HYPERANDROGENIZATION AND LATE GESTATIONAL INFECTION RESULT IN AN AUTISM-LIKE PHENOTYPE IN A RODENT MODEL

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Abstract

Autism spectrum disorder (ASD) is characterized by persistent difficulties with communication, social behaviour, and repetitive behaviour, and disproportionately affects males compared to females. Maternal immune activation (MIA) resulting from gestational infections can disrupt fetal neurodevelopment, increasing the risk for neurodevelopmental disorders such as ASD. Rodent models of MIA produce offspring with an ASD-like phenotype and demonstrate a sex bias mirroring the human condition. Sex differences in brain and behaviour are largely attributable to the prenatal androgen surge during late gestation which acts to masculinize the male brain. In the current study, I assessed whether prenatal androgens mediate the sex bias in MIA-associated deficits. I manipulated MIA both prior to (embryonic day [E] 12.5) and during (E17.5) the prenatal androgen surge (i.e., early and late gestation, respectively), and in the presence or absence of exogenous androgen treatment. Offspring of both sexes were assessed for communicative, social, and repetitive behaviour deficits. The findings support male vulnerability to MIA deficits, that is dependent on the timing of gestational infection, such that the greatest deficits were found when MIA coincided with the prenatal androgen surge. Exogenous androgens did not have an additive effect of exacerbating deficits in late gestational MIA; instead, hyperandrogenization in the absence of MIA resulted in behavioural deficits comparable to those of MIA offspring. These results suggest that high androgen signalling alone is capable of producing an ASD-like phenotype, supporting the use of hyperandrogenization as a rodent model of ASD. Overall, these findings indicate that the maternal immune system can influence offspring brain and behaviour in a sex- and time-dependent manner, and suggest that prenatal androgens contribute to the greater risk of ASD in males than females.

Keywords: gestational infection, androgens, autism, social development, sex bias

Declaration of Co-Authorship

I hereby declare this thesis incorporates materials that resulted from joint research supervised by Dr. Ashlyn Swift-Gallant. Specifically, Yellow Martin aided in breeding of stimulus animals, behavioural testing, dissections, and provided guidance and support as laboratory manager. Kerri Sparkes aided in behavioural testing and coding of three-chamber social paradigm and marble burying tests. Stephanie Salia and Meagan Hinks aided in behavioural testing, dissections, and neonatal dissections. Stephanie Salia also aided in the scoring of neonatal ultrasonic vocalization recordings. Alison Randell aided in the scoring of ultrasonic vocalization recordings and neonatal dissections. Zachary Porter, under the supervision of Dr. Deepak Kaushik (Faculty of Medicine, Memorial University) aided in cytokine (TNFα) analysis. Experimental designs were developed in conjunction with Dr. Swift-Gallant. In all cases, I performed the primary contributions, data analysis, interpretation, and writing for this project.

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List of Abbreviations

ANOVA	Analysis of Variance
AR	Androgen Receptor
ASD	Autism Spectrum Disorder
CSF	Cerebrospinal Fluid
dsRNA	Double-Stranded RNA
E	Embryonic Day
E2	Estradiol
ELISA	Enzyme-Linked Immunosorbent Assay
EMB	Extreme Male Brain
FMR1 gene	Fragile X Messenger Ribonucleoprotein 1 gene
FXS	Fragile X Syndrome
GABA	Gamma-Aminobutyric Acid
IBA	Ionized Calcium Binding Adaptor Molecule
IFN	Interferon
IL	Interleukin
IP	Intraperitoneal
LPS	Lipopolysaccharide
MIA	Maternal Immune Activation
NDD	Neurodevelopment Disorder
P4	Progesterone
PCOS	Polycystic Ovarian Syndrome
PND	Postnatal Day
Poly I:C	Polyinosinic:polycytidylic acid
Q-CHAT	Quantitative Checklist for Autism in Toddlers
SV	Seminal Vesicles
T	Testosterone

TLR	Toll-Like Receptor
TNF	Tumor Necrosis Factor
TP	Testosterone Propionate
USV	Ultrasonic Vocalization
VPA	Valproate

1.0 Maternal Immune Activation as a Risk Factor for Autism Spectrum Disorder

Prenatal brain development is exceptionally vulnerable to environmental influences that can shape the trajectory of neural development (reviewed in Miguel et al., 2019). One example is that of maternal immune activation (MIA) resulting from gestational infection (as reviewed in Haddad et al., 2020). During MIA, inflammatory markers, such as cytokines, are transferred from mother to fetus via amniotic fluid, plasma, and/or the placenta, with the potential for long-lasting effects on the fetal central nervous system (CNS) (Haida et al., 2019; Minakova & Warner, 2018). In recent years, MIA has been identified as a major risk factor for the onset of various neurodevelopmental disorders (NDDs), including autism spectrum disorder (ASD) (Atladóttir et al., 2010; Kneusel et al., 2014; Patterson, 2009). Indeed, maternal infection during the first trimester of pregnancy has been associated with increased prevalence of ASD in children (Antoun et al., 2021; Atladóttir et al., 2010), although the largest meta-analysis to date suggests an equal risk across trimesters (Tioleco et al., 2021). Given that one in 66 children and youth in Canada are diagnosed with ASD (Ofner, 2018), it is of critical interest to investigate the underlying mechanisms of MIA as they pertain to ASD development.

1.1 Sex Bias in ASD and MIA Rodent Model

ASD symptomology is defined by three core characteristics: namely, impaired communication (e.g., verbal and non-verbal deficits), social challenges (e.g., difficulty expressing or recognizing emotions; social avoidance), and restricted and repetitive behaviours (e.g., movements such as flapping, spinning, and rocking; narrow or specific interests; resistance to change) (American Psychiatric Association, 2013). There are a wide variety of preclinical rodent models of ASD, including inbred (e.g., BTBR T+ Itpr3tf/J mice), chemically induced (e.g., valproate (VPA), lipopolysaccharide (LPS), and polyinosinic:polycytidylic acid (poly I:C),

and genetic mutation models (e.g., *Shank3* and *Fmr1)*, that all lend insight into the complex mechanisms of the human condition (reviewed in Panzenhagen et al., 2022). There is a prominent sex disparity in ASD diagnoses, whereby males are four times as likely to be diagnosed with ASD compared to females (Fombonne, 2009; Loomes et al., 2017). It has been posited that females tend to receive an ASD diagnosis later in life than males, or go undiagnosed, as females may actively "camouflage" their symptoms, or present with symptoms outside of typical diagnosis criteria (Loomes et al., 2017; Zener, 2018). Genetic factors, such as fragile X syndrome (FXS) have also been attributed to the skewed sex ratio in ASD diagnosis (Schaafshma & Pfaff, 2014). In FXS, silencing of the Fragile X Messenger Ribonucleoprotein 1 (FMR1) gene on the X chromosome results in developmental challenges including learning disabilities and cognitive impairment. As females possess two X chromosomes, they are less likely to develop FXS than males, which may, in part, account for the sex disparity in diagnosis (Garber et al., 2008). Even so, rodent models of MIA have consistently demonstrated a sex bias where males are more susceptible to neurological and developmental deficits following MIA, while females generally experience fewer and less severe deficits (e.g., Carlezon et al., 2019; Haida et al., 2019; Malkova et al., 2012). This sex disparity in MIA susceptibility in rodents closely mirrors the sex bias present in the human condition. As such, it has been suggested that other biological underpinnings aside from genetic factors contribute to the higher susceptibility of males for the development of ASD, such as prenatal exposure to androgens (Carlezon et al., 2019; Haida et al., 2019).

The extreme male brain (EMB) theory posits that hypermasculinization of the brain resulting from high androgen signalling can lead to ASD symptomology (Auyeung et al., 2009; Baron-Cohen, 2002; Baron-Cohen et al., 2011). The EMB may be an exaggeration of sex

differences during neurodevelopment to produce a hypermasculinized brain (Baron-Cohen, 2002). Using amniocentesis, multiple studies have found an association between elevated androgens in amniotic fluid and ASD in offspring. Indeed, excess testosterone (T) and androstenedione (a precursor of T) in amniotic fluid was correlated with ASD diagnoses in male offspring (Baron-Cohen et al., 2015). Male children with elevated amniotic androgens were found to score higher for autism traits on the Quantitative Checklist for Autism in Toddlers (Q-CHAT) as well as demonstrate social difficulties and increased repetitive behaviours at 4 years of age (Auyeung et al., 2012; Knickmeyer et al., 2005). The EMB theory has also been investigated in rodents, however, research is limited. For example, there is some correlational work such as a recent study in which plasma T was found to be elevated in male mice treated with VPA, an epilepsy medication that is associated with increased risk of ASD when exposed in utero (Grgurevic, 2023).

Preclinical rodent models are a valuable tool in investigating MIA processes, as offspring display neurological and behavioural deficits that are analogous to ASD symptomology (Careaga et al., 2017; Haida et al., 2019; Malkova et al., 2012). Poly I:C, a synthetic double-stranded RNA (dsRNA) analog and immunostimulant, is often used to mimic viral infection in MIA rodent models (Haida et al., 2019). Poly I:C is an agonist of toll-like receptor 3 (TLR3), a pattern recognition receptor associated with the innate immune response against viral infections (Reisinger et al., 2015). To detect the presence of viral pathogens, TLR3 recognizes dsRNA, which is commonly produced during viral replication. As poly I:C is comprised of dsRNA, it activates TLR3, triggering the maternal immune response (Reisinger et al., 2015). In mouse models, poly I:C is commonly administered to pregnant dams at an early gestational timepoint of embryonic day (E) 12.5; fetal neurodevelopment is especially susceptible to environmental

insults at this point in gestation, as neural tube closure and progenitor migration are underway (Haida et al., 2019; Naviaux et al., 2014). Severe MIA-associated social and developmental deficits have reliably been reported using this protocol in mice (Haida et al., 2019; Hsiao & Patterson, 2010; Naviaux et al., 2014; Shi et al., 2008). For instance, Haida et al. (2019) found that a single injection of poly I:C on E12.5 in mice was sufficient to have detrimental effects on developmental milestones (e.g., righting reflex, opening of eyes), social behaviour and repetitive behaviour.

The influences of MIA in later gestation are understudied compared to earlier gestational timepoints, despite evidence that MIA onset during late gestation affects social behaviour, working memory, and motor development (Arsenault et al., 2013; Connor et al., 2012; Meyer et al., 2006). Thus, a greater emphasis on understanding MIA in later prenatal development is warranted, and given that sex differences largely do not emerge until later in gestation, studying late-gestational MIA may also provide insight as to why males are disproportionately susceptible to MIA deficits compared to females. Specifically, prenatal androgens are the primary driver of sexual differentiation of the brain and behaviour (George & Ojeda, 1982), and in male mice, androgens do not begin to rise until around E16, peak at E18 and subside following the day of birth (Konkle & McCarthy, 2011; Weisz & Ward, 1980). Androgens during this time are necessary and sufficient to permanently masculinize the brain and behaviour (Bakker et al., 2006; Phoenix et al., 1959; Zuloaga et al., 2008). As such, I hypothesized that androgens increase vulnerability to MIA-associated deficits in males, and thus males will exhibit greater severity of symptoms with gestational infection coinciding with prenatal androgens, than prior to prenatal androgens. Prior to the prenatal androgen surge, the sexes differ in their sex

chromosome complement – males XY and females XX. Thus, any sex differences prior to the prenatal androgen surge are likely attributable to sex chromosomes.

1.2 Evaluating MIA-Associated Deficits in Mice

An ASD-like phenotype can be assessed in MIA offspring through an array of behavioural tasks focused on communicative, social, repetitive, and anxiety-like behaviours (Chang et al., 2017; Haida et al., 2019; Malkova et al., 2012). Further, rodent MIA offspring experience changes in inflammatory processes and neurology, such as alterations in cytokine levels and microglia activation and density, that parallel those observed in the human condition (reviewed in Careaga et al. 2017; Choi et al., 2016; Smith et al., 2007).

1.2.1 Communication Deficits in the MIA Model

Mice produce ultrasonic vocalizations (USV) as an essential intraspecies mode of communication (Caruso et al., 2022; reviewed in Premoli et al., 2021). During the first two weeks of life, mouse pups produce ultrasonic calls, often referred to as isolation calls, to attract the attention of the dam. Such calls aid in eliciting a retrieval response in the dams to collect pups that have been separated from the nest, and encourages the feeding and grooming of pups (D'Amato et al., 2005; Hahn et al., 1998). As one of the earliest possible measures of social and communicative behaviour in mice, neonatal USVs are an essential means of assessing an ASD-like phenotype in rodent models (Jouda et al., 2019; Wöhr & Scattoni, 2013). Ultrasonic communication of MIA neonatal offspring has been consistently reported to deviate from that of controls; however, there is much variability in the nature of these differences. A number of studies have demonstrated an increase in duration, frequency, and number of USVs produced by neonatal MIA offspring compared to saline controls (Choi et al., 2016; Kim et al., 2017; Schwartzer et al., 2013), while a reduction in USV production and frequency has been reported

by others (Baharnoori et al., 2012, Carlezon et al., 2019; Malkova et al., 2012). The divergent directionality of these communicative measures across studies is likely partially attributed to variability in the gestational day at which MIA was initiated, differences in poly I:C dose and timing of USV recordings. As such, the mechanisms of MIA on neural development is likely highly sensitive in relation to timing and dose of gestational infections.

Throughout juvenile development and into adulthood, USVs serve as a critical means of conveying socially relevant information to conspecifics (Malkova et al., 2012; Scattoni et al., 2008). USV production among juvenile mice is correlated with social bonding and motivation (Panskepp et al., 2007; Peleh et al., 2019). Likewise, USVs are related to prosocial interactions, such as facial and anogenital sniffing (Moles et al., 2007; Sungur et al., 2018). Typically, USVs at the juvenile and adult stages are predominantly produced by males in the presence of a stimulus female; however, USV emission also occurs in male-male (e.g., mediation of competition) and female-female (e.g., affiliative) social interactions (Moles et al., 2007; Zala et al., 2017). In the MIA model, deficits in USV communication have been reported when juvenile and adult mice are presented with same- or opposite-sex conspecifics. Males exhibit greater communicational deficits than females following MIA, such that adult MIA males have reduced USV production in the presence of both sexes (Hsiao et al., 2013; Malkova et al., 2012).

USV calls can be categorized by the shape of common call types, such as frequency steps, chevron, complex, and upward and downward calls, to name a few (Grimsley et al., 2011; Premoli et al., 2021; Scattoni & Crawley, 2011). These call types are conserved from neonatal development to adulthood, but call patterns vary with age and context (Premoli et al., 2021). MIA has been reported to alter the types of calls produced by offspring. For example, Malkova et al. (2012) found that MIA adult males had increased production of short and complex calls, but a

reduction in calls with harmonic elements, in a social situation with a male conspecific. Similarly, adult MIA males had reduced 2-frequency-step and chevron calls when interacting with a female stimulus animal (Malkova et al., 2012). It is important to note that the interpretation of these individual call types has yet to be deciphered within differing contexts from early postnatal life to adulthood (Premoli et al., 2021). In future research, delineating the meaning of each call type in various contexts/scenarios will be a key factor in understanding the development of communication in rodent models of NDDs.

1.2.2 Social Deficits in the MIA Model

Social challenges such as social avoidance, limited eye contact, difficulty interpreting another's emotions, and disinterest in social interactions are often present in ASD (American Psychiatric Association, 2013). While social behaviour may be considered less complex in rodent models, mice are highly social animals and thus investigating social deficits in the MIA model may inform our understanding of social challenges in ASD. In mice, an ASD-like phenotype is characterized by a decrease in sociability and social novelty preference (Haida et al., 2019). Sociability refers to the tendency to spend time with a conspecific as opposed to spending time alone, while social novelty preference is the tendency to investigate an unfamiliar rather than a familiar conspecific (Haida et al., 2019; Moy et al., 2004). These social behaviours are most commonly evaluated using the three-chamber social paradigm (Chang et al., 2017; Haida et al., 2019; Malkova et al., 2012). Compared to typically-developing mice, MIA offspring generally choose to spend more time in an empty chamber than with an unfamiliar stimulus. Likewise, MIA offspring will prefer a familiar stimulus mouse over an unfamiliar mouse (Chang et al., 2017; Haida et al., 2019; Malkova et al., 2012). Other social paradigms, such as partition tests and social/partner preference tests (reviewed in Silverman et al., 2010), have also been

implemented to assess social development in rodent models of ASD and report similarly diminished social behaviour (McFarlane et al., 2008; Spencer et al., 2008). In line with the sex bias in this model, MIA males exhibit impaired sociability and weakened preference for social novelty compared to females (Haida et al., 2019; Malkova et al., 2012), while MIA females do not differ from control females on such measures.

1.2.3 Repetitive Behaviour in the MIA Model

Restricted and repetitive behaviours are a central component of ASD symptomology, and often consist of stereotypic movements such as hand flapping and body rocking, narrow or specific interests, and resistance to change (American Psychiatric Association, 2013). In rodent models of ASD, an array of repetitive or persistent behaviours can be objectively measured, including obsessive self-grooming, jumping, and circling, and limited exploration during a nose-poke task (Malkova et al., 2012; Moy et al., 2008; Silverman et al., 2010). A marble burying task is most commonly used to measure repetitive behaviour in MIA offspring (Chang et al., 2017; Deacon, 2006). During this task, an ASD-like phenotype is characterized as an increase in digging and burying of marbles in bedding (Choi et al., 2016; Malkova et al., 2012). The sex bias in MIA vulnerability is evident through measures of repetitive behaviour. Specifically, MIA male offspring are consistently reported to engage in significantly more marble burying than MIA females (Carlezon et al., 2019; Haida et al., 2019).

1.3 Cytokines and MIA

Cytokines are small cell-signalling proteins that promote the effective coordination of immune responses (Minakova & Warner, 2018; Paraschivescu et al., 2020). These immune molecules play an important role in the regulation of processes such as neurogenesis, synaptogenesis, myelination, and apoptosis, that are crucial to typical prenatal and early

postnatal neurodevelopment (Paraschivescu et al., 2020). Following MIA, maternal cytokines transmitted to the fetus are posited to disrupt the balance of essential pre-existing fetal cytokines (Minakova & Warner, 2018). The presence of maternal cytokines may also alter the development of fetal neural circuitry through excessive synaptic pruning, which may contribute to the onset of ASD development (Fernández de Cossío et al., 2017). In line with this hypothesis, children with ASD have been found to have irregular proinflammatory cytokine profiles in the brain, cerebrospinal fluid (CSF), and serum (Chez et al., 2007; Zimmerman et al., 2005), and abnormal peripheral cytokine profiles have been proposed as possible biomarker for ASD diagnosis (reviewed in Zhao et al., 2021)

As cytokine action is essential in the immune response, exploring proinflammatory cytokine activity in both the mother and offspring (pre- and postnatally) is paramount to our understanding of MIA-associated deficits (Minakova & Warner, 2018). Through rodent models, an array of proinflammatory cytokines have been identified as key mediators of fetal neurodevelopment following MIA (Choi et al., 2016; Minakova & Warner, 2018; Smith et al., 2007). Elevated levels of tumor necrosis factor (TNF)-α, interleukin (IL)-6, IL-17a, IL-1β, and interferon (IFN)-β, among others, have been consistently reported in MIA offspring (or dams) (Arrode-Brusés & Brusés, 2012; Choi et al., 2016). TNF-α is of particular interest; in humans, elevations in this cytokine can cause complications in pregnancy, including abnormal placental growth, restricted blood supply, and necrosis of the embryo (reviewed in Alijotas-Reig et al., 2017). Further, excessive TNF-α has been associated with impaired social functioning and increased repetitive and stereotyped behaviours (Wang et al., 2012)

Findings of cytokine changes in the MIA model tend to be influenced, at least partially, by the gestational timing of immune insult. Currently, there is minimal knowledge of how the

timing of MIA onset (i.e., early vs late gestational infection) affects fetal and maternal cytokine profiles. While the majority of rodent studies reporting elevations in cytokines induced MIA in early gestation (e.g., E12.5), less is known about the effects of MIA in late gestation for the mediation of cytokines. The studies that have been performed in late MIA provide sufficient grounds for further investigation at this timepoint; for example, Gilmore et al. (2005) found that while poly I:C on E16 increased TNF-α in maternal serum and amniotic fluid, levels were significantly decreased in the neonatal brain. Similarly, Potter et al. (2023) found that offspring of MIA dams receiving poly I:C on E15 experienced behavioural and cognitive deficits that correlated with elevated maternal TNF-α.

The majority of studies do not examine male and female fetuses/pups separately, making it impossible to discern which sex (if either) is driving the effect of cytokine elevations. It is possible that altered cytokine levels following MIA demonstrate sex-dependency. For instance, in humans, T has been found to increase proinflammatory cytokines associated with MIA, such as TNF-α, IL-1β, IL-6 (reviewed in Bianchi, 2019). Similarly, in patients with atherosclerosis (plaque build-up in the arteries), levels of TNF-α, IL-1β, and IL-6 were significantly greater in males than females, with this elevation in cytokines suggested to be associated with androgens (Bernardi et al., 2020). As such, investigation of how androgen-MIA interactions influence cytokine action is essential to our understanding of the sex bias in deficits in the MIA model. More broadly, knowledge is limited regarding the mechanism by which heightened cytokine activity translates to behavioural deficits in offspring (Arrode-Brusés & Brusés, 2012; Carpentier et al., 2011). To date, research is limited regarding the role cytokines play in the development of behavioural deficits following MIA, and whether androgen-cytokine interactions contribute to

the sex bias in MIA susceptibility. Hence, studying the role of androgens in the immune response may provide important insight into the sex bias in vulnerability to ASD.

1.4 Study Overview

Although it is well-established that maternal infection is a risk factor for NDDs including ASD, few studies have considered the mechanisms underlying the sex bias in MIA/ASD risk. As the prenatal androgen surge takes place during late gestation, and androgens have been found to interact with the immune system, manipulating both MIA and androgens during late gestation is a necessary step in understanding increased vulnerability to MIA deficits among males. Here, I examined whether sex differences in prenatal androgen exposure influence communicative, social, and anxiety-like behavioural deficits following MIA in a sex-dependant manner, while exploring the underlying mechanisms of this relationship through the assessment of neural pro-inflammatory responses in the neonatal brain and sex steroid hormones in neonatal serum.

