# THE ROLE OF THE GUT MICROBIOME ON SOCIO-SEXUAL BEHAVIOURS IN MICE

by © Stephanie Salia

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## Abstract

The gut microbiome is host to trillions of microorganisms that influence the brain and behaviour via the gut-brain axis. Gonadal hormones drive sex differences in the gut microbiota composition that translates into sex-dependent effects on behaviour when depleted. To date, these studies have primarily examined the gut's depletion on psychiatric disorders, including anxiety and depressive-like behaviours in rodents. The current study explored the role of gut microbiota on socio-sexual behaviours in male and female mice. Broad-spectrum antibiotic (ABX) in drinking water was used to deplete the microbiota in either early development (embryonic day 16 to postnatal day 21) or adulthood (day 60 to 81) while the control group received normal drinking water. Compared to control males, early and adult ABX decreased male territorial aggression, while adulthood ABX also decreased sexual odor preferences among males. Next, I examined whether these decreases in socio-sexual behaviour among males following ABX resulted from the depletion of the gut microbiota, rather than other non-specific effects of antibiotics, and/or whether these behavioural deficits could be due to decreases in androgens. To do so, cecal microbiota transplantation with same and opposite-sex control cecum contents or testosterone treatment was provided to adult antibiotic-treated males. Microbiota transplant with male cecum restored both olfactory preference and male aggression among adult ABX males. Female microbiota partially restored olfactory preference but not aggression among ABX males, while testosterone treatment was insufficient to rescue any of these behaviours. In adult ABX females, male microbiota transplant did not alter socio-sexual behaviours, but testosterone treatment increased male-typical sexual behaviours. Together, the results suggest a sex-dependent role for the gut microbiome in the display of sex-typical behaviours in mice that is independent of androgen.

## Declaration of Co-Authorship

I hereby declare that this thesis incorporates material that results from joint research; Specifically, Experiment 1 encompasses unpublished material co-authored with Francine B. Burke and Leah Myles (both honours students) and MSc student Yellow Martin under the supervision of Dr. Ashlyn Swift-Gallant. In all cases, primary contributions, experimental designs, data analysis, interpretation, and writing were performed by myself.

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## List of Abbreviations

| | |
|---|---|
| ABX | Antibiotic Treatment |
| AR | Androgen Receptor |
| ARKO | Androgen Receptor Knock-Out |
| ArKO | Aromatase Knock-Out |
| AFP | Alpha-Fetoprotein |
| ANOVA | Analysis of Variance |
| ANS | Autonomic Nervous System |
| AOB | Accessory Olfactory Bulb |
| BBB | Blood-Brain Barrier |
| BNST | Bed Nucleus Of The Stria Terminalis |
| CMT | Cecal Microbiota Transplant |
| CNS | Central Nervous System |
| DHT | Dihydrotestosterone |
| E | Estradiol |
| ER | Estrogen Receptor |
| ERα | Estrogen Receptor Alpha |
| ERβ | Estrogen Receptor Beta |
| ErαKO | Estrogen Receptor Alpha Knock-Out |
| ERβKO | Estrogen Receptor Beta Knock-Out |
| ENS | Enteric Nervous System |
| EPM | Elevated Plus-Maze |
| FPR | Formyl Peptide Receptor |

| | |
|---|---|
| GBA | Gut-Brain Axis |
| GIT | Gastrointestinal Tract |
| GF | Germ-Free |
| HPA | Hypothalamic-Pituitary Adrenal |
| LS | Lateral Septum |
| MePD | Medial Amygdala Posterodorsal Division |
| MOE | Main Olfactory Epithelium |
| MPOA | Medial Preoptic Area |
| NAcc | Nucleus Accumbens |
| OR | Olfactory Receptor |
| PBS | Phosphate-Buffered Saline |
| PND | Post-Natal Day |
| SPF | Specific-Pathogen Free |
| T | Testosterone |
| TAAR | Trace Amine-Associated Receptors |
| VMH | Ventromedial Hypothalamus |
| VNO | Vomeronasal Organ |
| VR | Vomeronasal Receptors |
| VTA | Ventral Tegmental Area |
| WT | Wild-Type |

## 1.0 The Role of the Gut-Microbiome on Socio-sexual Behaviours in Mice

The gut microbiome comprises bacteria, viruses, fungi, and their genetic makeup in the gastrointestinal tract (GIT) (Cresci & Izzo, 2019). Microbe-host interactions, including microbial mediation of metabolism, immune, and neuroendocrine responses, have been discovered and explored in the past decade (Cresci & Izzo, 2019). Specifically, these studies report that the microbiome, immune system, and brain regularly communicate (Sylvia & Demas, 2018). This bidirectional communication via the gut-brain axis has significant consequences for the brain and its function, starting in early development (Clarke et al., 2013). The gut microbiome has several benefits for physiological well-being, such as maintaining intestinal homeostasis and protecting against pathogens (Tetel et al., 2018). Conversely, an imbalance or absence in gut microbiota composition alters the immune system's capacity to fight infections and interferes with normal brain functions and behaviours (Clarke et al., 2013). Recent findings have shown sex-dependent effects of the gut microbiome on behaviour, including anxiety-related behaviours in rodent models (Clarke et al., 2013; Dempsey et al., 2019; Desbonnet et al., 2015; Jasarevic et al., 2016; Sylvia & Demas, 2018).

Despite evidence of sex-dependent effects on anxiety-related behaviours in rodents, the microbiome's influence on other sexually differentiated behaviours, including social and sexual behaviours, is unclear (Sylvia et al., 2017). Androgens are the primary hormone responsible for sex differences in neurodevelopment and behaviour, and links between the microbiome and androgens have been reported (e.g., the gut microbe *Clostridium scindens* converts glucocorticoids into androgens at a high affinity; Ridlon et al., 2013). Collectively, the literature suggests an exciting interaction between the gut microbiome and hormones. For example, androgens could be acting upon the gut microbiome to impact its composition (He et al., 2019),

which, in turn, affects sex hormones and behaviour. Alternatively, sex differences in the gut microbiome may affect the brain and behaviour independent of sex hormones through direct communication via the vagus nerve or the immune system (Zhu et al., 2017). Thus, the present studies explored the gut-endocrine interaction and its effect on behaviours.

### 1.1 Socio-sexual Behaviours in Mice

Socio-sexual behaviours occur in broader social contexts and are critical for reproductive success in rodents. Sexual behaviours encompass appetitive behaviours (e.g., ear wiggling by females), investigative behaviours (e.g., anogenital investigation of an opposite-sex partner by both sexes), and consummatory behaviours such as mounting and intromissions in males or lordosis in females (McCarthy, 2012). These behaviours and their neural substrates exhibit robust sex differences; for instance, the medial preoptic area (MPOA), a brain region critical for the display of sexual behaviours in rodents, is larger in males than in females (Breedlove, 1994). Territorial aggression is a social behaviour that is also highly sexually differentiated; typically, resident males will show aggressive behaviours, including chasing, attacks and biting, towards a male intruding on their territory (Soma et al., 2008; Takahashi & Miczek, 2013). Territorial aggression is more prevalent in males than females, while females tend to show high levels of aggression when caring for their pups (i.e., maternal aggression; Beach, 1976; Olivier & Young, 2002).

The development of sex differences in the brain and behaviour depends heavily on androgens, such as testosterone (T) and its metabolites, including estradiol (E) (Beach, 1976). T and its metabolites can act directly via the androgen receptor (AR) or can be converted to E by the enzyme aromatase in the brain and then act upon estrogen receptors (ER) (Bodo & Rissman, 2008; Kudwa et al., 2006). These hormonal processes are critical in the differentiation of neural

structures and pathways responsible for expressing sex-specific behaviours in rodents (Bodo & Rissman, 2008; Juntti et al., 2010; Kudwa et al., 2006). The traditional theory for the development and expression of these socio-sexual behaviours is centred around the organizational-activational hypothesis dating back to Phoenix et al. (1959) (reviewed in Arnold, 2009).

### 1.1.1 Sexual Differentiation of Brain and Behaviour

The organizational-activational framework presents evidence for a critical period for T's action on the brain. One well-illustrated critical period in rodents begins in perinatal development when males have a surge in androgens around the time of birth lasting through the first 24 hours of postnatal development (Corbier et al., 1978). This exposure to androgens permanently organizes the nervous system and the display of adult male-typical sexual and aggressive behaviours. A disruption of this androgen exposure during critical periods in development can result in a lasting decrease in male-typical behaviours and/or increases in the female-typical central nervous system (CNS) and behaviour (Corbier et al., 1983; Phoenix et al., 1959). Exposure to androgens in adulthood acts in an activational manner, or transiently, to further activate or enhance male-typical sexual and aggressive behaviours in male rodents (Bodo & Rissman, 2008; Martel & Baum, 2009; Simon et al., 1985 and Swift-Gallant, 2016). Although much of the literature has focused on the critical perinatal period, recent work suggests that puberty is a second critical window in development where sex hormones further organize the brain and behaviour (Schulz et al., 2009). For the full extent of male-typical behaviour, androgens are required during both organizational periods in development, as well as activationally in adulthood. Conversely, the absence of androgens in organizational periods and the exposure to estrogen and progesterone in puberty and adulthood lead to the full extent of

female-typical behaviours (Schulz et al., 2009). In other words, sex hormones must exert organizational effects during the critical periods of development on the CNS structures and must be continuously present to mediate components of these structures and the resulting behaviours for the full extent of sex-typical behaviour (reviewed in Swift-Gallant et al., 2012).

The aromatization hypothesis further explains the mediation of gonadal hormones in sexual differentiation of the brain and behaviour. Upon entering the brain, the high T levels in males can be converted into E by the aromatase enzyme. Then, E can bind to either estrogen alpha (ERα) or beta (ERβ) receptors to masculinize or defeminize the male brain (Bakker et al., 2006; Konkle & McCarthy, 2011). The male-typical brain is mediated by both androgen's action directly on AR and estrogen's action on ER via aromatization (Naftolin,1994). For example, in androgen receptor knock-out (ARKO) male mice, male-typical sexual and aggressive behaviours are decreased and show a female-like olfactory preference (Sato et al., 2003). Likewise, studies on aromatase knock-out (ArKO) and ER knock-out (ERKO) mice have shown the role of estrogen and ERs in expressing sexual and aggressive behaviours, respectively. Specifically, the ArKO male mouse displays a significant decrease in sexual (e.g. mount, intromission) and aggressive male behaviours due to the lack of a functional *Cyp19* gene, which encodes aromatase rendering the ArKO male mouse unable to synthesize estrogens (Matsumoto et al., 2003). ERαKO mice of both sexes are infertile, and males display reduced territorial aggression and show no partner preference when simultaneously presented with an unfamiliar same and opposite-sex conspecific (Ogawa et al., 1997; Wersinger et al., 1997; Wersinger & Rissman, 2000; reviewed in Bonthuis et al., 2010). These findings suggest a role for ERα in the expression of masculine behaviours. Conversely, ERβKO males do not differ from wild-type (WT) males in male sexual behaviour tests. Instead, they show increases in female-typical behaviours such as

lordosis, suggesting a role of ERβ in the male brain's defeminization (Kudwa et al., 2006). While females are exposed to low levels of E in utero via maternal estrogens, alpha-fetoprotein (AFP) prevents estrogen from entering and masculinizing the female fetus (Bakker et al., 2006). This has been experimentally tested by knocking out AFP (AFP-KO). AFP-KO female mice show increases in male-typical behaviours such as increased mounting and thrusting towards female stimulus animals (Bonthuis et al., 2010) (summary of KOs in Table 1). Together, the evidence suggests that androgens, acting via AR and ER, are required during both early organizational periods in development, as well as in adulthood, for the full display of male-typical behaviours. Conversely, female socio-sexual behaviours depend upon the absence of sex hormone action in early development and estrogen and progesterone in adulthood.

**Table 1**

*Summary of genetic KOs and their respective phenotypes*

| Genotype | Phenotype | Reference |
|---|---|---|
| ARKO ♂ | Female-like olfactory preference | Sato et al., |
| | Decreased mounting/thrusting and territorial aggression | 2003) |
| ArKO ♂ | Decreased mounting/thrusting and territorial aggression | Matsumoto et |
| | | al., 2003 |
| ERαKO ♂ & ♀ | Infertile | For review, see |
| ERαKO ♂ | Decreased territorial aggression; No partner preference | Bonthuis et al., |
| | | 2010 |
| ErβKO ♂ | Increased female-typical behaviours; e.g., lordosis | Kudwa et al., |
| | | 2006 |

| | | |
|---|---|---|
| AFP-KO ♀ | Increased mounting/thrusting/intromission towards female stimulus | Bonthuis et al., 2010 |

### 1.1.2 Neural Structures Underlying Socio-sexual Behaviours

During social interaction, mice primarily process pheromonal sensory cues from conspecific mates via a hormone-dependent neural circuit, the accessory olfactory system (Schellino et al., 2016), although the main olfactory system also contributes to the discrimination of conspecifics (Mori & Sakano, 2011). Notably, the main distinction between the two olfactory systems is that the main olfactory epithelium (MOE) of the main olfactory system detects volatile odors through the expression of olfactory receptors (ORs) or trace amine-associated receptors (TAARs) (Ishii & Touhara, 2019; Sanchez-Andrade & Kendrick, 2009). The vomeronasal organ (VNO), a paired structure located in the nasal cavity, on the other hand, expresses vomeronasal receptors (VRs) and formyl peptide receptors (FPRs), which detect non-volatile odors known as pheromones, as well as some volatile odors (Greer et al., 2016; Ishii & Touhara, 2019). Thus, while the main olfactory system can discriminate between male and female odors, the accessory olfactory system processes pheromonal cues that stimulate the sexually dimorphic neural circuitry underlying socio-sexual behaviours (see Figure 1).

The accessory olfactory system (AOS) relies on sensory input from the VNO to convey pheromonal signals to the accessory olfactory bulb (AOB), which then communicates to downstream neural regions responsible for displaying sex-typical socio-sexual behaviour (Ishii & Touhara, 2019; Wysocki et al., 1982). They include the posterodorsal medial amygdala (MePD), bed nucleus of the stria terminalis (BNST), medial preoptic area (MPOA), ventromedial hypothalamus (VMH), ventral tegmental area (VTA) and the nucleus accumbens that receive

projections from the VTA. Androgens sexually differentiate the AOS pathway and downstream neural structures in early development (Domínguez-Salazar et al., 2002), while gonadal hormones acting upon this sexually dimorphic neural circuit mediate socio-sexual behaviour in adulthood (Schellino et al., 2016). Particularly, these brain regions that receive projections from the AOB display increased neuronal activity in response to opposite-sex odors, and gonadectomy decreases both olfactory preference for opposite-sex odors and neural activity in this circuitry (Baum, 2009; Bodo & Rissman, 2007; Maras & Petrulis, 2010; Pfaus & Heeb, 1997).

Interestingly, aromatase, AR and ER are expressed at high levels throughout this neural circuitry, and androgen action via both AR and ER is responsible for the sexual differentiation of this circuit (Ishii & Touhara, 2019; Soma et al., 2008). The MePD, for instance, gets direct inputs from the MOB and AOB, is linked to male sexual behaviour, and is larger in males than females (Ishii & Touhara, 2019; Morris et al., 2005). Castration in adult male rats eliminates the sex difference found in the MePD size; however, treatment with E increases both volume and soma size of MePD cells compared to untreated castrates, while treatment with dihydrotestosterone (DHT) only sustains MePD soma size but not volume (Cooke et al., 2003). Models of AR insensitivity, such as the testicular feminization mutant (*Tfm*), with a global loss of AR function (Lyon & Hawkes 1970), show a partially masculinized MePD, even though these animals have normal ER binding in the MePD (Attardi et al., 1976; Morris et al., 2005). Together these findings suggest that both AR and ER mediate the masculinization of the MePD.

Receiving direct projections from the MePD, the MPOA is also sexually differentiated in volume and soma size, favouring males, and its ablation results in decreased male-typical mounting behaviours (Zuloaga et al., 2008). The MPOA is a critical site of aromatization, and evidence supports a role for aromatized T via ERs rather than ARs in the masculinization of the

volume of the MPOA (Dohler et al., 1986). For instance, the *Tfm* male rats have a fully masculinized volume in the MPOA, although smaller soma size than wild-type males (Morris et al., 2005). However, there is reduced aromatase activity in the MPOA of *Tfm* male rats, indicating a reduced ability to convert T to E, which may account for this incomplete masculinization of the MPOA (Roselli et al., 1987; Zuloaga et al., 2008).

