mediation of socio-sexual behaviour by investigating the VNO as a possible site of androgen action for the sexual differentiation of sex-typical behaviours.

4.1 The VNO is a Site of Androgen Action

Our first experiment examined whether the VNO is a site of androgen action during the organizational period by providing a microinjection of testosterone to the VNO on the day of birth. We found that a single dose of testosterone on the day of birth was indeed sufficient to alter the displays of socio-sexual behaviours in male mice. Specifically, we found that males given testosterone spent less time investigating the anogenital region and decreased sexual

behaviour (mounting and thrusting) towards an intruder male when compared to male controls. Instead of performing these affiliative/sexual behaviours, males with VNO testosterone treatment displayed increased territorial aggression when exposed to a male intruder compared to controls. Testosterone treatment to the VNO did not appear to influence odor preferences, as only the expected sex effect for female odor preference was found. Further, the testosterone treatment did not appear to have an effect on adult females as no socio-sexual behaviour changes were observed among females treated during the neonatal period with testosterone compared to control females.

From here, I designed Experiment 2 to assess whether androgens were acting via ARs and/or ERs within the VNO to affect change in socio-sexual behaviours during the organizational period. To accomplish this, we administered a local dose of a vehicle, estradiol, or the non-aromatizable androgen DHT on PND1 in mice. We found that a single microinjection of DHT on PND1 was sufficient to increase socio-sexual behaviours in males but not females. Specifically, we observed an increase in male territorial aggression in response to a male intruder when compared to the control group. From this we deduced that androgens may act via AR in the VNO to mediate socio-sexual behaviour in male mice.

Finally, in Experiment 3 I took a complementary approach by examining whether blocking ARs with flutamide or blocking androgen action via ER via inhibition of estradiol production with letrozole was sufficient to inhibit male-typical socio-sexual behaviours. To do so, I administered a microinjection of either letrozole, flutamide, or an oil vehicle on PND1 in the VNO of male mice. To ensure that the effects of our manipulations were indeed working locally, I administered the same injections systemically in a separate group for comparison. As a result of the VNO manipulations, I unexpectedly found that a single microinjection of letrozole

on PND1 was sufficient to *increase* the number of thrusts per mount towards a female intruder when compared to the control group; these results suggest that instead of inhibiting male-typical behaviour as predicted, letrozole led to an increase in male-typical sexual behaviour. Possible explanations for these findings are detailed below. In our systemically injected groups, we discovered differences between treatment groups with flutamide treated mice displaying increased grooming in response to a male intruder compared to the letrozole treated groups. Importantly, neither of these groups differed from controls. From this we concluded that our manipulations were indeed acting locally within the VNO.

4.2 Masculinizing Effects on Adult Behaviour

The results from the present study indicate that local androgen administration to the VNO of males on the day of birth is producing a masculinizing effect on adult behaviour. As the first day of birth resides within a critical period of development in rodent models (Corbier et al., 1992), it is likely that the masculinizing effects are due to a male-typical organization of the VNO and its associated subcortical pathways (Guillamón et al., 1988; Guillamón & Segovia, 1997). Generally, when encountering a novel conspecific, a mouse will investigate the anogenital and face/body regions of another mouse to determine their status (threat or potential mate), which will then be followed by attempts to mate or aggressive behaviour to defend their territory. However, with a microinjection of testosterone in Experiment 1, we observed decreased anogenital investigation and increased aggression towards a male intruder. Therefore, it is possible that the increase in androgens during the critical period re-organized the male's neural circuitry to promote a heightened response to male pheromonal cues, thus prompting them to switch from investigative behaviour to aggression more quickly. There are multiple possible explanations for this heightened response. First, the VNO may have become sensitized during

exposure to the initial surge of androgens which resulted in higher detection of pheromonal cues and therefore a heightened response. Additionally, downstream VNO neural input structures involved in aggression such as the amygdala may influence a greater aggressive response to pheromonal input (Nelson & Trainer, 2007). Studies involving electrophysiology in response to pheromone exposure and brain region analyses should be explored to determine androgen contributions to aggressive behaviours. For example, it is possible that androgen action via the VNO contributes to the brain's response to pheromonal cues and/or affects the sexual differentiation of these downstream neural targets.

4.3 AR Influence on Socio-Sexual Behaviour

In Experiments 1 and 2 we found that manipulations of both testosterone and DHT within the VNO produced changes in aggressive behaviour and investigative behaviours. Conversely, estradiol administration to the VNO did not induce any behavioural changes in Experiment 2. These results suggest that androgens are acting on ARs within the VNO to affect aggressive and investigative behavioural displays. This finding is in line with previous ARKO and AR overexpression studies showing a role for AR in socio-sexual behaviour. When Sato et al. (2004) produced a global ARKO model, male mice with the AR-null mutation demonstrated a complete termination of both male-typical sexual and aggressive behaviours. Although sexual behaviours were not rescuable, administration of DHT to the AR-null mutated males was able to partially restore aggressive behaviours, demonstrating the necessity of the interplay between androgens acting on ARs specifically. In investigating neural and global AR overexpression on socio-sexual behaviours, Swift-Gallant et al. (2016a) generated a loxP transgenic mouse that overexpressed ARs upon activation by Cre. Upon exposure to intruder animals in the resident intruder paradigm, both global and neural AR overexpressed animals displayed decreases in aggressive

behaviours, with global AR overexpressed mice also showing increased same sex anogenital investigation. The authors concluded that excess AR expression can lead to a reduction of male-typical aggressive behaviours. Taken together, prior research indicates that AR mediates socio-sexual behaviour in male mice, and the current work indicates that the effects of AR on socio-sexual behaviour is at least in part mediated via the VNO.

4.4 Local Versus Systemic Androgen Manipulations

In previous research that has investigated androgen action on both AR and ERs, it was determined that the non-aromatizable androgen DHT, but not estradiol, was sufficient to masculinize olfactory preference in female mice when administered on PND1, suggesting that androgens act on ARs for sexual odor preferences (Bodo & Rissman, 2008b). Other socio-sexual behaviours, such as mounting or thrusting can also be masculinized among females with neonatal administration of androgens (Gandelman & Kozak, 1988). In Experiment 1 and 2 we found evidence that androgens in the VNO affect male, but not female socio-sexual behaviours. One possible explanation is the site of action – namely, in the current studies we administered microdoses to the VNO, which based on the results of Experiment 3, are unlikely to have global effects on the animal. Indeed, we used $1/10^{th}$ of the dose used by Bodo and Rissman (2008a) (100 µg of DHT per 0.01 mL sesame oil vs 10 µg in 4 µL in current study). Thus, larger doses administered systemically can act upon the brain as well as other non-neural targets, whereas the microdoses in the present study would be too dilute by the time they reached targets outside of the VNO. Thus, the effects of androgens administered systemically in females on the day of birth may be acting in other targets than the VNO to affect socio-sexual behaviours. Alternatively, androgens in the VNO may be able to affect socio-sexual behaviour among females, however the length of exposure may not have been sufficient to see any effects. Males during the early critical

period are exposed to androgens for several days, and thus a microinjection was likely sufficient to amplify the effects within the VNO. Conversely, females do not have any sex hormone exposure during this early neonatal period, thus the microdose in the VNO on a single day may not be sufficient to see any effects in adulthood. Future studies should investigate whether increasing the length of exposure of androgens in the VNO during the neonatal period and/or in adulthood would be sufficient to increase male-typical behaviours among females.

In Experiment 3 we evaluated the first possibility by administering systemic injections of aromatase inhibitors and AR antagonists to observe whether the microdoses provided to the VNO would be sufficiently potent to work on sites of action outside of the VNO to affect change. While systemically treated mice displayed minor differences in socio-sexual behaviour (i.e., grooming) between letrozole and flutamide groups, these treatment groups did not differ from controls. Thus, while it is possible that minor differences in behaviour could result due to the microdoses in the current study, the fact that these treatment groups did not differ from controls suggest that the microdoses administered systemically largely did not affect socio-sexual behaviour. Similar to Experiment 1 and 2, the doses administered in Experiment 3 were $1/10^{th}$ of the dose administered systemically to see behavioural differences, therefore this dose is likely too dilute to have global effects on the animal. This is supported by the finding that greater differences are observed when microdoses are administered directly to the VNO than when administered systemically. The results of the current study suggest that the resultant behavioural changes in the VNO androgen manipulation studies can be directly attributed to local androgen manipulations within the VNO.

4.5 Inhibition of Estradiol Production Increases AR Activity

In Experiment 3, we found a significant difference in the sexual behaviour displayed by VNO treated letrozole males compared to controls. Specifically, we observed an increase in thrusts per mount in the letrozole group compared to the control group in the presence of a female intruder. This result was surprising as we had hypothesized that administration of letrozole would *decrease* male-typical behaviours as letrozole acts to inhibit the conversion of testosterone to estradiol, therefore decreasing androgen action on ERs. Two possibilities could explain this finding. First, estradiol action on ERs within the brain typically increases masculinization; females are exposed to low levels of maternal estradiol, but alpha-fetoprotein binds to peripheral estradiol, inhibiting estradiol from entering the brain and masculinizing female brains (Keller et al., 2010). Indeed, female AFP knockout mice show increases in male-typical behaviours (Keller et al., 2010). Outside of the brain, since both males and females are exposed peripherally to estradiol, ARs may be the pathway through which masculinization occurs. This could potentially explain why increased male-typical behaviours are seen when inhibiting ER action within the VNO. Second, as letrozole acts to inhibit the conversion of testosterone to estradiol, we may have increased the amount of circulating androgens and thus inadvertently produced increased androgen action upon AR (Loves et al., 2008; Kauffman et al., 2015). In turn, this may have caused an increase in male typical behaviours such as the thrusts per mount that were displayed by our PND1 letrozole VNO males. Similar findings have previously been reported in multiple mammalian studies. Human studies have observed males treated with an aromatase inhibitor displaying supraphysiological levels of free-floating testosterone (Loves et al., 2008; De Ronde & De Jong, 2011). Similarly, in a female mice model of polycystic ovary syndrome, letrozole systemically treated mice displayed a fourfold increase

in circulating testosterone levels (Kauffman et al., 2015). Taken in conjunction with our findings, there is support that a local administration of letrozole to the VNO has an effect on sexual behaviours in males, however, it is unclear whether this is due to lower action via ER or increased action via AR. Future studies may consider using viral vectors to upregulate or downregulate AR and ER within the VNO to address this question.

4.6 FOS Expression Coincides with Sexual Odor Preference

Before dissection we exposed the experimental animals to opposite sex soiled bedding to examine FOS immunoreactivity along the accessory olfactory pathway. This was examined in conjunction with the olfactory preference test to establish the sexual preferences of the animals and neural activity in response to such bedding stimuli. In adult VNO ablation studies, decreases have been observed in both sexual odor preferences and FOS in the brain regions including the MePD and along the accessory olfactory pathway (Pankevich et al., 2004; 2006; Samuelsen & Meredith, 2009). Furthermore, FOS-IR along the accessory olfactory pathway often corresponds with odor preferences (Swift-Gallant et al., 2016a), such that increased preference for a bedding stimulus is accompanied by an increase in neural activity, approximated by FOS, along the accessory olfactory pathway. Males from all manipulations demonstrated a preference for female odors during the olfactory preference test, and we found no differences between treatment groups in FOS expression in areas including the NAcc, NAs, BNST, and MPOM. The BNST and MPOA were critical brain regions chosen for examination as they are some of the first regions to receive input from the VNO neural circuit and are areas critical for socio-sexual behaviours. We decided to examine the NA as it falls on the later end of the VNO neural circuit and is involved in rewarding aspects of socio-sexual behaviour. As males did not differ in their olfactory preference, nor in FOS expression, we can conclude that behaviour and subsequent neural

activity in response to opposite-sex odors remains consistent amongst manipulations. In future work, it may be of interest to collect brains for FOS analysis following male-male interactions, as the majority of the behaviour effects observed in Experiment 1 and 2 due to androgen manipulations via the VNO consisted of alterations in territorial aggression.