A pilot study was first conducted to establish the MIA model in our laboratory and to assess whether social deficits and anxiety-like behaviour would be greater when MIA was induced prior to (i.e., E12.5) or during (i.e., E16.5 and E18.5) the prenatal androgen surge. While the late MIA timepoints, E16.5 and E18.5, did not differ, both led to greater deficits in neonatal USVs (e.g., decrease in average call frequency) compared to early MIA (E12.5), and saline controls for communicational deficits. This supported the hypothesis that more severe social deficits would occur when MIA coincided with the prenatal androgen surge and provided a foundation for further exploration.

Next, I conducted a full-scale experiment to assess behaviour development, including communicative, social, and repetitive behaviour, in both sexes with MIA at an early and a late timepoint in gestation, and in the presence or absence of exogenous androgens. MIA was

stimulated in pregnant dams prior to (E12.5) or during the organizational prenatal androgen surge (E17.5) or saline was administered at both timepoints as a control. Exogenous androgens or an oil vehicle was administered to pregnant dams at timepoints corresponding to the male-typical androgen surge (E16, E18, E20 and postnatal day (PND)1). I assessed whether hyperandrogenization and/or MIA prior to or during the prenatal androgen surge exacerbated ASD-like behaviours.

Hypotheses and Predictions: In male offspring, testosterone propionate (TP) administration was expected to increase social behaviour deficits in the MIA model, with late MIA males receiving TP expected to experience the most severe deficits overall. Specifically, for the three-chamber social paradigm, it was hypothesized that late MIA males, compared to both early MIA and controls, would spend less time in the social chambers in phase 1 and the novel chamber in phase 2; a corresponding increase in time spent in the non-social (phase 1) and familiar (phase 2) chambers of the apparatus was predicted, together indicative of reduced sociability and social novelty preference. I anticipated that prenatal androgen treatment would further exacerbate these social deficits, especially in late MIA males. Similarly, late MIA males were hypothesized to exhibit greater deficits, as defined as more repetitive burying behaviour, on the marble burying task compared to both early MIA and control males. This repetitive behaviour was anticipated to be exacerbated in late MIA males receiving prenatal androgen treatment. In response to brief maternal separation, altered USVs were expected such that late MIA males were anticipated to experience reduced call number and duration; prenatal androgen treatment was hypothesized to exacerbate these communicational deficits in late MIA males. It was anticipated that over time (i.e., from PND 4-8), differences in communicational deficits between groups would be reduced, as previously reported with MIA deficits (Malkova et al.,

2012). As juveniles, late MIA males were hypothesized to produce fewer calls and calls of shorter duration in the presence of a same or opposite sex conspecific than early MIA and control males, with prenatal androgen exposure expected to exacerbate deficits. For both neonatal and juvenile USVs, it was hypothesized that late MIA males would differ from controls in the types of calls produced; however, this was exploratory as information on specific USV call types is limited (Premoli et al., 2022). On all behaviour tests, early MIA males were expected to be intermediate between late MIA and control males, indicative of an ASD-like phenotype, but of a less severe nature than late MIA.

I evaluated females in response to the same treatment conditions to determine if androgenization, likened to male-typical levels, would increase the prevalence and severity of MIA-associated deficits. As such, female offspring receiving TP were anticipated to display greater behavioural deficits than oil controls. While I did not anticipate differences in early vs late MIA without androgenization, I expected late MIA females that were androgenized to display greater deficits than other treatment groups. Specifically, I hypothesized that MIA females would spend less time in the social and novel chambers and more time in the non-social and familiar chambers of the three-chamber social paradigm apparatus, and that prenatal androgen exposure would exacerbate social deficits, especially in the late MIA females. MIA females were anticipated to demonstrate increased repetitive marble burying behaviour compared to saline controls, with T administration increasing the number of marbles being buried. Neonatal and juvenile MIA females were hypothesized to produce fewer calls and shorter calls compared to controls. Females exposed to prenatal androgens were anticipated to produce high-frequency calls, as previously reported (Kikusui et al., 2021).

Interestingly, the behavioural results indicated that hyperandrogenization alone (i.e., without MIA) and late MIA alone (i.e., without hyperandrogenization) led to the greatest deficits in ASD phenotype among males, while a combination of hyperandrogenization and late MIA did not further exacerbate the severity of behavioural deficits. Next, I led an investigation into the mechanisms underlying the relationship between MIA and androgens via neonatal steroid hormone and brain cytokine analyses. Given the behavioural results, late MIA and hyperandrogenization were expected to be accompanied by significantly elevated pro-inflammatory cytokine levels compared to early MIA and controls. As an exploratory analysis, steroid hormones, including T, estradiol (E2), progesterone (P4), cortisol and the thyroid hormones T3 and T4, were evaluated as differences in androgen-sensitive somatic measures suggested MIA and hyperandrogenization may alter circulating androgen levels. As such, to understand how these manipulations affected steroid hormones, I asked whether hyperandrogenization, early and/or late MIA altered steroid hormones in neonatal males.

Overall, the goals of this study were to delineate the role of gestational infection and prenatal androgens in mediating the susceptibility of MIA male offspring for developing an ASD-like phenotype, as well as elucidate the mechanisms underlying the sex bias in ASD. As detailed below, the novel findings here suggest that late MIA, coinciding with the prenatal androgen surge, leads to the greatest behavioural deficits. Further, hyperandrogenization, in the absence of MIA, can lead to an ASD-like phenotype in male offspring.

2.0 Methodology

2.1 Subjects

Wildtype C57BL/6 mice were obtained from Charles River Laboratories (St. Constant, QC, Canada) and paired for breeding in Tecniplast GM500 individually ventilated cages (311 x

159 x 132 mm) containing corncob bedding, nesting, and enrichment materials. Animals were kept in standard lighting conditions on a 12-hour light-dark cycle with lights on at 0700 and were provided *ad libitum* access to food and water. Ethical approval was obtained from the Institutional Animal Care Committee at Memorial University of Newfoundland.

Breeding females were examined daily for seminal plugs following pairing to approximate the first day of gestation, with the presence of a seminal plug marking embryonic day (E) 0.5. Males were separated from females upon observation of a seminal plug. Dams were weighed daily to confirm pregnancy. On the day of birth (PND1), litters were culled to two males and two females to mitigate litter effects. Pups were selected to be culled at random, with the exception of pups that appeared especially unhealthy or malformed (e.g., abnormal body colouring, severely underweight, missing limbs, etc). Pups were weaned and individually housed at PND21. Neonatal behaviour testing was conducted on PND 4, 6 and 8, and juvenile behavioural testing between PND 35-45 during the hours of 0800–1700.

Juvenile work followed a 2 x 2 x 3 between-subjects factorial design. Groupings were assigned based on sex (male or female), androgen treatment (testosterone propionate [males n = 38; females n = 33] or corn oil vehicle [males n = 36; females n = 34]), and MIA treatment (poly I:C injection on E12.5 (males n = 25; females n = 24), poly I:C on E17.5 (males n = 24; females n = 21), or saline vehicle on E12.5 and E17.5 (males n = 25; females n = 22)). A total of 141 experimental mice (male n = 74; female n = 67) were used in the behavioural study.

2.2 Stimulus Animals

Stimulus C57BL/6 mice (males n = 31, females n = 31) were bred to use during juvenile behavioural testing. Stimulus mice were unmanipulated, age-matched to experimental animals, and singly housed.

2.3 MIA Manipulation

MIA was induced in pregnant dams through intraperitoneal (IP) administration of poly I:C (1.25mg/mL of 0.9% NaCl) at a volume of 5mg/kg on E12.5 (early MIA) or E17.5 (late MIA). Poly I:C (Sigma-Aldrich, Cat# P1530-100MG) was stored at -20°C and mixed fresh on day of use. Control dams received saline (0.9% NaCl, volume equivalent to poly I:C weight dependent dosage) on both E12.5 and E17.5.

2.4 Hormone Manipulation

Testosterone propionate (TP) (100µg; 1mg/mL of corn oil) was administered subcutaneously to pregnant dams on E16, 18, and 20, and to pups on the first day of birth (PND1) at a volume of 0.1mL. Pups sacrificed at PND1 did not receive postnatal hormone treatment. This dose falls within the typical range administered to see physiological effects and/or masculinization among female mice (Armoskus et al., 2014; Ghahramani et al., 2014; Hisasue et al., 2010). Control dams and pups received corn oil at a volume of 0.1mL following the same schedule.

2.5 Behavioural Testing

2.5.1 Developmental Milestones

Developmental milestones were assessed as motor impairments are often associated with MIA (Haida et al., 2019). Eye-opening was assessed daily from PND11-17 and was scored as 0 = “both eyes closed”, 1 = “one eye open”, or 2 = “both eyes open”. To assess righting reflexes, pups were positioned supine, and the time taken to right was recorded on PND 4, 6, and 8 to 16, until righting was achieved in less than 0.5 seconds.

2.5.2 Marble Burying

A Tecniplast GM500 cage (331 mm x 159 mm x 132 mm) containing 4 cm of corncob bedding was covered by ventilated plexiglass. Sixteen marbles of approximately 19 mm in diameter were arranged atop the bedding in a 4 x 4 grid. Each experimental animal was recorded in the apparatus for 30 minutes. The apparatus and marbles were cleaned with 70% EtOH and allowed to dry between trials. Videos were manually scored for the number of marbles buried at 5 min intervals (i.e., 5, 10, 15, 20, 25, and 30 minutes). A marble was considered to be buried if it was at least 2/3 covered with bedding, as previously described (Chang et al., 2017).

2.5.3 Neonatal Ultrasonic Vocalizations

Neonatal ultrasonic vocalizations (USVs) were recorded on PND 4, 6, and 8 in response to a brief maternal separation. Pups were individually removed from the home cage for testing. Pups were placed in a 1L glass beaker resting on an electric heating mat. An iPad (model #A1822) was vertically nestled on top of a Styrofoam box (26.5 cm x 23 cm x 23.5 cm) with an opening to connect an Echo Meter Touch 2 PRO Ultrasonic Module (for IOS, Wildlife Acoustics) with the microphone facing the opening of the beaker. Using the Echo Meter Touch app, USVs were recorded for three minutes. Following PND 4 recordings, pups received paw tattoos for identification using green ink (Ketchum Manufacturing Inc.) and were returned to the nest. The apparatus was cleaned with 70% ethanol (EtOH) and allowed to dry between trials.

Prior to coding, sounds under 40kHz were filtered from the USV audio files using an RStudio script to reduce background noise and uploaded to Raven Lite 2 software (version 2.0.1, Cornell Lab of Ornithology). The background colour was set to “cool”, and the brightness value set between 55 – 70, and contrast set to a value of 50 to achieve a high contrast of neon green coloured calls on a dark blue coloured background. At a scale increasing by 50 ms, the highest and lowest frequency of each call was manually selected in a box. For each file, average low and

high call frequency, average call frequency, average call duration, total call duration, and number of calls were calculated. Syllable classification analyses were conducted on male PND 4 and PND 6 files, given that more prominent communication deficits were observed in males. Calls were classified manually based on syllable type for a 1.5-minute segment (0 – 30 seconds, 1 – 1.5 minutes, and 2 – 2.5 minutes) of the 3-minute recording using a modified method adapted from Scattoni et al., (2011), Minakova et al., (2012), and Grimsley et al., (2011). Categories include 1-frequency-step, 2-frequency-step, chevron, reverse chevron, downward, upward, flat, complex, short, and unstructured (Figure 1).

2.5.4 Juvenile USVs (Interaction with Same- or Opposite-Sex Conspecific)

Juvenile USVs were recorded in response to a three-minute encounter with an age-matched same and opposite sex conspecific in a counterbalanced manner. At PND 35-45, experimental mice were placed in an empty cage (44.5 cm x 20.5 cm x 23 cm). A ventilated plexiglass covering was outfitted with an iPad stand and an opening (50 cm x 28 cm) for connecting the Echo Meter. The experimental mouse habituated to the cage for 10 minutes. A stimulus conspecific was then placed in the apparatus and USVs were recorded during the three-minute interaction. The apparatus was cleaned with 70% EtOH and allowed to dry between trials. The coding process was as described above for neonatal USVs. A syllable classification analysis, following the same procedure as for neonatal USVs, was conducted for recordings of experimental males interacting with stimulus males, as the most severe juvenile communication deficits were observed during male-male encounters.

2.5.5 Three-Chamber Social Paradigm

An apparatus (61.2 cm x 40.6 cm x 22.5 cm) featuring three chambers (39.4 cm x 19.7 cm) and a ventilated plexiglass covering was used to assess social behaviour in the three-

chamber social paradigm. Subjects could freely move between chambers through 3.8 cm x 3.5 cm openings between each chamber. A cylindrical cage (11 cm high, 7.6 cm in diameter) was placed in both the left and right chamber to hold stimulus animals. A perimeter of 1.27 cm around the base of each cylindrical cage was designated as an interaction zone.

Testing of juveniles (PND 35-45) consisted of a 10-minute habituation followed by two 10-minute experimental phases: sociability and social novelty. Subjects were habituated to the apparatus whilst both cages were empty. During the sociability phase, an age- and sex-matched stimulus animal was placed in either the left or right cage (counterbalanced across subjects). A second stimulus animal was then placed in the remaining empty cage for the social novelty phase, while the first stimulus mouse also remained in the apparatus. A video camera mounted above the apparatus was used to record the sociability and social novelty phases. The apparatus was cleaned with 70% EtOH and allowed to dry between experimental animals. ANY-Maze software (version 7.0.6, Stoelting Co.) was used to score videos for 1) duration spent in the left, center, and right chambers, 2) duration spent in left and right interaction zones, 3) entries into the left, center, and right chambers, and 4) entries into the left and right interaction zones. Assignment of the left and right chamber (social, non-social, familiar, and novel) for stimulus placement was counterbalanced across subjects, with the first stimulus animal inhabiting the social chamber during the sociability phase (familiar chamber during social novelty phase). For the sociability phase, we calculated 1) duration spent in the social and non-social chambers, 2) duration spent in social and non-social interaction zones, 3) entries into social and non-social chambers, and 4) entries into social and non-social interaction zones. For the social novelty phase, we calculated 1) duration spent in the familiar and novel stimulus chambers, 2) duration spent in familiar and novel stimulus interaction zones, 3) entries into familiar and novel stimulus

chambers, and 4) entries into familiar and novel stimulus interaction zones. A sociability score and a social novelty score were calculated using a sociability index (time spent in social chamber - time spent in non-social chamber/time spent in social chamber + time spent in non-social chamber) and social novelty index (time spent in novel stimulus chamber - time spent in familiar stimulus chamber / time spent in novel stimulus chamber + time spent in familiar stimulus chamber), respectively, as described by Haida et al. (2019).

2.6 Dissections and Sample Processing

2.6.1 Juveniles

Dissections occurred the day following the final behavioural test. All experimental mice were weighed, overdosed with Avertin (40mg/kg) via IP injection, and decapitated. Trunk blood was collected, refrigerated for 4 hours, and centrifuged at 2000 rcf at 8°C for 20 minutes. Serum was collected from the supernatant and stored at -20°C. Gonads and male seminal vesicles (SV) were dissected and weighed. Cerebellums were separated from the rest of the brain, flash-frozen using 2-methtylbutane, cooled on dry ice, and stored at -80°C for future analyses. The remaining brain tissue was post-fixed in 4% paraformaldehyde for 4 hours, transferred to 20% sucrose, and refrigerated until sectioning. Brains were sectioned on a sliding freezing microtome at 30 μm, sectioned into 4 series, and stored in cryoprotectant at -20°C.

2.6.2 Neonates

Male pups were euthanized on the first day of birth (PND 1) via rapid decapitation. A total of 37 litters were used for the neonatal work. Brains were flash-frozen using 2-methylbutane on dry ice, wrapped in foil, and stored at -80°C. Trunk blood was collected, refrigerated for 4 hours, and centrifuged at 2000 rcf at 8°C for 20 minutes. Serum was collected and stored at -20°C.

Homogenization of neonatal male brain tissue (n = 35) was performed for utilization in cytokine and hormone analyses: Saline/oil (n = 6); saline/T (n = 6); early MIA/oil (n = 6); early MIA/T (n = 6); late MIA/oil (n = 6); late MIA/T (n = 5). Brains were removed from storage at -80°C and allowed to thaw on wet ice. Digestion buffer was prepared using Pierce RIPA Buffer (Cat. 89900) and Halt Protease and Phosphatase inhibitor (Cat. 1861281 100X) at a final concentration of 1x and kept on ice. Each brain was homogenized in 200 µL digestion buffer using a handheld motorized tissue grinder until no solid tissue was visible. The samples were rotated at 4°C for 1 hour to ensure complete digestion of tissue. Samples were centrifuged at 15000 rcf for 10 minutes at 4°C. The supernatant of each sample was transferred to new 1.5mL tubes and stored at -20°C for future use.

2.7 Enzyme-Linked Immunosorbent Assays (ELISAs)

In this study, male, but not female, samples were considered for ELISAs due to the prominent behavioural deficits observed in males, and the relevance of male data in evaluating the effects of hyperandrogenization on an ASD-like phenotype. However, it is possible that MIA also effects endocrine production in females, and thus should be included in future studies.

2.7.1 Quantification of TNF-α in Neonatal Male Brain Tissue

TNF-α was quantified in neonatal male homogenized brain tissue using a Mouse TNF-α ELISA kit (Invitrogen, Cat # BMS607-3) according to the manufacturer's protocol. A bicinchoninic (BCA) assay of each brain sample (1:20 dilution in dH_2O) was performed using the Pierce BCA Protein Assay Kit (Thermo Scientific, Cat # 23227) according to the manufacturer's protocol. The total protein concentration of each sample was calculated based on absorbance values and the standard curve. Averages of the duplicate concentrations

corresponding to the standard curve were divided by the total protein concentration determined via BCA assay to determine normalized TNF-α concentrations (μg/mL) for each sample.

2.7.2 Quantification of Steroid Hormones in Neonatal Male Serum

A hormone array was conducted by Eve Technologies (Calgary, Alberta, Canada), to measure concentrations of cortisol, progesterone, E2, T, T3, and T4 in neonatal (PND1) male serum (STTHD-Serum-Plasma Array). To prepare for shipment, the serum samples were removed from -20°C and allowed to thaw on wet ice. Three samples at a minimum volume of 75 μL were prepared for each of the male groups (i.e. saline/oil, saline/T, early MIA/oil, early MIA/T, late MIA/oil, late MIA/T), totalling 18 samples. To meet the minimum requirements of 75 μL of serum for steroid multiplex assay, the serum of males were pooled by litter, and up to two litters were pooled per sample assayed. As such, while only three samples were analyzed per group, each sample consisted of serum collected from three to six PND 1 males from one to two litters. The samples were shipped overnight on dry ice. Samples were tested in duplicate, and the observed concentrations (ng/mL) were averaged.

2.8 Statistical Analyses

Post-hoc comparisons using Fisher's LSD were conducted for all significant omnibus effects. Marginal interactions were explored ($p \leq .08$).Effect size was measured using Cohen's *d* for significant post-hoc tests. Interactions of sex by MIA treatment by hormone treatment were not considered; As males naturally have higher levels of perinatal androgens compared to females, the administration of excess androgens results in hyperandrogenization of males and androgenization of females. As such, these treatment conditions are not comparable in males and females. All statistical analyses for this study were performed using Jamovi (version 2.3.18). Significance was set at $\alpha = .05$.

2.8.1 Developmental Milestones

Due to the restricted range of values for righting reflex (0 – 60 seconds) and eye-opening (0, 1, or 2 eyes open), these data were analysed using Kruskal-Wallis non-parametric tests. MIA treatment (3 groups; saline, early MIA, late MIA) by hormone treatment (2 groups; corn oil and testosterone propionate) was analysed separately for each sex. Righting reflex compared groups on latency to self-right at PND 4, 6, and 8 to 16. Eye-opening compared groups on number of eyes open daily from PND 11-17. Effect sizes were determined using epsilon-squared (ε^2).

2.8.2 Somatic and Behavioural Measures

Between-subjects Analysis of Variance (ANOVA) was used to compare groups on all remaining dependent measures. Male groups were compared on gonadal and seminal vesicle weight as these somatic measures are androgen-dependent and provide an approximation of androgen sensitivity. Female groups were also compared on gonadal weight, while body weight was compared for both sexes. Consistent with prior research (e.g., Haida et al., 2019), the sociability phase (phase I) of the three-chamber social paradigm (i.e., one non-familiar conspecific present) compared groups on 1) duration in social and non-social chambers, 2) duration in social and non-social interaction zones, 3) entries into social and non-social chambers, 4) entries into social and non-social interaction zones, and 5) sociability index scores. The social novelty phase (phase II) of the three-chamber social paradigm (i.e., one non-familiar (novel) and one familiar conspecific present) compared groups on 1) duration in familiar and novel stimulus chambers, 2) duration in familiar and novel stimulus interaction zones, 3) entries into familiar and novel stimulus chambers, 4) duration in familiar and novel stimulus interaction zones, and 5) social novelty preference index scores. In the marble-burying test, groups were compared on the number of marbles at least 2/3 buried in bedding at consecutive 5-min intervals

from 0-30 minutes (i.e., 5, 10, 15, 20, 25, and 30 minutes), as previously described (e.g., Chang et al., 2017). For neonatal USVs at PND 4, 6, and 8, and juvenile USVs in the presence of a male or female conspecific, groups were compared on 1) average low call frequency, 2) average high call frequency, 3) overall average call frequency, 4) average call duration, 5) total call duration, and 6) total number of calls. Additionally, male and female neonatal USVs at PND 4 and 6, as well as male juvenile USVs in response to a male conspecific, were scored for the number of calls of each classification type (i.e., 1-frequency-step, 2-frequency-step, chevron, reverse chevron, downward, upward, flat, complex, short, and unstructured).