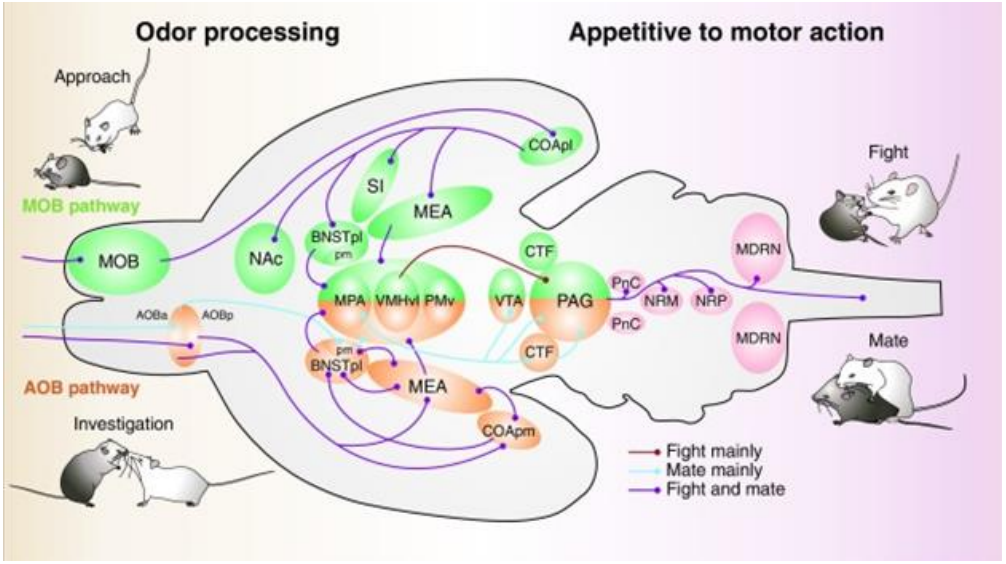
The BNST is also linked to the control of sexual behaviours due to its connection to the MePD, receiving direct and indirect inputs from the AOB (Giardino et al., 2018; Jennings & de Lecea, 2020). The aromatase neurons expressed in the BNST enable naïve male mice to discriminate male versus female conspecifics; inhibiting these neurons eliminates mate preference and diminishes consummatory sexual behaviours (Bayless et al., 2019). Furthermore, a critical role for the BNST in sex recognition and the appropriate social response has been reported. For example, stimulating the BNST neurons in males increases mounting towards male conspecifics (Bayless et al., 2019).

The role of the VMH in the control of sex-typical behaviours in rodents is duly noted in the literature. The ventrolateral subdivision of the VMH (VMHvl) is an ovarian hormone-sensitive locus that expresses progesterone receptor neurons ($VMHvl^{PR}$) (Jennings & de Lecea, 2020; Rubin et al., 1983). In female mice, $VMHvl^{PR}$ neurons are active during mating and investigating a male conspecific while lesions/ablations of these neurons have been shown to diminish lordosis (Griffin & Flanagan-Cato, 2011; Yang et al., 2013). The VMH also receives projection from the MePD, and ablation in males reduces mating and aggressive behaviours (Ishii & Touhara, 2019; Yang et al., 2013). Remarkably, the connection between the lateral septum (LS) and VMH has been directly implicated in the display of aggressive behaviours without affecting sexual behaviour (Wong et al., 2016).

The rewarding component of socio-sexual behaviours is linked to the mesolimbic system (dopamine release by the VTA into the NAcc) (Gunaydin et al., 2014; Watabe-Uchida et al., 2017). The VTA receives significant input from the MPOA, MePD, and BNST in both sexes and its activation is associated with social interest and mate preference (Chung et al., 2017). For example, the NAcc show increases in FOS in response to opposite-sex odor exposures (Bressler & Baum, 1996). On the contrary, lesioning the VTA-NAcc pathway or blocking dopamine receptor 1(i.e., postsynaptic or heterosynaptic receptor that couples to the heterotrimeric G proteins to stimulate adenylate cyclase activity and cyclic AMP accumulation; Undieh, A. S, 2010) signalling in the NAcc eliminates mate preference (Beny-Shefer et al., 2017). Altogether, these findings suggest that androgens acting via both AR and ER are necessary for the full masculinization of the male brain, translating into sex-typical socio-sexual behaviours (Trainor et al., 2003).

**Figure 1**

*Neural structures underlying socio-sexual behaviours*




Hashikawa et al. (2016)

*Note*: Neuronal circuits implicated in the display of socio-sexual behaviours in mice.

### 1.2 The Gut Microbiome: An Emerging System

There is a growing appreciation for the microbiome's role in mediating health, disease, CNS, and behaviour. The gut microbiome, which is a host to trillions of microorganisms (bacteria, fungi, viruses), colonize the host organism, including the oral cavity, placenta, vagina, skin, and gut-intestinal tract (GIT), with the majority residing within the GIT (Cresci & Izzo, 2019). Four major phyla have been identified in the gut microbiome, namely, *Firmicutes, Bacteroidetes, Actinobacteria*, and *Proteobacteria* (Belizario & Napolitano, 2015). Apart from the gut microbiome's role in metabolism, it significantly affects physiology and mental health and has been suggested to influence diabetes, depression and anxiety via interaction with the immune and endocrine system (Jašarević et al., 2016; Markle et al., 2013; Sarafian et al., 2017; Tetel et al., 2018). For instance, increased permeability of the intestinal epithelium permits bacteria secretions that affect the brain by producing cytokines that prompt immune responses (Cryan & Dinan, 2012; Oh & Cheon, 2020). There is also a suggestive role of the commensal microbiota on the postnatal development of the stress response hypothalamic-pituitary-adrenal axis (HPA) (Sudo et al., 2004). Furthermore, sex differences in the gut microbiota composition have been associated with obesity, asthma and disorders of the brain, such as autism, anxiety and motor deficits in Parkinson's diseases (Sampson et al., 2016; Tetel et al., 2018), suggesting that sex hormones may interact with the gut microbiome to influence the brain and behaviour.

#### 1.2.1 Factors Affecting the Gut Microbiome

The microbiota acquired through bacterial colonization from the early postnatal period is stable and resistant to transient interruptions (Mondot et al., 2013; Sudo et al., 2004). Nonetheless, its composition can be altered by a range of factors, including endocrine changes or external

influences such as diet, stress and oral antibiotics (Cresci & Izzo, 2019; Tetel et al., 2017; Ursell et al., 2012). Dietary components, including fat and fibre, significantly impact the gut microbiota (Buettner et al., 2018; Pellizzon & Ricci, 2018). For example, the soluble fibre found in standard chow is converted to short-chain fatty acids, promoting the growth of commensal microbiota and decreasing local and systemic inflammation compared to a refined low soluble fibre diet (den Besten et al., 2013; Kuo, 2013; Morrison et al., 2020). Also, a high-fat diet (e.g., 45% fat diet) enhances gut permeability and inflammation, thus inducing dysbiosis (Murphy et al., 2015; Voigt et al., 2014).

The impact of stressors on the microbiome, such as restraint and maternal separation, is also profound in both early and later life. Prenatal stress on pregnant Sprague-Dawley rat dams lowered the abundance of *Streptococcus* and *Lactobacillus*, increasing the HPA response to stress in their male offspring at four months of age (Golubeva et al., 2015). Sudo et al. (2004) also observed similar exaggerated HPA stress responses in germ-free male mice lacking all microorganisms when subjected to restraint. This was, however, partly reversed by the reconstitution with *Bifidobacterium infantis* from specific-pathogen-free (SPF) mice with the normal gut microbiome. These studies only looked at males; hence, the sex-dependent effect of stress on the gut microbiota composition is not known. The use of antibiotics also depletes the gut microbiome and can have significant impacts on behaviour. Broad-spectrum antibiotics deplete the gut microbiota composition on a short-term basis (Desbonnet et al., 2015; Sylvia et al., 2017). This unnatural depletion can be reversed with the use of probiotics like *Lactobacillus paracasei*, or the microbes can regenerate with time (Desbonnet et al., 2015; Verdu et al., 2006). However, it should be noted that the maximum duration required for the microbiota's complete regeneration to that of baseline level is yet to be reported. Altogether, these exogenous and

endogenous factors alter the gut microbiome's composition, affecting the brain and behaviour via the gut-brain axis.

### 1.2.2 The Gut-Brain Axis

The gut-brain axis (GBA) refers to the bidirectional communication between the gut and the brain (Carabotti et al., 2015; Zhu et al., 2017). The microbiome facilitates this cross-talk by integrating immune, metabolic, and endocrine signals (Mayer et al., 2015). The GBA comprises the CNS, the autonomic nervous system (ANS), the enteric nervous system (ENS), as well as the HPA (Carabotti et al., 2015). None of these multi-organ systems work in isolation, but instead, each communicates and influences the other (Jašarević et al., 2016). Through physical (e.g., via the vagus nerve) and biochemical (e.g., neurotransmitter activities, endocrine signalling) connections, the gut can influence brain development and function, while the brain can also affect the gut's microenvironment (Bonaz et al., 2018; Yano et al., 2015). Several approaches, including the use of germ-free animals, probiotic agents, antibiotics, and animals exposed to pathogenic bacterial infections, have been used to probe this axis (Vissavajjhala, 2017).

### 1.2.3 Mouse Microbiota Models

Recent studies on the microbial effect on physiology and behaviour have resorted to germ-free (GF) or antibiotic-treated mice (Kennedy et al., 2018). The breeding procedure used with GF mice renders them free of microorganisms, thereby allowing researchers to assess how the microbiome's absence influences the CNS and behaviour, including the possible mechanisms that underlie these influences. While GF mice offer an ideal means of evaluating the long-term effects of gut dysbiosis on brain development and behaviour, they require strict housing conditions to maintain their germ-free status (Desbonnet et al., 2015).

Antibiotic treatment (ABX) has proven to be effective in depleting the gut microbiome. Previous studies using a combination of broad-spectrum antibiotics such as ampicillin, neomycin and vancomycin report decreased fecal microbial diversity, resulting in a dramatic shift in the gut microbiota's composition (Becattini et al., 2016; Desbonnet et al., 2015; Reikvam et al., 2011; Sampson et al., 2016). These antibacterial agents do not easily cross the blood-brain barrier (BBB) and ensure animals' health while rendering them in a germ-free state (Reikvam et al., 2011; Sylvia et al., 2018). Therefore, broad-spectrum antibiotic treatment in mice offers a more flexible approach for studying the role of the microbiome in CNS and behaviour. Also, ABX offers the opportunity to deplete the microbiome at different life stages, such as early critical periods in development or adulthood. For instance, giving ABX via drinking water to pregnant dams limits the maternal transfer of microbes to pups, enabling studies to examine the effects of bacterial depletion in early development (Gonzalez-Perez et al., 2016; Li et al., 2017). Alternatively, the microbiome's transient effect in adulthood can be studied via ABX administration in drinking water for 2-3 weeks to temporarily deplete the microbiome in animals that otherwise had an unaltered microbiome throughout their lifespan (Desbonnet et al., 2015). Hence, ABX treatment offers a more suitable model for assessing the microbiome's effects during early critical periods versus adulthood and is a more cost-effective model for investigating bacterial depletion effects on behaviour.

### 1.3 Sex Differences in the Gut Microbiota's Composition: Role of Gonadal Hormones

Sex differences are prevalent in various anatomical, physiological, and behavioural traits, including gut microbiota composition. In mice, there have been mixed findings in relation to the sex difference in gut microbiome composition. Sex differences in the gut microbiota composition do not appear until puberty, supporting the role of sex hormones in shaping the gut microbiota

composition (Markle et al., 2013; Yurkovetskiy et al., 2013). Overall microbial richness and diversity tend to be lower in males than females, though males have an increased *Firmicutes* ratio to *Bacteroidetes* (Elderman et al., 2018). Administering T propionate to females in early development causes an increase in the *Firmicutes/Bacteroidetes* ratio (i.e., more male-typical) in adulthood (Moreno-Indias et al., 2016), suggesting that the sex differences in the gut microbiome that emerge at puberty are at least in part mediated by gonadal hormones during critical periods of development. Menon et al. (2013) found a role of ERβ in shifting the Firmicutes/Bacteroidetes ratio in female mice using knock-out technology. However, ERβKO female mice have reduced ovarian function; thus, it is possible ovarian hormone action via other receptors (e.g., ER-alpha or progesterone receptor) may have also accounted for the observed gut dysbiosis (Tetel & Pfaff, 2010). On the other hand, Yurkovetskiy et al. (2013) observed no significant sex differences in prepubescent mice's gut microbiota, and in adulthood, males showed a higher abundance of *Bacteroidetes, Firmicutes, Proteobacteria, and Actinobacteria* than females. Castration, which eliminated the androgen source, brought the males' microbiota closer to females than in intact males (Yurkovetskiy et al., 2013). The latter study used non-obese diabetic (NOD) mice, which may explain the discrepancy between studies. Nevertheless, the research supports the role of sex hormones in mediating the gut microbiome composition.

There may also be discrepancies in the literature with respect to sex differences in the gut microbiota due to strain differences. For instance, Org et al. (2016) evaluated a total population of 689 mice and reported significant differences between strains due to genetic variations. The C57BL/6J mouse strain, for instance, exhibited a clear sex difference compared to other strains like the DBA/2J mice, with C57BL/6J males having more abundance of *Coprococcus* and *Bacteroides* than their female counterparts (Org et al., 2016). They also report that diet (chow or

high-fat diet) and hormonal status (gonadectomy or sham control) significantly affected male mice's microbiota composition while the female microbiota composition was more responsive to dietary changes than gonadal hormones. T treatment significantly altered these changes in castrated C57BL/6J and C3H/HeJ male mice, but this was ineffective in DBA/2J mice. Interestingly, Elderman et al. (2018) found that BALB/c mice (males and females) exhibit a higher *Firmicutes*/*Bacteroidetes* ratio than C57BL/6J mice. Together, these studies suggest that sex differences in the gut microbiota may depend on sex, steroid hormones and the genetic differences between strains of mice (Elderman et al., 2018; Sylvia et al., 2017).

Mixed results on sex differences in the gut microbiota have been reported in humans as well. While Zhernakova et al. (2016) found moderate to no effect of sex on the human microbiota, Shin et al. (2019) reported sex by sex hormone differences in a human sample; overall, men and women with high levels of sex hormones exhibited a higher diversity of gut microbial communities than those with low sex hormone levels. Within sex, comparisons revealed that women with high E had higher levels of *Bacteroidetes* and lower levels of *Firmicutes* phyla than women with low E, while T levels among men were related to higher *Acinetobacter*, *Dorea*, *Ruminocaccus* and *Megamonas*. Others have also reported sex hormone effects on the gut microbiome among humans; for example, Koren et al. (2012) found a significant shift in the gut microbiota from the first to the third trimester of pregnant women. Together, this research suggests an influence of sex hormones on the gut microbial community.

### 1.4 The Gut Microbiome Can Affect Gonadal Hormones

While gonadal hormones can modify the gut microbiota composition, the gut microbiome can also alter hormonal levels. Novel findings from Colldén et al. (2019) suggest that the microbiome regulates intestinal androgen, particularly DHT. The authors report that GF mice

were deficient in free DHT levels in the distal intestine (colon). It is hypothesized that the cecum, which has higher bacterial and metabolic activity, regulates glucuronidated DHT and T, which results in increased free levels of the potent androgen DHT of mice with a healthy microbiome (Colldén et al., 2019). Findings from Markle et al. (2013) revealed that early-life microbial exposure determines sex hormone levels among non-obese diabetic (NOD) mice. Of note, T and commensal bacteria are implicated in the development and modification of diabetes in the NOD strain, respectively (Markle et al., 2013; Tai et al., 2016). Castration, for instance, increases disease incidence in male NOD mice while specific-pathogen-free (SPF)conditions protect these mice relative to their female counterparts (Markle et al., 2013), further supporting the interaction between the gut microbiome and androgens in male mice. In their study, the level of 17β-estradiol was not different between GF and SPF NOD male and female mice; however, T was higher in the GF females than in the SPF females and lower in the GF males than in the SPF males. The sex difference in T levels remained in GF mice, with GF males having higher serum T levels than GF females. When the cecal content from SPF male NOD mice was administered to weaning (22-26 days old ) GF female mice, the testosterone levels elevated in the recipient GF females mice and persisted during the adult stage; T levels were not affected in GF female mice that received cecal content from a female mouse (Markle et al., 2013). Thus, it is possible that the male-typical gut microbiota increases circulating T; indeed, it has been shown that the gut microbe *Clostridium scindens* converts glucocorticoids into androgens at a high affinity (Ridlon et al., 2013), and thus may contribute to the increases in T among female recipients of male gut microbiota. These findings suggest that the microbiome can regulate the production and metabolism of androgens, the primary sex hormone that mediates the sexual differentiation of the brain and socio-sexual behaviours.