4.7 Limitations

The results of this study are novel and address a gap in current literature regarding the VNO as a site of androgen action, however there are a number of limitations that were present in our design that should be considered when implementing future studies. One such limitation is that we did not assess the female estrus cycle of experimental female mice nor did we present them with an intact, sexually experienced male, therefore we are unable to draw conclusions regarding female sexual behaviour. It is therefore possible that when in behavioural estrus and when presented with a sexually experienced male, one may see the emergence of female socio-sexual behaviours, such as lordosis.

Similarly, the Resident Intruder paradigm assesses male typical aggression and does not present females with an instance in which females typically show aggression, such as maternal aggression in the presence of pups. Therefore, it is possible that VNO manipulations alter female aggression. Future studies may wish to explore the effect of androgen action within the VNO on female aggressive behaviours through sex specific tests of aggression; indeed, maternal behaviours including aggression have previously been found to be dependent upon a functional VNO (Hasen & Gammie, 2009), demonstrating a critical role for the VNO in such behaviours.

We observed interesting alterations in socio-sexual behaviours in response to androgen manipulations across all three experiments, however it is important to note that the VNO is only one part of the circuit, and thus the effects of androgen manipulation via the VNO may be in part

masked by compensatory mechanisms in other downstream neural targets that mediate socio-sexual behaviours. In other words, the role of androgens via the VNO may in actuality be larger than what is observed in the current studies. Relatedly, the androgen manipulations in the current work only took place on PND1, while the early androgen surge begins earlier in prenatal development, starting around embryonic day 18. Thus, it is possible that longer androgen exposure to the VNO spanning the full duration of the male-typical surge in androgens, may increase behavioural alterations and/or affect other behaviours than what is reported in the current studies.

While consistent with prior published protocols (Kimchi et al., 2007; Cross et al., 2021), it is possible that differences among groups could be attributable to single housing of mice. For example, singly housing mice may cause an increase in anxious/depressive behaviours, and it is possible that manipulating neonatal androgens could produce an anxiolytic effect on the animal by effecting their emotionality rather than socio-sexual behaviour. However, given that androgens were administered locally to the VNO, it is unlikely that the androgen manipulations affected the upstream neural circuits involved in anxious/depressive behaviour.

An additional limitation of the current study is in regard to the timing of our behavioural testing which occurred during the animal's light cycle. Typically, behavioural testing is conducted during the dark cycle as this is a period in which rodents display higher activity levels, however we were unable to do this due to facility limitations. With that said, the current work, as well as previous research conducted by Cross et al. (2021) has shown comparable levels of sexual behaviour during the light cycle to that of dark cycle behaviours from Swift-Gallant et al. (2016). As well, the majority of the foundational work evaluating the role of the VNO on socio-sexual behaviour was conducted in the light cycle. For example, Clancy et al. (1984)

demonstrated decreases sexual and aggressive behaviours in VNO-ablated mice compared to controls in the light portion of the light/dark cycle.

It is also possible that our study was limited as some animals may have had an impaired ability to sense odours as all were not successful in retrieving the buried food in the allotted time (see Table 1). In particular, impairments may have existed in Experiment 2's DHT group as when testing the efficacy of the main olfactory system in the buried food test, the DHT group spent significantly more time searching for the food compared to controls. However, males in Experiment 2 displayed a preference for female-soiled bedding consistent with males from both Experiments 1 and 3, suggesting that these mice were able to smell. In addition, no differences in the buried food test was found in Experiment 1, and there were consistencies in the socio-sexual behavioural results between Experiments 1 and 2; both found increases in aggressive behaviours in androgen administered groups. Taken together, we can infer that any group differences are likely not due to impaired olfaction alone. A possible alternative explanation for being unsuccessful in the test may be that the animals were not sufficiently motivated to find the food. The experimenters also noted that some animals began digging near the food stimuli at the beginning of the test session, but ended up burying the food stimulus deeper making it more difficult to retrieve.

Lastly, the use of aromatase inhibitors and androgen antagonists may not be precise and may be inhibiting more than just the receptors within the VNO, including those in surrounding tissues. Therefore, future research should further examine receptor necessity within the VNO through differing methods such as viral vectors which may provide more accuracy and precision.

Overall, the present study does provide evidence for the VNO as a site of androgen action and for this action to affect socio-sexual behaviours during critical periods of development;

however, future work may consider these methodological limitations in their experimental designs.

5.0 Conclusion and Future Directions

The work presented in this study has for the first time indicated that androgens can act within the VNO to mediate sex-typical behaviour. This suggests that androgens may affect pheromone processing, which in turn affects how the brain processes sexual odor cues and mediates socio-sexual behaviour. Results from Experiments 1 and 2 suggest a role for androgen action via AR within the VNO for male territorial aggression. Experiment 1 and 3 results suggest that androgens may also affect sexual behaviour, although the receptor upon which androgens are acting is unclear. Specifically, letrozole increased sexual behaviour, suggesting that this antagonist is either increasing available testosterone to act upon AR, or that inhibiting action via ERs is increasing sexual behaviours.

Future research should explore whether activational hormones can increase male-typical behaviours in females treated neonatally with androgens to the VNO, as it is likely that both neonatal and adult hormones are required for the display of these behaviours. Furthermore, it would be of interest to investigate whether androgens during the second critical period in development, namely puberty, also plays a role in socio-sexual behaviour.

The current study sets the stage for future work directed at understanding how the VNO is mediating behaviour – is it that this sensory organ is processing the sexual odor cues differently depending on the neonatal endocrine milieu, such that different information is being sent to the neural circuit controlling socio-sexual behaviour? And/or is androgen action via the VNO causing changes in the VNO which then affect the development of the brain? In other words, is the sexual differentiation of the VNO affecting the sexual differentiation of

downstream neural circuits? Further research in this field is warranted to enhance our understanding of the interplay between hormones and chemicals signals and their influence on adult behaviour and neuroanatomical organization.

References

Albonetti, M. E., & Dessì-Fulgheri, F. (1990). Female-female mounting in the European rabbit. *Zeitschrift für Säugetierkd, 55*, 128-138.

Bailey, N. W., & Zuk, M. (2009). Same-sex sexual behavior and evolution. *Trends in Ecology and Evolution, 24*, 439-446.

Bateman, H. L., & Patisaul, H. B. (2008). Disrupted female reproductive physiology following neonatal exposure to phytoestrogens or estrogen specific ligands is associated with decreased GnRH activation and kisspeptin fiber density in the hypothalamus. *NeuroToxicology, 29*(6), 988-997.

Beny, Y., & Kimchi, T. (2016). Conditioned odor aversion induces social anxiety towards females in wild-type and TrpC2 knockout male mice. *Genes, Brain & Behavior, 25*(8), 722-732.

Baum, M. J., & Bakker, J. (2013). Roles of sex and gonadal steroids in mammalian pheromonal communication. *Frontiers in Neuroendocrinology, 34*(4), 268-284.

Baum, M. J., & Kelliher, K. R. (2009). Complementary roles of the main and accessory olfactory systems in mammalian mate recognition. *Annual Review of Physiology, 71*, 141-160.

Bodo, C. (2008). A role for the androgen receptor in the sexual differentiation of the olfactory system in mice. *Brain Research Review, 57*(2), 321-331.

Bodo, C., & Rissman, E. (2007). Androgen receptor is essential for sexual differentiation of responses to olfactory cues in mice. *European Journal of Neuroscience, 25*(7), 2182-2190.

Bodo, C., & Rissman, E. (2008a). The organizational-activational hypothesis as the foundation

for a unified theory of sexual differentiation of all mammalian tissues. *Hormones & Behavior, 55*(5), 570-578.

Bodo, C., & Rissman, E. (2008b). The androgen receptor is selectively involved in organization of sexually dimorphic social behaviors in mice. *Endocrinology, 149*(8), 4142-4150. doi: 10.1210/en.2008-0183

Booth, J. E. (1978). Effects of the aromatization inhibitor androst-4-ene-3,6,17-trione on sexual differentiation induced by testosterone in the neonatally castrated rat. *Journal of Endocrinology, 79,* 69-76.

Brand, T., Kroonen, J., Mos, J., & Slob, A. K. (1991). Adult partner preference and sexual behaviour of male rats affected by perinatal endocrine manipulations. *Hormones and Behaviour, 25*, 323-341.

Breedlove, S. M. (1994). Sexual differentiation of the human nervous system. *Annual Review of Psychology, 45,* 389-418.

Caminero, A. A., Segovia, S., & Guillamón, A. (1991). Sexual dimorphism in accessory olfactory bulb mitral cells: A quantitative Golgi study. *Neuroscience, 45*, 663-670.

Clancy, A. N., Coquelin, A., Macrides, F., Gorski, R. A., and Noble, E. P. (1984). Sexual behavior and aggression in male mice: involvement of the vomeronasal system. *Journal of Neuroscience*, *4*, 2222–2229.

Clark, C. R., & Nowell, N. W. (1980). The effect of the non-steroidal antiandrogen flutamide on neural receptor binding of testosterone and intermale aggressive behavior in mice. *Psychoneuroendocrinology, 5*(1), 39-45.

Corbier, P., Edwards, D., & Roffi, J. (1992). The neonatal testosterone surge: A comparative study. *Archives Internationales De Physiologie, De Biochimie Et De*

Biophysique, *100*(2), 127–131.

Cross, K. J. S., Martin, Y. H., Salia, S., Gamba, I., Major, C. A., Hassan, S., … Swift-Gallant, A. (2021). Puberty is a critical period for vomeronasal organ mediation of socio-sexual behavior in mice. *Frontiers in Behavioural Neuroscience, 14*, 1-12.

Cunningham, R. L., Lumia, A. R., & McGinnis, M. Y. (2012). Androgen receptors, sex behaviour, and aggression. *Neuroendocrinology, 96*, 131-140

De Ronde, W., & de Jong, F. H. (2011). Aromatase inhibitors in men: effects and therapeutic options. *Reproductive Biology and Endocrinology, 9*, 93. doi: https://doi.org/10.1186/1477-7827-9-93

Domínguez-Salazar, E., Portillo, W., Baum, M. J., Bakker, J., & Paredes, R. G. (2002). Effect of prenatal androgen receptor antagonist or aromatase inhibitor on sexual behavior, partner preference and neuronal FOS responses to estrous female odors in the rat accessory olfactory system. *Physiology & Behaviour, 75*, 337-346.

Dulac, C., & Axel, R. (1998). Expression of Candidate Pheromone Receptor Genes in Vomeronasal Neurons. *Chemical Senses, 23*(4), 467-475.

Edwards, D.A., & Burge, K.G. (1971). Early androgen treatment and male and female sexual behavior in mice. *Hormones & Behavior, 2*, 49– 58.

Edwards, D. A., & Herndon, J. (1970). Neonatal estrogen stimulation and aggressive behavior in female mice. *Physiology & Behavior, 5*(9), 993-995.