2.8.3 Inter-Rater Reliability

For the marble burying and USV tests, 2-4 raters scored the videos/files. As such, a subset of files (3-5 files per test) were scored by all raters to calculate inter-rater reliability using Pearson's *r* coefficient. In all cases, $r^2 \geq .9$.

2.8.4 ELISAs

Between-subjects ANOVA was used to compare male neonatal brain samples on normalized TNF-a concentrations.

Planned comparisons were made between oil-treated saline ($n = 3$) and T-treated saline ($n = 3$) neonatal (PND 1) males to assess the effect of hyperandrogenization on cortisol, progesterone, E2, T, T3, and T4 serum concentrations. Planned comparisons were also made between oil-treated saline ($n = 3$), oil-treated early MIA ($n = 3$), and late MIA ($n = 3$), to assess the influence of MIA treatment on the same hormones.

3.0 Results

3.1 MIA and Hyperandrogenization Independently Reduced SV and Body Weight

In line with prior work (e.g., Izvolskaia et al., 2019), MIA altered seminal vesicle (SV) weight. Specifically, a significant MIA by hormone treatment interaction was found for SV weight, $F(2, 68) = 8.558$, $p < .001$, such that both oil-treated late MIA males, $p < .001$, $d = 1.675$, and oil-treated early MIA males, $p = .036$, $d = .872$, had lower SV weight than oil-treated saline controls (Figure 2a). T-treated saline controls had significantly lower SV weight compared to oil-treated saline controls, $p < .001$, $d = 2.605$. T-treated early MIA males had a greater SV weight compared to T-treated saline controls, $p = .032$, $d = -.930$. Among early MIA males, oil-treated had a greater SV weight compared to T-treated, $p = .049$, $d = .803$.

A significant MIA by hormone treatment interaction was found for body weight, $F(2, 68) = 5.670$, $p = .005$. Prenatal T decreased body weight compared to saline controls, $p < .001$, $d = 1.836$. T-treated early MIA had a greater body weight than both T-treated late MIA, $p = .033$, $d = .870$, and T-treated saline controls, $p = .018$, $d = -.949$. Oil-treated late MIA males had a significantly lower body weight than both oil-treated early MIA males, $p = .011$, $d = 1.071$, and oil-treated saline controls, $p < .001$, $d = 1.671$ (Table 1).

A main effect of hormone treatment was found for gonadal weight, $F(1, 68) = 79.510$, $p < .001$, where prenatal T treatment decreased gonadal weight compared to oil-treated males, $p < .001$, $d = 2.075$ (Figure 2b). This effect remained when accounting for body weight (i.e., gonadal weight/body weight), $F(1, 68) = 65.667$, $p < .001$, such that T-treated males had a lower gonadal weight/body weight compared to oil-treated males, $p < .001$, $d = 1.885$.

3.2 Male Behaviour

3.2.1 Hyperandrogenization Improved Righting Reflex in Absence of MIA

Differences in latency to self-right were found in late neonatal development (i.e., PND 10-14). At PND 10, T-treated saline controls were faster to self-right than T-treated early MIA

males, $\chi^2(1) = 4.531$, $p = .033$, $\varepsilon^2 = .181$. At PND 11, T-treated saline controls were faster to self-right than oil-treated saline controls, T-treated early MIA, and T-treated late MIA males, $\chi^2(1) = 5.655$, $p = .017$, $\varepsilon^2 = .236$, $\chi^2(1) = 7.868$, $p = .005$, $\varepsilon^2 = .315$, and $\chi^2(1) = 4.334$, $p = .037$, $\varepsilon^2 = .181$, respectively (Figure 3). T-treated saline control males continued to right faster than T-treated early MIA and T-treated late MIA neonatal males on subsequent days: T-treated saline vehicles were faster than T-treated early MIA at PND 14, $\chi^2(1) = 4.506$, $p = .034$, $\varepsilon^2 = .180$, and compared to T-treated late MIA at PND 12 and 14, $\chi^2(1) = 5.872$, $p = .015$, $\varepsilon^2 = .156$, and $\chi^2(1) = 8.019$, $p = .005$, $\varepsilon^2 = .334$, respectively. Among late MIA males, T-treated were slower to self-right than oil-treated at PND 14, $\chi^2(1) = 5.630$, $p = .019$, $\varepsilon^2 = .245$. No differences in righting latency were found among oil-treated groups (ps > .05) (Table 2). There were no significant differences in eye-opening among male groups across neonatal development (ps > .05) (Table 3).

3.2.2 MIA During Prenatal Androgen Surge Increased Repetitive Behaviour

A 30-minute marble burying task, previously described by Chang et al. (2017), was used to assess repetitive digging behaviour. Late MIA males displayed more repetitive behaviours than Early MIA and saline controls. Specifically, a significant main effect of MIA treatment was found at the 10-min timepoint, $F(2, 68) = 3.811$, $p = .027$, and post hoc tests indicated that late MIA males buried more marbles than both saline controls, $p = .023$, $d = -.664$. and early MIA males, $p = .016$, $d = -.705$ (Figure 4; Table 4), while early MIA and saline controls did not differ on this measure.

3.2.3 Hyperandrogenization Increased Call Frequency During Maternal Separation

PND 4. A significant MIA by hormone treatment interaction was found for 2-frequency-step calls, $F(2, 62) = 3.759$, $p = .029$, and downward calls, $F(2, 62) = 3.981$, $p = .024$. While oil-treated early MIA males produced more 2-frequency-step calls compared to oil-treated saline

controls, $p = .040$, $d = -.842$, T-treated early MIA males produced fewer 2-frequency-step calls than their oil-treated counterparts, $p = .020$, $d = .937$. For downward calls, T-treated saline controls produced fewer downward calls compared to their oil-treated counterparts, $p = .042$, $d = .829$. Conversely, T-treated early MIA males produced more downward calls than both T-treated saline controls, $p = .006$, $d = -1.123$, and T-treated late MIA males, $p = .018$, $d = 1.058$. A main effect of hormone treatment was found for short calls, $F(2, 62) = 4.373$, $p = .041$, such that oil-treated males produced more short calls than T-treated males, $p = .041$, $d = .517$. A marginal MIA by hormone treatment interaction was also found for complex calls, $F(2, 62) = 2.958$, $p = .059$, such that T-treated late MIA males produced fewer complex calls compared to oil-treated late MIA, $p = .011$, $d = 1.271$, although they did not differ from controls (Figure 5). There were no differences in frequency, call number, call duration.

PND 6. A significant main effect of hormone treatment was found for average low call frequency, $F(1, 66) = 6.397$, p = .014, average high call frequency $F(1, 66) = 9.784$, $p = .003$, and overall average call frequency, $F(1, 66) = 8.641$, $p = .005$ (Figure 6). T-treated males produced calls with a higher average low frequency, $p = .014$, $d = -.598$, higher average high frequency, $p = .003$, $d = -.739$, and produced higher-frequency calls, $p = .005$, $d = -.694$, compared to oil-treated control males (Table 5). A marginal MIA by hormone treatment interaction was found for flat calls, $F(2, 64) = 2.677$, $p = .076$. Both T-treated early MIA, $p = .015$, $d = -1.027$, and T-treated late MIA, $p = .011$, $d = -1.046$, produced more flat calls than T-treated saline controls. A significant main effect of hormone treatment was found for 2-frequency-step calls, $F(1, 64) = 6.254$, $p = .015$, such that prenatal T decreased 2-frequency-step calls when compared to oil-treated males, $p = .015$, $d = .600$ (Figure 7; Table 6).

PND 8. A significant main effect of MIA treatment was found for total call duration, $F(1, 70) = 3.508$, $p = .035$), such that early MIA males produced longer calls than late MIA males, $p = .010$, $d = .739$ (Figure 8), though neither MIA group differed from controls. A significant main effect of hormone treatment was found for call number, $F(1, 70) = 11.710$, $p = .001$, average call duration, $F(1, 70) = 10.265$, $p = .002$, total call duration, $F(1, 70) = 11.158$, $p = .001$, average low call frequency, $F(1, 70) = 20.543$, $p < .001$, average high call frequency, $F(1, 70) = 12.484$, $p < .001$, and overall average call frequency, $F(1, 70) = 16.498$, $p < .001$ (Figure 9). T-treated males produced fewer calls, $p = .001$, $d = .786$, produced shorter calls, $p = .002$, $F = 10.265$, spent less time calling, $p = .001$, $d = .767$, produced calls with a higher average low frequency, $p < .001$, $d = -1.041$, and higher average high frequency, $p < .001$, $d = -.811$, and produced higher-frequency calls, $p < .001$, $d = -.932$, compared to oil-treated control males (Table 5).

3.2.4 MIA Impaired Communication with Same-Sex Conspecific

A significant MIA by hormone treatment interaction was found for call number, $F(2, 56) = 4.371$, $p = .017$, and total call duration, $F(2, 56) = 3.877$, $p = .026$ (Figure 10a). Among saline controls, T treatment reduced the number of calls produced, $p = .002$, $d = 1.434$, and time spent calling, $p = .004$, $d = 1.340$, compared to oil-treated. Compared to oil-treated saline controls, oil-treated early MIA and late MIA males produced fewer calls ($p = .023$, $d = 1.001$; $p = .001$, $d = 1.445$), and spent less time calling ($p = .002$, $d = 1.387$). A significant main effect of MIA treatment was found for average call duration, $F(2, 56) = 7.309$, $p = .002$, such that early and late MIA males produced shorter calls than saline controls in the presence of a male conspecific, $p = .011$, $d = .814$, and $p < .001$, $d = 1.168$, respectively. No significant differences were found for prenatal T treatment in MIA groups (ps > .05).

Significant MIA by hormone treatment interactions were found for the following call types: 1-frequency-step, $F(2, 56) = 3.941$, $p = .025$; downward, $F(2, 56) = 5.403$, $p = .007$; upward, $F(2, 56) = 3.621$, $p = .033$; flat, $F(2, 56) = 4.296$, $p = .018$; short, $F(2, 56) = 5.166$, $p = .009$; and unstructured, $F(2, 56) = 5.241$, $p = .008$. Further, marginal MIA by hormone treatment interactions were found for 2-frequency-step, $F(2, 56) = 3.168$, $p = .050$, chevron, $F(2, 56) = 3.014$, $p = .057$, reverse chevron, $F(2, 56) = 2.964$, $p = .060$, and complex calls, $F(2, 56) = 2.884$, $p = .064$ (Figure 11). Among saline controls, prenatal T treatment reduced production of 1-frequency-step, $p = .004$, $d = 1.362$, 2-frequency-step, $p = .007$, $d = 1.284$, downward, $p < .001$, $d = 1.632$, upward, $p = .037$, $d = .987$, chevron, $p = .004$, $d = 1.376$, reverse chevron, $p = .021$, $d = 1.086$, flat, $p < .001$, $d = 1.593$, short, $p < .001$, $d = 1.653$, complex, $p = .005$, $d = 1.337$, and unstructured calls, $p < .001$, $d = 1.676$, , and compared to oil-treated counterparts.

MIA treatment reduced call production compared to saline controls. Oil-treated early MIA and oil-treated late MIA produced less 1-frequency-step ($p = .049$, $d = .838$; $p = .002$, $d = 1.389$), 2-frequency-step ($p = .019$, $d = 1.009$; $p = .003$, $d = 1.317$), downward ($p = .011$, $d = 1.099$; $p < .001$, $d = 1.632$), chevron ($p = .033$, $d = 9.12$; $p = .002$, $d = 1.386$), flat ($p = .010$, $d = 1.115$; $p = .010$, $d = 1.137$), short ($p = .017$, $d = 1.027$; $p = .002$, $d = 1.377$), and unstructured calls ($p < .001$, $d = 1.494$; $p = .001$, $d = 1.463$), , and, compared to oil-treated saline controls. Additionally, oil-treated late MIA produced less upward, $p = .007$, $d = 1.197$, reverse chevron, $p = .003$, $d = 1.328$, and complex calls, $p = .003$, $d = 1.328$, than oil-treated saline controls. Early and late MIA males did not significantly differ for any call type (p's > .05). Further, prenatal T treatment did not significantly influence call type production among MIA treated males (ps > .05).

Main effects of hormone treatment were found for average low call frequency, $F(1, 56) = 6.109$, $p = .017$, average high call frequency, $F(1, 56) = 8.267$, $p = .006$, and overall average call frequency, $F(1, 56) = 6.838$, $p = .011$, such that T-treated males produced calls with a lower average low, $p = .017$, $d = .630$, high, $p = .006$, $d = .733$, and overall call frequency, $p = .011$, $d = .667$, when compared to oil-treated males (Figure 12; Table 7). Main effects of hormone treatment were found for chevron calls, $F(1, 56) = 4.709$, $p = .034$, and complex calls, $F(2, 56) = 5.229$, $p = .026$, such that prenatal T reduced the production of these call types compared to oil-treated males, $p = .034$, $d = .556$, and $p = .026$, $d = .586$, respectively.

3.2.5 Late MIA Hindered Ultrasonic Communication During Opposite-Sex Interactions

A significant MIA by hormone treatment interaction was found for call number, $F(2, 56) = 4.467$, $p = .016$, and for total call duration, $F(2, 56) = 4.157$, $p = .021$, while a marginal MIA by hormone treatment interaction was found for average high call frequency, $F(2, 56) = 2.709$, $p = .075$. Oil-treated late MIA males produced fewer calls than both oil-treated early MIA, $p = .017$, $d = 1.102$, and oil-treated saline controls, $p < .001$, $d = 1.851$. Oil-treated late MIA spent less time calling than both oil-treated early MIA, $p = .024$, $d = 1.039$, and oil-treated saline controls, $p < .001$, $d = 1.760$. Among early MIA males, perinatal T treatment reduced the number of calls produced, $p = .003$, $d = 1.357$, time spent calling, $p = .008$, $d = 1.198$, and lowered the average high call frequency, $p = .044$, $d = .900$, compared to oil-treated counterparts. Similarly, T treatment reduced the number of calls and duration of calls produced by saline controls, $p < .001$, $d = 2.100$, and $p < .001$, $d = 1.964$, respectively. Among oil-treated males, late MIA reduced average high call frequency compared to saline controls, $p = .044$, $d = .881$ (Figure 10b; Table 7).

3.2.6 Perinatal T Reduced Sociability in Non-MIA and Early MIA, but not Late MIA

A significant MIA by hormone treatment interaction was found for number of entries into the social chamber, $F(2, 68) = 5.332$, $p = .007$ (Figure 13a), and social interaction zone, $F(2, 68) = 5.133$, $p = .008$ (Figure 13b). Prenatal T led to decreased entries into the social chamber and interaction zone among both saline controls and early MIA groups. Specifically, T-treated saline controls had reduced entries into the social chamber, $p = .002$, $d = 1.316$, and interaction zone, $p = .001$, $d = 1.360$, compared to oil-treated saline controls, and T-treated early MIA males entered the social chamber less often than oil-treated early MIA males, $p = .003$, $d = 1.248$. The reverse effect was found among Late MIA males, where a decrease among oil-treated late MIA males and an increase among T-treated late MIA males on these measures resulted in no difference between these groups. Specifically, oil-treated late MIA males showed reduced entries into the social chamber compared to oil-treated early MIA males, $p = .018$, $d = .991$, and T-treated late MIA males showed an increase in the social chamber and social interaction zone entries compared to T-treated saline males, $p = .028$, $d = .900$ and $p = .012$, $d = -1.030$, respectively.

A significant main effect of MIA treatment was found for entries into the non-social chamber, $F(2, 68) = 4.003$, $p = .023$ (Figure 13c), and interaction zone, $F(2, 68) = 4.194$, $p = .019$ (Figure 13d), where late MIA males entered the non-social chamber, $p = .007$, $d = .792$, and interaction zone, $p = .005$, $d = .828$, less often than saline controls. A significant main effect of hormone treatment was found for duration in the non-social chamber, $F(1, 68) = 4.882$, $p = .031$, such that T-treated males spent more time in the non-social chamber compared to oil-treated males, $p = .022$, $d = -.514$. Similarly, a significant main effect of hormone treatment was found for the sociability index, $F(1, 68) = 6.763$, $p = .011$, such that prenatal T treatment reduced sociability in males, $p = .011$, $d = .605$ (Table 8).

3.2.7 MIA During Prenatal Androgen Surge Reduces Preference for Social Novelty

During phase II of the three-chamber social paradigm, a significant MIA by hormone treatment interaction was found for entries into the novel stimulus chamber (Figure 14a), $F(2, 67) = 5.131$, $p = .008$, and interaction zone (Figure 14b), $F(2, 67) = 7.187$, $p = .001$. T-treated saline controls entered the novel stimulus chamber, $p = .005$, $d = 1.167$, and novel interaction zone, $p < .001$, $d = 1.439$, less often than oil-treated saline controls. Compared to oil-treated saline controls, oil-treated late MIA made fewer entries into the novel stimulus chamber, $p = .002$, $d = 1.306$, and interaction zone, $p = .001$, $d = 1.357$. Interestingly, T-treated late MIA entered the novel stimulus interaction zone more often than T-treated saline controls, $p = .047$, $d = -.809$.

A significant main effect of hormone treatment was found for entries into the familiar stimulus chamber (Figure 15a), $F(1, 67) = 16.893$, $p < .001$, and interaction zone (Figure 15b), $F(1, 67) = 10.023$, $p = .002$, such that T-treated males entered the familiar chamber and interaction zone less often than oil-treated males, $p < .001$, $d = .964$, and $p = .005$, $d = .742$, respectively. Further, a significant main effect of hormone treatment was found for duration in the familiar stimulus chamber (Figure 15c), $F(1, 67) = 5.344$, $p = .024$, and familiar interaction zone (Figure 15d), $F(1, 67) = 6.102$, $p = .016$, such that T-treated males spent less time in the familiar stimulus chamber, $p = .024$, $d = .542$, and interaction zone, $p = .016$, $d = .579$, compared to oil-treated males. A marginal MIA by hormone treatment interaction for duration in the familiar stimulus chamber, $F(2, 67) = 2.627$, $p = .080$, revealed that, compared to oil, T treatment led to a reduction in time spent in the familiar stimulus chamber in both saline and late MIA males, $p = .028$, $d = .896$, and $p = .023$, $d = .951$, respectively. Oil-treated late MIA spent more time in the familiar stimulus chamber than oil-treated early MIA, $p = .017$, $d = -1.021$, while oil-treated saline controls were intermediate between these groups on this measure.

A significant main effect of hormone treatment for duration in the novel stimulus chamber (Figure 14c), $F(1, 67) = 8.430$, $p = .005$, revealed that T-treated males spent more time in the novel stimulus chamber compared to oil-treated males, $p = .005$, $d = -.681$. Similarly, a marginal MIA by hormone treatment interaction for duration in the novel stimulus interaction zone, $F(2, 67) = 2.879$, $p = .063$, revealed oil-treated late MIA males spent less time in the novel stimulus interaction zone than oil-treated saline controls, $p = .043$, $d = .844$. Lastly, a significant main effect of hormone treatment was found for the social novelty index (Figure 14d), $F(1, 67) = 7.941$, $p = .006$, where T-treated males had a higher social novelty index than oil-treated males, $p = .006$, $d = -.661$ (Table 8).

3.3 Androgenization Reduced Ovarian Weight

A significant MIA by hormone treatment interaction was found for gonad weight, $F(2, 61) = 3.887$, $p = .026$, such that T-treated saline controls had a greater gonad weight compared to oil-treated saline controls, $p < .001$, $d = -1.505$ (Figure 16). T-treated late MIA females had a significantly lower gonad weight compared to T-treated early MIA, $p = .049$, $d = .862$, and T-treated saline controls, $p < .001$, $d = 1.669$. This effect remained when accounting for body weight, $F(2, 61) = 4.006$, $p = .023$). Specifically, T-treated late MIA, $p < .001$, $d = 1.669$, and T-treated early MIA, $p = .049$, $d = .840$, as well as oil-treated saline controls, $p < .001$, $d = -1.493$, had lower gonad weight / body weight compared to T-treated saline female. A main effect of hormone treatment was found for body weight, $F(1, 61) = 7.598$, $p = .008$, such that T-treated females had greater body weight compared to oil-treated females, $p = .008$, $d = -.675$ (Table 9).

3.4 Female Behaviour

3.4.1 Late MIA Adversely Influenced Developmental Milestones

Differences in latency to self-right were found at PND 6 and PND 10. At PND 6, oil-treated late MIA female mice were slower to self-right than oil-treated saline control mice, $\chi^2(1) = 4.308$, $p = .038$, $\varepsilon^2 = .253$. At PND 10, T-treated saline controls were slower to self-right than oil-treated saline controls, $\chi^2(1) = 4.554$, $p = .033$, $\varepsilon^2 = .217$ (Table 10). Prenatal T did not influence the righting reflex speed in MIA groups. Oil-treated MIA females differed for eye-opening in late neonatal development. Specifically, at PND 14, oil-treated late MIA demonstrated delayed eye-opening compared to oil-treated early MIA, $\chi^2(1) = 3.923$, $p = .048$, $\varepsilon^2 = .178$, but neither MIA group differed from oil-treated saline controls. No significant differences were found at other timepoints measured (Table 11).

3.4.2 Perinatal Testosterone Reduced Repetitive Behaviour in Late MIA

A significant MIA by hormone treatment interaction was found for marbles buried at the 10-minute timepoint, $F(2, 61) = 3.739$, $p = .029$, such that oil-treated late MIA females had buried more marbles than oil-treated saline controls, $p = .032$, $d = -.939$, and oil-treated early MIA, $p = .004$, $d = -1.252$, at this timepoint. Similarly, a significant MIA by hormone treatment interaction was found for marbles buried at the 30-minute timepoint, $F(2, 61) = 3.845$, $p = .027$, where oil-treated late MIA females had buried more marbles than oil-treated saline controls, $p = .020$, $d = -1.016$, and oil-treated early MIA, $p = .005$, $d = -1.205$. Oil-treated early MIA did not differ from oil-treated saline controls throughout the marble-burying task (p's > .05). Interestingly, T-treated late MIA females had buried fewer marbles than oil-treated late MIA females at 30 minutes, $p = .036$, $d = .936$, indicative of reduced anxiety-like behaviour (Figure 17; Table 12).