### 1.5 Role of the Gut Microbiome on Social Behaviours

Evidence over the last decade implicates the gut microbiome in anxiety and depressive-like behaviours, as well as specific bacterial strains (*Bifidobacteria infantis* and *Lactobacillus rhamnosus*) with beneficial roles in mediating these behaviours (Bellono et al., 2017; Bercik et al., 2011; Bravo et al., 2011; Desbonnet et al., 2008; Tetel et al., 2017). Specifically, reduced anxiety-like behaviours have been reported among males of GF mice models when assessed on various behavioural paradigms (e.g., open field test, light-dark box and the elevated plus-maze; Clarke et al., 2013; Heijtz et al., 2011; Neufeld et al., 2011). The studies suggest that the absence of the gut microbiota has a sex-specific anxiolytic effect in GF mice. In contrast, Ceylani et al. (2018) found decreased locomotor activity and higher anxiety levels in antibiotic-treated BALB/c male mice tested on the elevated plus-maze and open field tests. Such conflicting findings may not be surprising in light of the fact that GF mice have no bacteria throughout the lifespan, while antibiotic-treated mice had bacteria up until the antibiotic treatment. Dysbiotic microbiota induced by either exogenous factors (e.g., stress, diet and antibiotics) or breeding conditions (e.g., GF state) have also been shown to have a causal role in the development of depressive-like behaviours in rodents (Chevalier et al., 2020; Gacias et al., 2016; Jianguo et al., 2019; Liu et al., 2020; Xie, 2017). Specifically, the gut microbiome mediates depression in a sex-dependent manner, as a dysbiotic gut appears to increase susceptibility in male mice compared to females (Bridgewater et al., 2017).

Given the sex-dependent effects of the gut microbiome on depression and anxiety-like behaviours, as well as the interaction of the gut microbiome with sex hormones, I hypothesized that there might be sex-dependent effects of the gut microbiome on sex-typical behaviours. The effects of microbiome disruption on socio-sexual behaviours such as sexual preferences and

aggression have yet to be explored among common rodent laboratory animals. To date, only two studies have asked whether there is an association between the gut microbiome and aggression, one in dogs (Kirchoff et al., 2019) and the other in hamsters (Sylvia et al., 2017). Kirchoff et al. (2019) examined fecal samples of dogs characterized as aggressive and non-aggressive, and specific gut microbiome composition was associated with aggressive behaviours in males. For example, they found a greater abundance of the phyla *Firmicutes* and the genus *Lactobacillus* in aggressive dogs, with non-aggressive dogs showing a greater abundance of *Proteobacteria* and *Fusobacteria* (Kirchoff et al., 2019). Sylvia et al. (2017) found decreased aggression in antibiotic-treated male and female hamsters. They assessed the effects of single versus the repeated antibiotic treatment and found that repeated (14 days; with a recovery phase after single treatment) but not single (7 days) treatments caused a marked decrease in the frequency of attacks and overall aggression scores in male hamsters, which returned to normal levels following recovery. However, a single treatment decreased aggression in the females, suggesting females are more susceptible to antibiotic treatment effects. Given the role of androgens on the display of male-typical aggression (e.g., castration of a male decreases aggression) and the associations between sex hormones and the microbiome, one plausible explanation for the results of Sylvia et al. is that microbiome depletion altered androgen levels. It is equally possible that the absence of the gut microbiota alone accounted for decreased aggression via direct and/or immune influences on the brain.

### 1.6 Study Overview

The current study explored whether depletion of the gut microbiota via antibiotics influences the display of socio-sexual behaviours in male and female mice. The first experiment examined socio-sexual and anxiety-related behaviours in male and female mice following

depletion of the gut microbiota in early development (gestational day 16 – PND 21) and adulthood (PND 60 – 85). Given the sex-dependent effect of the microbiota depletion on anxiety-related behaviours and aggression (Desbonnet et al., 2015; Sylvia et al., 2017), coupled with the role it plays in the regulation of androgens (Colldén et al., 2019; Ridlon et al., 2013), it was hypothesized that microbiota depletion would affect androgen-dependent socio-sexual behaviours in a sex-dependent manner. Specifically, early and adult ABX was hypothesized to decrease male-typical androgen-dependent behaviours such as sexual behaviour towards a female and male territorial aggression. On the other hand, females were hypothesized to not show any differences in their behaviour compared to control females, as females do not depend upon androgens for the display of female-typical socio-sexual behaviour.

In Experiment 1, socio-sexual behaviours in males were found to be impeded by early and adult ABX treatment. Experiment 2 was designed to assess the direct and indirect (endocrine) effects of the gut microbiome on socio-sexual behaviours; Adult ABX males were treated with 1) male cecum transplantation, 2) female cecum transplantation, or 3) T treatment. Given that there can be a direct mediation of the gut microbiome on behaviours, it was hypothesized that a healthy/normal microbiota (e.g. re-colonizing the gut microbiota) would reverse antibiotic-induced behaviour changes in male mice. Indirectly, the gut microbiome may mediate socio-sexual behaviour by altering androgens, and as such, it was hypothesized that T treatment would rescue male-typical behaviours in adult antibiotic-treated males. Finally, the study followed up on Markle et al.'s (2013) report by comparing male microbiota transplant and T treatment in ABX female mice. It was hypothesized that both male microbiota transplant and T treatment would induce male-typical sexual behaviours in ABX adult females. Overall, the present studies will advance our understanding of the role and extent to which the gut

microbiome mediates socio-sexual behaviours in mice and as well elucidate the mechanism underlying such mediation.

## 2.0 Method

### 2.1 Experiment 1

#### 2.1.1 Subjects

Experiment 1 followed a 2 x 3 between-subjects factorial design in which mice were assigned to groups based on sex (male or female) and treatment (Early ABX, Adult ABX or control conditions). Male and female wild-type C57bl/6 mice were obtained from Charles River, QC and paired with opposite-sex conspecifics for breeding. Early ABX dams were examined for a seminal plug to approximate the first day of gestation. Breeding males were then separated from the pregnant dams before antibiotic treatment (i.e., embryonic day 16). All offspring were weaned at 21-22 days of age and mice from the same litter were divided among experimental conditions, resulting in the following sample sizes: Early ABX (males n = 18; females n = 13), Adult ABX (males n = 14; females n = 11), and control group (males n = 11; females n = 8). All mice were singly housed between PND 40 – 50. Early ABX treatment was defined as embryonic day 16 to postnatal day 21, while adulthood was defined as postnatal day 60 to 85 for ABX treatment conditions. Mice had *ad libitum* access to a standard chow diet (Teklad 2018, Envigo) that contains a rich source of soluble fibre and water and were kept on a 12- hour light-dark cycle with all behaviour testing performed during the light phase (3 – 6 hours after lights on). Ethical approval was obtained from the Institutional Animal Care Committee at Memorial University of Newfoundland and Labrador, and all procedures outlined followed the Canadian Council on Animal Care (CCAC) guidelines.

### 2.1.2 Stimulus Animals

Four males and three female gonadectomized C57BL/6 mice were utilized as stimuli during the resident intruder test. At 8-12 weeks of age, stimulus males were castrated, while stimulus females were ovariectomized and implanted with a Silastic capsule (1.98 mm id/ 3.17 mm od) containing dissolved 17β-estradiol in sesame oil (50 µg in 0.025 ml) and sealed with Silastic Medical Adhesive Silicone (Dow Corning, Midland, MI, USA; previously described in Swift-Gallant et al., 2016). Stimulus females were injected with progesterone (500 µg in 0.1 ml of corn oil) 2 to 5 hours before testing to induce behavioural estrus. Gonadectomized stimulus males were swabbed on the lower back/near their tail with sexually experienced male urine (pooled urine of 5 - 8 male mice; aliquots were frozen at -20°C; thawed on testing days) before each behavioural test. Such urine contains pheromones capable of eliciting inter-male aggression (Chamero et al., 2011). Soiled bedding used in the olfactory preference test was collected from sexually experienced males and estrus-induced females 48 hours following cage change. The bedding was stored in resealable bags at -20°C and was allowed to reach room temperature before use on testing day,

### 2.1.3 Antibiotic Treatment

A combination of antibiotics was chosen to deplete the gut microbiota based on a previous report that such combination reduced the fecal bacterial DNA load by 400-fold while ensuring the animals' health (Reikvam et al., 2011; Sampson et al., 2016). The antibiotic cocktail consisted of 1 g/L ampicillin (-20°C), 1 g/L neomycin sulphate (22°C), 500 mg/L vancomycin (-20°C), and 10 mg/L erythromycins (-20°C) from Cayman Chemical Company, as well as 100 mg/L gentamicin (4°C) from Tocris Bioscience. The broad-spectrum antibiotics (powder form) were weighed ahead of time and stored in 50 ml tubes at -20°C. Drinking water was added to the

powder for easier dissolving and vortexed on the day of use. The cocktail of antibiotics was administered in place of drinking water, and bottles were wrapped in aluminum foil and changed three times a week (Mondays/Tuesdays/Fridays). For early treatment, antibiotics were administered to pregnant females starting on embryonic day 16. This would limit the exposure of pups to maternal microbes during birth and nursing. Pups from the Early ABX condition continued to receive antibiotics through breastmilk of the mother until weaning (postnatal day 21), while the Adult ABX mice received antibiotics via drinking water from postnatal day 60 to 85 (i.e., at least 20 days prior to the onset of behaviour testing, as well as throughout behaviour testing). It should be noted that 2 – 4 weeks of antibiotic treatment duration does not affect host metabolism (Battson et al., 2019; Gacias et al., 2016). Control animals received normal drinking water containing no antibiotics.

### 2.1.4 Behaviour Testing

Behavioural testing was conducted between 9 am and 4 pm on four separate days, with a minimum of one day of rest in between tests. The olfactory preference test was carried out on day 1 of testing, Resident-Intruder and the sexual behaviour test counterbalanced between day 2 and 3 and the Elevated plus-maze was conducted on day 4. All mice were sexually naïve at the time of testing.

#### 2.1.4.1 Olfactory Preference Test

Preference for the opposite - and same-sex odors was investigated using the Olfactory Preference paradigm. Experimental animals were placed in a clear container (42 cm × 25 cm × 20 cm) with clean bedding covering the bottom and three ramekins (4 cm in height x 8 cm in diameter) containing clean bedding for a 5-minute habituation phase. In the testing phase, the ramekins were replaced with male-soiled, female-soiled and clean bedding in a quasi-

randomized order. Upon experimenters leaving the testing room, trials were video-recorded for a 10-minute period. The video recordings of behaviour tests were later coded by a blind experimenter using Behavioural Observation Research Interaction Software (BORIS©, Torino, Italy) for the duration of time spent investigating each ramekin. Each ramekin was later matched with the corresponding bedding type using the testing records, and a female preference score was calculated (time spent in female bedding minus time spent in male bedding). The female preference score is often used for analyses when assessing results from the olfactory preference tests as it takes into consideration the preference for female over male sexual odor cues within the same test (Bodo & Rissman, 2007; Swift-Gallant et al., 2016).

#### 2.1.4.2 Resident-Intruder Paradigm and Sexual Behaviour Test

Sexual and aggressive behaviours toward male and female stimulus animals were investigated using the resident-intruder paradigm. Each animal underwent two tests with live stimulus mice: once with a female intruder and once with a male intruder. Tests were conducted in the experimental animal's home cage by taking out all enrichment/food leaving only the bedding remaining along the cage's bottom, and the cage lid was replaced with a transparent Plexiglas lid with air holes. Behaviours were video-recorded for a 15-minute trial while experimenters left the room. The frequency, duration and latency of socio-sexual behaviours, including anogenital investigation, face/body investigation, self-grooming, mounting, thrusting, intromission), were measured. Aggression was defined as the number and duration of chasing, pounce/attack, tumbling, boxing, and biting, previously shown to be typical in intermale aggression (Ogawa et al., 1998). Latencies to the first sexual and aggressive behaviour were recorded with the maximum score (900 seconds, i.e., 15 minutes) given to mice that did not show

any sexual or aggressive behaviour, respectively. An experimenter blind to the sex of the experimental and stimulus mice coded all videos for behaviours using BORIS.

#### 2.1.4.3 Elevated Plus-maze (EPM)

Since the effect of the GBA on anxiety-like behaviours is well established in the literature (Bercik et al., 2011; Neufeld et al., 2011), the EPM was used to assess whether the mice in the present study also showed anxiety-like behaviour (Ceylani et al., 2018). The elevated plus maze is a four-arm maze raised from the ground; two arms are closed on all sides, and two arms are open. When mice are anxious, they tend to hide and do not like open spaces, especially in high height, which is incorporated in this test as the maze is "elevated" (Adamec et al., 2005; Neufeld et al., 2011). Increased proportion of time spent in the open arms (time in open arms/total time in open or closed arms) and an increase in the proportion of entries into the open arms (entries into open arms/total entries into open or closed arms) was used as an indication of anti-anxiety behaviour (Walf & Frye, 2007). During testing, mice were placed at the junction between the open and closed arms, facing the open arm opposite to where the experimenter stood (Walf & Frye, 2007). Each trial lasted 10 minutes under video recording using a standard video camera; these videos were analyzed using Noldus EthoVision XT version 15 (Noldus Information Technology©, USA).

### 2.1.5 Dissections

Following the completion of the behavioural tests, mice were exposed to a ramekin filled with opposite-sex soiled bedding for 10 minutes in their home cage. Ninty minutes after the exposure, mice were weighed, then overdosed with Avertin (40mg/100g); blood was collected and stored at 4 °C for 24 hours, then spun at 3000 rcf for 20 minutes. The serum was transferred to a new tube and stored at -20 °C for further potential analysis. Following blood collection,

mice were perfused with 0.1 M phosphate buffer followed by 4% paraformaldehyde in 0.2M phosphate-buffered saline (PBS). Ceca were removed from all experimental animals during dissection to validate the depletion of the gut microbiome. Stool samples were also collected from all mice and stored at - 80 °C for future bacterial sequence analysis. Somatic measures for androgen-sensitive tissues such as seminal vesicles, testes and female uterine horn/ovaries were also weighed. Brains were extracted and post-fixed for 2 hours in 4% paraformaldehyde, then transferred to 20% sucrose. Brains in sucrose were stored at 4 °C until sectioning. All brains were coronally sectioned within two weeks of dissection on a sliding freezing microtome at 30 µm into four series; tissue was stored in cryoprotectant at -20 °C for future histological processing.

### 2.1.6 Cecum Weight

To validate antibiotic treatment as an effective process for gut microbiota depletion, animals that received antibiotic cocktails during early development and adulthood were compared to the vehicle-treated controls. Prior research has indicated that an enlarged cecum is expected when the microbiota is depleted in both antibiotic-treated and germ-free mice (Reikvam et al., 2011; Zaborin et al., 2016). Hence, ceca were extracted during dissection and weighed using an analytical balance for further analysis.

## 2.2 Experiment 2

### 2.2.1 Subjects

Male and female wild-type C57bl/6 mice from the Charles River, QC, were paired with opposite-sex conspecifics for breeding. Offspring were weaned at 21-22 days of age, and all mice were singly housed between 45 - 55 days of age. Male and female littermates were randomly assigned to one of the following experimental groups: 1) Males that received only

ABX treatment (ABX, n = 12); 2) ABX males that received cecal contents from control males (A+MC, n = 12); 3) ABX males that received cecal contents from control females (A+FC, n = 9); 4) ABX males with T treatment (A+T, n = 12); 5) ABX females that received cecal contents from control males ($A+MC_f$, n = 10); 6) ABX females with T treatment ($A+T_f$, n = 11); 7) Males control (no antibiotic treatment, vehicle gavage / blank capsule implant, n = 12) and 8) Female control (no antibiotic treatment, vehicle gavage/blank capsule implant, n = 11). Mice had *ad libitum* access to food and water and were kept on a 12- hour light-dark cycle with all behaviour testing performed during the light phase (3 – 6 hours after lights on). A total of 89 mice were utilized in this experiment. All procedures adhered to the Canadian Council on Animal Care (CCAC) guidelines with approval from the Institutional Animal Care Committee at Memorial University of Newfoundland and Labrador.