Gandelman, R., & Kozak, M. (1988). Neonatal testosterone administration, but not *in utero* contiguity to males, augments the display of male sexual behavior by testosterone-treated adult female mice. *Physiology & Behavior, 42*(5), 453-456.

Gladue, B. A., & Clemens, L. G. (1978). Androgenic influences on feminine sexual behavior in

male and female rats: Defeminization blocked by prenatal antiandrogen treatment. *Endocrinology, 103*(5), 1702-1709.

Guillamón, A., Segovia, S., & Abril, A. (1988). Early effects of gonadal steroids on the neuron number in the medial posterior region and the lateral division of the bed nucleus of the stria terminalis in the rat. *Developmental Brain Research*, *44*(2), 281–290. doi: 10.1016/0165-3806(88)90226-x

Guillamón, A., & Segovia, S. (1997). Sex differences in the vomeronasal system. *Brain Research Bulletin*, *44*(4), 377–382. doi: 10.1016/s0361-9230(97)00217-7

Halem, H. A., Baum, M. J., & Cherry, J. A. (2001). Sex difference and steroid modulation of pheromone-induced immediate early genes in the two zones of the mouse accessory olfactory system. *Journal of Neuroscience, 21*(7), 2474-2480.

Hofmann, T., Schaefer, M., Schultz, G., & Gudermann, T. (2000). Cloning, expression and subcellular localization of two novel splice variants of mouse transient receptor potential channel 2. *Biochemical Journal, 351*(1), 115-122.

Hasen, N. S., & Gammie, S. C. (2009). Trpc2 gene impacts on maternal aggression, accessory olfactory bulb anatomy and brain activity. *Genes, Brain & Behavior, 8*(7), 639-649.

Huber, S. E., Zoicas, I., Reichel, M., Mühle, C., Büttner, C., Ekici, A. B., … Müuller, C. P. (2018). Prenatal androgen receptor activation determines adult alcohol and water drinking in a sex-specific way. *Addiction Biology, 23*(3), 904-920.

Illendula, M., Osuru, H. P., Ferrarese, B., Atluri, N., Dulko, E., Zuo, Z., & Lunardi, N. (2020). Surgery, anesthesia, and intensive care environment induce delirium-like behaviors and impairment of synaptic function-related gene expression in aged mice. *Frontiers in Aging Neuroscience. 12*. doi: 10.3389/fnagi.2020.542421

Juntti, S. A., Tollkuhn, J., Wu, M. V., Fraser, E. J., Soderborg, T., Tan, S., ... & Shah, N. M. (2010). The androgen receptor governs the execution, but not programming, of male sexual and territorial behaviors. *Neuron*, *66*(2), 260-272.

Kammel, L. G., & Correa, S. M. (2019). Selective sexual differentiation of neuron populations may contribute to sex-specific outputs of the ventromedial nucleus of the hypothalamus. *Journal of Neuroendocrinology, 32*(1).

Kauffman, A. S., Thackray, V. G., Ryan, G. E., Tolson, K. P., Glidewell-Kenney, C. A, Semaan, S. J., … Mellon, P. L. (2015. A novel letrozole model recapitulates both the reproductive and metabolic phenotypes of polycystic ovary syndrome in female mice. *Biology of Reproduction, 93*(3), 1-12.

Keller, M., Pawluski, J. L., Brock, O., Douhard, Q., Bakker, J. (2010). The α-fetoprotein knock-out mouse model suggests that parental behavior is sexually differentiated under the influence of prenatal estradiol. *Hormones and Behavior, 57*(4-5), 434-440.

Kikusui, T., Kajita, M., Otsuka, N., Hattori, T., Kumazawa, K., Watarai, A., … Mogi, K. (2018). Sex differences in olfactory-induced neural activation of the amygdala. *Behavioural Brain Research, 346*, 96-104.

Kimchi, T., Xu, J., & Dulac, C. (2007). A functional circuit underlying male sexual behaviour in the female mouse brain. *Nature, 448*(7157), 1009-1014.

Kudwa, A. E., Michopoulos, V., Gatewood, J. D., & Rissman, E. F. (2006). Roles of estrogen receptors α and β in differentiation of mouse sexual behaviour. *Neuroscience, 138*, 921-928.

Kwiatkowski, C. C., Akaeze, H., Ndlebe, I., Goodwin, N., Eagle, A. L., Moon, K., … Robinson,

A. J. (2021). Quantitative standardization of resident mouse behavior for studies of aggression and social defeat. *Neuropsychopharmacology,* 1-10.

Lilley, M. K., Ham, J. R., & Hill, H. M. (2020). The development of socio-sexual behavior in belugas (Delphinapterus leucas) under human care. *Behavioural Processes, 171*, 104321.

Liman, E. R., Corey, D. P., & Dulac, C. (1999). TRP2: a candidate transduction channel for mammalian pheromone sensory signaling. *Proceedings of the National Academy of Sciences of the United States of America, 96*(10), 5791-5796.

Liman, E. R., & Dulac, C. (2007). TRPC2 and the Molecular Biology of Pheromone Detection in Mammals. In Liedtke, W. B., & Heller, S. (Eds.), *TRP Ion Channel Function in Sensory Transduction and Cellular Signaling Cascades*. CRC Press/Taylor & Francis.

Loves, S., Ruinemans-Koerts, J., & de Boer, H. (2008). Letrozole once a week normalizes serum testosterone in obesity-related male hypogonadism. *European Journal of Endocrinology, 158*, 741-747. doi: 10.1530/EJE-070663

Lucas, P., Ukhanov, K., Leinders-Zufall, T., & Zufall, F. (2003). A diacylglycerol-gated cation channel in vomeronasal neuron dendrites is impaired in TRPC2 mutant mice: Mechanism of pheromone transduction. *Neuron, 40*(3), 551-561.

Lyon, M. F., & Hawkes, S. G. (1970). X-linked gene for testicular feminization in the mouse. *Nature*, *227*(5264), 1217-1219.

Martel, K., & Baum, M. (2009). Adult testosterone treatment but not surgical disruption of vomeronasal function augments male-typical sexual behavior in female mice. *The Journal of Neuroscience, 29*(24), 7658-7666.

Maseroli, E., Santangelo, A., Lara-Fontes, B., Quintana, G. R., Mac Cionnaith, C. E.,

Casarrubea, M… Ricca, V. (2020). The non-aromatizable androgen dihydrotestosterone (DHT) facilitates sexual behavior in ovariectomized female rats primed with estradiol. *Psychoneuroendocrinology, 115*, 104606.

Massa, R., Stupnicka, E., Kniewalkd, Z., & Martini, L. (1972). The transformation of testosterone into dihydrotestosterone by the brain and the anterior pituitary. *Journal of Steroid Biochemistry, 3*(3), 385-399.

McCarthy, M. M. (2012). Sexual differentiation of brain and behaviour. In *Handbook of Neuroendocrinology* (pp. 393-413). Academic Press. Elsevier, London, Waltham, San Diego.

Meisel, R. L., & Sachs, B. D. (1994). The physiology of male sexual behaviour. *The Physiology of Reproduction, 2*, 3-105.

Monfils, M. H., & Agee, L. A. (2019). Insights from social transmission of information in rodents. *Genes, Brain & Behaviour, 18*(1), 1-13.

Morris, J., Jordan, C., & Breedlove, S. (2004). Sexual differentiation of the vertebrate nervous system. *Nature Neuroscience, 7*(10), 1034-1039.

Nelson, R. J., & Trainer, B. C. (2007). Neural mechanisms of aggression. *Nature Reviews Neuroscience, 8*, 536-546.

Ogawa, S., Chester, A. E., Hewitt, S. C., Walker, V. R., Guastafsson, J, Smithies, O…Pfaff, D. W. (2000). Abolition of male sexual behaviors in mice lacking estrogen receptors α and β (αβERKO). *Neurobiology, 97*(26), 14747-14741.

Ohno, S., & Geller, L. N. (1974). Tfm mutation and masculinization versus feminization of the mouse central nervous system. *Cell, 3*(3), 235-242.

Olivier, B., & Young, L. J. (2002). Animal models of aggression. *Neuropsychopharmacology:*

The Fifth Generation of Progress, *118*, 1699-1708.

Olvera-Hernández, S., Chavira, R., & Fernández-Guasti, A. (2015). Prenatal letrozole produces a subpopulation of male rats with same-sex preference and arousal as well as female sexual behavior. *Physiology & Behaviour, 139*, 403-411.

Pankevich, D., Baum, M., & Cherry, J. (2004). Olfactory sex discrimination persists, whereas the preference for urinary odorants from estrous females disappears in male mice after vomeronasal organ removal. *Journal of Neuroscience, 24*(42), 9451-9457.

Pankevich, D. E., Cherry, J. A., and Baum, M. J. (2006). Effect of vomeronasal organ removal from male mice on their preference for and neural FOS responses to female urinary odors. *Behavioural Neuroscience, 120*, 925–936. doi: 10.1037/0735-7044.120.4.925

Penatti, C. A. A., Costine, B. A., Porter, D. M., & Henderson, L. P. (2009). Effects of chronic exposure to an anabolic androgenic steroid cocktail on α_5-receptor-mediated GABAergic transmission and neural signaling in the forebrain of female mice. *Neuroscience, 161*, 526-537.

Phoenix, C., Goy, R., Gerall, A., & Young, W. (1959). Organizing action of prenatally administered testosterone propionate on the tissues mediating mating behavior in the female guinea pig. *Endocrinology, 65*, 369-382.

Raskin, K., De Gendt, K., Duittoz, A., Liere, P., Verhoeven, G., Tronche, F., & Mhaouty-Kodja, S. (2009). Conditional inactivation of androgen receptor gene in the nervous system: effects on male behavioral and neuroendocrine responses. *Journal of Neuroscience*, *29*(14), 4461-4470.

Rissman, E. F., Early, A. H., Taylor, J. A., Korach, K. S., & Lubahn, D. B. (1997). Estrogen receptors are essential for female sexual receptivity. *Endocrinology, 138*(1), 507-510.

Rissman, E. F., Wersinger, S. R., Fuggar, H. N., & Foster, T. C. (1999). Sex with knockout models: Behavioral studies of estrogen receptor α. *Brain Research, 835*(1), 80-90.

Robinson, S., Penatti, C. A. A., & Clark, A. S. (2012). The role of the androgen receptor in anabolic androgenic steroid-induced aggressive behavior in C57BL/6J and Tfm mice. *Hormones and Behavior, 61*(1), 67-75.

Romeo, R. D. (2003). Puberty: A period of both organizational and activational effects of steroid hormones on neurobehavioural development. *Journal of Neuroendocrinology, 15*(12), 1185-1192.

Samuelsen, C. L., and Meredith, M. (2009). The vomeronasal organ is required for the male mouse medial amygdala response to chemical-communication signals, as assessed by immediate early gene expression. *Neuroscience, 164*, 1468–1476. doi: 10.1016/j.neuroscience.2009.09.030

Sato, T., Matsumoto, T., Kawano, H., Watanabe, T., Uematsu, Y., Sekine, K., ... & Kato, S. (2004). Brain masculinization requires androgen receptor function. *Proceedings of the National Academy of Sciences*, *101*(6), 1673-1678.

Scordalakes, E. M., & Rissman, E. (2004). Aggression and arginine vasopressin immunoreactivity regulation by androgen receptor and estrogen receptor alpha. *Genes, Brain and Behavior, 3*(1), 20-26.

Segovia, S., & Guillamón, A. (1982). Effects of sex steroids on the development of the vomeronasal organ in the rat. *Developmental Brain Research, 5*, 209-212.