3.4.3 Androgenization Increased Frequency of Calls Emitted by Neonatal Females

PND 4. A significant main effect of hormone treatment was found for average low call frequency, $F(1, 54) = 5.994$, $p = .018$, average high call frequency, $F(1, 54) = 10.126$, $p = .002$, and overall average call frequency, $F(1, 54) = 8.511$, $p = .005$ (Figure 18). T-treated females produced calls with a higher average low frequency, $p = .018$, $d = -0.654$, high frequency, $p = .002$, $d = -.849$, and overall frequency, $p = .005$, $d = -.779$, compared to oil-treated females.

PND 6. A significant main effect of MIA treatment was found for average call duration at PND 6, $F(1, 56) = 5.380$, $p = .007$, such that early MIA females produced shorter calls than late MIA females, $p = .002$, $d = -1.045$, and saline controls, $p = .048$, $d = .612$ (Figure 19).

PND 8. A significant main effect of hormone treatment was found for overall average call frequency, $F(1, 60) = 4.033$, $p = .049$, such that T-treated females produced calls with a higher overall frequency compared to oil-treated females, $p = .049$, $d = -0.497$ (Figure 18; Table 13).

3.4.4 MIA Reduced USV Call Production in Juvenile Females

A significant main effect of MIA treatment was found for the number of calls produced in the presence of a male stimulus conspecific, $F(2, 52) = 3.840$, $p = .028$. Specifically, early MIA and late MIA females produced fewer calls than saline controls, $p = .012$, $d = .867$, and $p = .044$, $d = .645$, respectively. No significant differences were found among female groups for juvenile USVs in the presence of a female stimulus conspecific (Table 14).

3.4.5 Perinatal Testosterone Increased Social Novelty Preference

There were no significant findings among female groups for phase I (sociability) of the three-chamber social paradigm (Table 15). During phase II (social novelty preference), a marginal MIA by hormone treatment interaction was found for duration in the novel stimulus chamber, $F(2, 61) = 2.713$, $p = .074$. T-treated saline controls spent more time in the novel

stimulus chamber than oil-treated saline controls, $p = .034$, $d = -.925$. T-treated saline controls also spent more time in the novel stimulus chamber than T-treated early MIA, $p = .018$, $d = 1.017$, and T-treated late MIA, $p = .035$, $d = .944$, suggesting reduced social novelty preference among T-treated MIA females; oil-treated controls and MIA groups did not differ on this measure. A marginal MIA by hormone treatment interaction was also found for duration in the novel stimulus interaction zone, $F(2, 61) = 2.920$, $p = .062$, such that T-treated late MIA females spent more time in the novel stimulus interaction zone than oil-treated late MIA, $p = .011$, $d = -1.153$, suggesting androgenization in late MIA females is increasing preference for social novelty.

3.5 Increased Sociability in Late MIA Males versus Females

A significant sex by MIA treatment interaction was found for duration in the social chamber, $F(2, 63) = 3.532$, $p = .035$. Post hoc analyses revealed late MIA males spent significantly more time in the social chamber compared to late MIA females, $p < .001$, $d = 1.486$. Similarly, a marginal interaction was found between sex and MIA treatment for the sociability index, $F(2, 63) = 2.803$, $p = .068$, with late MIA males having a higher sociability index than late MIA females, $p = .002$, $d = 1.347$. Conversely, no sex differences were found for social behavior in saline controls or early MIA mice (Figure 20). The sexes did not differ on any other behavioural measure (i.e., USVs or marble burying).

3.6 Hyperandrogenization Reduced TNF-α in MIA, but Elevated Levels in Non-MIA

A significant interaction was found between MIA treatment and hormone treatment for brain TNF-α concentration in neonatal male brain tissue, $F(2, 29) = 22.591$, $p < .001$ (Figure 21). Hyperandrogenization significantly increased TNF-α levels in saline controls, $p = .001$, $d = -2.090$. In contrast, hyperandrogenization significantly decreased TNF-α among early MIA males,

$p < .001$, $d = 3.363$. Compared to hyperandrogenized saline controls, T-treated early MIA, $p < .001$, $d = 3.899$, and T-treated late MIA, $p < .001$, $d = 3.562$, had significantly lower brain TNF-α levels. Among oil-treated males, TNF-α was elevated in early MIA compared to both late MIA, $p = .004$, $d = 1.832$, and saline controls, $p = .012$, $d = -1.554$.

3.7 MIA did not Affect Steroid Hormones on First Day of Birth

A significant main effect of hormone treatment was found in saline control males for serum cortisol concentration, $F(1, 4) = 19.597$, $p = .011$, such that hyperandrogenization elevated cortisol in saline controls. Hyperandrogenized and oil-treated saline controls did not differ for T, P4, E2, T3, or T4 concentrations (p's > .05).

No main effect of MIA treatment was found for steroid hormones. Specifically, oil-treated early MIA and saline control males did not differ for cortisol, T, P4, E2, T3, or T4 concentrations (p's > .05). Oil-treated late MIA and saline controls did not differ for cortisol, T, P4, E2, T3, or T4 concentrations (p's > .05). Oil-treated early and late MIA males did not differ for cortisol, T, P4, E2, T3, or T4 concentrations (p's > .05) (Table 16).

4.0 Discussion

4.1 Overview

In the present study, I used a mouse model of MIA to investigate mechanisms underlying the greater susceptibility of males than females to developing an ASD-like phenotype. To do so, I assessed whether in male offspring, late MIA, coinciding with the prenatal androgen surge, would result in greater MIA-associated deficits than early MIA, prior to testicular androgen production. I then investigated whether hyperandrogenization during this critical organizational period would exacerbate MIA-associated deficits. The findings revealed that, as anticipated, late gestational MIA resulted in the most severe behavioural deficits among male offspring.

Hyperandrogenization did not further exacerbate deficits in male offspring born to MIA dams as expected. Instead, high androgen signalling, in the *absence* of MIA, was sufficient in producing an ASD-like phenotype, lending support to the EMB theory of ASD.

In female offspring, I assessed whether perinatal androgenization (likened to male-typical levels) would result in susceptibility to MIA deficits that is comparable to males. While late MIA female offspring presented with some behavioural deficits, they were minimal relative to those seen in males, and androgen treatment did not further exacerbate deficits in females. Instead, perinatal androgen exposure in females resulted in increased repetitive and social behaviours in a manner comparable to male-typical behaviour (not necessarily an ASD-like phenotype). Taken together, these findings suggest that the timing of gestational infection is directly related to the severity of ASD-like behavioural deficits in offspring, and this ASD-like phenotype is influenced by exposure to prenatal androgens, with greater than male-typical levels (i.e., hyperandrogenization) leading to deficits.

4.2 MIA and Hyperandrogenization Shaped Male Behaviour

4.2.1 Developmental Milestones

Developmental delays such as increased latency to self-right and delayed eye-opening have been reported in male pups following MIA (Arsenault et al., 2014; Haida et al., 2019) and is thought to be related to impairments in sensorimotor abilities, muscle function, and coordination in adulthood (Arsenault et al., 2014; Haida et al., 2019). Developmental challenges in MIA neonatal offspring may be associated with reductions of neurons in the motor cortex and Purkinje cells (PC) in the cerebellum (Haida et al., 2019). In the human condition, reductions in PC have been reported in postmortem analyses (Whitney et al., 2008). In the current study,

hyperandrogenization alone led to a decrease in latency to self-right, but in MIA groups, hyperandrogenization was not sufficient to accelerate motor development on this measure.

4.2.2 Male Neonatal USVs

Neonatal USVs are one of the earliest possible measures of communication development in rodent models of ASD (Jouda et al., 2019; Wöhr & Scattoni, 2013). MIA neonatal offspring commonly present with deficits in ultrasonic communication during brief maternal separation (reviewed in Premoli et al., 2021). While some research has been conducted on the influence of prenatal/perinatal androgen exposure on adult USV production, little is known regarding how perinatal androgens mediate neonate USV production during maternal separation. For example, previous research has shown that T treatment in prenatal and peripubertal development results in increased call frequency in adulthood (Kikusui et al., 2021). Consistent with this work, we found that call frequency was higher in hyperandrogenized male pups at PND 6 and 8 compared to oil-treated. Hyperandrogenization largely impaired communication in neonatal males. Specifically, elevated perinatal androgens reduced call number, average call duration, and total call duration in male pups at PND 8 regardless of MIA treatment.

Analysis of neonatal male USV call classifications yielded mixed findings. At PND 4, production of downward calls was increased in hyperandrogenized early MIA compared to oil-treated early MIA males. T-treated early MIA also produced more downward calls than T-treated late MIA and T-treated saline controls. Given these results, it is possible that perinatal T treatment is enhancing communication in early MIA males by increasing these calls; alternatively, there may be a threshold at which overproduction of calls may be abnormal and deter, rather than encourage, the dam to retrieve the pup. In non-MIA (saline) males, the opposite effect was observed, as hyperandrogenization was found to decrease the number of downward

calls produced at PND 4. Overall, fewer calls with hyperandrogenization suggest deficits in communication; however, more work is needed to understand whether specific call types, such as downward calls, encourage or discourage maternal care.

A reduction in the production of complex calls was found at PND 4 in hyperandrogenized late MIA males. Previous studies have shown dams will respond to both simple and complex sequences of isolation calls in a similar manner when pups are presented individually (Wöhr & Scattoni, 2013). However, in a litter, it is difficult for the dam to seek out specific pups when all are producing similar vocalizations. Thus, pups may try to increase the complexity of their call sequences to increase their likelihood of being groomed, fed, and retrieved by the dam (Wöhr & Scattoni, 2013). As call complexity is reduced in hyperandrogenized late MIA male pups, late gestational MIA in the presence of excess androgens may be reducing the efficacy of USV communication.

To date, the meaning behind the various call types has yet to be interpreted or understood (Premoli et al., 2022). The same call types are used by neonates and adults and in a variety of circumstances. Emphasis should be placed on trying to determine the meaning of the call types in different contexts so clearer interpretations of neonatal USVs can be drawn in NDD mouse models (Wöhr & Scattoni, 2013). Nevertheless, our results suggest that perinatal hyperandrogenization in both late MIA and saline controls impairs neonatal communication.

4.2.3 Male Juvenile USVs

Hyperandrogenization reduced the number of calls produced by saline controls in the presence of a stimulus female, as well as the total time spent calling. Indeed, hyperandrogenization resulted in similar deficits as oil-treated late MIA males, suggesting that hyperandrogenization on its own produces MIA-like deficits in communication. While prior

work has found that hyperandrogenization increases call frequency (Kikusui et al., 2021), we did not observe group differences on this measure in the presence of a female conspecific. However, Kikusui et al. (2021) administered T during neonatal and peripubertal development and recorded USVs in adulthood, whereas in the present study, T was administered prenatally and on the day of birth and the animals were tested as juveniles. Further, the stimulus females in the current study were sexually naïve and unmanipulated, whereas Kikusui et al. (2021) used ovariectomized females primed with 17β-estradiol and P4 to induce behavioural estrus. Prenatal T treatment led to a reduction in call frequency compared to oil-treated males. This is in contrast to neonatal USV results, where perinatal T treatment increased call frequency. This reduction in call frequency in juvenile males when interacting with a stimulus male may be explained by power dynamics. Sangiamo et al. (2020) found that non-dominant males produce higher frequency USVs while aggressors produce lower frequency USVs in male-male encounters. In the current study, the hyperandrogenized experimental male may be asserting dominance over the stimulus male, thus lowering their USV frequency. In future studies, the encounters should be video recorded to assess prosocial and aggressive behaviours.

Juvenile late MIA males spent less time calling and produced fewer calls compared to both early MIA and saline controls in the presence of a stimulus female. These results were anticipated as late MIA males were expected to display greater communicational deficits. Similar findings have been reported in MIA mice in the late juvenile stage (i.e., 8-10 weeks old), such that MIA reduced the number and duration of calls produced (Hsiao et al., 2013; Malkova et al., 2012). However, to my knowledge, this study is novel in evaluating the USVs of MIA mice in the early juvenile stage (i.e., 5-7 weeks old). Among oil-treated males, early MIA and late MIA produced fewer calls and produced shorter calls compared to saline controls. Similarly, oil-

treated early MIA and late MIA produced fewer of each call classification type compared to oil-treated saline controls. This was anticipated, as we hypothesized ultrasonic communication would be impaired in the MIA groups (Malkova et al., 2012; Wöhr & Scattoni, 2013). Hyperandrogenization was found to reduce the number of calls and time spent calling for early MIA in the presence of a female conspecific. Oil- and T-treated late MIA males did not differ on these measures, suggesting hyperandrogenization in late MIA did not further exacerbate communication deficits toward the opposite sex.

4.2.4 Male Social Behaviour

Consistent with our hypothesis, decreased sociability was found in late MIA males compared to early MIA and saline controls, as demonstrated by reduced entries into the social chamber and interaction zone. Unexpectedly, hyperandrogenization in saline controls was sufficient to produce similar deficits, while hyperandrogenization in late MIA males did not further exacerbate deficits. Overall, hyperandrogenized males spent more time in the non-social chamber and interaction zone and had a lower sociability index compared to oil-treated males. These results suggest that hyperandrogenization reduces sociability in males, which has been shown previously in the literature (Grgurevic, 2023; Swift-Gallant et al., 2016). Specifically, in a preclinical VPA mouse model of ASD, males were found to have elevated plasma T and reduced social behaviour, demonstrating a connection between excess androgens and social behaviour deficits (Grgurevic, 2023). Additionally, overexpression of AR, and thus increased androgen signalling, has been found to decrease male-typical social behaviour, such as intermale aggression (Swift-Gallant et al., 2016).

In the social novelty phase of the three-chamber social paradigm, oil-treated late MIA males entered the novel stimulus interaction zone and chamber less often than oil-treated saline

controls. Further, oil-treated late MIA males spent less time in the novel stimulus interaction zone compared to oil-treated saline controls. As well, compared to oil-treated early MIA, oil-treated late MIA spent more time with the familiar stimulus. These results demonstrate a reduction in social novelty preference in MIA male offspring that is in line with previous findings (Chang et al., 2017; Haida et al., 2019; Malkova et al., 2012). Specifically, as hypothesized, late MIA males experienced the greatest social deficits.

Hyperandrogenized males spent more time in the novel stimulus chamber compared to oil-treated males. Additionally, T-treated males had a higher social novelty preference score compared to oil-treated males. This opposes findings from the sociability phase, where hyperandrogenization was largely found to *decrease* sociability. This was unexpected, as the T-treated groups demonstrating reduced sociability in Phase I were anticipated to also show reduced social novelty preference in Phase II. The opposite influence of hyperandrogenization observed between sociability and social novelty preference may be explained by the nature of the two phases; for instance, the social novelty preference introduces elements that can be affected by prenatal androgens, notably social memory and social recognition, that are absent in the sociability phase (reviewed in Aspesi et al., 2023; Yang et al., 2011). It should be noted that social novelty preference is thought to be less relevant than sociability in rodent models of ASD, as sociability more closely resembles symptomology of the human condition (Yang et al., 2011).

4.3 Summary of Male Results

In comparison to early MIA, late MIA resulted in deficits of a more severe nature in males. This was seen across communication and social behaviour, as well as repetitive behaviour. These findings support the hypothesis that MIA during the prenatal androgen surge would lead to more severe deficits. It was hypothesized that if androgens are important in sex

differences in MIA, providing more androgens during prenatal androgen surge would have an additive effect on MIA deficits. Interestingly, administration of exogenous androgens to late MIA males **did not** worsen deficits. Given that late MIA males presented with such low communication and social behaviour, there may be a floor effect, such that perinatal T could not further worsen these deficits. Alternatively, it may be possible that T administration ~24-30 hours prior to MIA exposure suppressed the fetal immune system and dampened the effect of MIA on development; the addition of perinatal androgens may have partially ameliorated deficits in late MIA males, such as by enhancing sociability and reducing anxiety-like behaviour.

The results of a hormone assay discerned that there was no difference in the serum T levels of oil-treated and hyperandrogenized late MIA neonatal males on the first day of birth. Neonatal mice euthanized on PND 1 did not receive a postnatal T injection, meaning at least 24 hours had passed between the time of the last maternal T injection and birth. It is possible that androgen-immune interactions are indeed taking place during the prenatal androgen surge; however, due to washout, T levels may have returned to baseline prior to sacrifice on the first day of birth. This study employed a hyperandrogenization protocol that is well-established (Armoskus et al., 2014; Ghahramani et al., 2014; Hisasue et al., 2010), and androgenized female offspring presented with male-typical behaviours, providing confidence that the T treatment was effective (Michard-Vanhée, 1988; Vom Saal & Bronson, 1980).

Intriguingly, hyperandrogenization in the absence of MIA resulted in behavioural deficits in males that were comparable to those of MIA offspring. These findings suggest that high androgen signalling alone is sufficient to produce an ASD-like phenotype in males. These findings align with Baron-Cohen's (2002) EMB theory which suggests elevated maternal androgens may increase the risk of ASD in offspring. Support for the EMB theory has been

garnered from various studies linking higher fetal androgens and androgen-related traits to greater risk of ASD (Auyeung et al., 2009; Auyeung et al., 2012; Baron-Cohen, 2002; Baron-Cohen et al., 2011; Knickmeyer et al., 2005). Underlying mechanisms of the EMB theory remain unclear, as such studies in humans must rely on correlational data due to ethical constraints in manipulating the prenatal endocrine environment. Nevertheless, we know that T influences synaptic plasticity, molecular structure, expression of proteins, and apoptosis of neurons, that permanently influence neurological organization (reviewed in Gore et al., 2014). T is also capable of influencing gamma-aminobutyric acid (GABA) and oxytocin, among a variety of other neurotransmitters and hormones, that mediate neurodevelopment (Baron-Cohen et al., 2020). Thus, while we do not yet know the mechanism by which T may be acting to increase risk, the results of the present study provide evidence for a causal relationship between hyperandrogenization and ASD-like phenotype. As such, future work may consider hyperandrogenization as a preclinical model of ASD, and evaluate the mechanisms further.

Increased fetal steroidogenic activity is known to be associated with ASD in humans (Baron-Cohen et al., 2015). To understand how hyperandrogenization and gestational infection can influence the hormone milieu in offspring, the steroid hormones cortisol, T, E2, P4, T3 and T4 were assayed in neonatal male serum (PND 1). Serum cortisol levels were not elevated in MIA offspring in the current study. This was unexpected, given that elevated cortisol has previously been reported in a preclinical rodent model of ASD; in a mouse model of MIA induced by LPS injection, cortisol was elevated in male offspring following stress in a resident-intruder paradigm (French et al., 2013). However, in contrast to the current study, French et al. (2013) assessed cortisol levels in adulthood, and not on the first day of birth – this could explain

why no difference in cortisol was found between MIA groups in the current study. Further investigation of HPA response to MIA in neonatal offspring is warranted.

Interestingly, hyperandrogenized saline males had elevated serum cortisol on the first day of birth. In the normally developing fetus, cortisol is known to increase as a result of increased T (Gitau et al., 2005). In the case of hyperandrogenization (and EMB), this effect may be exaggerated. Studies of EMB theory have found increased steroidogenic activity, including cortisol; elevated cortisol levels were reported in the amniotic fluid of male offspring who were later diagnosed with ASD (Baron-Cohen et al., 2015). Further, the HPA axis is significantly more reactive in children with ASD. For instance, in response to stress, children with ASD have significantly more serum cortisol than neurotypical children, and this elevation in cortisol persists for longer than normal (Spratt et al., 2012). This increase in cortisol may be related to the ASD-like behavioural phenotype observed in the hyperandrogenized non-MIA males in the current study.

The proinflammatory cytokine TNF-α has been found to be elevated in the brain tissue, plasma, and cerebrospinal fluid (CSF) of individuals with ASD (Ashwood et al., 2011; Vargas et al., 2005; Xie et al., 2017). In this study, TNF-α levels were *elevated* in the neonatal brain tissue of oil-treated early MIA males, while late MIA and saline controls were comparable for TNF-α levels. Interestingly, among early MIA, hyperandrogenization significantly *reduced* TNF-α. TNF-α is capable of both neurotoxic and neuroprotective effects depending on a variety of factors, including receptor action and timing of immune insult (Gilmore et al., 2015). In a rat model of MIA, Gilmore et al. (2005) administered poly I:C at E16 and found that TNF-α was significantly increased in the placenta and amniotic fluid, as well as in maternal plasma. However, TNF-α levels were decreased in the neonatal brain (Gilmore et al., 2005). Given that

TNF-α is important in mediating cortical and neuronal development, it is possible that a reduction in TNF-α expression in neurons or supporting glial cells is affecting typical neurodevelopment in MIA neonates and increasing susceptibility to NDDs (Gilmore et al., 2005). Conversely, hyperandrogenization increased TNF-α compared to oil controls. Hyperandrogenization produced greater deficits than early MIA, perhaps due to elevated proinflammatory cytokines. It is possible there are no effects of MIA and hyperandrogenization together because interactions between MIA and T paradoxically lead to decreases in cytokines; meanwhile MIA and T alone tend to increase cytokines.

4.4 Effects of MIA and Androgenization on Female Behaviour

4.4.1 MIA and Androgenization Influenced Female Development

MIA had little effect on the developmental milestones in neonatal females. Compared to oil-treated early MIA females, oil-treated late MIA had delayed eye-opening during neonatal development; however, the MIA groups did not differ from saline controls, suggesting that saline was intermediate to early and late MIA. Androgenization influenced developmental milestones in neonatal females, such that androgenized females took longer to self-right than oil-treated controls. It may be possible that T-treated female pups had increased body weight compared to oil-treated as pups, which impeded their ability to self-right. In future studies, pups should be weighed each day of neonatal development. Perinatal androgen treatment increased body and ovarian weight in juvenile female offspring. Among late MIA females, androgenization significantly reduced juvenile ovarian weight. Rodent models of polycystic ovarian syndrome (PCOS), characterized by androgen excess, often report reductions in ovarian weight that are largely attributed to a reduction in or the absence of corpora lutea (Aflatounian et al., 2020; Chen et al., 2015; de Souza et al., 2018). Conversely, increases in ovarian weight due to the

development of ovarian cysts have also been reported when T is administered within 24 hours of birth (Jang et al., 2015), which may explain increased ovarian weight in hyperandrogenized saline females.