### 2.2.2 Stimulus Animals

Nine to twelve-week-old C57bl/6 mice were gonadectomized and utilized as stimuli during behaviour testing. Stimulus males (n = 4) were castrated, and females (n = 5) were ovariectomized and implanted with a Silastic capsule (1.98 mm id/ 3.17 mm od) containing dissolved 17β-estradiol in sesame oil (50 µg in 0.025 ml) and sealed with Silastic Medical Adhesive Silicone (Dow Corning, Midland, MI, USA). On testing days, stimulus females were injected with progesterone (500 µg in 0.1 ml of corn oil) 2 to 5 hours before testing to induce behavioural estrus. Castrated stimulus males were swabbed on the lower back/near their tail with the urine of sexually experienced male mice (pooled urine of 5-10 male mice; aliquots were frozen at -20 °C /thawed on testing days) before each behavioural test.

### 2.2.3 Testosterone Treatment

Before antibiotic treatment, at 60 days of age, Silastic capsules containing T (1.6 mm inner diameter, 3.2 mm outer diameter; 6 mm effective release length) were implanted in both male and female A+T groups. Capsules of this length produce T levels at near-normal circulating levels in mice (Chen et al., 2014; Zuloaga et al., 2008). All other mice had blank capsule implants to control for surgical implant stress. For this surgery, animals were anesthetized with 1-2% isoflurane and provided with slow-release Meloxicam (5mg/kg). Prior to making a 2-3 mm incision, the incision site between the shoulder blades was plucked to remove fur, and the area was cleaned with isopropyl alcohol and iodine. The capsule was inserted, and the incision site was closed with tissue adhesive.

### 2.2.4 Antibiotic Treatment

Antibiotic treatment followed the same procedure outlined in Experiment 1 via drinking water to deplete the gut microbiota, whereas controls received regular drinking water. Antibiotic treatment started four days following the capsule implant. The A+MC and A+FC conditions received two weeks of antibiotic treatment aimed to induce a germ-free state to enhance colonization of donor microbiota during transplantation (Battson et al., 2019; Gacias et al., 2016). The ABX only and A+T conditions of both male and female groups received antibiotics until the end of behaviour testing (22 days).

### 2.2.5 Cecal Microbiota Transplantation (CMT)

Cecal content was used for microbiota transplantation in the current study based on a previous report that fecal and cecal microbiota cluster differently in mice, and thus, sampling from the cecum is more suitable for gut-specific studies (Kozik et al., 2019; Pang et al., 2012). Here, the cecum of 8–9-week-old control male and female C57bl/6 mice (n = 6-8/group) was

resected and content collected into sterile 2 ml microcentrifuge tubes and diluted with phosphate-buffered saline (PBS) (0.5ml cecum / 0.5 PBS) as previously described (Gacias et al., 2016; Ubeda et al., 2013); these aliquots were mixed to ensure homogeneity between aliquots. The aliquots (1 ml) were then stored at -80˚C until the day of oral gavage. Prior to oral gavage, all aliquots were thawed and further diluted with PBS in a 1:5 ratio (total ratio 1:10). To re-colonize, the gut, male and female recipient mice (A+MC and A+FC) were provided with 200 µl of cecal content via oral gavage over the subsequent 12 days every other day (for a total of 6 treatments) following two weeks of antibiotic treatment. Following a 1-day rest period of ending oral gavage, behaviour testing was conducted on the two subsequent days, and on the fourth day following the last oral gavage, dissections were performed.

### 2.2.6 Behaviour Testing

A battery of tests assessing sexual and aggressive behaviours was conducted on the days following a 1-day rest period of ending oral gavage. Behavioural testing took place between 9 am and 4 pm on two separate days, with the olfactory preference test on the morning of the first day. Resident-Intruder and the sexual behaviour tests were counterbalanced between the afternoon of day 1 (i.e., separated by 2 -3 hours) and day 2. Mice were between 90 and 100 days of age and sexually naïve at the time of testing (see Experiment 1 for details on behaviour tests and dissections).

## 2.3 Statistical Analysis

Experiment 1 followed a 2 x 3 between-subjects design, and the Analysis of Variance (ANOVA) for sex (male and female) by treatment (Early ABX, Adult ABX and control) was conducted for somatic measures and behavioural tests. Specifically, these groups were compared on cecum weight, time spent investigating female bedding over male bedding on the olfactory

preference test, sexual and aggressive behaviours during the Resident-Intruder paradigms in response to either stimulus male and female conspecifics, and for anxiety-related behaviours on the elevated plus-maze test. In Experiment 2, the effects of cecal microbiota transplant (CMT) and T treatment following antibiotic treatment were assessed for somatic measures and behavioural tests using ANOVA. Separate ANOVAs were conducted for the male-only (ABX, A+MC, A+FC, A+T and male control) and female-only ($A+MC_f$, $A+T_f$ and female control) groups. All significant omnibus effects were followed with Tukey post hoc analyses, and effect sizes were determined using partial eta squared ($\eta p^2$). Alpha was set at $p < .05$. Jamovi version 1.1.9.0 was used for all statistical analyses.

## 3.0 Results

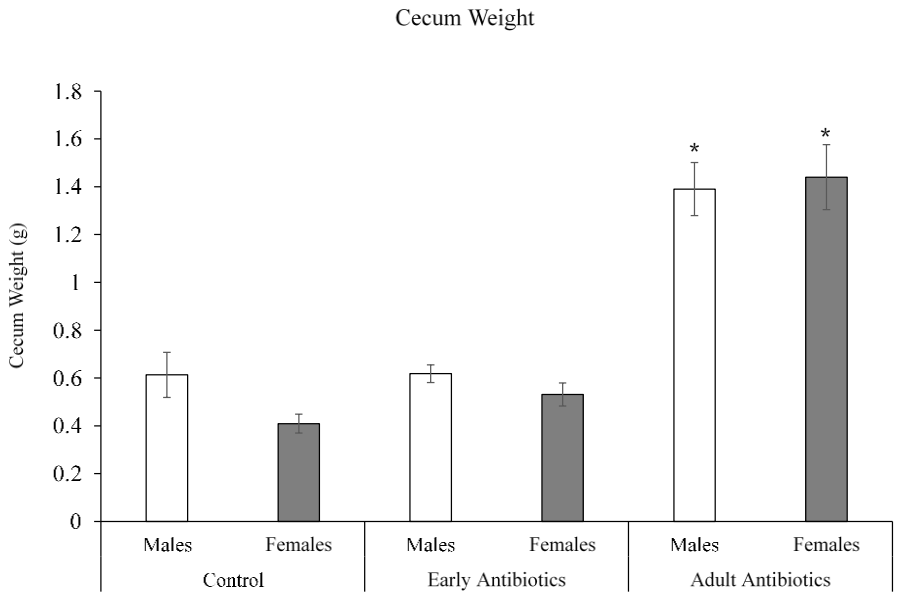
### 3.1 Experiment 1

#### 3.1.1 Cecum Enlargement: A Macroscopic Measure for Gut Microbiota Depletion

A main effect of antibiotic treatment was found for cecum weight, $F(2, 67) = 70.89$, $p < .001$, $\eta p^2 = .679$, suggesting that antibiotic treatment induced an enlargement of the cecum. Post hoc analyses showed that Adult ABX mice had a significantly higher cecum weight compared to Early ABX and controls ($p < .001$), as seen in Figure 2. Early ABX mice did not differ from controls ($p > .05$). There were no significant effects of sex, $F(1, 67) = 1.27$, $p = .264$, $\eta p^2 = .019$ or sex by treatment interaction, $F(2, 67) = 1.05$, $p = .357$, $\eta p^2 = .030$ on cecum weight. Enlarged and heavier ceca are a macroscopic sign associated with gut microbiota depletion (Reikvam et al., 2011), suggesting the antibiotic treatment was effective in depleting the gut microbiota in adulthood, while those with early ABX display evidence of a recolonized microbiome.

**Figure 2**

*Cecum weight by treatment condition (Mean +/- Standard Error of the Mean [SEM]).*




*Note.* Cecum weight is significantly heavier in the Adult ABX condition compared to Early ABX and control conditions. * = $p < .001$;*Error bars = +/-SEM.*

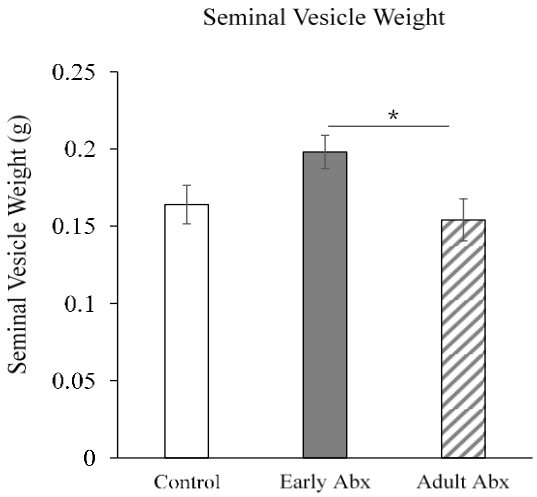
### 3.1.2 Somatic Measures

The expected sex difference was found on body weight, $F(1, 68) = 65.445$, $p < .001$, $\eta p^2 = .490$, with males weighing more than females. Body weight did not significantly differ by ABX condition, $F(2, 68) = .977$, $p = .382$, $\eta p^2 = .028$, nor was there a significant interaction of sex by treatment, $F(2, 68) = 1.518$, $p = .226$, $\eta p^2 = .043$ (see Table 2 for means and SEM). One subject (ID#472) was identified as an outlier in body weight (i.e. exceeded two standard deviations above the mean), and thus was excluded from all further analyses.

Overall, the results suggest that gut microbiome depletion may affect androgen-sensitive organs; males across antibiotic treatment conditions differed in seminal vesicle weight, $F(2, 40) = 4.02$, $p = .026$, $\eta p^2 = .167$. While control males did not differ from either Early or Adult ABX conditions, $p = .149$ and $p = .839$ respectively, a significant increase in seminal vesicle weight was found in Early ABX males compared to Adult ABX males ($p = .027$; see Figure 3). However, depleting the gut microbiota in either early development or adulthood did not affect gonad weight ( $p > .05$). Similarly, the ovary and uterine horns in the antibiotic-treated females were comparable to control females ( $p > .05$).

**Figure 3**

*Seminal vesicle weight by treatment condition (Mean +/- SEM)*




*Note.* Seminal vesicle weight is significantly higher in the Early ABX males compared to the Adult ABX males, * $p < .05$.

**Table 2**

*Somatic measures at the time of dissection: Mean (SEM)*

| | | Bodyweight (g) | Gonads weight (g) |
|---|---|---|---|
| Early ABX | Male | 26.06 (0.586) | 0.139 (0.004) |
| | Female | 21.50 (0.933) | 0.097 (0.013) |
| Adult ABX | Male | 26.36 (0.372) | 0.136 (0.006) |
| | Female | 23.00 (0.447) | 0.110 (0.012) |
| Controls | Male | 26.60 (0.733) | 0.153 (0.004) |
| | Female | 21.36 (0.565) | 0.096 (0.011) |

*Note.* Statistical differences were found between males and female mice on measures of body weight and gonad weight.

### 3.1.3 Antibiotic Treatment Altered Sexual Odor Preference in Male Mice

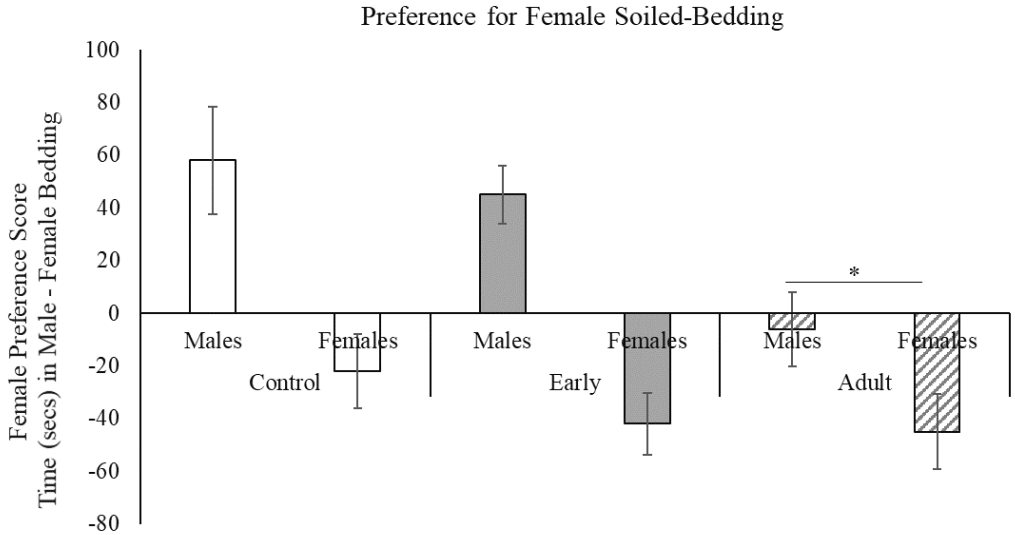
The expected sex effect was found on the female preference score, $F(1, 69) = 38.43$, $p < .001$, $\eta p^2 = .358$, such that males displayed a greater preference than females for female-soiled bedding ($p < .001$). Interestingly, antibiotic treatment significantly affected female preference score, $F(2, 69) = 5.44$, $p = .006$, $\eta p^2 = .136$, such that the Adult ABX mice showed a significant decrease in female preference score compared to the Early ABX and control mice, $p = .046$ and $p = .007$, respectively (see Figure 4A). Given our *a priori* predictions regarding sex and antibiotic treatment, further analyses were conducted on time spent in each soiled bedding (i.e., male-soiled, female-soiled and neutral bedding) to explore whether Adult ABX males or females were driving the main effect of antibiotic treatment on the female preference score. The Adult ABX

males did not differ from Early ABX and control males on time spent investigating female-soiled, $F(2, 40) = 2.37$, $p = .107$, $\eta p^2 = .106$, or neutral bedding, $F(2, 40) = 2.19$, $p = .125$, $\eta p^2 = .099$, respectively. However, on time spent investigating male-soiled bedding, males differed by antibiotic treatment, $F(2, 40) = 5.33$, $p = .009$, $\eta p^2 = .210$, such that Adult ABX males spent more time in the male-soiled bedding compared to Early ABX males ( $p = .056$) and control males ($p = .007$) (see Figure 4B). Females did not differ by ABX treatment in time spent investigating male, female or neutral bedding ($p < .05$). Altogether, these results suggest that Adult ABX males show a decrease in female sexual odor preferences on the olfactory preference test that is driven by an increase in the investigation of male-soiled bedding rather than a decrease investigation of female-soiled bedding.