Segovia, S., Paniagua, R., & Guillamón, A. (1984). Effects of postpubertal gonadectomy on the neurosensorial epithelium of the vomeronasal organ in the rat. *Developmental Brain Research, 14*(2), 289-291.

Segovia, S., Guillamón, A., & Del Abril, A. (1988). Early effects of gonadal steroids on the neuron number in the medial posterior and the lateral divisions of the bed nucleus of the stria terminalis in the rat. *Developmental Brain Research, 44*, 281.

Segovia, S., & Guillamón, A. (1993). Sexual dimorphism in the vomeronasal pathway and sex differences in reproductive behaviors. *Brain Research Reviews, 18*, 51-74.

Soma, K. K., Scotti, M. A. L., Newman, A. E., Charlier, T. D., & Demas, G. E. (2008). Novel mechanisms for neuroendocrine regulation of aggression. *Frontiers in Neuroendocrinology*, *29*(4), 476-489.

Swift-Gallant, A., Coome, L. A., Ramzan, F., and Monks, D. A. (2016a). Nonneural androgen receptors affect sexual differentiation of brain and behavior. *Endocrinology 157*, 788–798. doi: 10.1210/en.2015-1355

Swift-Gallant, A., Coome, L., Srinivasan, S., and Monks, D. (2016b). Non-neural androgen receptor promotes androphilic odor preference in mice. *Hormones and Behavior, 83*, 14–22.

Swift-Gallant, A., & Monks, D. (2017). Androgenic mechanisms of sexual differentiation of nervous system and behaviour. *Fronteirs in Neuroendocrinology, 46*, 32-45.

Swift-Gallant, A., Coome, L., Ramzan, F., & Monks D. (2020). Non-androgenic testicular mediation of androphilia in male mice with global overexpression of androgen receptors. *Behavioural Brain Research, 391.* doi: 10.1016/j.bbr.2020.112694

Takahashi, A., & Miczek, K. A. (2013). Neurogenetics of aggressive behavior: studies in rodents. *In Neuroscience of Aggression* (pp. 3-44). Springer, Berlin, Heidelberg.

Toran-Allerand, C. D. (1976). Sex steroids and the development of the newborn mouse hypothalamus and preoptic area in vitro: Implications for sexual differentiation. *Brain*

Research, 106(2), 407-412.

Vagell, M. E., & McGinnis, M. Y. (1998). The role of gonadal steroid receptor activation in the restoration of sociosexual behavior in adult male rats. *Hormones and Behavior, 33*, 163-179.

Wilson, E. V., & Bossert, W. H. (1963). Chemical communication among animals. *Recent Progress in Hormone Research, 19*, 673-716.

Wissenbach, U., Schroth, G., Philipp, S., & Flockerzi, V. (1998). Structure and mRNA expression of a bovine trp homologue related to mammalian trp2 transcripts. *FEBS Letters, 429*(1), 61-66.

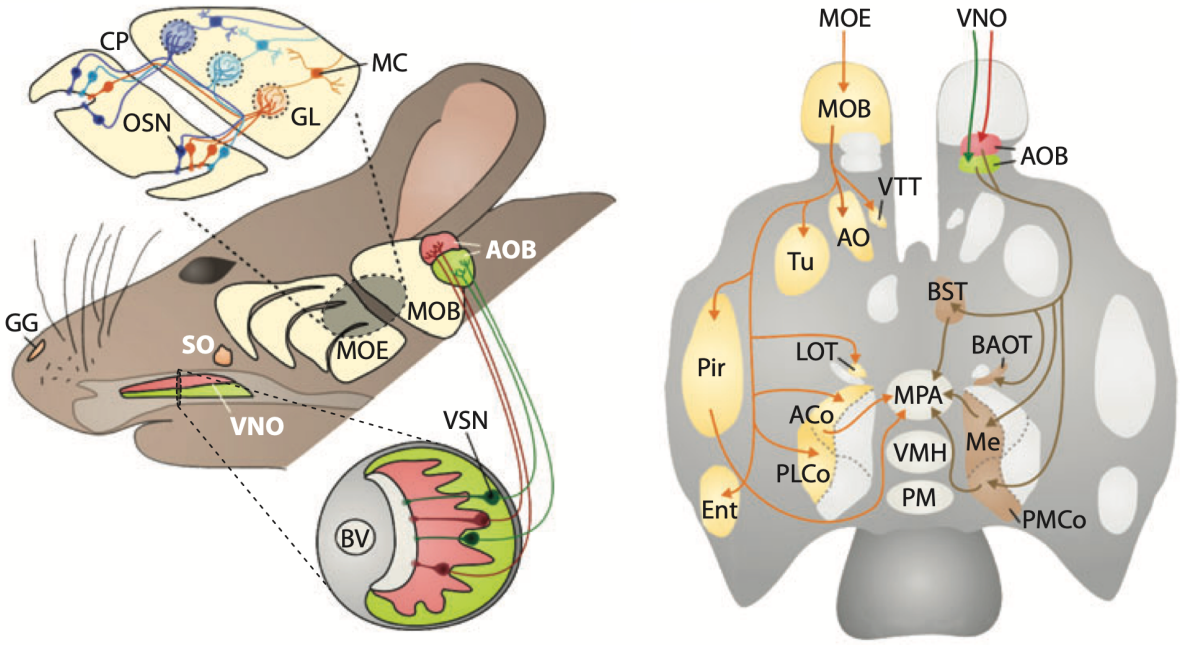
Zhao, J., Bian, C., Liu, M., Zhao, Y., Sun, T., Xing, F., & Zhang, J. (2018). Orchiectomy and letrozole differentially regulate synaptic plasticity and spatial memory in a manner that is mediated by SRC-1 in the hippocampus of male mice. *Journal of Steroid Biochemistry and Molecular Biology, 178*, 354-368.

Zuloaga, D. G., Puts, D. A., Jordan, C. L., & Breedlove, M. (2008). The role of androgen receptors in the masculinization of brain and behavior: What we've learned from the testicular feminization mutation. *Hormones and Behavior, 53*(5), 613-626.

Appendix A

Figure 1

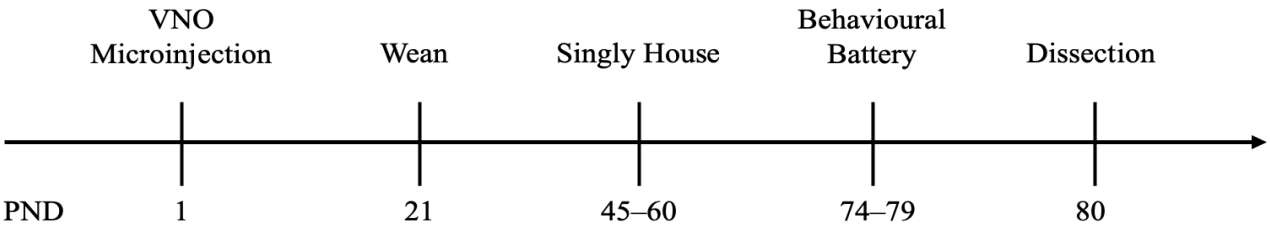
Main Olfactory Epithelium (MOE) and Vomeronasal Organ (VNO) Projection Pathways



Note. Diagram depicting the projection pathways of the MOE and VNO. The left image shows olfactory sensory neurons (OSN) located within the MOE projecting through the cribriform plate (CP) to glomeruli (GL) within the main olfactory bulb (MOB). Mitral cells (MC) aid in transmitting information from GL to the forebrain. Vomeronasal sensory neurons (VSN) transmit information from the VNO to the accessory olfactory bulb (AOB). The right image demonstrates the projection pathways from the MOE and VNO to the forebrain. The current studies evaluated a marker of neural activity (FOS) in response to opposite-sex odors in the bed nucleus of the stria terminalis (BST) and medial preoptic area (MPA). All other abbreviations can be found on page xii. Images adapted from Baum, M. J., & Kelliher, K. R. (2009). Complementary roles of the main and accessory olfactory systems in mammalian mate recognition. *Annual Review of Physiology, 71*, 141-160.

Figure 2

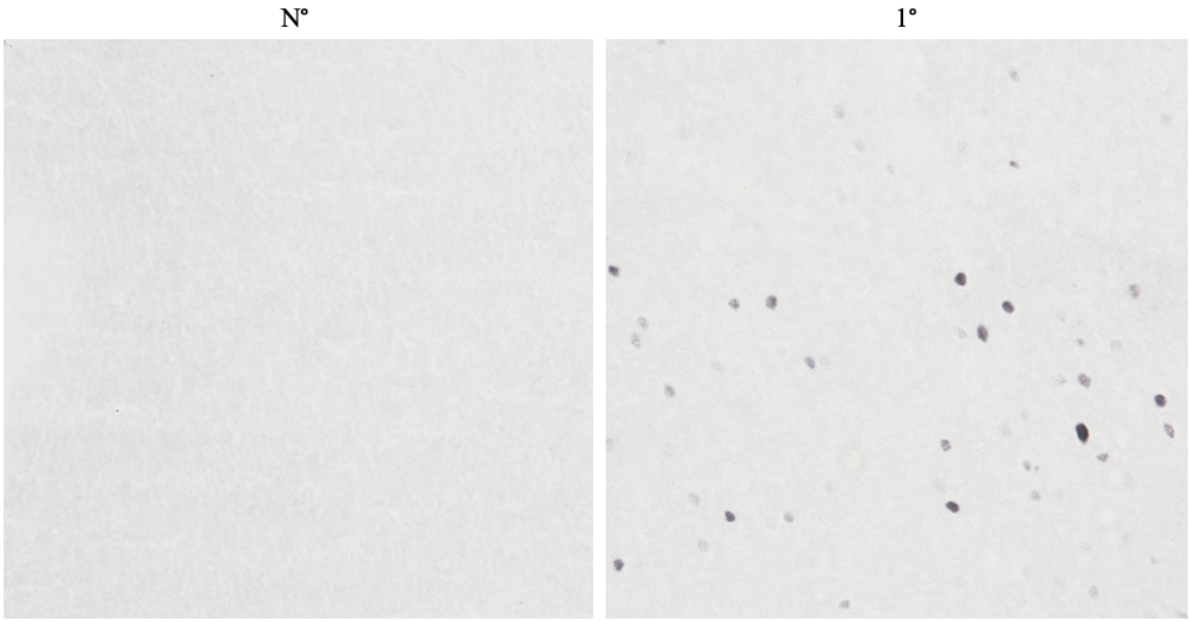
Timeline and Groupings for Experiments 1-3



	Experiment 1	Experiment 2	Experiment 3
VNO	Oil	Oil	Oil
	Testosterone	DHT	Flutamide
		Estradiol	Letrozole
SUBQ			Oil
			Flutamide
			Letrozole