4.4.2 Androgenization and MIA Influenced Ultrasonic Communication in Females

At PND 4 and PND 8, perinatal androgenization increased call frequency in neonatal females. Similar findings have been previously reported, as Kikusui et al. (2021) found that T treatment during the neonatal and peripubertal periods increased high-frequency calls in female mice. Exogenous perinatal androgen exposure may be making females more male-typical. At PND 6, findings demonstrate a possible communicational deficit in early MIA females, as they produced shorter calls in response to maternal separation compared to late MIA and saline control females. MIA offspring have been found to produce significantly more short calls compared to controls (Malkova et al., 2012). In the presence of a stimulus male, both oil-treated early MIA and late MIA juvenile females produced fewer calls than oil-treated saline controls, suggesting that MIA can negatively influence communication in female offspring during opposite-sex interactions. Neither MIA condition nor hormone treatment influenced female USVs in the presence of another female, which may be attributed to the low numbers of calls produced. Overall, hyperandrogenization increased communication in females, while early and late MIA produced similar deficits in communication in the juvenile period.

4.4.3 Androgenized Females Displayed Male-Typical Social Behaviour

Among late MIA females, prenatal androgen treatment resulted in an increased preference for social novelty. Androgenized females may be exhibiting increased social novelty preference because the prenatal androgen treatment is making females more male-typical for social behaviour. Previous studies have shown that males tend to be more social on tasks such as

three-chamber social paradigm compared to females (Cox & Rissman, 2011; Karlsson et al., 2015). On the other hand, sociability was not affected by hyperandrogenization, nor MIA in females.

4.4.4 Androgenization Reduced Anxiety-Like Behaviour in MIA Females

Repetitive marble-burying behaviour was influenced by MIA condition, with significantly more marbles being buried by females exposed to MIA during late gestation. Interestingly, androgenization in late MIA significantly reduced marble burying compared to oil-treated late MIA. These findings may suggest an anxiolytic effect of T when MIA is induced during late gestation. Under varying circumstances T has been demonstrated to have both anxiogenic and anxiolytic properties (reviewed in Domonkos et al., 2018), and perinatal T treatment was previously found to have an anxiogenic effect of increasing marble burying in female mice (Goel & Bale, 2008). In the current study, given that T treatment reduced anxiety-like behaviour in late MIA females, it is possible that an interaction between MIA and T is having an anxiolytic effect.

4.5 Females May be Protected Against ASD

MIA-associated deficits were minimal in female offspring. Given the heightened male susceptibility for deficits in this preclinical ASD model, it was anticipated that behavioural development of female offspring would be largely unaltered by exposure to MIA in this study (e.g., Carlezon et al., 2019; Haida et al., 2019; Malkova et al., 2012). Here, I was particularly interested in examining the effects of MIA on behavioural development in relation to the prenatal androgen surge. As this surge in androgens during late gestation is experienced only by male fetuses, and prenatal androgens are not necessary for female prenatal development (George & Ojeda, 1982) it is unsurprising that minimal MIA deficits were observed in female offspring.

It is possible that females may possess protective factors, such as resistance to genetic mutations and naturally elevated levels of the social bonding hormone oxytocin, that reduce the risk of ASD development (Miller et al., 2013). For instance, the female protective effect suggests that females are biologically guarded and require a greater genetic burden or additional environmental risk factors to develop ASD, whereas males are far less robust (Dougherty et al., 2022; Robinson et al., 2013; Wigdor et al., 2022). It should also be noted that the dose of poly I:C administered here was lower than most studies. In the current study, a dose of 5mg/kg was administered, while 20mg/kg is commonly used in the MIA mouse model (e.g. Choi et al., 2016; Haida et al., 2019; Kim et al., 2017). The fact that MIA deficits were observed in male offspring with this lower dose of poly I:C demonstrates the sensitivity of this model. Even so, it may take a more extreme immune insult to cause the onset of an ASD-like phenotype in female offspring. Overall, early and late MIA largely did not produce differences in females, and androgenization increased some male-typical behaviours but overall did not produce an ASD-like phenotype.

4.6 Limitations

The current study has produced novel findings that garner interest in understanding the relationship between MIA and androgens for offspring behavioural development. This was an exploratory study with limitations that should be addressed in future investigations. All experimental and stimulus animals were singly-housed, with no exposure to social interactions following weaning. Previous studies have found that isolation can impact social development, suggesting that singly- versus group-housed animals may perform differently on social tasks (Bouet et al., 2011; Kercmar et al., 2011). Similarly, prior social experience plays a role in the normal production of murine USVs in adulthood (Burke et al., 2018; Screven & Dent, 2019). To assess the impact of prior social experience on social performance and communication in the

MIA mouse model, the present study should be replicated with group-housed animals. Additionally, maternal behaviour of MIA dams toward pups was not assessed in this study. Limited research has been conducted on how MIA influences maternal care; however, poly I:C-treated dams have been found to favor exploratory behaviours over pup retrieval (Zambon et al., 2022). Further research is warranted regarding how MIA influences dam-offspring interactions, as it is possible that altered behaviour in dams can influence offspring behaviour (e.g., neonate USVs).

The three-chamber paradigm employed in the current study is well-validated and commonly used in rodent models of ASD and other NDDs to assess social behaviour (reviewed in Jabarin et al., 2022). For a more in-depth evaluation of social behaviour, multiple tasks should be evaluated. While we also evaluated USVs in response to same- and other-sex conspecifics, future studies may consider evaluating the interaction between the animals, to include measures of anogenital investigation, aggression and sexual interest (Koolhaas et al., 2013; McFarlane et al., 2008; Wang et al., 2011). Additionally, behaviour testing was performed during the light phase of the light-dark cycle. Mice are generally more active during the dark phase; as such, one may contend that social behaviour tests conducted during the light phase are less representative of true behaviour. However, a review by Yang et al. (2008) found that social behaviour scores produced by studies using both light and dark phase testing yield similar results, suggesting that circadian rhythm may not influence social activities in laboratory mice as greatly as was once believed.

While several ASD-like behavioural deficits were examined in this study, learning and memory abilities were not assessed. This omission was largely due to the time constraint of testing within the juvenile period. Memory deficits may influence behaviours such as social

preference during the social novelty phase of the three-chamber social paradigm. As learning and memory deficits are present in both the human condition and in MIA mouse models, tests of learning and memory (e.g., fear conditioning) should be included in future studies (reviewed in Desaunay et al., 2020; Pasciuto et al., 2015).

Finally, while the present work evaluated sex as a biological variable, future work may also consider circulating hormones during juvenile behaviour testing by tracking the estrous cycles. The estrous cycles of experimental female offspring and unmanipulated stimulus females were not assessed prior to testing. In this study, experimental juvenile mice underwent behavioural testing at PND 35-45. Given that first estrus in mice generally begins 10 days following the opening of the vagina around PND 25 (Ismail et al., 2011; Nelson et al., 1982), it is possible some mice in this study were sexually mature. As female behaviour, especially in social interactions, can vary throughout the estrous cycle, tracking this in future studies can help control for any variability in social behaviour (Chari et al., 2020).

4.7 Future Directions

4.7.1 Cytokine Activity in MIA

Cytokine analyses have been performed frequently during fetal development (e.g., Arrode-Brusés & Brusés, 2012), while fewer studies have assessed cytokines in early neonatal development (e.g., first day of birth). Further, it is unclear how cytokine action varies in the fetal brain when MIA is induced at an early versus a late timepoint in gestation. To supplement the findings presented here, other key cytokines, including IL-6, IL-17a, and IL-1β are being assayed in male neonatal (PND 1) brain tissue. In rodents, IL-6 serves to promote neural growth, cell differentiation and migration, and provide protection against excitotoxicity in the developing brain (Minakova & Warner, 2018; Smith et al., 2007; Wei et al., 2012). Overactivity of IL-6 in

mice has reliably been found to contribute to neurodevelopmental abnormalities, including impairments in synaptic plasticity and altered neural circuitry, that can later contribute to behavioural deficits (Smith et al., 2007, Wei et al., 2012). Increased levels of IL-6 have been reported in postmortem brain tissue of ASD patients (Ashwood et al., 2011; Goines & Ashwood, 2013; Li et al., 2008; Vargas et al., 2005). Similarly, elevated levels of the proinflammatory cytokine IL-17A have been reported in serum samples from children diagnosed with ASD (Al-Ayadhi & Mostafa, 2012). In the MIA rodent model, IL-17A has been shown to promote abnormal cortical development, decrease social approach behaviour, and increase marble burying in offspring (Choi et al., 2016). Additionally, maternal exposure to poly I:C on E16 was found to significantly increase IL-1β, IL-7, and IL-1 in the fetal brain (Arrode-Brusés & Brusés, 2012). As such, studying an array of cytokines will help to identify potential candidate cytokines underlying the sex and/or gestational timing differences with MIA. In the present study, emphasis was placed on investigating the immune environment in neonatal offspring. In future studies, these investigations should be expanded to include maternal and fetal cytokine action to provide an overview of immune interactions at various stages during MIA.

4.7.2 Late MIA and Microglia

Brain tissue of juvenile offspring from the current study has undergone immunohistochemical staining for ionized calcium binding adaptor molecule (Iba)-1, a calcium-binding protein specific to microglia and macrophages. Stereology will be used to assess microglial density in the hippocampus. Microglia, the resident immune cells of the CNS, can produce and release pro-inflammatory cytokines to defend against pathogens (reviewed in Mosser et al., 2017). Additionally, microglia play a crucial role in neurodevelopment through pruning of excess synaptic spines, promotion of neuronal connectivity, and maintenance of

homeostasis in the brain (Fernández de Cossío et al., 2017; Smolders et al., 2018). Postmortem neuroanatomical analyses have revealed immune overactivity in individuals with ASD, characterized by elevated microglial activation and density (Morgan et al., 2010; Vargas et al., 2005). Altered microglial activity has been consistently reported following MIA in rodents (Fernández de Cossío et al., 2017; Murray et al., 2019; O'Loughlin et al., 2017; Smolders et al., 2018). For instance, heightened microglial activity has been observed to disrupt synaptic pruning, thereby contributing to behavioural abnormalities in offspring, including decreased sociability and increased marble burying (Fernández de Cossío et al., 2017; Murray et al., 2019). The hippocampus is of particular interest for studying MIA-associated microglial abnormalities as this brain region is especially vulnerable to inflammatory insult (Hitti & Siegelbaum, 2014; Williamson & Bilbo, 2013). Evidence of increased microglia activation has been found in the hippocampus of neonatal and newly weaned rodents following a prenatal immune challenge (Li et al., 2014, Murray et al., 2019; Ratnayake et al., 2012).

The timing at which poly I:C is administered and when microglia alterations are investigated are of critical importance to understanding the role microglia play in MIA-associated deficits. Many of the studies that have found MIA-associated microglial abnormalities did so when MIA onset occurred in early development and microglia were assessed later than the first day of birth, such as at weaning (PND 21) or in adulthood (e.g. Hui et al., 2018; Murray et al., 2019). Consequently, there is a gap in knowledge pertaining to the effect of late MIA on microglia. This is of relevance to the sex bias in MIA vulnerability. Sex differences in activated microglia have also been noted following MIA, where decreased synaptic pruning was reported only in males (Fernandez de Cassio et al., 2017). In humans, microglial activity increases following the surge in prenatal testosterone in males (Lenz et al., 2013). As such, the relationship

between MIA and microglia as well as affiliated sex differences is a lucrative area of research for exploring the mechanisms of MIA on NDDs. Following from the current study, our lab is further exploring the role of androgens in sex biased MIA deficits by investigating whether disabling androgen receptors (AR) neurally or in microglia will lessen MIA-associated deficits in males when MIA coincides with the prenatal androgen surge.

4.8 Conclusions

To my knowledge, this study is the first to investigate the role of prenatal androgens and timing of gestational infection in a rodent model in an effort to elucidate the underlying mechanisms contributing to the sex disparity in ASD diagnosis. MIA was manipulated both prior to and during the prenatal androgen surge (i.e., early and late gestation, respectively) that acts to organize the brain and behaviour of developing male fetuses, in the presence or absence of exogenous androgens. As anticipated, manipulation in late gestation resulted in the most severe behavioural deficits in male offspring, suggesting a relationship between MIA and androgens for developing an ASD-like phenotype. Exogenous androgens did not have an additive effect of exacerbating deficits in late MIA. Interestingly, hyperandrogenization in the absence of MIA resulted in behavioural deficits comparable to those of MIA offspring, suggesting a role for high androgen signalling in the development of an ASD-like phenotype. Given that MIA and perinatal hyperandrogenization did not differ, hyperandrogenization on its own may be used as a preclinical model of ASD.

Taken together, the findings of this study can be divided in two overarching themes: 1) timing of gestational infection is critical for influencing the severity of MIA-associated behavioural deficits and 2) exposure to prenatal androgens significantly influences the development of an ASD-like phenotype. The findings of this study will help provide a greater

understanding of how the maternal immune system can influence communication, social, and anxiety-like behaviour of offspring in a sex-dependant manner. Many avenues of exploration remain, including analysis of microglia and cytokines, as well as the use of transgenic animals, that will help to bring us closer to resolving the enigma of ASD etiology between the sexes.

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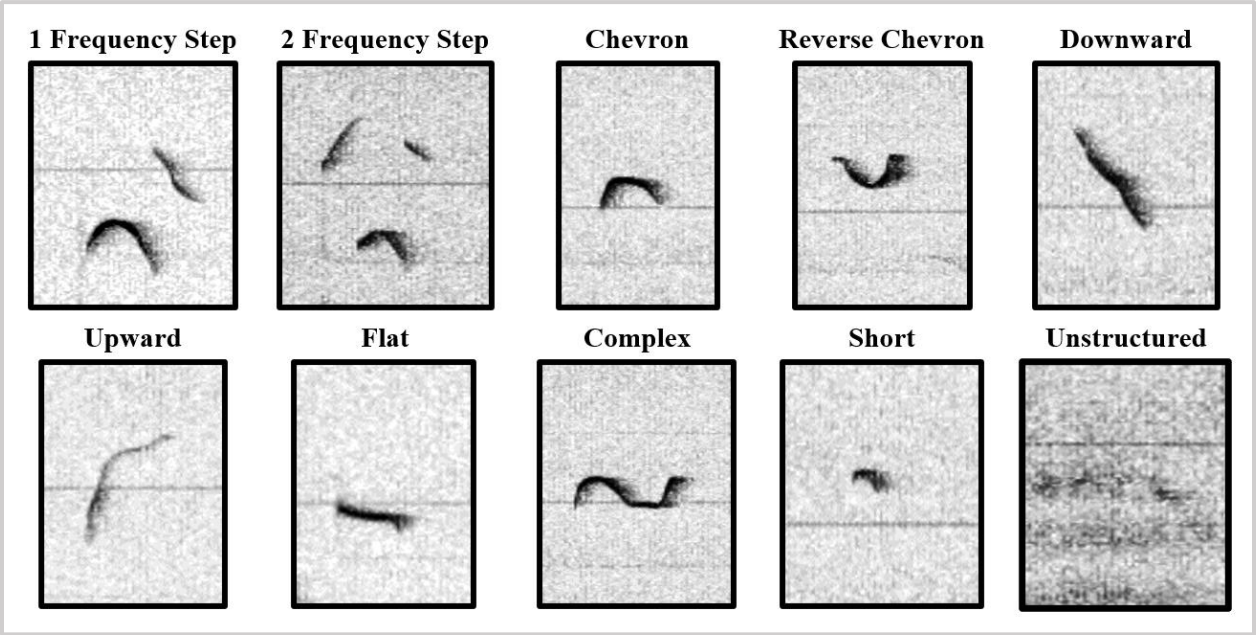
Appendix A - Figures

Figure 1

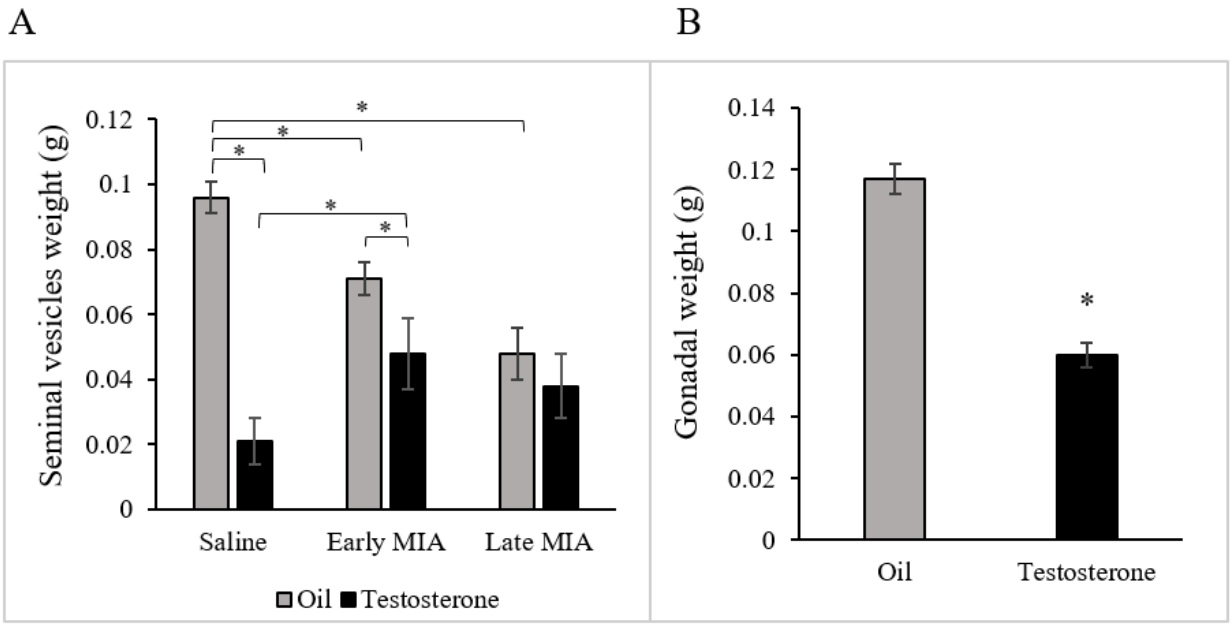
Typical sonograms of ultrasonic vocalization (USV) syllable classifications



Note. USV syllable categories were defined using previously described classification schema (Grimsley et al., 2011; Malkova et al., 2012; Scattoni et al., 2008). **1-frequency-step**: Two components; a flat or downwards main call with an additional punctuated component preceding the main call; no separation in time between components. **2-frequency-step:** Three components; a main call flanked by two discontinuous steps; no separation in time between components. **Chevron:** Inverted-U shape; highest frequency is a least 6 kHz greater than starting and ending frequencies. **Reverse Chevron:** U shape; lowest frequency is a least 6 kHz lower than starting and ending frequencies. **Downward:** Frequency at end is a minimum of 6 kHz less than frequency at beginning. **Upward:** Frequency at end is a minimum of 6 kHz greater than frequency at beginning. **Flat:** Modulation less than 6 kHz. **Complex:** One element with two or more directional changes in frequency of greater than 6 kHz. **Short:** Less than or equal to 5 ms in duration. **Unstructured:** No main sound component; shape is not consistent with any other category

Figure 2

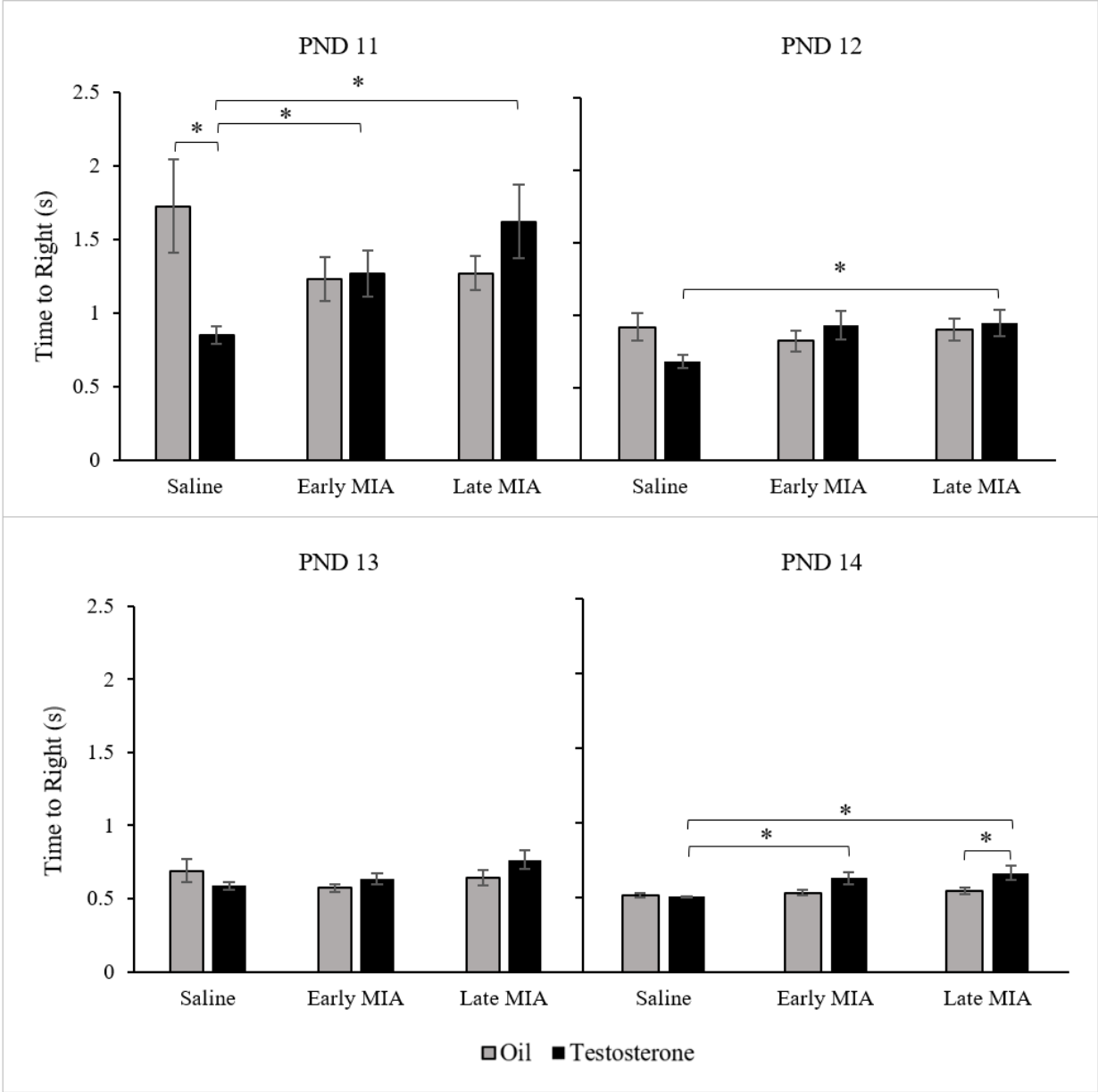
Seminal vesicles weight and gonadal weight in grams (g) as a function of MIA and hormone treatment.