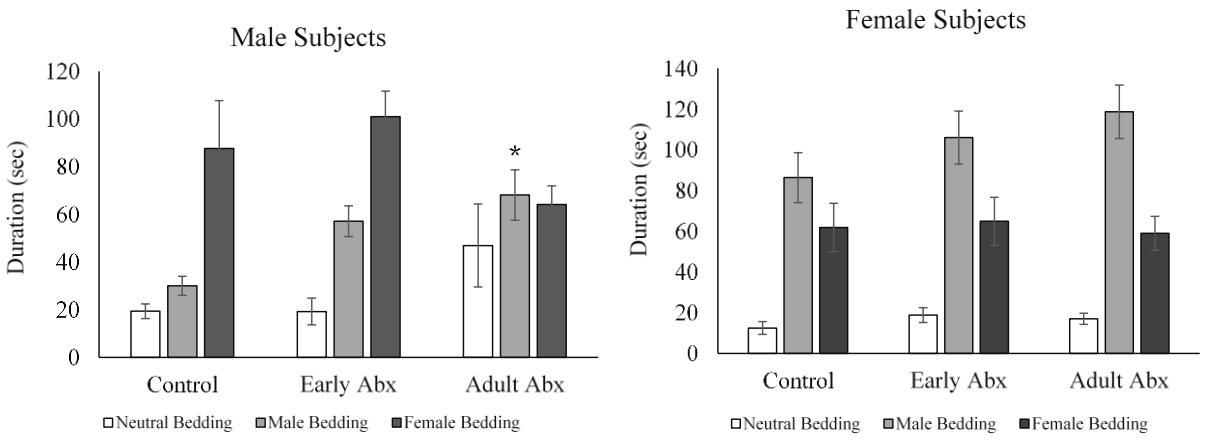
**Figure 4**

*Mean female preference score and Time spent investigating bedding type (Mean +/- SEM)*

**A.** Female Preference Score



**B.** Time Spent in Bedding Type




*Note.* **A.** Adult ABX males displayed decreased female preference scores compared to control and Early ABX males. * indicates a significant difference from control and early ABX groups, $p < .05$. **B.** Duration of time spent investigating male-soiled, female-soiled, and neutral bedding for male and female subjects, +/- SEM. Adult ABX mice differed significantly from control mice in their investigation of male bedding. Adult ABX males spent a comparable duration of time investigating male, female, and neutral bedding. * indicates a significant difference between control and early ABX males, $p < .05$.

### 3.1.4 Anogenital and Face/Body Investigation of Estrus Female and Male Intruders

The frequency and duration of anogenital investigation of an estrus female intruder did not differ by sex, ABX treatment or sex by treatment (all $ps \geq .1$). A main effect of sex was found for the number of face/body investigations of a female intruder, such that female mice performed this behaviour more than male mice, $F(1, 68) = 8.55$, $p = .005$, $\eta p^2 = .112$; no antibiotic treatment or sex by treatment effects were found for this measure, $F(2, 68) = 1.60$, $p = .211$, $\eta p^2 = .045$ and $F(2, 68) = .074$, $p = .929$, $\eta p^2 = .002$, respectively. No significant effects of

sex, ABX treatment or sex by treatment interaction were found for the duration of face/body investigation of a female intruder (all *ps* ≥ .1).

In response to a male intruder, no significant effects of sex, ABX treatment or sex by treatment interaction were found for the number of anogenital investigations ($p > .05$). A main effect of sex, however, was found for the duration of anogenital investigations such that males investigated the anogenital region of male intruders for a longer duration than females, $F(1, 68) = 11.77$, $p = .001$, $\eta p^2 = .148$. The main effect of antibiotic treatment or sex by treatment interaction were not significant for this measure, $F(2, 68) = .15$, $p = .865$, $\eta p^2 = .004$ and $F(2, 68) = .17$, $p = .841$, $\eta p^2 = .005$, respectively. An effect of antibiotic treatment was found for the number of face/body investigation of a male intruder, $F(2, 68) = 3.56$, $p = .034$, $\eta p^2 = .095$, such that Adult ABX mice investigated male intruders more than Early ABX and control mice ($p < .05$); Early ABX did not differ from controls on this measure. No effects of sex or sex by antibiotic interaction were found on this measure, $F(1, 68) = 1.54$, $p = .219$, $\eta p^2 = .022$ and $F(2, 68) = .76$, $p = .472$, $\eta p^2 = .022$, respectively. No significant sex, antibiotic treatment or sex by treatment effects were found for the duration of face/body investigation of a male intruder (all *ps* > .05), (see Table A1 in Appendix A for means and SEM).

### 3.1.5 Sexual Responses to Estrus Female and Male Intruders Remained Unaltered

Overall, sexual behaviours in response to an estrus female intruder were not affected by the gut microbiome depletion via antibiotic treatment. The expected main effect of sex was found such that male mice mounted, thrust, and intromitted more than female mice in response to an estrus-induced female intruder (all *ps* < .001). Similarly, resident males showed a lower latency to mount female estrus intruders than female residents ($p < .001$). No significant effects of sex, AB treatment or sex by AB treatment were found for sexual behaviours in response to a

male intruder (all *ps* > .1) (see Table A1 in Appendix A for sexual behaviours in response to a male intruder).

### 3.1.6 Antibiotic Treatment in Early Development and Adulthood Decreased Male Aggression

Aggressive behaviours were observed in response to male intruders and not towards female intruders, as expected. Main effects of sex were found for the occurrence and duration of aggressive acts and the latency to aggress a male intruder (all *ps* < .05). Specifically, male mice displayed more chasing, attacks, tumbling, boxing and biting, and performed these behaviours for longer durations than female mice. There were no main effects of antibiotic treatment for the frequency and duration of aggressive behaviours (all *ps* > .05), except for latency to aggress, $F(2, 68) = 3.39$, $p = .039$, $\eta p^2 = .091$. Early ABX males showed an equivalent latency to aggress compared to the Adult ABX and control males (all *ps* > .1); however, Adult ABX mice showed a higher latency to aggress compared to control males ($p = .044$; see Figure 5).

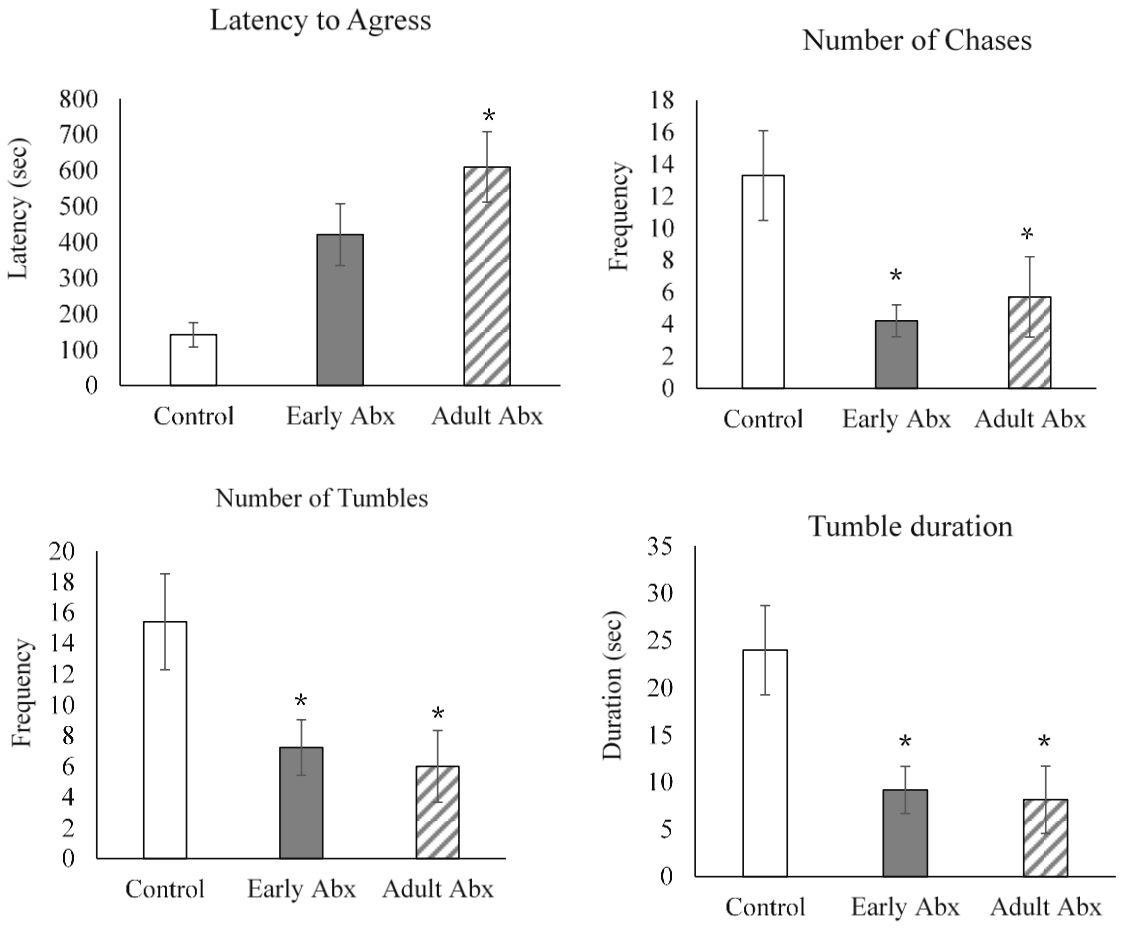
Interestingly, significant interactions of sex and antibiotic treatment were found for chasing frequency, $F(2, 68) = 4.29$, $p = .018$, $\eta p^2 = .112$; tumbling frequency, $F(2, 68) = 3.57$, $p = .034$, $\eta p^2 = .095$; tumbling duration, $F(2, 68) = 4.68$, $p = .012$, $\eta p^2 = .123$ and the latency to aggress, $F(2, 68) = 3.50$, $p = .036$, $\eta p^2 = .093$. Specifically, both Early and Adult ABX males displayed lower levels of these aggressive behaviours than control males (all $p < .05$), with the exception of latency to aggress – only Adult ABX males had a higher latency to aggress compared to both controls and Early ABX males on this measure (see Figure 5).

In comparison to female groups, control males displayed significantly more aggression on these measures (*ps* < .001), but both Early and Adult ABX males did not differ significantly from control or ABX females (all *ps* > .1). No effects of antibiotic treatment were found for

biting or boxing in response to a male intruder, although it should be noted that these behaviours were rarely observed in all groups (see Table A1 in Appendix A for means and SEM).

**Figure 5**

*Aggressive behaviours in response to a male intruder (Mean +/- SEM)*




*Note.* Early and Adult ABX resulted in a significant decrease in inter-male aggressive behaviours compared to control males. The frequency and duration of aggressive bouts (i.e. chasing bouts, and tumble bouts) decreased in both the Adult and Early ABX compared with control males, while Adult ABX had a greater latency to aggress compared to both control and Early ABX males * indicates $p < .05$.

### 3.1.7 **Anxiety-like behaviours Were Not Affected by Antibiotic Treatment**

No significant effects of sex, antibiotic treatment or sex by treatment interactions were found on anxiety-like behaviours on the EPM. Specifically, there was no difference in the total distance travelled (all *ps* > .1). There were also no significant effects of sex, $F(1, 68) < .001$, $p = .977$ , $\eta p^2 = .000$, antibiotic treatment, $F(2, 68) = 1.95$, $p = .150$ , $\eta p^2 = .054$ or sex by treatment interaction, $F(2, 68) = .22$, $p = .804$ , $\eta p^2 = .006$ on the ratio of time spent on the open arms to the time spent on the closed arms. Likewise, there were no significant effects of sex, antibiotic treatment or sex by treatment interaction on the ratio of the open arm to close arm entries, duration in the closed arm, duration and frequency of entry in open arm, the percent difference in the open and closed arms, and percent duration in the closed arm and open arms (all *ps* > .1). However, there was a significant effect of sex for frequency in the closed arm, such that females retreated to the closed arms more than males, $F(1, 68) = 7.18$, $p = .009$, $\eta p^2 = .096$ (see Table A1 in Appendix A for means and SEM).

## 3.2 Experiment 2

### 3.2.1 Cecal Microbiota Transplantation (CMT) Reverses Antibiotic-Induced Cecum Enlargement

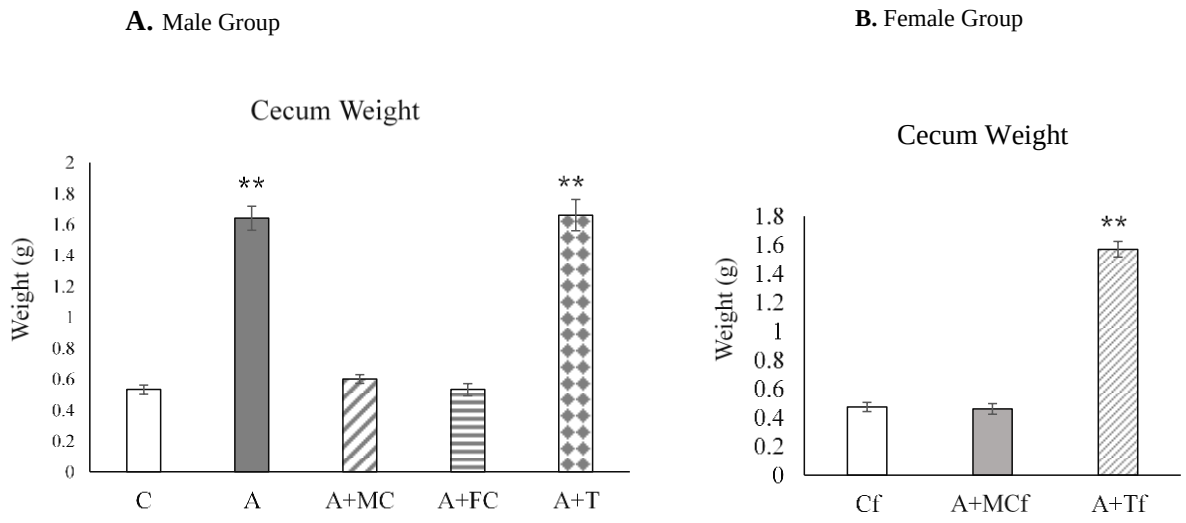
Among male groups, a significant effect of treatment was found for cecum weight, $F(4, 52) = 87.56$, $p < .001$, $\eta p^2 = .871$, such that the ceca of ABX and A+T male mice weighed more than A+MC, A+FC and control males (all *ps* < .001). As expected, both A+MC and A+FC groups did not differ from controls on cecum weight (all *ps* >.5), providing a macroscopic sign for successful recolonization of the depleted microbiota (see Figure 6A).

Likewise, in the female group, a significant effect of treatment was found for cecum weight, $F(2, 29) = 226.19$, $p < .001$, $\eta p^2 = .940$, such that a heavier cecum was found in A+$T_f$

females compared to A+$MC_f$ and controls ($p < .001$). Again, no significant difference was found between A+$MC_f$ and control females ($p > .5$), suggesting male microbiota successfully recolonized depleted microbiota in female recipients (see Figure 6B).

**Figure 6**

*Cecum weight by treatment condition (Mean +/- SEM)*




*Note.* **A.** Antibiotic treatment resulted in a heavier cecum weight among A and A+T treated groups. Both male and female cecal transplants decreased the cecum weight to control levels. **B.** The female group shows a significant increase in cecum weight in the A+$T_f$ condition compared to the A+$MC_f$ and controls. ** indicates $p < .001$.

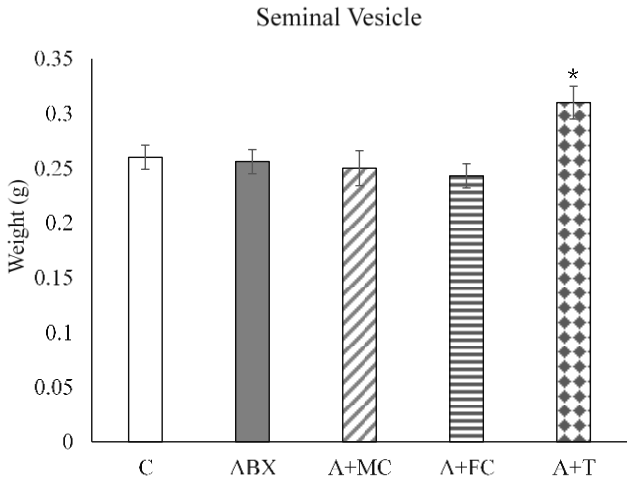
### 3.2.2 Somatic Measures

Overall, cecal transplantation or T treatment following antibiotic treatment did not affect body weight, $F(4, 52) = .74$, $p = .571$, $\eta p^2 = .054$, or testes weight, $F(4, 52) = 1.66$, $p = .173$, $\eta p^2 = .113$ among the male subjects. There was however, an effect of treatment condition on seminal vesicle weight, $F(4, 52) = 4.08$, $p = .006$, $\eta p^2 = .239$, such that A+T resulted in increased seminal vesicle weight compared to ABX, A+MC and A+FC (all *ps* $< .05$) (see Figure 7). Controls did

not significantly differ from A+T on this androgen-sensitive organ ($p$ = .064). Likewise, males with ABX only and cecal transplant (A+MC and A+FC) did not differ from controls on seminal vesicle weight ($p$ >.05).

**Figure 7**

*Seminal vesicle weight by treatment condition (Mean +/- SEM)*




*Note.* Increased seminal weight was found for the A+T condition compared to ABX, A+MC and A+FC males. A+T marginally differed from controls ($p$ = .064). * indicates a significant difference from ABX, A+MC and A+FC, $p$ < .05.