Figure 3

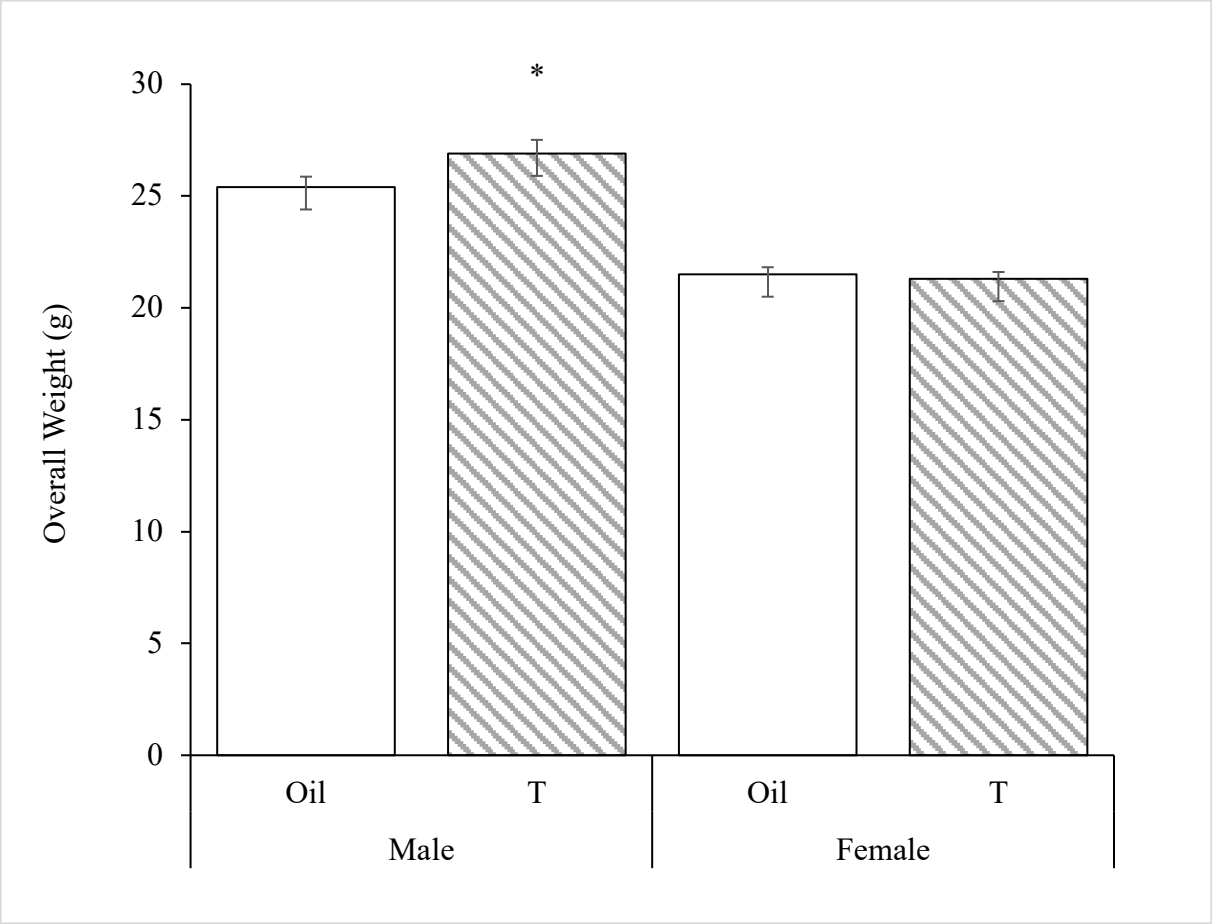
Representative Images of No Primary (N°) and Primary (1°) FOS Immunohistochemistry Results



Note. Representative comparison images of primary and no primary FOS-ir staining in the nucleus accumbens (NA). Overall, FOS analyses conducted for all three experiments indicated no effects of treatment, sex, or sex by treatment effects on markers of neural activity/FOS expression in response to opposite-sex odours in any brain regions analyzed (MPOA, BNST, NAcc, and NAs) (p's > .05).

Figure 4

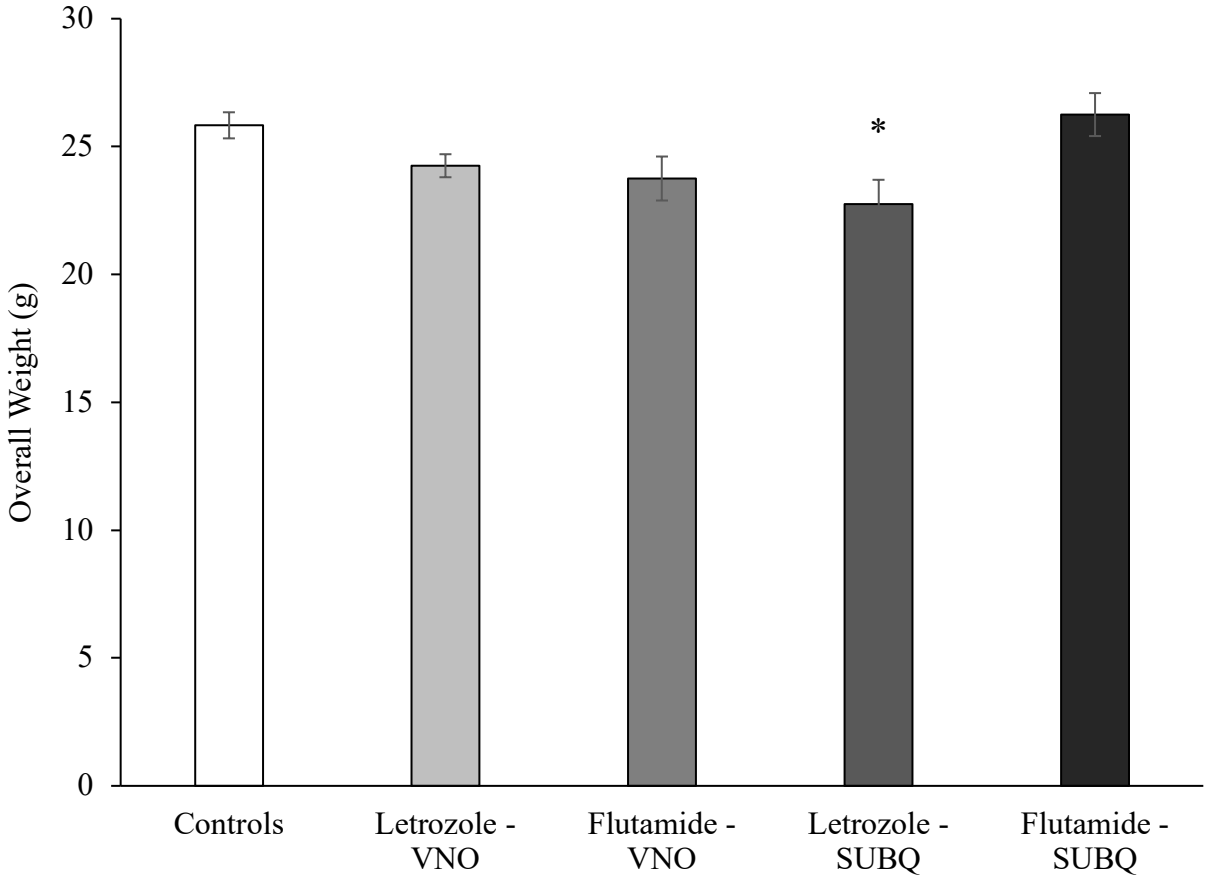
Experiment 1: Overall Body Weight of Mice by Treatment Group in Grams (g)



Note. Average overall body weight of mice at time of dissection by treatment group measured in grams (g), +/- SEM. The overall body weight of males treated with testosterone (T) (n = 12) was significantly higher than that of females treated with testosterone (n = 12) or an oil vehicle (oil) (n = 12) but did not significantly differ from vehicle males (M, Oil) (n = 8); * indicates $p < .001$.

Figure 5

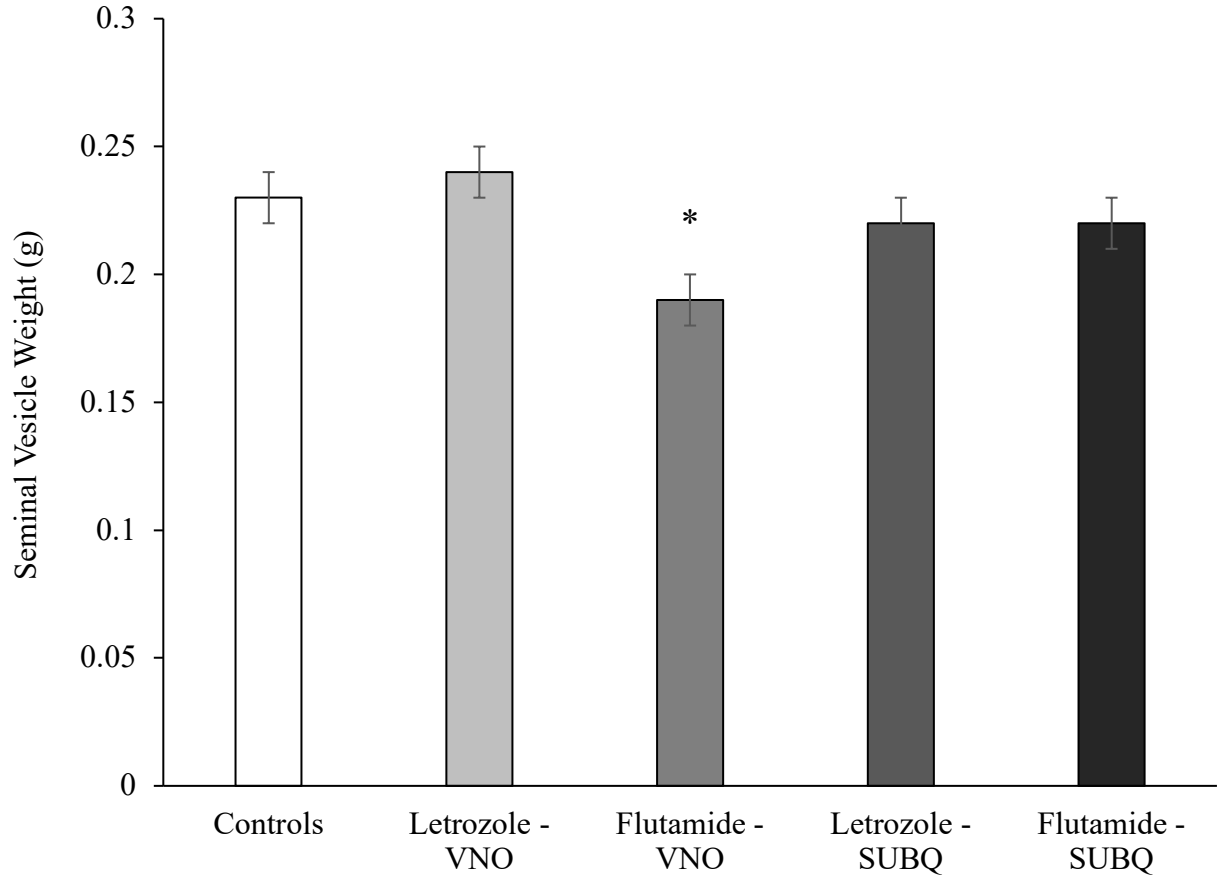
Experiment 3: Mean Overall Weight of Mice by Treatment Group Measured in Grams (g)



Note. Mean overall weight in grams of mice injected in the VNO and subcutaneously, +/- SEM. * indicates significant difference between letrozole subcutaneously (SUBQ) injected mice compared to controls and mice with SUBQ flutamide injections, $p < 0.01$, ($n = 12$ for all groups).