Note. Data shown as mean ± standard error of the mean (SEM) of juvenile male seminal vesicle weight (g) (**A**) and gonadal weight (g) (**B**). **A**. Among oil-treated males, early MIA and late MIA had lower SV weight than saline controls. T treatment lowered SV weight in saline controls, but increased SV weight in early MIA males compared to T-treated saline controls, *$p < .05$. **B**. Prenatal T treatment reduced gonadal weight, *$p < .05$.

Figure 3

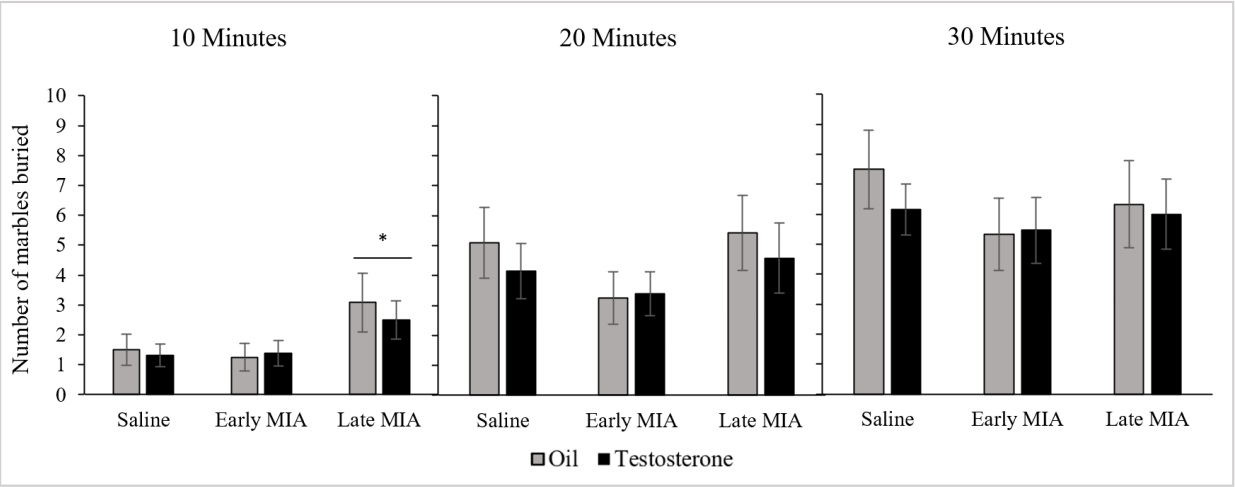
Latency to self-right in seconds (s) as a function of MIA and hormone treatment.



Note. Data shown as mean ± SEM of latency to self-right of neonatal males at PND 11 – 14. T-treated early and late MIA males tended to self-right slower than T-treated saline controls, while T treatment resulted in faster righting among saline controls compared to oil-treated, *$p < .05$.

Figure 4

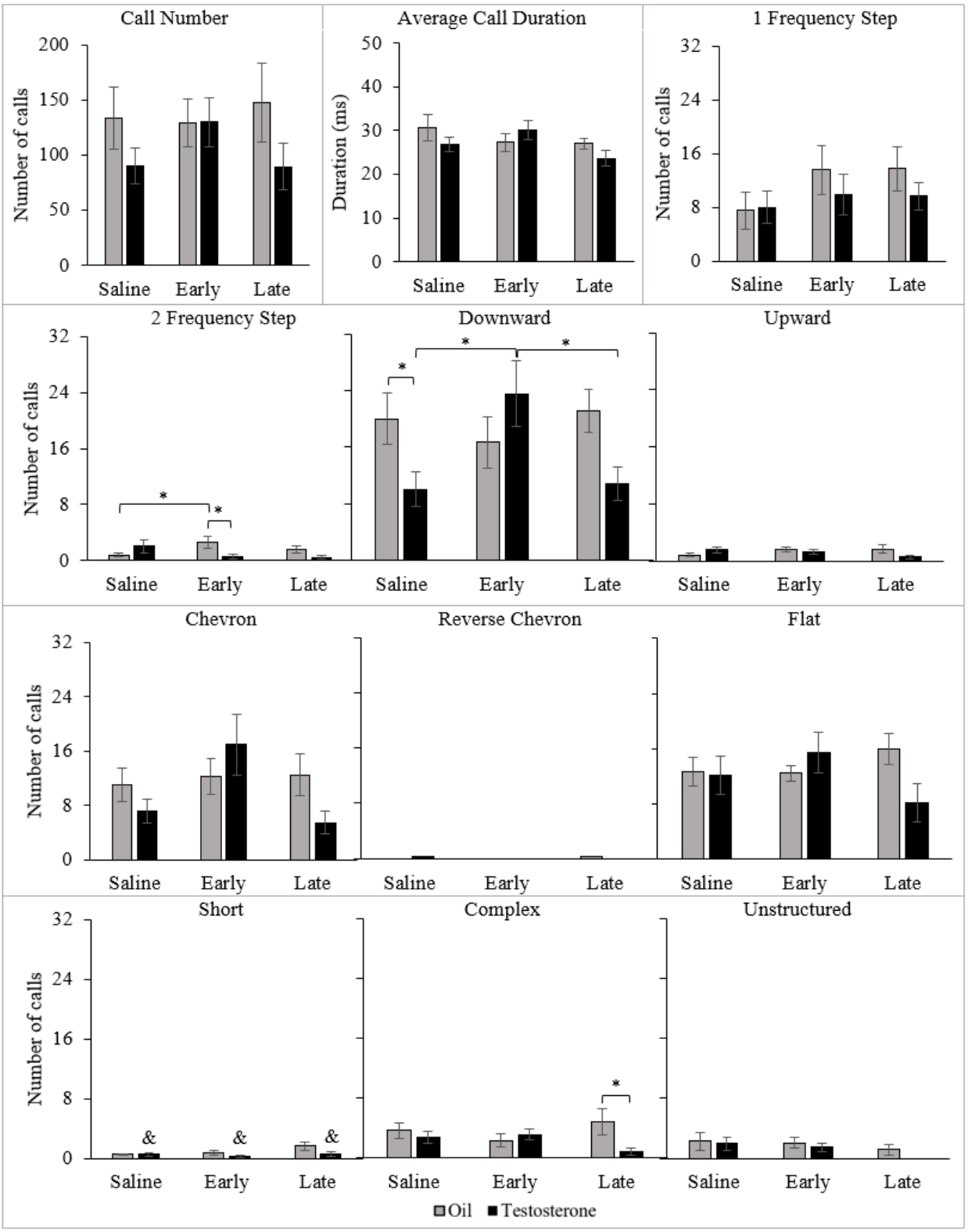
Number of marbles buried as a function of MIA and hormone treatment.



Note. Data shown as mean ± SEM of the number of marbles buried by juvenile males at the 10, 20, and 30-min timepoints of the marble burying task. Marbles were considered to be buried if they were at least 2/3 covered in bedding. Late MIA males buried more marbles than both early MIA males and saline controls at the 10-minute timepoint, *$p < .05$.

Figure 5

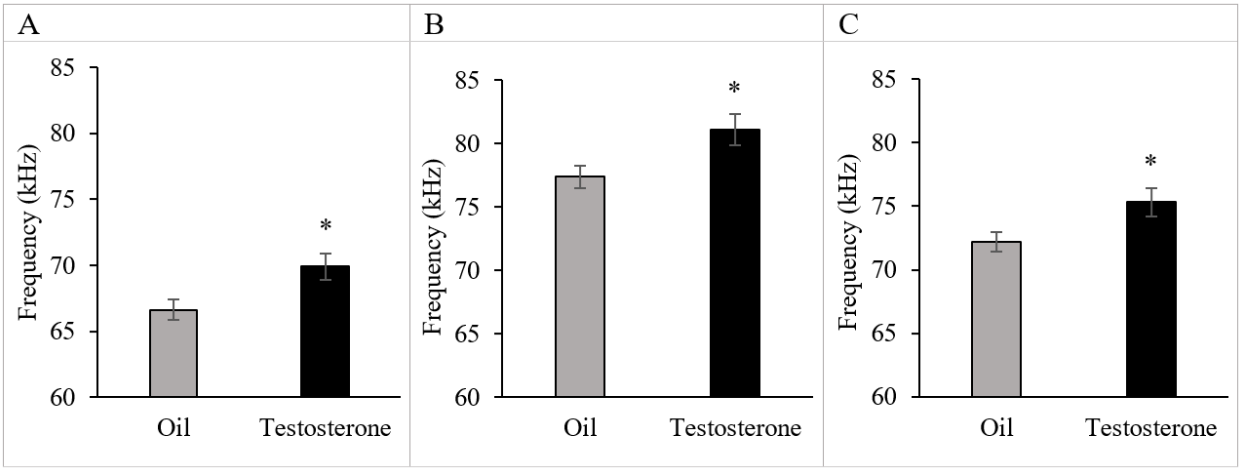
Characteristics of isolation USVs produced by neonatal males on PND 4.



Note. Data shown as mean ± SEM of overall call number, average call duration (ms), and number of calls per classification type, of neonatal males at PND 4. There were no significant findings for overall call number or average call duration (p's > .05). T treatment reduced 2-frequency-step calls produced by early MIA males. Oil-treated early MIA produced more 2-frequency-step calls than oil-treated saline controls, *p < .05. T-treated early MIA produced more downward calls than T-treated saline controls and T-treated late MIA. However, oil-treated saline controls produced more downward calls than their T-treated counterparts, *p < .05. T-treated males produced less short calls than oil-treated males, & indicates a significant difference from the corresponding oil group. Prenatal T reduced the production of complex calls among late MIA males (interaction of MIA by hormone treatment was marginal), *p < .05.

Figure 6

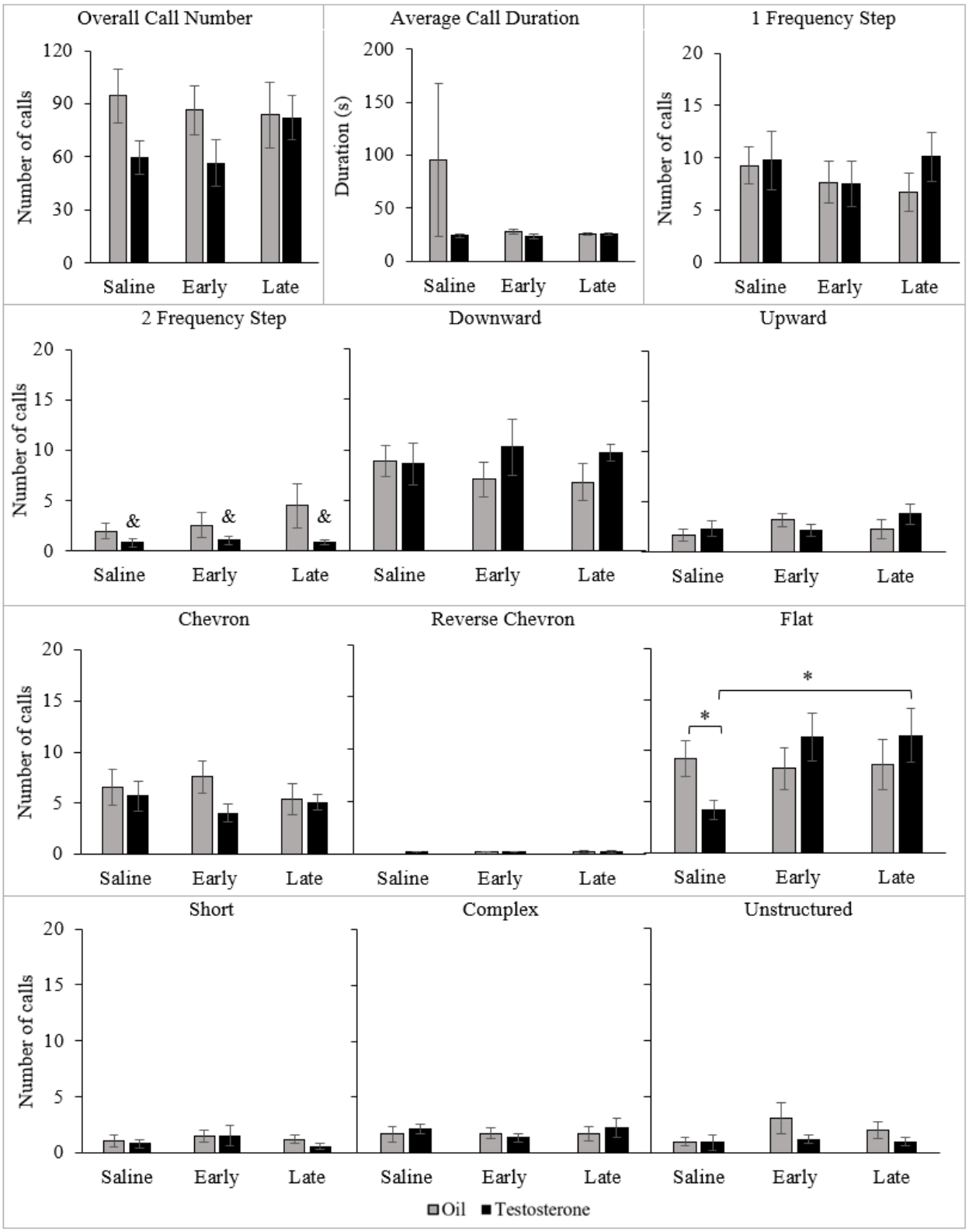
Average low call frequency, average high call frequency, and overall average call frequency in kilohertz (kHz) at PND 6 as a function of hormone treatment.



Note. Data shown as mean ± SEM of average low call frequency (kHz) (**A**), average high call frequency (kHz) (**B**), and overall average call frequency (kHz) (**B**) at PND 6. Compared to oil-treated males, T-treated males produced calls with a higher average low, high, and overall frequency, *****$p < .05$.

Figure 7

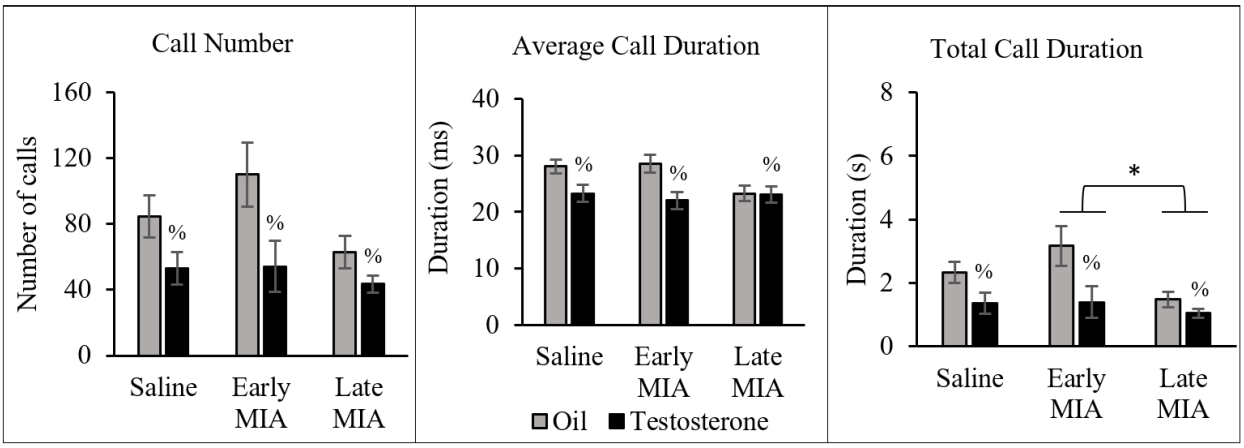
Characteristics of isolation USVs produced by neonatal males on PND 6.



Note. Data shown as mean ± SEM of overall call number, average call duration (ms), and number of calls per classification type, of neonatal males at PND 6. There were no significant findings for overall call number or average call duration (p's > .05). T-treated males produced less 2-frequency-step calls than oil-treated males, & indicates a significant difference from the corresponding oil group. Among T-treated males, saline controls produced less flat calls than both early MIA and late MIA (interaction of MIA by hormone treatment was marginal, p = .076), *p < .05.

Figure 8

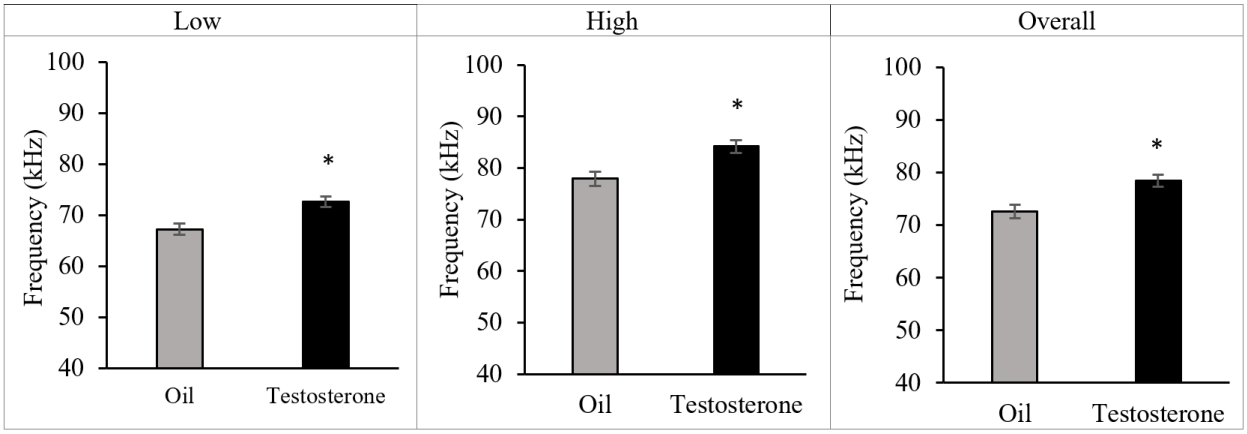
Call number, average call duration in milliseconds (ms), and total call duration in seconds (s) of neonatal males at PND 8.



Note. Data shown as mean ± SEM of the call number, average call duration (ms), and total call duration (s) of neonatal males at PND 8. Hyperandrogenized males produced less calls, shorter calls, and spent less time calling than oil-treated males (denoted by %). Late MIA males spent less time calling than early MIA males, $*p < .05$.

Figure 9

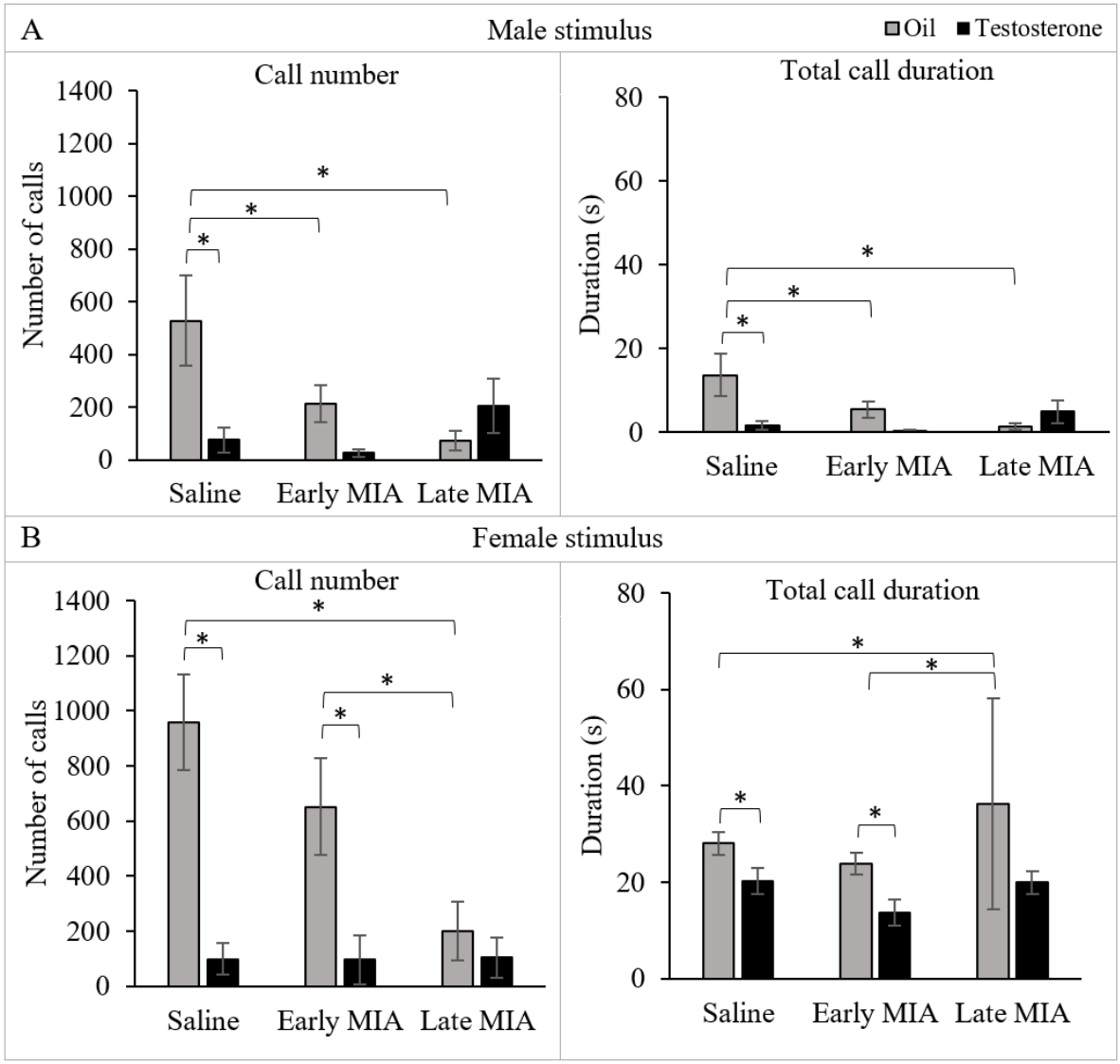
Average low call frequency, average high call frequency, and overall average call frequency in kilohertz (kHz) at PND 8 as a function of hormone treatment.