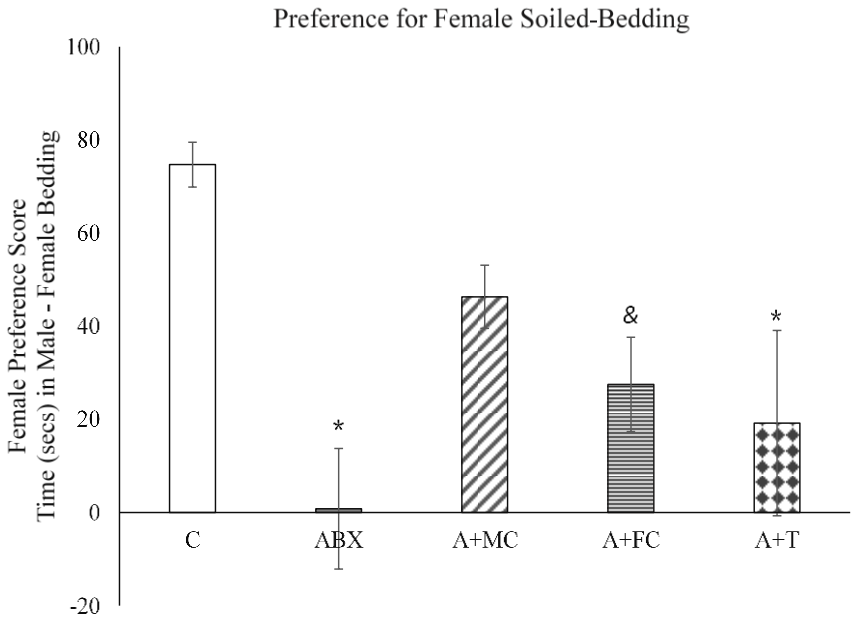
### 3.2.3 Male Microbiota Reverses Antibiotic-Induced Olfactory Preference in Male Recipients

Among the male groups, a significant effect of treatment condition was found on female preference score, $F(4, 52) = 5.63$, $p < .001$, $\eta p^2 = .302$, such that ABX males showed a decreased female preference score compared to A+MC and control males, $p$ = .049 and $p$ < .001, respectively. ABX males did not differ from A+FC or A+T (all $ps$ >.05). Interestingly, while

A+MC and A+FC were comparable to controls (*ps* >.05), A+T showed a significant decrease in the female preference score than controls, $p = .015$. All mice, regardless of condition, showed a preference for soiled bedding over clean bedding, even though the ABX condition spent more time in clean bedding compared to all other groups (see Figure B2 in Appendix B for time spent in each bedding type). The result suggests that T treatment was not sufficient to rescue antibiotic-induced olfactory preference phenotype in males. Male microbiota restored olfactory preference, whereas female microbiota partially did so, such that A+FC males were intermediate in that they did not differ from ABX or controls in their female preference scores (Figure 8).

**Figure 8**

*Mean female preference score +/- SE*




*Note.* The female preference score was calculated by subtracting the duration of time spent in female bedding from the duration of time spent in male bedding. ABX and A+T males displayed a decreased female preference score than controls and A+MC. A+FC were intermediate in that

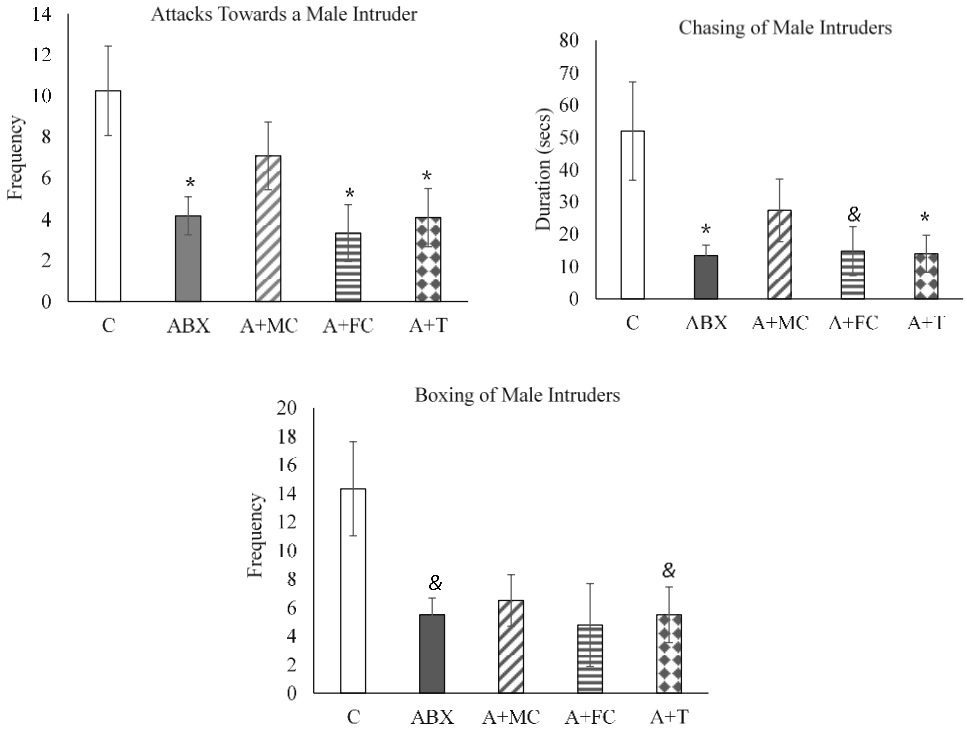
they did not differ from controls or ABX and A+T males. * indicates a significant difference from controls, & indicates the marginal difference from controls, $p < .1$

### 3.2.4 Male, But Not Female Microbiota Restores Aggression Phenotype in Male Recipients

In response to male intruders, a significant effect of treatment condition was found for aggressive behaviours. Specifically, treatment effects were found on the number of attacks, $F(4, 52) = 3.28$, $p = .018$, $\eta p^2 = .202$, frequency of boxing, $F(4, 52) = 3.02$, $p = .026$, $\eta p^2 = .189$ and duration of chasing, $F(4, 52) = 3.15$, $p = .022$, $\eta p^2 = .195$. Post hoc analyses showed a decrease in these aggressive bouts in ABX, A+T and A+FC conditions compared to control males (all *ps* $\leq .05$). Interestingly, A+MC did not differ from controls ($p > .05$), indicating the rescue of aggressive behaviour in this group. However, A+MC males did not significantly differ from ABX, A+T or A+FC conditions on these aggressive behaviours ($p > .05$) (see Figure 9). No significant effect of treatment condition was found for latency to aggress, frequency of tumbling and number of bites ($p > .05$), although it should be noted that tumbling and biting behaviours were rarely observed in all groups (i.e., only 13 animals showed any biting). These results suggest that male microbiota partially restored male aggression, while T treatment and female microbiota were insufficient to rescue the male-typical aggression phenotype in antibiotic-treated males.

**Figure 9**

*Frequency and duration of aggression bouts (Mean +/- SEM)*




*Note.* Significant effects were found for aggressive bouts' frequency and duration (i.e. chases, attacks and boxes). ABX, A+FC and A+T showed decreased aggression compared to control males. A+MC did not differ from controls, * $p < .05$ and & $p < .1$

### 3.2.5 Sexual Behaviours Remained Unaltered Following CMT and Testosterone Treatment in Male Mice

Sexual behaviours were found in response to female intruders, but none were observed in response to male intruders. No significant effects of treatment conditions were found among the male groups for sexual behaviour. Specifically, no significant effect was found in the latency to mount or in frequency and duration of mounting, thrusting and intromission (all *ps* > .05).

Likewise, investigative behaviours towards an estrus-induced female or urine-swabbed male intruders remained unaltered in all conditions. Specifically, no significant effect of treatment condition for the frequency and duration of both anogenital and face/body investigation were found (all *ps* > .1) (see Table B1 in Appendix B for all sexual behaviours).

### 3.2.6 Male Microbiota Partially Induced Lower Body Weight in Antibiotic-treated Females

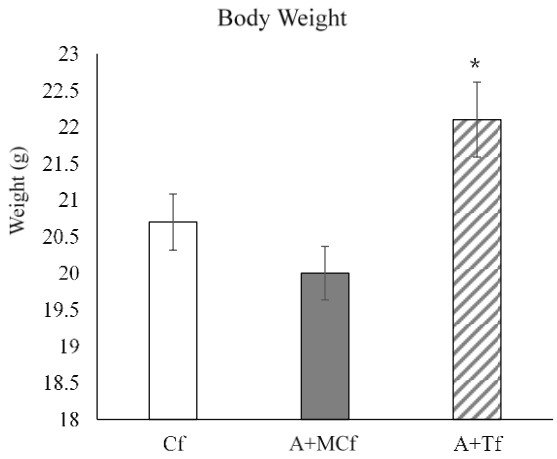
Among the female groups, a significant effect of treatment on body weight was found, $F(2, 29) = 6.07$, $p = .006$, $\eta p^2 = .295$, such that female mice that received male microbiota showed a lower body weight compared to $A+T_f$ ($p = .005$); Both $A+MC_f$ and $A+T_f$, however, did not differ from controls on body weight, $p = .471$ and $p = .075$, respectively (see Figure 10). Ovary weight did not significantly differ by treatment condition, $F(2, 29) = .53$, $p = .594$, $\eta p^2 = .035$.

No significant effect of treatment was found on female preference score, $F(2, 29) = .03$, $p = .972$, $\eta p^2 = .002$; suggesting that male cecal transplant or T treatment following antibiotic treatment did not alter female olfactory preference (See Table 3 for means and SEM).

Sexual behaviours remained unaltered, though there was a trend for mounting, such that the $A+T_f$ condition mounted estrus-induced female intruders more than $A+MC_f$ and controls, $p = .075$; this finding suggests that the T treatment was sufficient to partially masculinize behaviours in females following antibiotic treatment. No differences were found between treatment conditions for aggressive behaviours among the female groups. Aggression was rarely observed except for three females with T treatment (all *ps* > .5).

**Figure 10**

*Bodyweight by treatment condition (Mean +/- SEM*




*Note.* Decreased body weight was found for the A+$MC_f$ condition compared to A+$T_f$, but neither group differed from controls. * indicates a significant difference from A+$MC_f$ females, $p < .05$.

### 3.2.7 Social Investigation of Estrus Female and Male Intruders

Among the female groups, a significant effect of treatment was found for the duration of anogenital investigation of a female intruder, $F(2, 29) = 4.42$, $p = .021$, $\eta p^2 = .234$. Post hoc tests revealed that A+$T_f$ females spent more time investigating female intruders' anogenital regions than A+$MC_f$ and control females, $p = .046$ and $p = .036$, respectively; controls and A+$MC_f$ females did not differ on this measure. No significant effects were found for the frequency of anogenital investigation and frequency and duration of face/body investigation (all *ps* > .1).

In response to a male intruder, no significant effect of treatment condition was found for frequency and duration of anogenital investigation (all *ps* > .1). Conversely, significant effects were found for the frequency, $F(2, 29) = 6.17$, $p = .006$, $\eta p^2 = .299$ and duration, $F(2, 29) = 4.29$, $p = .023$, $\eta p^2 = .228$ of face/body investigation in response to a male intruder. Post hoc analyses

showed that A+$MC_f$ investigated male intruders more than A+$T_f$ and control females (all *ps* < .05). The A+$T_f$ investigated male intruders less than control females ($p = .034$). These results suggest that while male microbiota increased male-investigation behaviour in females, T treatment decreased this behaviour (See Table 3 for means and SEM).

**Table 3**

*Means (SEM) for female group's olfactory preference and social investigative behaviours*

| | C ($n = 11$) | A+$MC_f$ ($n = 10$) | A+$T_f$ ($n = 11$) |
|---|---|---|---|
| Female preference score | -39.43 (5.69) | -38.37 (14.80) | -34.33 (23.05) |
| Anogenital investigation duration (female intruder) | 43.43 (8.59) | 44.59 (8.09) | 102.14 (24.51) |
| Face/body investigation duration (male intruder) | 198.86 (23.54) | 231.36 (36.86) | 106.78 (32.72) |
| Face/body investigation frequency (male intruder) | 25.36 (3.74) | 28.50 (3.24) | 14.36 (1.61) |

*Note.* Statistical differences between treatment groups were not found for the female preference score. Significant differences between treatment conditions were found for investigative behaviours towards male and female intruders: A+$MC_f$ females displayed an increased face/body investigation of a male intruder compared to A+$T_f$ and control. In response to a female intruder, the A+Tf females showed an increased anogenital investigation compared to A+$MC_f$ and control females, $p < .05$.

## 4.0 Discussion

The present study explored whether gut microbiota depletion affects socio-sexual behaviours in mice and whether sex-dependent effects of the gut microbiome on behaviour are a result of gut microbiome interactions with androgen signalling. Two experimental designs were used to address these research questions. In Experiment 1, I investigated whether depletion of the microbiota via broad-spectrum antibiotic treatment in early development and adulthood would alter sex-typical socio-sexual behaviours. Depletion of the male gut microbiota in adulthood resulted in decreased sexual odor preferences and decreased territorial aggression in male mice, while the behaviour of female mice remained unaltered. Similar to adult depletion of the microbiota, depletion in early development also impeded inter-male aggression, although sexual odor preferences remained unchanged. Anxiety and sexual behaviours towards male and female stimulus animals remained unaltered in all groups. Experiment 2 assessed whether replenishing the gut microbiota or androgen treatment in adult antibiotic-treated males was sufficient to restore male-typical socio-sexual behaviours. Microbiota transplantation with male cecum contents into antibiotic-treated male recipients restored sexual odor preferences, while microbiota transplant with female cecum contents partially restored this behaviour. For aggression, male but not female microbiota partially restored aggression in gut-depleted male mice. T treatment was not sufficient to rescue either olfactory preference or aggression in antibiotic-treated males. Finally, male microbiota transplant in adult antibiotic-treated female recipients did not alter socio-sexual behaviours. Instead, T treatment increased male-typical sexual behaviours in antibiotic-treated females. Together, these results suggest that antibiotic treatment has a lasting sex-dependent effect on mice, and having a male-typical gut microbiome composition is required for the full display of sex-typical behaviours in male mice. These

findings also suggest that these effects of the gut microbiome on male-typical behaviour are independent of androgens.

### 4.1 Mechanism for the Gut Microbiome's Mediation on Socio-sexual Behaviours

#### 4.1.1 Microbiota Depletion and Olfactory Preference

On the olfactory preference test, antibiotic treatment in adulthood altered preference for sexual odor cues in males. Specifically, adult antibiotic-treated males spent comparable time investigating male and female-soiled bedding, contrary to the male-typical preference for female-soiled bedding (Bodo & Rissman, 2007). In mice, preference for opposite-sex odors is largely dependent on sex hormone action within the neural pathways responsible for sexual behaviour (Schellino et al., 2016). For example, both males and females exhibit an increase in neural activity along the AOS in response to opposite-sex odors. Gonadectomy decreases preference for opposite-sex odors and neural activity along this neural circuit (Bodo & Rissman, 2007). Given prior research linking the gut microbiome and androgens, decreased androgens levels were hypothesized to account for the altered olfactory preference in adult antibiotic-treated males. However, in Experiment 2, T treatment in adult antibiotic-treated males was not sufficient to restore male-typical sexual odor preferences. Another possible explanation for the altered olfactory preference is that the gut microbiome may impact sexual odor preferences independent of sex hormones. For example, a thinner olfactory cilia layer and decreased cellular level transduction have been reported in GF mice compared to conventional mice with a healthy microbiome (François et al., 2016). Hence, future work may investigate the olfactory epithelium and vomeronasal organs of antibiotic-treated mice. Alternatively, antibiotics may affect other aspects of the animal, such as anxiety or depression, that could impact their interest in sexual stimuli; however, this seems unlikely in the current study given that ABX males showed levels

of sexual behaviour consistent with control males. Thus, these findings suggest that the decrease in olfactory preference may not be indicative of a general decrease in sexual interest and thus may be more related to odor or pheromone processing. Of note, time spent in neutral/clean bedding did not differ from time spent in female or male soiled-bedding in ABX males, suggesting deficits in odor discrimination. However, in Experiment 2, ABX males show greater time in male and female soiled bedding compared to clean bedding, suggesting that the deficit is more so associated with decreases in female bedding compared to controls rather than deficits in odor discrimination.