Figure 6

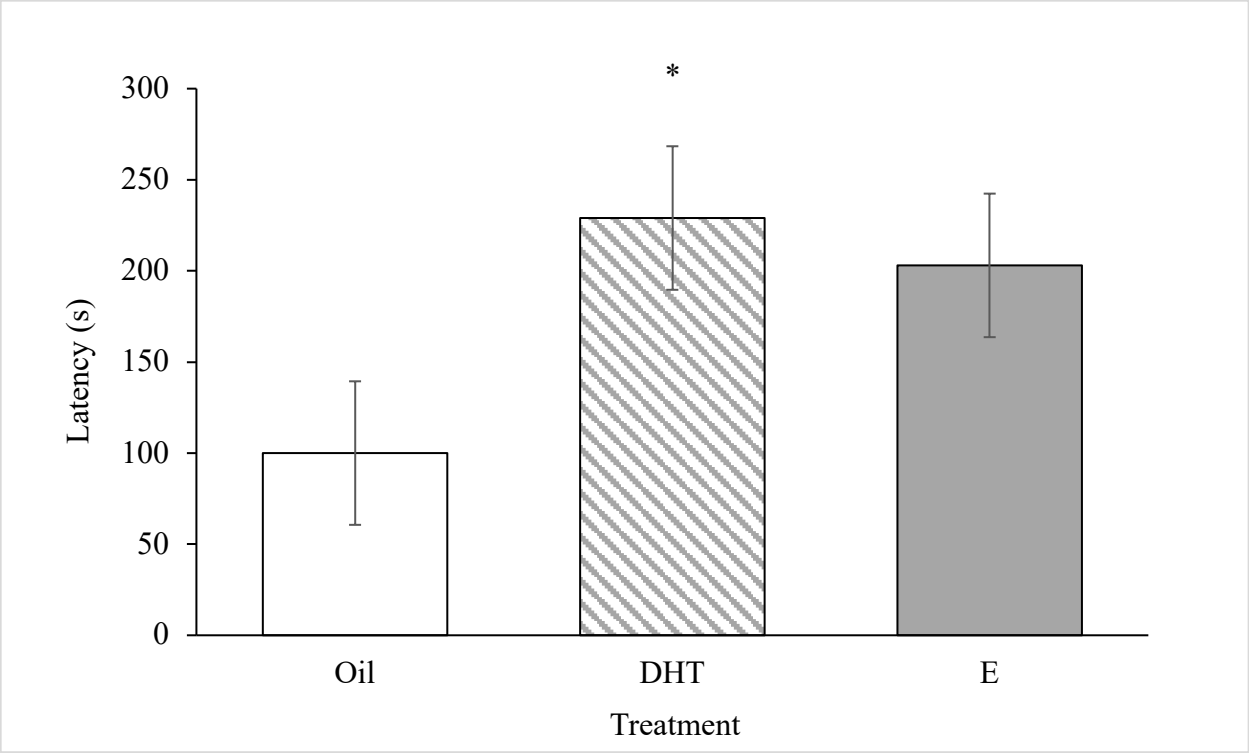
Experiment 3: Weight of Seminal Vesicles Measured in Grams (g)



Note. Seminal vesicle weight in grams of VNO injected and SUBQ injected mice, +/- SEM. * indicates that seminal vesicles of mice injected in the VNO with flutamide weighed significantly less than those of mice injected in the VNO with letrozole, $p < .05$, ($n = 12$ for all groups).

Figure 7

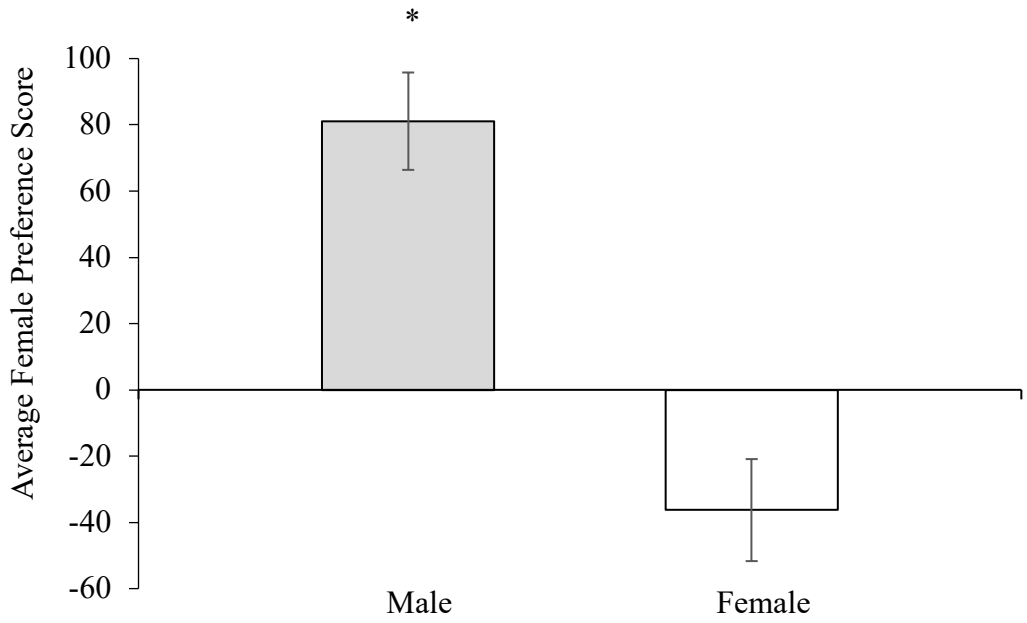
Experiment 2: Latency to Discover Buried Food by Treatment Group Measured in Seconds (s)



Note. Average latency to retrieve food during the buried food test by treatment groups in seconds (s), +/- SEM. Mice injected with dihydrotestosterone (DHT) (n = 9) took significantly longer to retrieve the buried food than the oil treated group (n = 10), * indicates p = .026; estradiol (E) (n = 9) treated mice did not significantly differ from controls (Oil) or DHT treated mice.

Figure 8

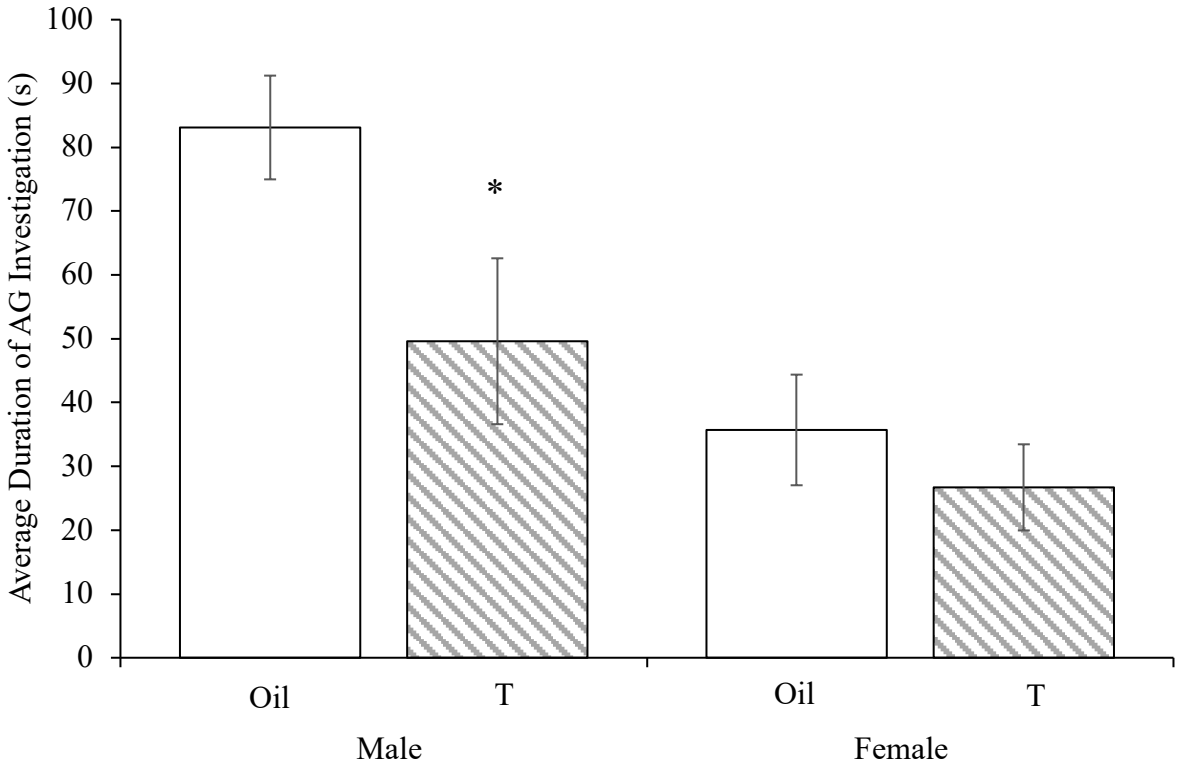
Experiment 1: Female Preference Score by Sex



Note. Average female preference scores for males (n = 20) and females (n = 24), +/- standard error of the mean (SEM). Males displayed a significantly greater preference for female bedding than females, * indicates $p < .001$. Treatment effects were not observed on this behavioural measure.

Figure 9

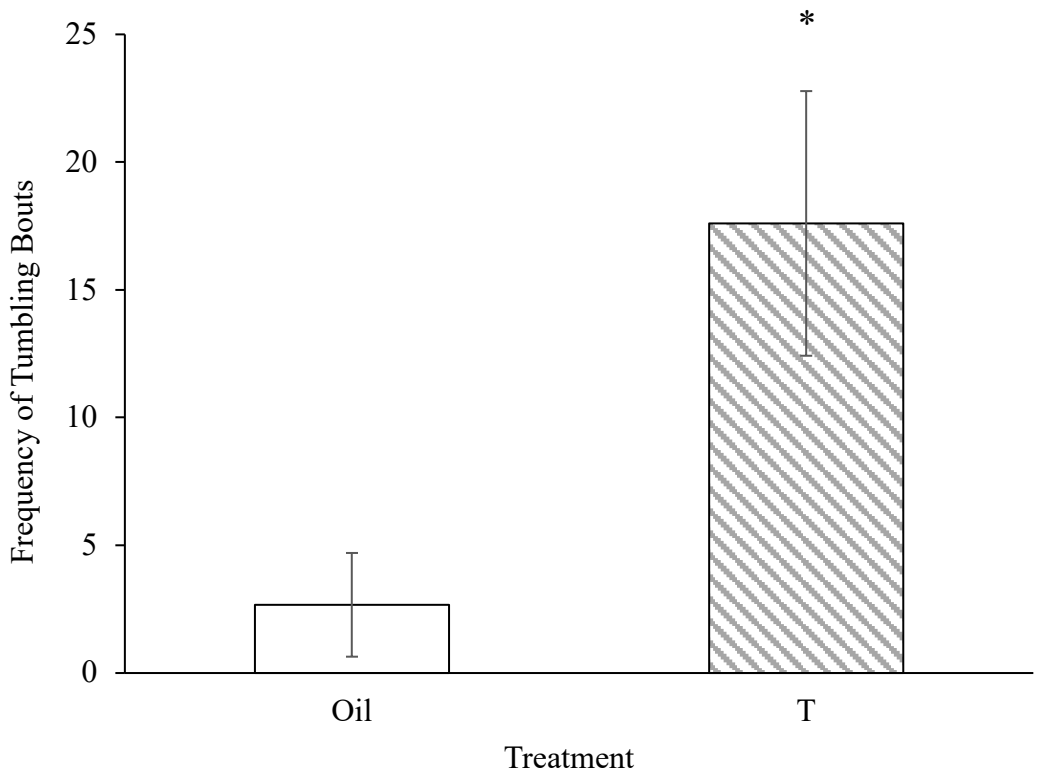
Experiment 1: Duration of Anogenital Investigation in Response to a Male Intruder



Note. Average duration of anogenital investigation by males and females in response to a male intruder, +/- SEM. Males (n = 20) spent significantly more time investigating the anogenital region of a male intruder compared to females (n = 24), $p < .001$. Male testosterone (T) treated mice displayed a significantly decreased average anogenital investigation duration compared to control males (Oil), * indicates $p = .035$.