Note. Data shown as mean ± SEM of average low call frequency (kHz), average low call frequency (kHz), and overall average call frequency of neonatal males on PND 8. T-treated males produced calls with a higher average high, low, and overall call frequency compared to oil-treated males, *$p < .05$.

Figure 10

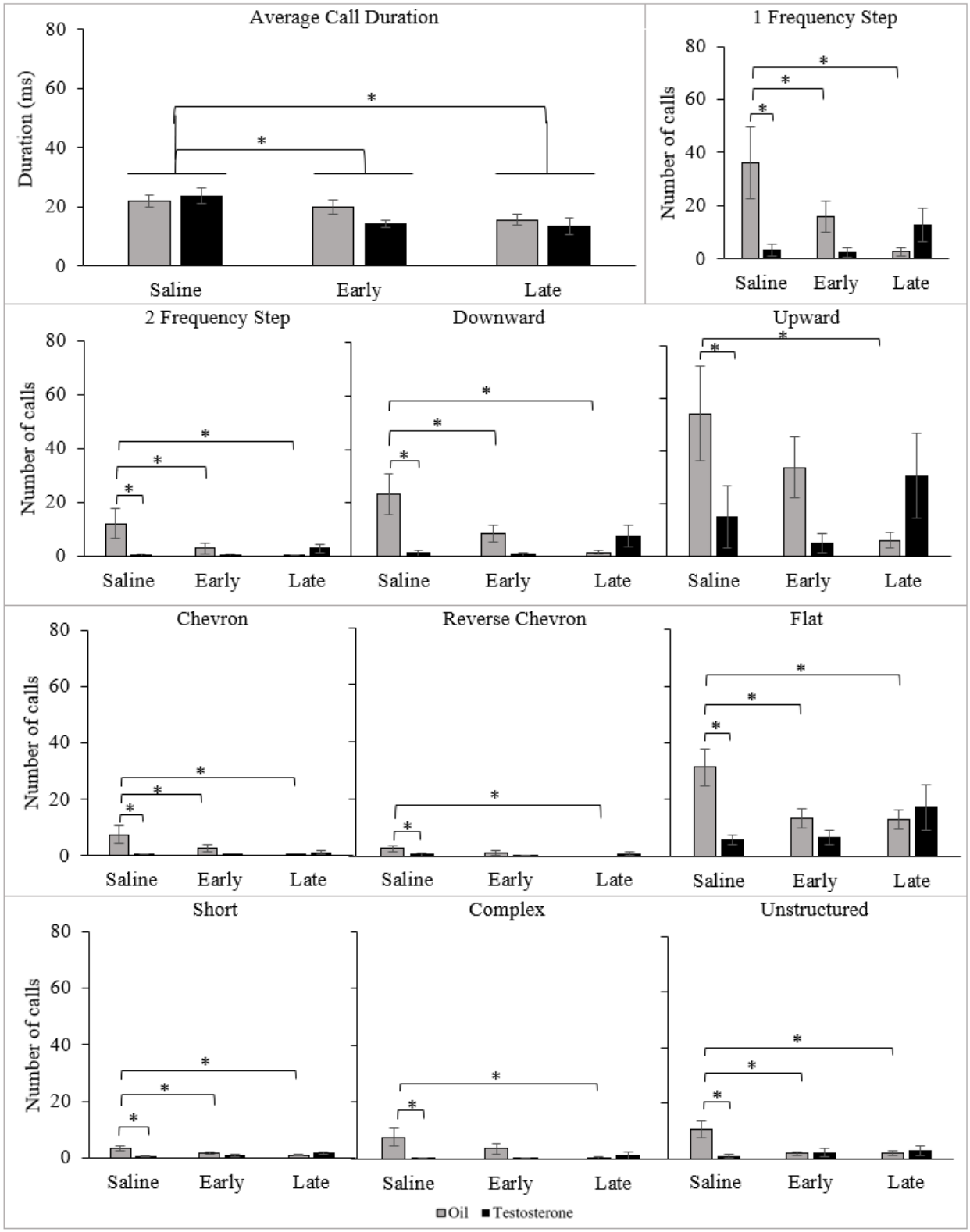
Call number and total call duration in seconds (s) of juvenile males interacting with a stimulus male or female as a function of MIA and hormone treatment.



Note. Data shown as mean ± SEM of call number and total call duration (s) of juvenile males interacting with a stimulus male (**A**) or a stimulus female (**B**). **A.** Prenatal T treatment reduced the number of calls produced and time spent calling among saline controls. Oil-treated early and late MIA produced fewer calls and spent less time calling than oil-treated saline controls, *$p <$ .05. **B.** Prenatal T treatment reduced the number of calls produced and time spent calling among saline controls. Among early MIA males, T-treated produced less calls and shorter calls than oil-treated. Oil-treated late MIA produced less calls and shorter calls than oil-treated early MIA and oil-treated saline controls, *$p < .05$.

Figure 11

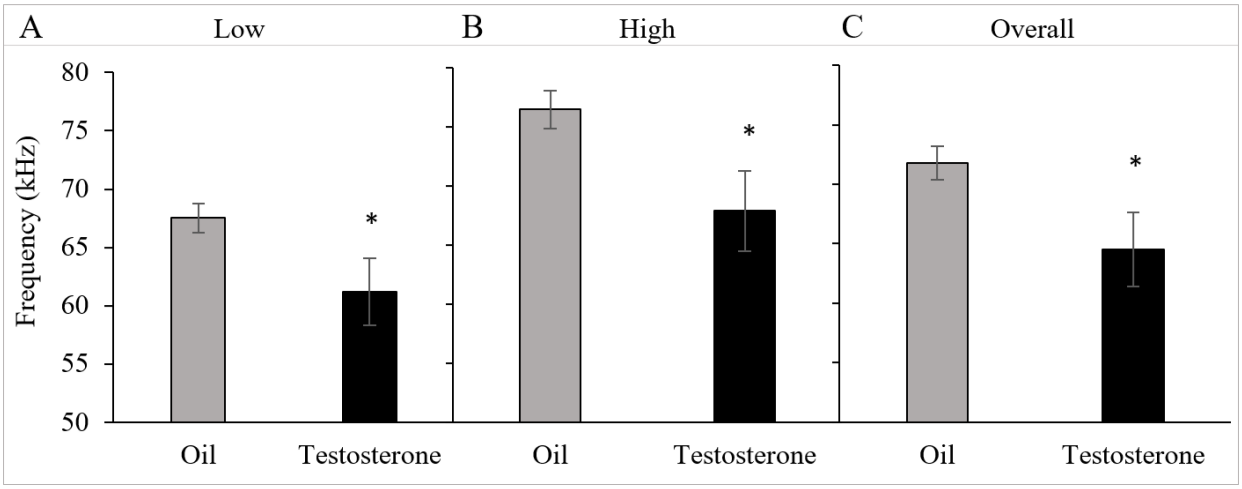
Characteristics of USVs produced by juvenile males in the presence of a male conspecific.



Note. Data shown as mean ± SEM of average call duration (ms) and number of calls per classification type of juvenile males interacting with a male conspecific. Late MIA and early MIA males produced longer calls than saline controls, *$p < .05$. For 1-frequency-step, downward, flat, short, and unstructured calls, oil-treated saline controls produced more calls compared to oil-treated early and late MIA, as well as T-treated saline controls, *$p < .05$. Similarly, oil-treated saline controls produced more upward calls than oil-treated late MIA, and T-treated saline controls, *$p < .05$. Oil-treated saline controls produced more reverse chevron (interaction $p = .060$) and complex calls (interaction $p = .064$) than oil-treated late MIA and T-treated saline controls, as well as compared to oil-treated early MIA males for 2-frequency-step (interaction $p = .050$) and chevron calls (interaction $p = .057$).

Figure 12

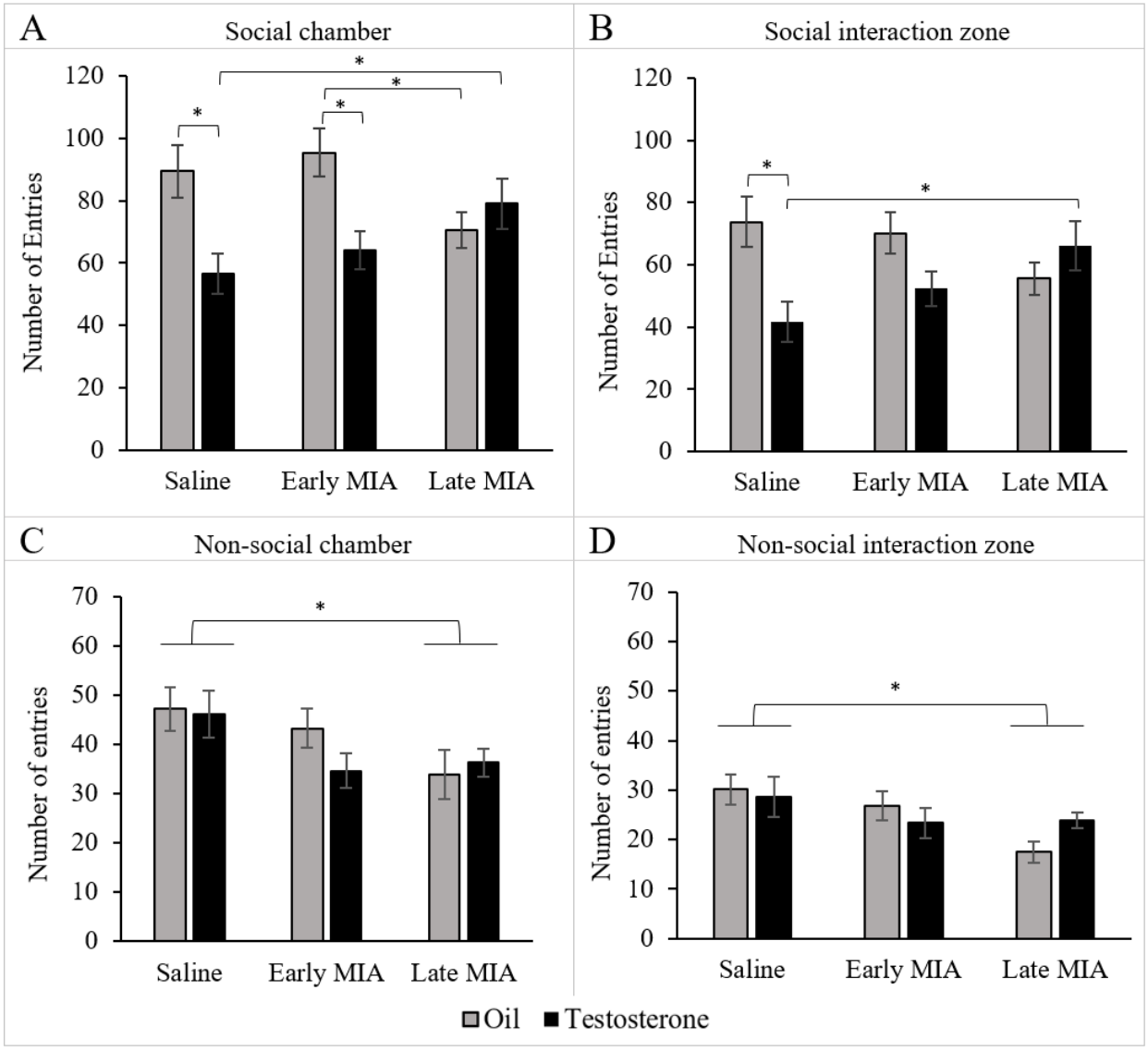
Average low call frequency, average high call frequency, and overall average call frequency in kilohertz (kHz) of juvenile males interacting with a stimulus male as a function of hormone treatment.



Note. Data shown as mean ± SEM of average low call frequency (**A**), average high call frequency (**B**), and overall average call frequency (**C**) of juvenile males interacting with a stimulus male. Compared to oil-treated males, T-treated males produced calls with a significantly lower average low, high, and overall call frequency when interacting with a male stimulus conspecific, ***$p < .05$.

Figure 13

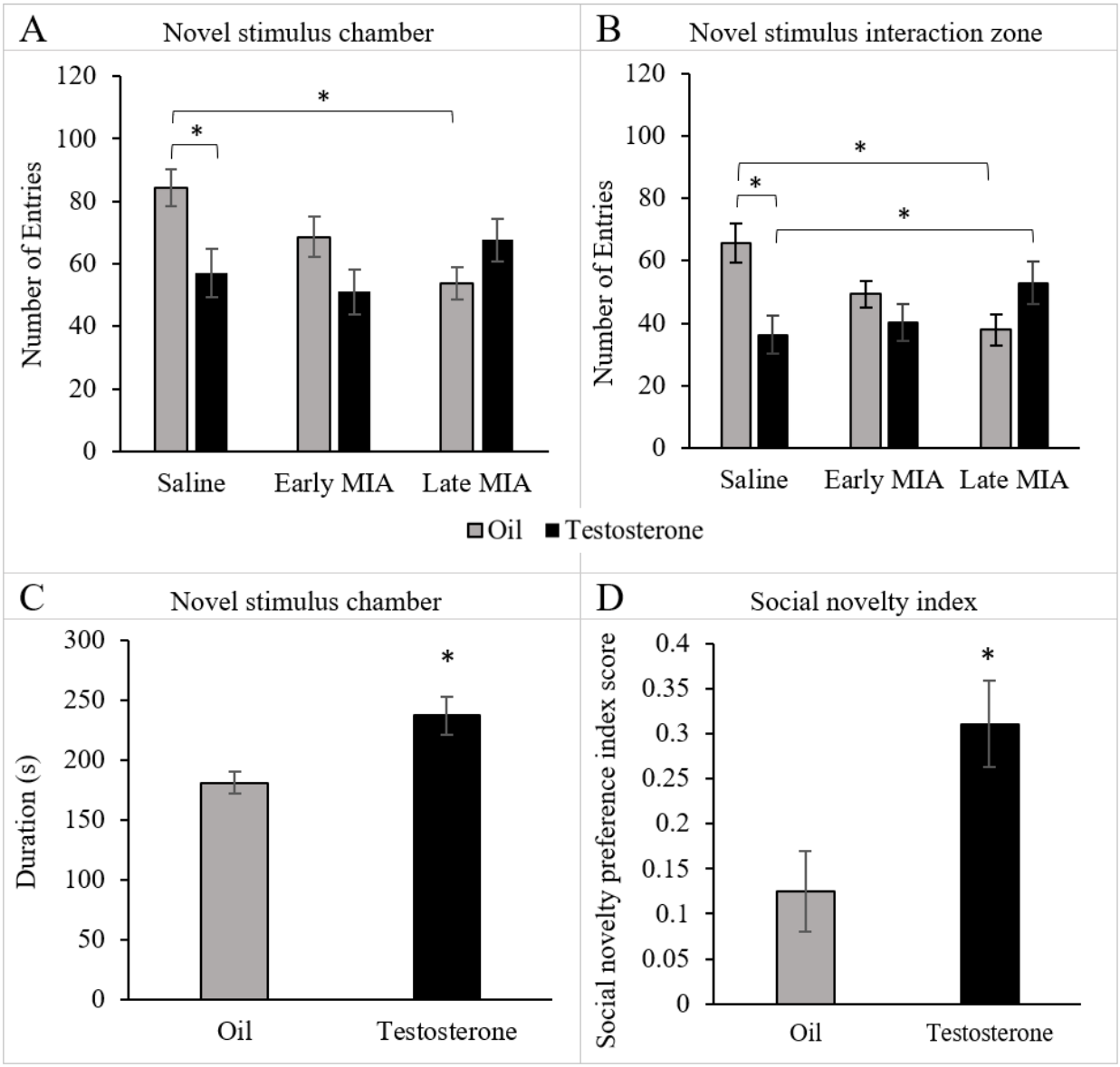
Entries into social and non-social chambers and interaction zones as a function of MIA treatment and hormone treatment.



Note. Data shown as mean ± SEM of entries into social chamber (**A**) and interaction zone (**B**), and entries into non-social chamber (**C**), and interaction zone (**D**), by juvenile males in Phase I of the three-chamber social paradigm. Prenatal T treatment reduced entries into the social chamber and interaction zone among saline controls. Prenatal T in late MIA increased entries into the social chamber and interaction zone compared to T-treated saline controls. Late MIA reduced entries into the non-social chamber and interaction zone compared to saline controls, $p < .05$.

Figure 14

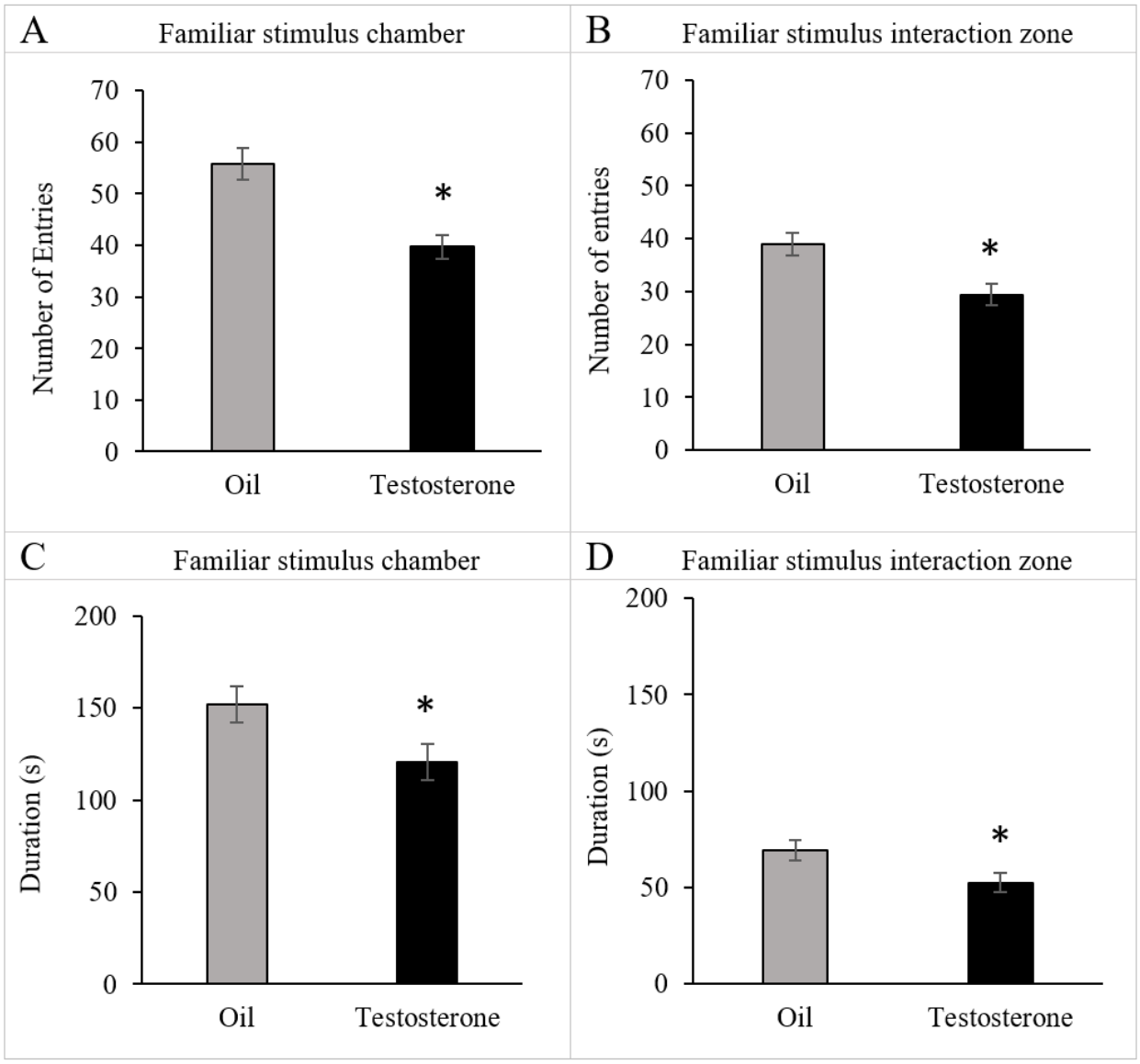
Entries in novel stimulus chamber and interaction zone, time in novel stimulus chamber, and social novelty preference index as a function of MIA and hormone treatment.



Note. Data shown as mean ± SEM of entries into novel stimulus chamber (**A**), entries into novel stimulus interaction zone (**B**), time in novel stimulus chamber (**C**), and social novelty preference (**D**) by juvenile males in Phase II of the three-chamber social paradigm. **A, B**. Oil-treated late MIA males entered the novel stimulus chamber and interaction zone less often than oil-treated saline controls. Prenatal T in late MIA increased entries into the novel stimulus interaction zone. **C, D**. Prenatal T increased time spent in the novel stimulus chamber, as well as preference for social novelty, compared to oil-treated males, *$p < .05$.