### 4.1.2 Gut Microbiota Depletion and Male Aggression

The results of the present studies, that antibiotic treatment in adulthood decreases inter-male aggression, are consistent with previous studies identifying a critical role for the gut microbiome in mediating the display of male aggression. For example, Sylvia et al. (2017) report decreased aggression in male and female Siberian hamsters following adult antibiotic treatment. In their study, two antibiotic treatments (each of 7 days) separated by a recovery phase caused a decrease in male aggression. We did not assess whether 7 days is sufficient to affect aggressive behaviour; however, 14-20 days of antibiotic treatment similarly decreased male aggression in our study. Sylvia et al. (2017) is the only rodent study available for reference, although studies in dogs suggest that the abundance of specific gut bacteria, including *Lactobacillus* and *Dorea*, are associated with aggressive behaviours (Kirchoff et al., 2019). Thus, future work may consider whether these bacteria also play a role in the display of male-typical aggression in mice.

I hypothesized that one way that the gut microbiome may be mediating aggressive behaviours is via the endocrine system. Namely, the gut microbe *Clostridium scindens* can produce androgens from glucocorticoids (Ridlon et al., 2013), and socio-sexual behaviours in

males is dependent upon androgens (e.g., Arnold, 2006; Ogawa et al., 1998); thus, depletion of the gut microbiome may result in a decrease in androgen signalling. In Experiment 2, I tested this hypothesis. The higher seminal vesicle weights in T-treated compared to cecal transplant males suggest that the T-treated group had higher circulating androgens. However, T treatment in antibiotic-treated males did not restore aggressive behaviours, suggesting that the role of the gut microbiome in aggressive behaviour in mice is independent of androgens. It is still possible that antibiotic treatment may have masked T's effect on behaviour, hence the insufficiency to restore male olfactory preference and aggression. For example, antibiotics may inhibit the abundance of circulating androgens and/or receptors, and simply increasing circulating androgens may not be sufficient to overcome these effects of the antibiotics. However, it is unlikely that the antibiotics could completely mask T's effects, as T in antibiotic-treated females increased male-typical socio-sexual behaviours, as detailed below (see section 4.3). Furthermore, this possible masking effect of antibiotics also seems unlikely in males, given the responsiveness of the seminal vesicles to the androgen treatment. Hence, these findings suggest a role of the gut microbiome in mediating socio-sexual behaviours that is independent of androgen signalling.

In the current study, cecal microbiota transplant from male and female untreated mice to antibiotic-treated male mice successfully recolonized the gut microbiota. This was evident via observation of cecum weight. An enlarged and heavier cecum is evident when the microbiota is depleted due to an increase in water content and disruption of the fermentation process (Reikvam et al., 2011); conversely, re-colonization of the gut in antibiotic-treated animals via microbiota transplantation results in a lower cecum weight comparable to untreated animals, suggesting the restoration of the microbial environment in these animals (Courtney, 2000; Savage & Dubos, 1968). Consistent with this prior work, control mice and recipients of either male or female

microbiota following antibiotic treatment did not differ in cecal weight, while antibiotic-treated mice had 2-3X larger cecum. These results provide a macroscopic sign of microbiota depletion in antibiotic-treated mice and microbiota recolonization in mice that received microbiota transplantation. This is consistent with prior work indicating that fecal microbiota transplantation restores the microbiota diversity and composition following antibiotic treatment (Le Bastard et al., 2018; Schmidt et al., 2020).

Indeed, previous studies using cecal/fecal transplantation have reported a reversal of psychiatric-like symptoms (e.g., depression and anxiety) in rodent models following gut dysbiosis (Chinna et al., 2020; Kelly et al., 2016; Zheng et al., 2016). Consistently, cecal microbiota transplantation reversed the antibiotic-induced behaviour changes in male mice in the current study. Thus, it provides evidence for gut microbes' direct role in mediating socio-sexual behaviours via a different mechanism (e.g., direct action via brain or immune response) than originally hypothesized (i.e., androgen). Specifically, male microbiota was sufficient to restore olfactory preference while partially restoring aggression in male recipients in the current study. Female microbiota only partially restored male olfactory preference and not aggression. These results suggest that sex differences in the gut microbiome contribute to sex differences in behaviour. Thus, it is possible that the male microbiota may contain a high abundance of specific microbes associated with male-typical olfactory preference and territorial aggression, as found in dogs (Kirchoff et al., 2019), and thus when transplanted into ABX males, reversed the antibiotic-induced behaviour changes. Alternatively, the absence of microbiota and/or a female-specific microbiota composition may decrease aggressive behaviours in mice. Future work investigating the microbiome composition of mice in the present work will shed light on which microbes may be responsible for the observed behavioural changes.

Another possibility is that sex-specific differences arising from the presence of X or Y chromosomes and/or sex hormones may explain the female microbiota's insufficiency to restore male recipients' behaviours. For example, Yurkovetskiy et al. (2013) report that irrespective of the sex of the donor, the gut microbiota, after puberty, distinctly segregates according to the sex of the recipient. In other words, male and female mice intestines accommodate microbiota differently (Wang et al., 2016); hence, the effects of female microbiota on male recipients may be compromised due to physiological sex differences. Moreover, sex differences in the immune system also impact microbiota transplantation. For example, males have a higher percentage of regulatory T cells irrespective of whether they received the microbiota from male or female mice (Elderman et al., 2018; Fransen et al., 2017). Thus, transplanting either male or female microbiota to sex-matched or opposite-sex mice may produce different behavioural changes. Further work is required to investigate these complex relationships between the gut, immune and endocrine systems to understand the mechanisms underlying the behavioural effects observed in the present study.

### 4.2 Maternal Antibiotic Exposure has Enduring Behavioural Effects in Offspring

The present results indicate that gut depletion in early development decreased adult male aggression, suggesting a long-lasting impact of early antibiotics on behaviour. The early antibiotic treatment was through maternal exposure to antibiotics from gestational day 16 to postnatal day 21. Notably, maternal gut microbiota during pregnancy or breastfeeding plays a crucial role in defining and shaping infant gut microbiota (Gonzalez-Perez et al., 2016; Nyangahu et al., 2018). Maternal antibiotic treatment during gestation has shown profound alterations in the composition of the GIT microbiota and immunity in offspring, translating into behavioural deficits both in early life and adulthood (Nyangahu et al., 2018; O'Connor et al.,

2021). It, thus, suggests that the early antibiotic group in the current study had an altered gut microbiota acquired indirectly via maternal dysbiosis (i.e., lack of microbiota transfer during gestation/delivery and lactation). Indeed, antibiotics, including ampicillin and vancomycin, have poor oral bioavailability and BBB permeability and, therefore, are unlikely to impact offspring microbiota directly or exert a direct effect on the brain (Grayson et al., 2010; Nyangahu et al., 2018). Fecal samples in neonatal development were acquired from mice in the Early ABX group and will be processed at a later date to assess whether the gut microbiome of Early ABX was depleted compared to controls in the present study.

The decreased aggression in the early antibiotic-treated males was somewhat surprising as they had nearly 50 days for the microbiota to regenerate (i.e., the ceca weight, a macroscopic sign for gut depletion, did not differ from control mice). This is, however, consistent with O'Connor et al. (2021), who observed persistent alterations in anxiety, sociability, and cognitive behaviours at seven weeks post-maternal antibiotic exposure (adulthood) even though cecal weight/ microbiota regeneration was not monitored in their study. The authors speculated a direct effect of the antibiotic used in their study (i.e., metronidazole) on the brain and behaviour as the drug crosses the BBB. Although the antibiotics (ampicillin, vancomycin, neomycin, gentamicin and erythromycin) used in the current study do not easily cross the BBB, we cannot rule out the possibility that traces may be absorbed and directly alter brain and behaviour. Alternatively, it is possible that early antibiotic treatment had long-lasting effects on gut microbiome composition – while the cecum weight of Early ABX was similar to controls, it is possible that the diversity and/or ratio of microbes differs, and this could result in differences in behaviour; analyses of fecal samples at dissection will help to address this possibility. Another possible explanation for the decreased aggression could be altered maternal care (e.g., decreased maternal licking and

grooming). Maternal care is known to alter offspring behaviour later in life (Lui et al., 2000), and it is possible that maternal antibiotic exposure altered the maternal gut microbiota and behaviour (Nyangahu et al., 2018). It will be of interest to explore antibiotic exposure on maternal behaviour in future work.

An increased seminal vesicle weight was found in the early antibiotic-treated males compared to adult-treated males, consistent with findings of Collden et al. (2019), where both seminal vesicle weight and androgen levels were elevated in GF mice, thus suggesting an increased circulating androgen level in the early-antibiotic treated males. Intuitively, a normal display of aggression would be expected, given a regenerated microbiota and increased seminal vesicle weight. It thus supports the idea that the decreased aggression is due to a lack of microbiota diversity when the microbiome regenerated, as reported with other behavioural deficits in response to antibiotic treatment (Desbonnet et al., 2015). Further research in the lab will assess the gut microbiome contents of early and adult antibiotic-treated males compared to controls to assess the diversity, or lack thereof, in these animals. T levels were not measured in the current study, and early antibiotic-treated males did not differ from controls in seminal vesicle weight; thus, it is still very speculative that these males exhibited an increase in circulating T levels. However, given the reported alterations in serum T levels in GF mice compared to control mice with normal microbiota, this warrants further investigation (Markle et al., 2013; Tung et al., 2017; Yurkovetskiy et al., 2013, except see Collden et al., 2019). Even though it remains unclear what mechanism is at play, the result suggests that gut microbiome depletion in early development (embryonic day 16 – PND 21) may have a long-lasting effect on the brain and behaviour. Replication of these findings and follow-up experiments with early ABX will be necessary.

While antibiotic treatment in both early development and adulthood altered male aggressive behaviours, antibiotic treatment did not affect aggression in female mice. This is in contrast to the results that Sylvia et al. (2017) reported in hamsters. In their study, a single antibiotic treatment (7 days) decreased females' aggression, whereas males only showed deficits in aggression following two 7-day treatments. However, these results may be attributable to species variation, as female Siberian hamsters tend to show higher rates of aggression than female mice (Scotti et al., 2008). The current study also used behavioural paradigms in which territorial aggression was measured, a type of aggression more typical of male mice. It will be of interest to further investigate the effect of gut microbiota depletion in female mice using paradigms where females typically show high aggression levels (e.g., maternal aggression tests).

### 4.3 Male Microbiota Transplantation and Testosterone Treatment in Antibiotic-treated Female Mice

Given the report of elevated serum T levels in GF female mice that received male microbiota (Markle et al., 2013), the current study tested whether microbiota transplantation with the male cecum and/or T treatment would be sufficient to induce male-typical behaviours in female mice.

The results of Experiment 2 suggest that T, but not male cecum transplantation, was capable of partially masculinizing behaviour in female mice. T-antibiotic-treated females showed an increased investigation of the anogenital region of female intruders and showed a trend of mounting towards female intruders compared to control females. These results are consistent with prior work showing that T treatment in adulthood can increase male-typical socio-sexual behaviours among females (e.g., Martel & Baum, 2009).

In contrast, male cecum transplants to antibiotic-treated females increased investigative behaviours towards male intruders compared to T-treated and control females, but no other behaviours were altered with cecum transplantation. This is surprising as Markle et al. (2013) found that male microbiota transplantation into females increases circulating T among these recipient females. However, there are some notable methodological differences between the current study and this prior work. In particular, the current study depleted the microbiome via antibiotics, and the gut's recolonization occurred in adulthood, while Markle et al.'s transplantation occurred in weaning GF female mice. As previously noted, GF mice are born in a microbe-free environment and thus had no microbiome throughout their lifespan until adulthood. Further, Markle et al. (2013) did their transplantation in puberty. It is possible that microbiota transplantation at puberty, during sexual maturation, may have a different effect on the animals' physiology than in adulthood. Alternatively, it may be that T did increase in the female mice receiving male cecum in the current study but did not reach sufficiently high levels to masculinize behaviour.

Evidence of decreased body weight was found in female recipients of male microbiota compared to T-antibiotic-treated females, although they did not differ significantly from controls. This is consistent with previous studies that GF females that received male microbiota lost significantly more weight than those that received female microbiota (Fransen et al., 2017). Fransen et al. suggest that the male microbiota induced more gut inflammation in GF female recipients. Alternatively, T-antibiotic-treated females may have a slightly increased body weight due to the engorged cecum's weight from depleting the gut microbiota (Chou et al., 2008; Reivkam et al., 2011). Nevertheless, both groups did not differ from controls, and thus it is

difficult to ascertain whether the transplantation group showed a decrease or whether the T group showed an increase in body weight.

### 4.4 Anxiety Behaviours Remained Unaltered

Anxiety-like behaviours on the elevated plus-maze remained unaltered in mice treated with antibiotics. This is in line with Gacias et al.'s (2016) report that anxiety-like behaviour was not affected by oral antibiotic treatment in mice but in contrast to others. For example, while Bercik et al. (2011) report that antibiotic treatment increased anxiety-like behaviours in conventionally raised mice, GF mice instead show reduced levels of anxiety-like behaviours compared to conventional mice (e.g., Clarke et al., 2012; Neufeld et al. 2011). The inconsistencies are not surprising, given that that the antibiotic-treated mice had intact microbiota at one point in time, which is not the case in GF mice. In the current study, exposure to experimenters and conspecifics in other behaviour test paradigms may have masked anxiety behaviours, if there were any. For instance, the elevated-plus maze was preceded by three unrelated behaviour tests (i.e., olfactory preference and two resident intruder tests); previous work did not couple other behaviour tests with the elevated plus-maze while others added additional tests of anxiety/depression-like behaviours, such as the forced swim test (Clark et al., 2013; Desbonnet et al., 2015; Gacias et al., 2016; Jasarevic et al., 2016). Thus, it is possible that anxiety was heightened among all groups in the present study due to frequent handling/behaviour testing and stress arising from single housing (i.e., PND 40 – 50) of experimental animals, masking any effects of antibiotic treatment. Indeed, all the experimental groups spent more time in the closed arms than the open arms in the current study, though this is consistent with the average response for untreated mice (Schrader et al., 2018). Although there was no antibiotic treatment effect, a sex difference was found such that females showed a higher frequency of

retreat to the closed arms than males, consistent with prior work (Tucker & McCabe, 2017). It will be interesting in future work to assess the effects of microbiota depletion in early development and adulthood in males and females on multiple anxiety tests to elucidate the gut depletion effect on anxiety, as well as assess whether cross-sex microbiota transfers reverse the observed sex differences on these tests.

### 4.5 Limitations and Future Direction

Though the present findings are novel and address a gap in the existing literature, there are some limitations to consider in interpreting the results and designing future studies. First and foremost, the unavailability of bacterial sequence analysis from stool samples obscures the microbiota transplantation's causal role in reversing antibiotic-induced phenotype. Specifically, it is unknown which specific microbes are present in male microbiota recipients and not in female microbiota recipients and/or T treatment males. Such data will provide insight into these microbes and characterize their corresponding behavioural roles.

Another limitation is that the current study did not assess the experimental females' estrus cycle, and these mice were not presented with a sexually experienced male in the resident intruder paradigm. Thus, it is possible that when in behavioural estrus and presented with a reproductive male, that alterations in female socio-sexual behaviours may emerge. In the same vein, the behavioural paradigm used to assess aggression is a paradigm in which male-typical aggression is observed. Thus, future studies may investigate the effect of gut microbiota depletion in female socio-sexual behaviours using paradigms where females typically show high aggression levels (e.g., maternal aggression tests). Also, microbiota transplantation in antibiotic-treated mice was only done in adulthood, yet it is likely that the microbiome during critical periods of brain development may hold different effects on behaviour. Future studies may

consider peripubertal microbiota transplantation and as well measure serum T levels in microbiota-depleted mice in early development to ascertain the role of the microbiota during critical periods in development on socio-sexual behaviours. Finally, the low sample size in the A+FC group (i.e., n = 8) limits the results and its interpretation. Increasing the sample size may offer a clear picture of this treatment, which future studies may consider.