Figure 10

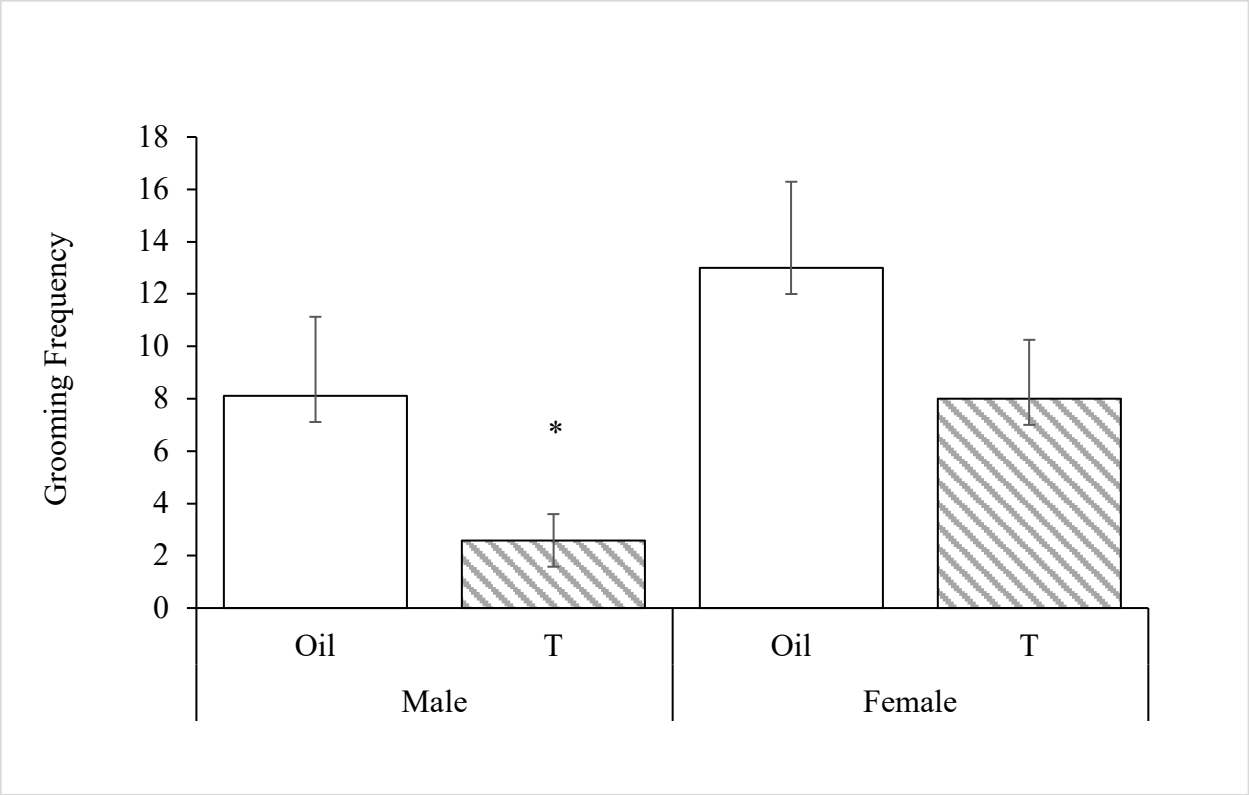
Experiment 1: Tumbling in Response to a Male Intruder



Note. Average number of tumbling bouts in males treated with oil ($n = 8$), or testosterone (T) ($n = 12$) in the VNO on PND1 in response to a male intruder, +/- SEM. Males given testosterone (T) displayed a higher frequency of tumbling when compared to oil treated males, * indicates $p = .006$.

Figure 11

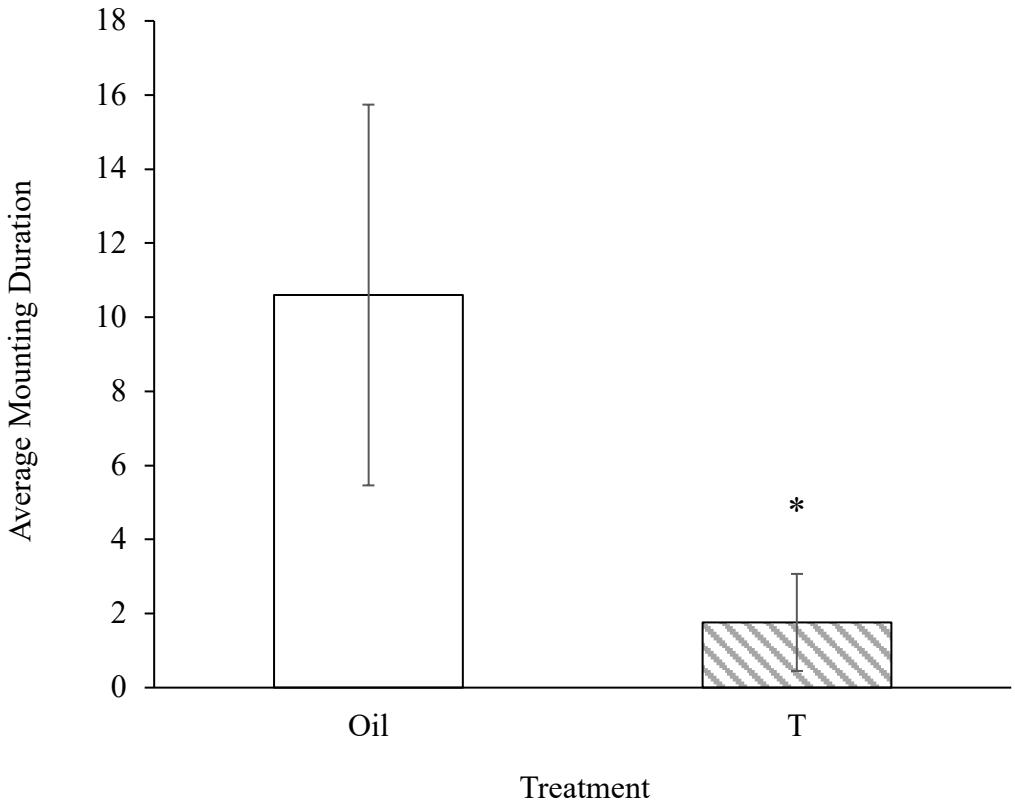
Experiment 1: Grooming in Response to a Female Intruder



Note. Average frequency of grooming displayed by oil (n_{Male} = 8, n_{Female} = 12) and testosterone (T) (n_{Male} = 12, n_{Female} = 12) VNO treated males and females in response to a female intruder, +/- SEM. Males treated with testosterone (T) demonstrated a significant decrease grooming in response to a female intruder, * indicates $p < .05$.

Figure 12

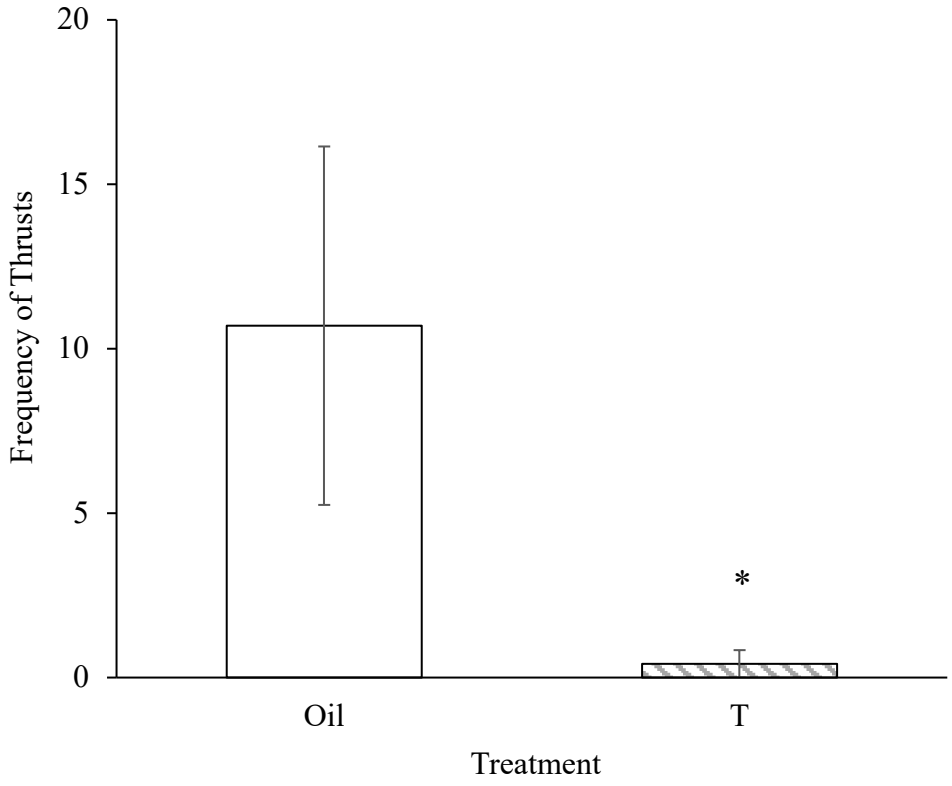
Experiment 1: Mounting in Response to a Male Intruder



Note. Average mounting duration displayed by oil (n = 8) and testosterone (T) (n = 12) VNO treated males in response to a male intruder, +/- SEM. Males treated with testosterone (T) demonstrated decreased sexual behaviour in the form of decreased average mounting duration in response to a male intruder, * indicates $p < .05$.