## 5.0 Conclusion

This study has, for the first time to my knowledge, explored the role of the gut microbiome in mediating socio-sexual behaviours in mice. The findings suggest that having a male-typical gut microbiome is required for the full display of sex-typical behaviours in male mice, thus providing strength for the existing argument that the gut microbiome plays an essential role in brain functioning and behaviour. The data provide insight into how sex differences in the gut microbiota composition affect behaviour and suggest that the gut microbiome influences sex-typical behaviours via alternative mechanisms than androgen signalling. This is surprising given the plethora of research pointing to androgens as the primary mediator of socio-sexual behaviour in male rodents. This suggests that the gut microbiome is either directly communicating with the brain to mediate behaviour or acting via other systems, such as the immune system, to mediate socio-sexual behaviours. Future work may consider the mechanism by which the gut microbiome mediates socio-sexual behaviour in mice. However, the current studies provide evidence that there are sex-specific factors in the gut microbiome composition that mediate socio-sexual behaviours in mice that is independent of androgen signalling.

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## Appendix A

**Figure A1**

*Timeline for Experiment 1*

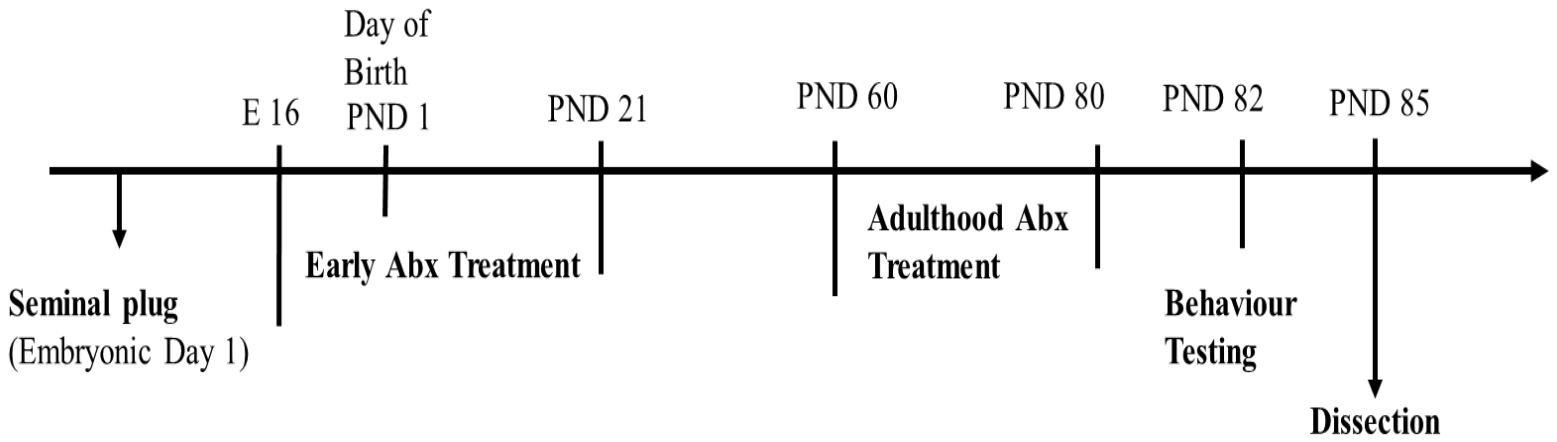


**Table A1**

*Mean (SEM) for all behavioural measures (dependent variables by treatment condition) - Experiment 1*

| | | Male | | | Female | | |
|---|---|---|---|---|---|---|---|
| | | Early Abx (n = 18) | Adult Abx (n =14) | Control (n = 10) | Early Abx (n = 13) | Adult Abx (n = 11) | Control (n = 8) |
| Olfactory Preference | Female preference score | 43.79 (10.97) | -3.99 (14.02) | 57.56 (20.52) | -41.07 (11.75) | -59.59 (14.33) | -24.50 (14.05) |
| | Duration in male bedding (s) | 57.20 (6.43) | 68.14 (10.59) | 30.07 (4.01) | 105.99 (13.03) | 118.65 (13.12) | 86.37 (12.26) |
| | Duration in female bedding (s) | 100.98 (10.71) | 64.15 (7.81) | 87.64 (20.14) | 64.92 (11.81) | 59.06 (8.29) | 61.88 (11.82) |
| | Duration in neutral bedding (s) | 19.26 (5.62) | 46.94 (17.41) | 19.40 (3.04) | 18.83 (3.66) | 17.04 (2.78) | 12.50 (3.11) |
| | Stimulus sex | | | | | | |

| | | | | | | | |
|---|---|---|---|---|---|---|---|
| Social | Anogenital | 14.11 (1.92) | 16.86 (3.54) | 14.10 (2.07) | 8.07 (2.12) | 3.54 (2.86) | 11.75 (2.01) |
| behaviours | Male | 18.83 (2.58) | 23.07 (3.67) | 24.50 (4.21) | 21.54 (3.12) | 24.73 (4.38) | 24.88 (5.88) |
| | Investigation (frequency) | | | | | | |
| | Female | | | | | | |
| | Face/Body | 26.50 (3.04) | 35.78 (5.58) | 24.40 (4.12) | 25.92 (4.12) | 39.46 (4.54) | 35.50 (6.24) |
| | Male | 25.22 (2.27) | 30.29 (3.92) | 27.00 (3.43) | 32.85 (4.03) | 40.46 (3.44) | 36.75 (6.47) |
| | Investigation (frequency) | | | | | | |
| | Female | | | | | | |
| Sexual | Mounting Frequency | 8.83 (2.49) | 17.21 (3.17) | 15.40 (3.62) | 2.23 (1.03) | 3.36 (1.67) | 2.13 (1.42) |
| Behaviours | Female | | | | | | |
| | Thrusting (frequency) | 39.11 (13.41) | 62.00 (18.14) | 59.50 (16.77) | 9.00 (5.42) | 7.82 (5.35) | 8.13 (5.42) |
| | Female | | | | | | |
| | Intromission (frequency) | 58.00 (20.04) | 46.43 (25.93) | 118.50 | 6.69 (5.20) | 4.36 (4.36) | 19.25 (18.13) |
| | Female | | | (42.63) | | | |
| | Aggression towards male intruders | | | | | | |
| Aggressive | Chasing frequency | 4.22 (1.00) | 5.71 (2.52) | 13.30 (2.81) | 1.23 (1.23) | .545 (.545) | .000 (.000) |

| | | | | | | | |
|---|---|---|---|---|---|---|---|
| behaviours | Duration | 8.02 (3.14) | 17.77 (10.70) | 68.59 (38.14) | 2.21 (2.21) | .504 (.504) | .000 (.000) |
| | Tumbling frequency | 7.22 (1.81) | 6.00 (2.34) | 15.40 (3.12) | 1.31 (1.31) | 2.18 (2.08) | 000 (.000) |
| | Duration | 9.12 (2.49) | 8.12 (3.55) | 23.96 (4.74) | 1.63 (1.63) | 2.17 (2.13) | .000 (.000) |
| | Attack frequency | 7.22 (1.81) | 6.00 (2.34) | 15.40 (3.12) | 1.31 (1.31) | 2.18 (2.08) | 0 (.0) |
| | Boxing Frequency | 9.83 (2.02) | 7.76 (3.21) | 11.50 (2.24) | 1.69 (1.17) | 2.45 (3.21) | 0 (.0) |
| | Biting Frequency | .056 (.056) | .714 (.714) | .100 (.100) | 0 (.0) | 0 (.0) | .100 (.125) |
| | Latency to Aggress | 420.91 (86.28) | 609.75 (98.56) | 141.38 (34.19) | 0 (.0) | 0 (.0) | 0 (.0) |
| | Anxiety measure | | | | | | |
| Elevated plus-maze | Closed Arm Frequency | 26.8 (1.91) | 25.5 (1.32) | 26.7 (2.2) | 31.5 (3.61) | 33.5 (1.46) | 29.6 (2.58) |
| | Closed Arm Duration | 457 (17.1) | 483 (10.4) | 488 (12.2) | 471 (12.9) | 463 (7.01) | 463 (12.7) |
| | % Duration Closed Arm | 76.2 (2.85) | 80.6 (1.71) | 81.3 (2.04) | 78.5 (2.13) | 77.2 (1.17) | 77.2 (2.12) |
| | Open Arm Frequency | 9.44 (1.42) | 6 (0.56) | 8.1 (1.35) | 8.69 (1.27) | 9.18 (0.86) | 8.25 (1.47) |
| | Open Arm Duration | 49.5 (10.5) | 28.5 (7.06) | 7 (8.14) | 49.4 (8.39) | 32.6 (6.81) | 42.1 (8.83) |
| | % Duration Open Arm | 8.25 (1.75) | 4.77 (1.18) | 6.17 (1.36) | 7.02 (1.47) | 8.25 (1.4) | 8.25 (1.4) |

| | | | | | | |
|---|---|---|---|---|---|---|
| Open Arm/Closed Arm Ratio | .132 (.041) | .063 (.017) | .080 (.018) | .111 (.022) | .072 (.016) | .094 (.020) |

## Appendix B

**Figure B1**

*Timeline for Experiment 2*

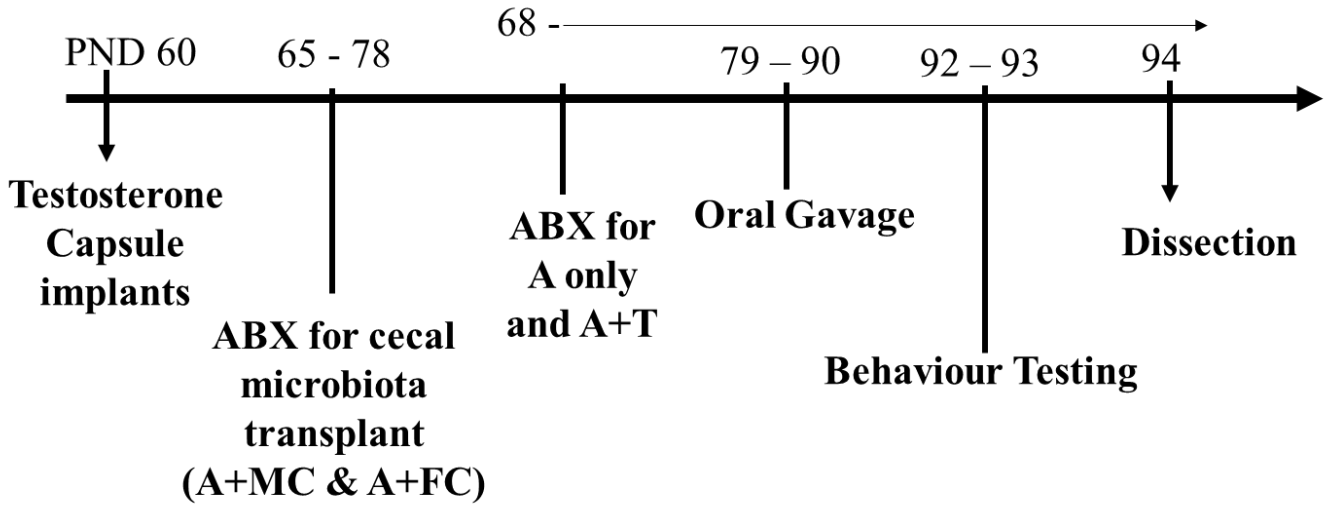
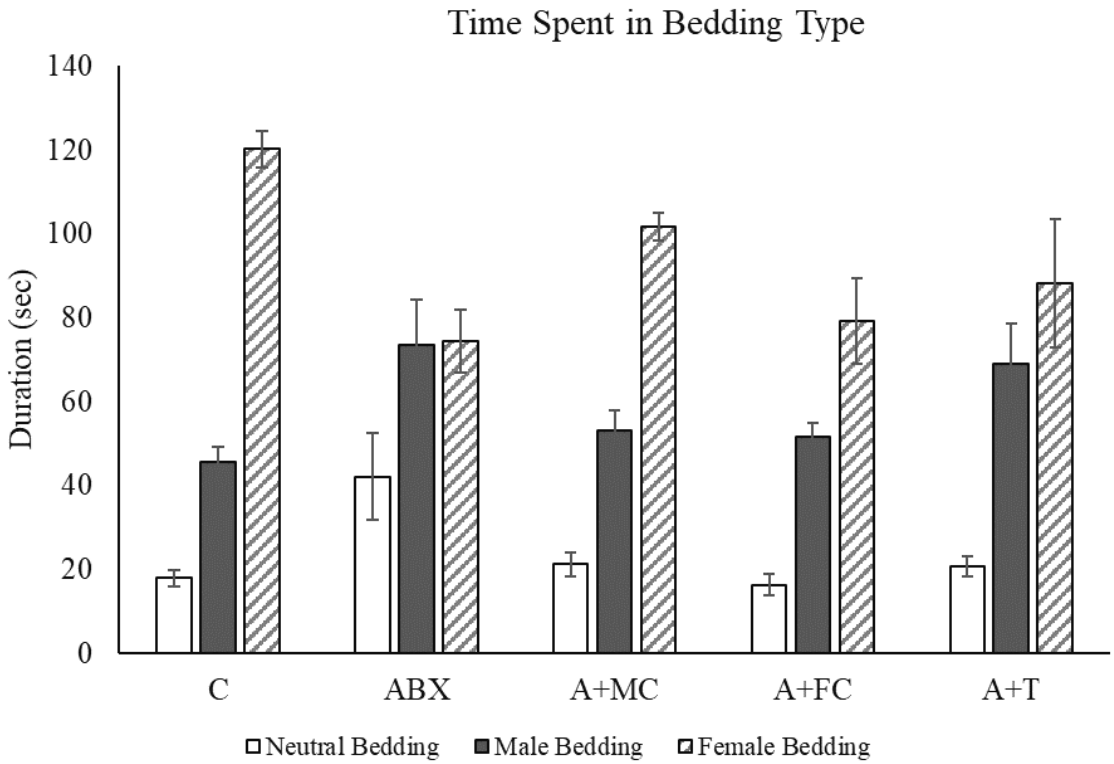


**Figure B2**

*Time spent investigating bedding type (Mean +/- SEM): Experiment 2*




*Note.* Duration of time spent investigating male-soiled, female-soiled, and neutral bedding for male and female subjects, +/- SEM. Antibiotic-only treated males spent more time in clean bedding even though all animals show a preference for soiled-bedding over clean bedding.

**Table B1**

*Sexual behaviours towards an estrus-induced female stimulus: Mean (SEM)*

| | | Anogenital Investigation Frequency | Anogenital Investigation Duration | Face/Body Investigation Frequency | Face/Body Investigation Duration | Latency to Mount (secs) | Mount Frequency | Thrust Frequency | Intromission Frequency |
|---|---|---|---|---|---|---|---|---|---|
| Males | A | 19.67 (2.34) | 109.49 (25.35) | 23.42 (2.84) | 103.49 (22.44) | 704 (81.44) | 4.67 (3.27) | 15.67 (12.90) | 2.42 (2.01) |
| | A+MC | 12.67 (2.06) | 71.90 (34.34) | 19.83 (2.97) | 86.72 (28.57) | 497 (105.1) | 4.42 (1.810 | 9.92 (4.80) | .00 (.00) |
| | A+FC | 19.11 (3.43) | 71.94 (21.02) | 22.33 (3.04) | 85.04 (23.19) | 806 (61.95) | 2.11 (1.36) | 4.89 (3.71) | .333 (.333) |
| | A+T | 22.08 (3.11) | 113.67 (26.71) | 25.75 (3.33) | 120.49 (23.38) | 813 (58.05) | 1.67 (1.13) | 5.92 (3.99) | .00 (.00) |
| | C | 17.33 (2.00) | 93.12 (29.10) | 20.08 (2.23) | 84.55 (22.18) | 701 (87.90) | 4.00 (2.26) | 14.58 (10.61) | 1.08 (.753) |
| Females | $A+MC_f$ | 15.10 (2.57) | 44.59 (8.09) | 18.40 (2.57) | 80.67 (17.35) | 853 (45.66) | 1.20 (.998) | 2.80 (2.01) | 1.10 (.823) |
| | $A+T_f$ | 18.182 (3.25) | 44.58 (24.51) | 28.27 (4.72) | 137.53 (25.91) | 758 (70.59) | 3.91 (1.71) | 6.09 (2.87) | 3.27 (2.26 |
| | C | 12.55 (2.39) | 43.43 (8.58) | 25.18 (3.38) | 117.72 (16.58) | 900 (.00) | .182 (.183 | .00 (.00) | .00 (.00) |