Figure 13

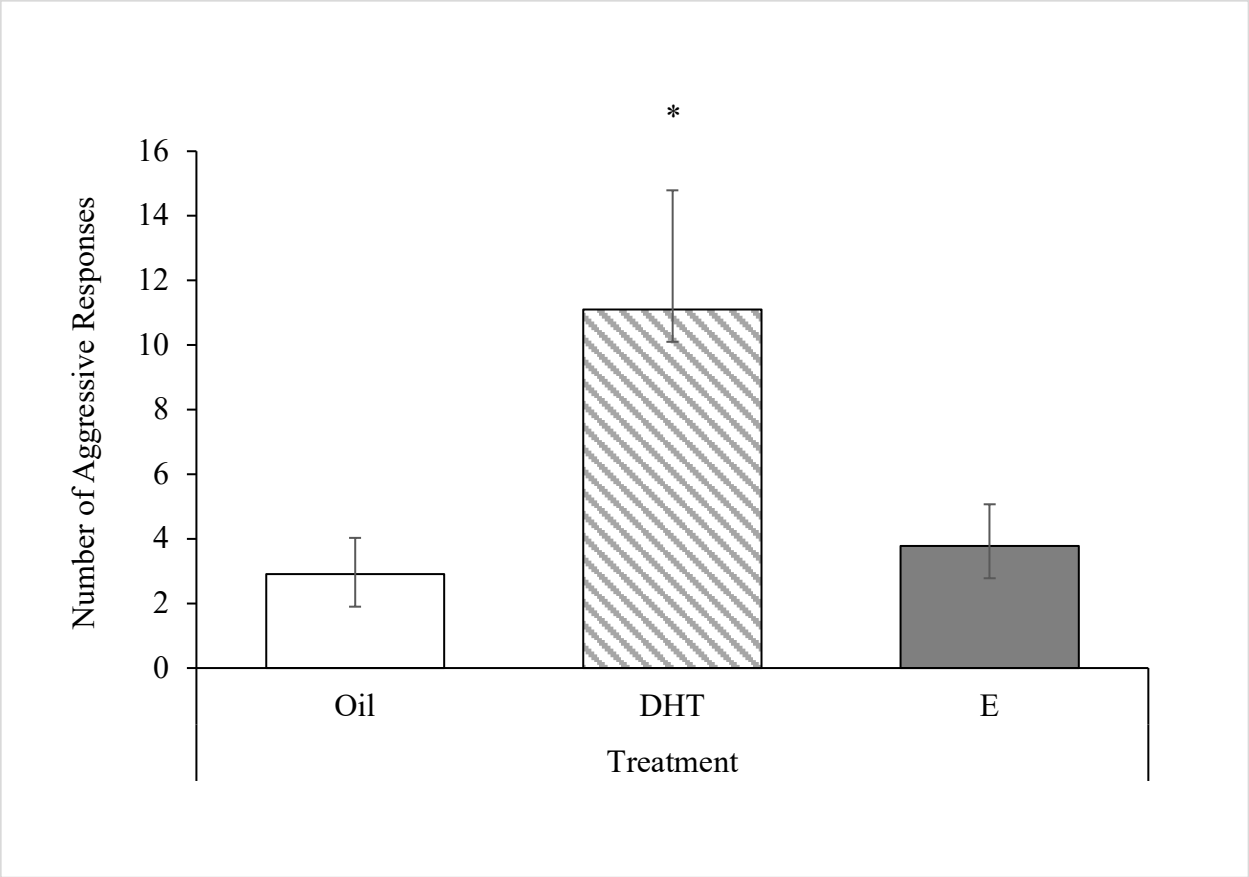
Experiment 1: Thrusting in Response to a Male Intruder



Note. Average number of thrusts displayed by oil (n = 8) and testosterone (T) (n = 12) VNO treated males in response to a male intruder, +/- SEM. Males treated with testosterone (T) demonstrated a significant decrease in sexual behaviour in the form of thrusting in response to a male intruder, * indicates $p < .05$.

Figure 14

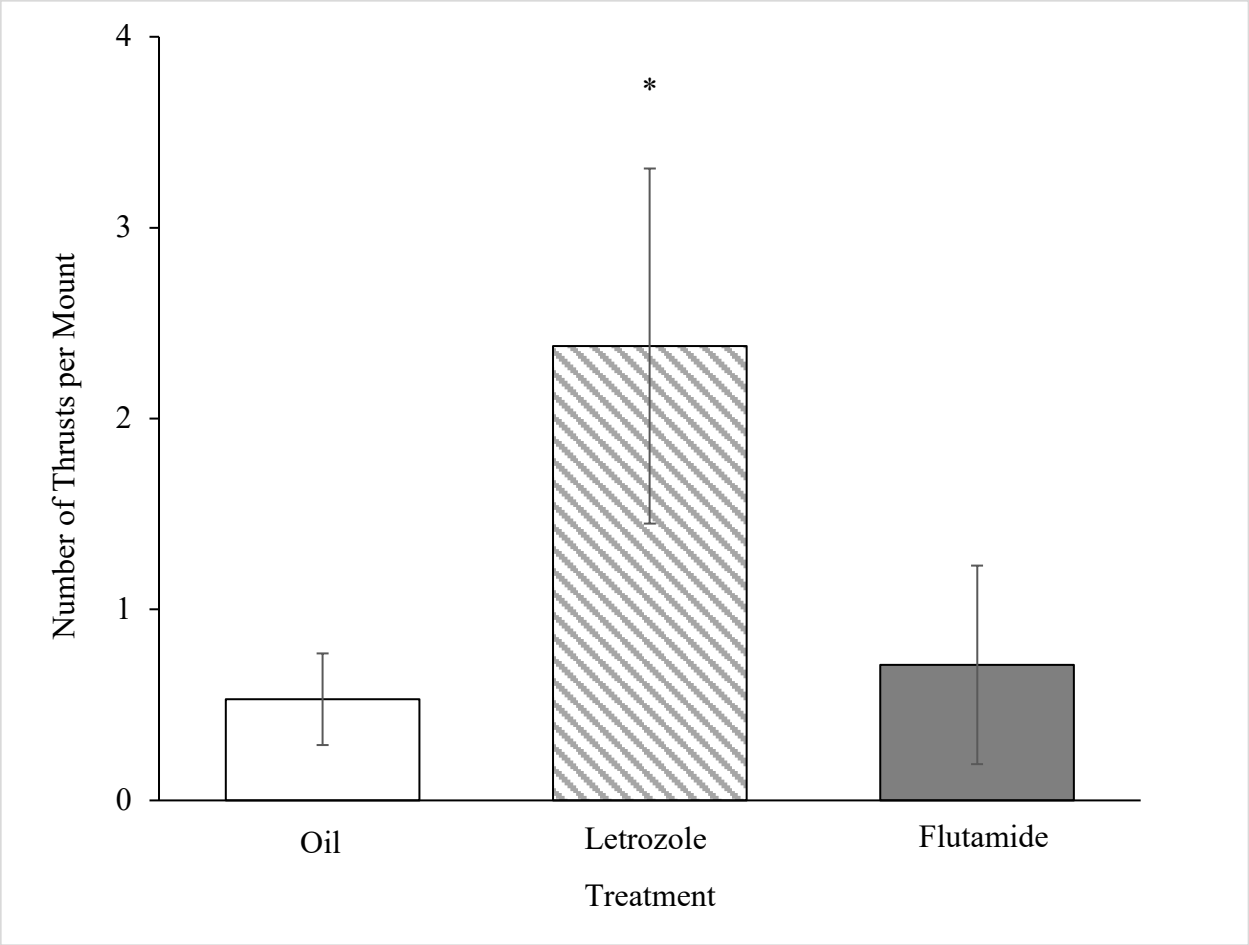
Experiment 2: Aggression in Response to a Male Intruder



Note. Average number of aggressive responses (bites, attacks, boxing) in males treated with oil (n = 10), dihydrotestosterone (DHT) (n = 9), and estradiol (E) (n = 9) in response to a male intruder, +/- SEM. Males exposed to DHT aggressed significantly more in response to a male intruder compared to oil and E treated males, * indicates $p < 0.05$. Female experimental animals did not display increased aggression towards any intruders.

Figure 15

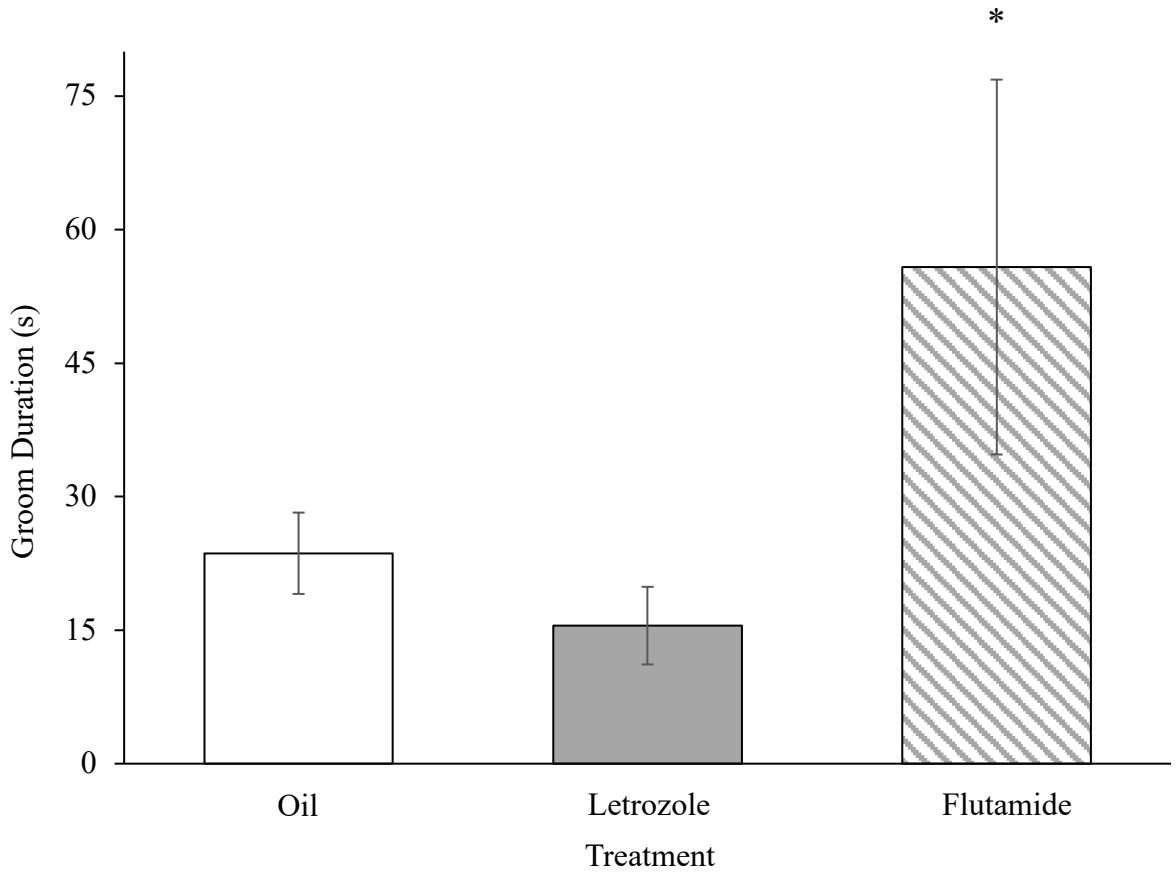
Experiment 3: Thrusts per Mount in Response to a Female Intruder



Note. Average number of thrusts per mount by males injected in the VNO with oil (n = 12), letrozole (n = 12), or flutamide (n = 12) in response to a female intruder, +/- SEM. Males exposed to letrozole displayed significantly more thrusts per mount when compared to controls in response to a female intruder. * indicates significant difference from controls, $p < .05$.

Figure 16

Experiment 3: Groom Duration in Response to a Male Intruder



Note. Average grooming duration by males injected subcutaneously (SUBQ) with oil (n = 12), letrozole (n = 12), and flutamide (n = 12) in response to a male intruder, +/- SEM. Males who received SUBQ injections of flutamide displayed a significant increase in grooming duration when exposed to a male intruder in comparison to males who received SUBQ injections of letrozole, * indicates significant difference from letrozole group, $p < .05$.

Appendix B

Table 1

Identification of Animals who did not Complete the Buried Food Test

	Identification Number	**Sex**	**Treatment Group**
Experiment 1	307	M	Oil-VNO
Experiment 2	332	M	Oil-VNO
	365	M	DHT-VNO
	595	F	DHT-VNO
Experiment 3	669	M	Flut-VNO
	675	M	Oil-VNO
	680	M	Let-SUBQ
	735	M	Let-VNO
	738	M	Flut-SUBQ
	758	M	Let-SUBQ
	760	M	Flut-VNO
	797	M	Let-SUBQ
	811	M	Oil-SUBQ
	822	M	Let-SUBQ
	828	M	Flut-VNO
	834	M	Oil-SUBQ
	840	M	Flut-SUBQ
	845	M	Oil-SUBQ