Figure 15

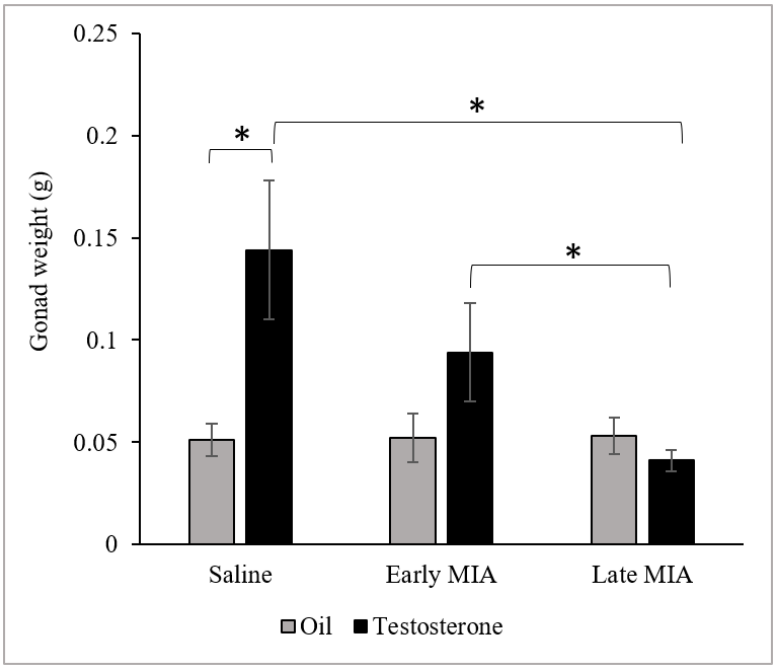
Entries and duration in seconds (s) in the familiar stimulus chamber and interaction zones by juvenile males as a function of hormone treatment.



Note. Data shown as mean ± SEM of entries into familiar stimulus chamber (**A**), entries into familiar stimulus interaction zone (**B**), time in familiar stimulus chamber (**C**), and time in familiar stimulus interaction zone (**D**) by juvenile males in Phase II of the three-chamber social paradigm. Prenatal T treatment reduced number of entries and time spent in the familiar stimulus chamber and interaction zone compared to oil-treated males, *$p < .05$.

Figure 16

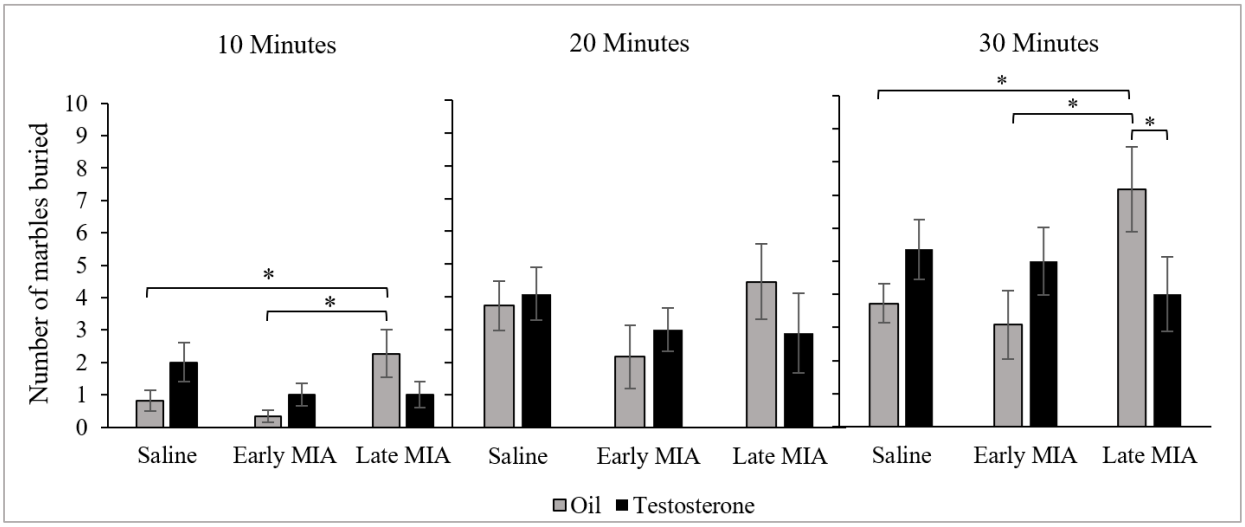
Female gonad weight in grams (g) as a function of MIA and hormone treatment.



Note. Data shown as mean ± SEM of juvenile female gonadal weight (g). Among T-treated females, late MIA had a significantly lower gonad weight compared to both early MIA and saline, while prenatal T increased gonad weight among saline controls, * $p < .05$.

Figure 17

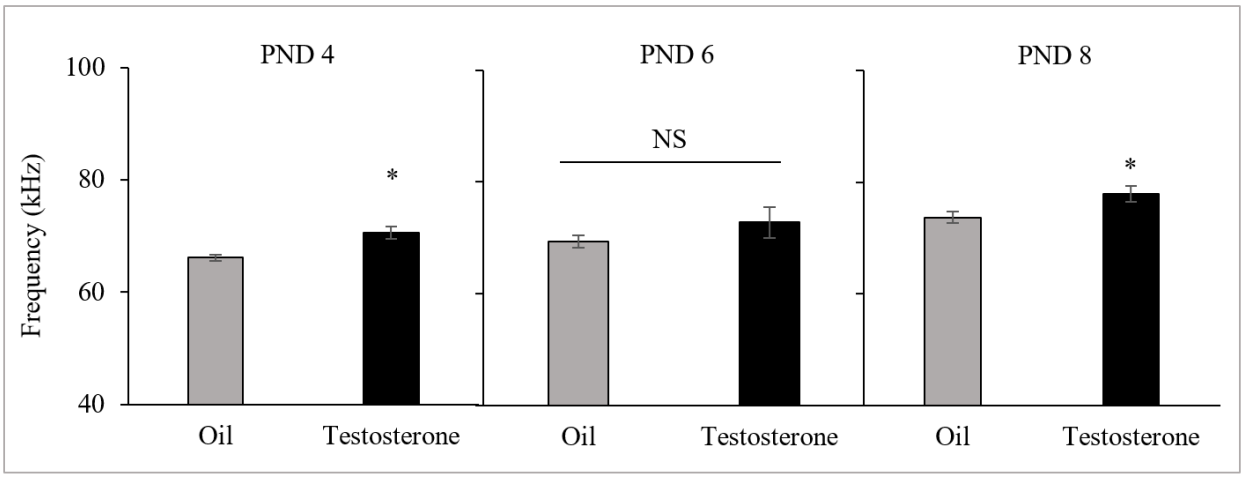
Number of marbles buried by females at the 10-, 20-, and 30-minute timepoints as a function of MIA and hormone treatment.



Note. Data shown as mean ± SEM of the number of marbles buried by juvenile females at the 10-, 20-, and 30-minute timepoints of the marble burying task. Marbles were considered to be buried if they were at least 2/3 covered in bedding. Oil-treated late MIA females buried more marbles than oil-treated early MIA and saline controls at the 10- and 30-minute timepoints. At 30 minutes, prenatal T reduced marble burying in late MIA females, $*p < .05$. No differences were found at the 20-minute timepoint.

Figure 18

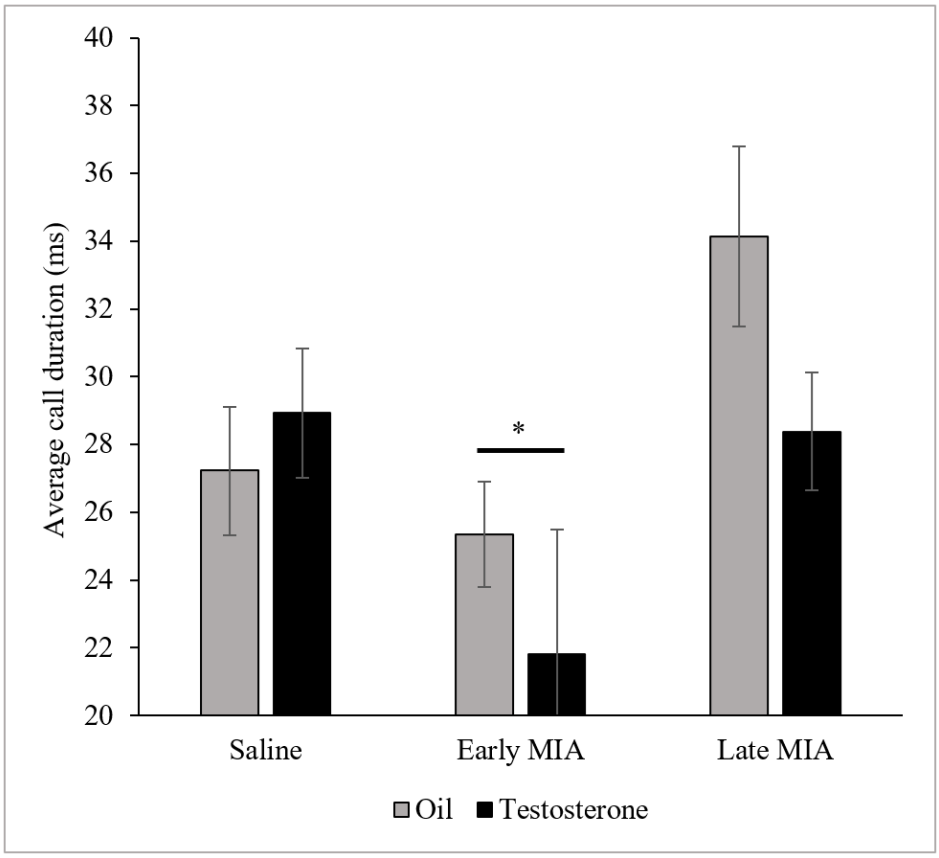
Overall average call frequency in kilohertz (kHz) of neonatal females as a function of hormone treatment.



Note. Data shown as mean ± SEM of overall average call frequency (kHz) of neonatal females at PND 4, 6, and 8. T-treated females called with a higher overall average call frequency compared to oil-treated females at PND 4 and 8, $*p < .05$.

Figure 19

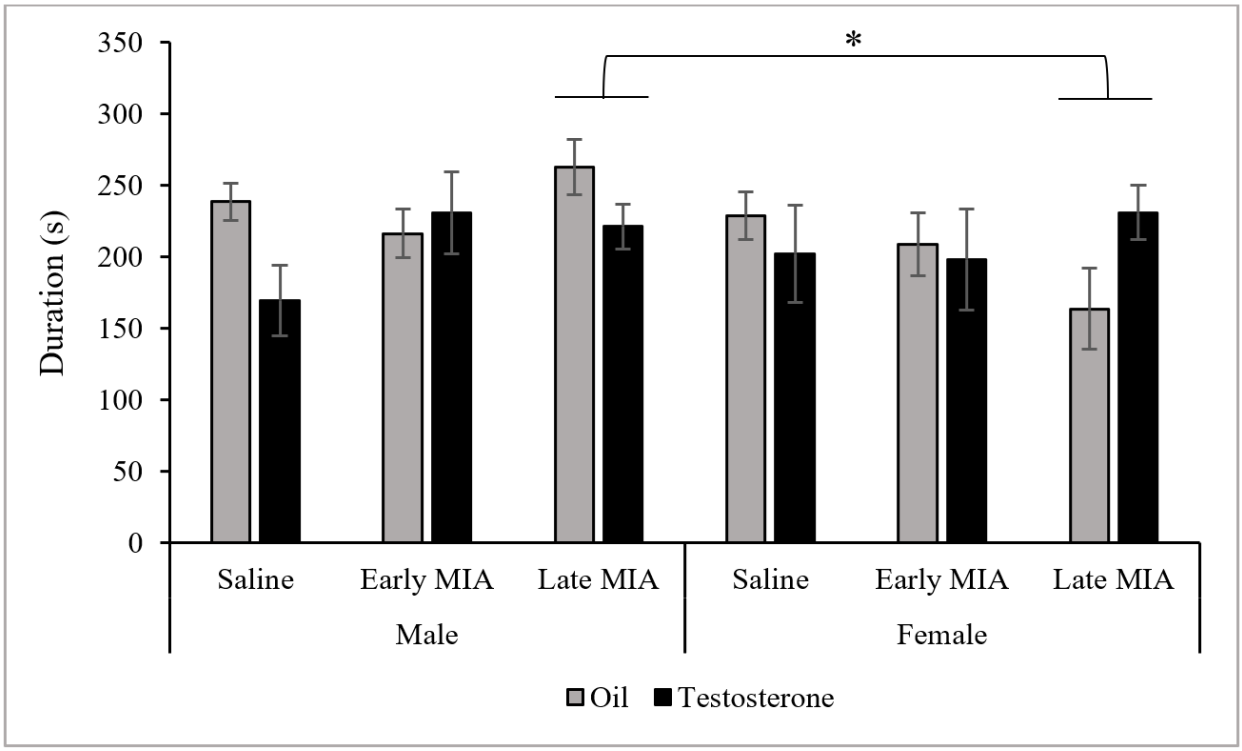
Average call duration in milliseconds (ms) of neonatal females at PND 6 as a function of MIA and hormone treatment.



Note. Data shown as mean ± SEM of average call duration (ms) of neonatal females at PND 6. A main effect of MIA treatment was found, such that early MIA females produced shorter calls than both late MIA and saline controls at this timepoint, $*p < .05$.

Figure 20

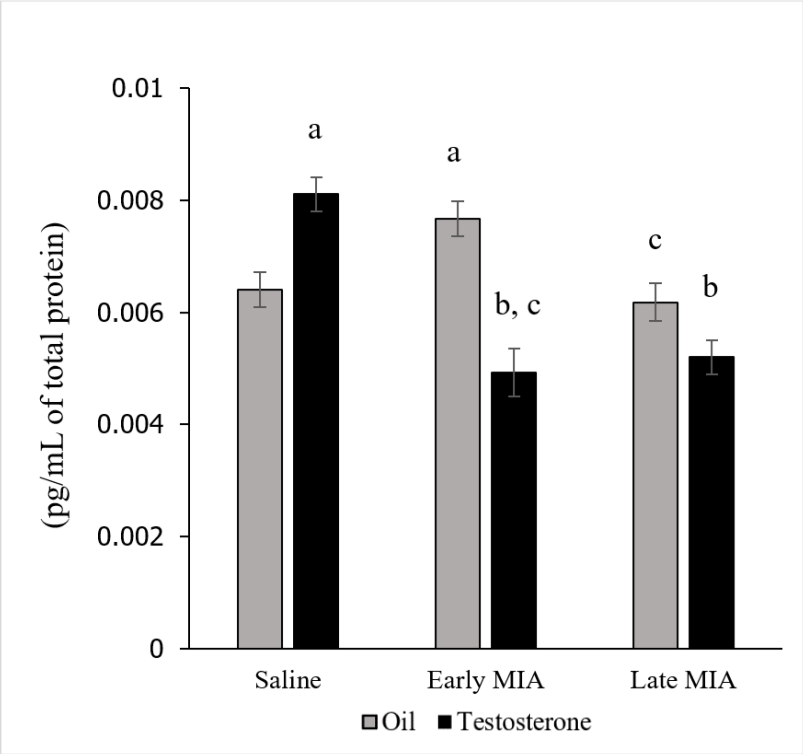
Duration in social chamber in seconds (s) as a function of sex, MIA treatment, and hormone treatment.



Note. Data shown as mean ± SEM of time spent in the social stimulus chamber (s) by juvenile males and females. Late MIA males spent more time in the social chamber than late MIA females, $p < .05$.

Figure 21

TNF-α concentrations measured as picograms/millilitre (pg/mL) of total sample protein in neonatal male brain tissue as a function of MIA and hormone treatment.



Note. Data shown as mean ± SEM of TNF-α concentration (pg/mL of total sample protein) in neonatal (PND 1) male homogenized brain samples. TNF-α concentrations were elevated in early MIA compared to late MIA and saline controls. Hyperandrogenization significantly reduced TNF-α in early MIA. Contrastingly, in saline controls, hyperandrogenization significantly increased TNF-α concentrations. 'a' indicates significant difference from saline/oil, $p < .05$; 'b' indicates significant difference from saline/T, $p < .05$; 'c' indicates significant difference from early MIA/oil, $p < .05$.

Appendix B - Tables

Table 1

Means (SEM) of juvenile male somatic measures by MIA treatment and hormone treatment.

	Saline		Early MIA		Late MIA	
Somatic measure	Oil (n = 12)	Testosterone (n = 13)	Oil (n = 12)	Testosterone (n = 13)	Oil (n = 12)	Testosterone (n = 12)
Body weight (g)	22.417 (0.668)	18.846 (0.678)	21.250 (0.429)	20.692 (0.603)	19.167 (0.386)	19.000 (0.444)
Gonadal weight (g)	0.114 (0.007)	0.051 (0.006)	0.126 (0.006)	0.069 (0.006)	0.112 (0.012)	0.061 (0.008)
Gonadal weight / body weight	0.005 (0)	0.003 (0)	0.006 (0)	0.003 (0)	0.006 (0.001)	0.003 (0)
Seminal vesicle weight (g)	0.096 (0.005)	0.020 (0.007)	0.071 (0.005)	0.048 (0.011)	0.048 (0.008)	0.038 (0.010)

Table 2

Means (SEM) for the righting reflex in neonatal males by MIA treatment and hormone treatment across postnatal day (PND) 4-15.

	Saline		Early MIA		Late MIA	
PND	Oil (n = 12)	Testosterone (n = 13)	Oil (n = 12)	Testosterone (n = 13)	Oil (n = 12)	Testosterone (n = 12)
4	48.617 (6.227)	58.252 (1.748)	59.250 (0.750)	54.098 (4.183)	(n = 8) 50.815 (6.044)	(n = 10) 53.712 (5.602)
6	19.446 (7.256)	16.109 (5.905)	14.034 (6.427)	(n = 11) 28.596 (9.069)	(n = 8) 28.146 (9.711)	(n = 10) 31.463 (9.456)
8	2.143 (0.193)	6.625 (4.464)	1.802 (0.131)	2.378 (0.387)	(n = 10) 2.954 (1.316)	2.113 (0.289)
9	2.017 (0.305)	2.778 (1.006)	1.908 (0.450)	2.808 (1.264)	2.341 (0.646)	(n = 10) 3.193 (1.013)
10	1.587 (0.322)	1.924 (0.441)	1.530 (0.418)	1.083 (0.121)	1.983 (0.492)	2.797 (0.977)
11	1.618 (0.311)	0.798 (0.068)	1.073 (0.160)	1.272 (0.125)	1.215 (0.134)	1.419 (0.240)
12	0.894 (0.166)	0.625 (0.040)	0.763 (0.104)	0.792 (0.069)	0.940 (0.129)	1.015 (0.138)
13	0.770 (0.139)	0.592 (0.040)	0.552 (0.031)	0.628 (0.051)	0.618 (0.053)	0.766 (0.063)
14	0.500 (0)	0.500 (0)	0.538 (0.028)	0.609 (0.050)	0.512 (0.013)	0.678 (0.062)
15	0.500 (0)	0.500 (0)	0.500 (0)	0.500 (0)	0.500 (0)	0.500 (0)

Note. Number of animals vary per measure due to experimental disruption or error.

Table 3

Means (SEM) for eye-opening in neonatal males by MIA treatment and hormone treatment across PND 11-17.

	Saline		Early MIA		Late MIA	
PND	Oil (n = 12)	Testosterone (n = 13)	Oil (n =12)	Testosterone (n = 13)	Oil (n = 12)	Testosterone (n = 12)
11	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)
12	0 (0)	0 (0)	0 (0)	0 (0)	0.083 (0.083)	0 (0)
13	0.750 (0.279)	0.538 (0.183)	0.917 (0.260)	0.615 (0.241)	0.667 (0.284)	0.417 (0.229)
14	1.750 (0.131)	1.769 (0.166)	1.667 (0.142)	1.769 (0.122)	1.500 (0.195)	1.750 (0.131)
15	2.000 (0)	2.000 (0)	2.000 (0)	1.923 (0.077)	1.917 (0.083)	2.000 (0)
16	2.000 (0)	2.000 (0)	2.000 (0)	2.000 (0)	1.917 (0.083)	2.00 (0)
17	2.000 (0)	2.000 (0)	2.000 (0)	2.000 (0)	2.000 (0)	2.000 (0)

Note. No eyes open = 0; one eye open = 1; both eyes open = 2.

Table 4

Means (SEM) for the marble burying task in juvenile males by MIA treatment and hormone treatment.

	Saline		Early MIA		Late MIA	
Timepoint (mins)	Oil (n = 12)	Testosterone (n = 13)	Oil (n = 12)	Testosterone (n = 13)	Oil (n = 12)	Testosterone (n = 12)
5	0.500 (0.289)	0.231 (0.166)	0.167 (0.112)	0.231 (0.122)	1.083 (0.452)	0.500 (0.195)
10	1.500 (0.529)	1.308 (0.382)	1.250 (0.463)	1.385 (0.417)	3.083 (0.981)	2.500 (0.645)
15	3.167 (0.895)	3.385 (0.764)	2.917 (0.941)	2.308 (0.644)	4.417 (1.083)	3.500 (0.996)
20	5.083 (1.184)	4.154 (0.926)	3.250 (0.880)	3.385 (0.738)	5.417 (1.258)	4.583 (1.171)
25	5.583 (1.145)	5.769 (1.033)	5.000 (1.015)	4.308 (0.804)	5.667 (1.333)	5.000 (0.992)
30	7.500 (1.311)	6.154 (0.846)	5.333 (1.202)	5.462 (1.107)	6.333 (1.443)	6.000 (1.168)

Table 5

Means (SEM) for isolation USV parameters in neonatal males by MIA treatment and hormone treatment at PND 4, 6, and 8.

		Saline		Early MIA		Late MIA	
Measure	PND	Oil PND 4 (n = 12) PND 6 (n = 12) PND 8 (n = 12)	Testosterone PND 4 (n = 14) PND 6 (n = 13) PND 8 (n = 13)	Oil PND 4 (n = 12) PND 6 (n = 12) PND 8 (n = 12)	Testosterone PND 4 (n = 13) PND 6 (n = 11) PND 8 (n = 13)	Oil PND 4 (n = 9) PND 6 (n = 11) PND 8 (n = 13)	Testosterone PND 4 (n = 11) PND 6 (n = 13) PND 8 (n = 13)
Number of calls	4	133.273 (28.141)	89.929 (16.593)	129.000 (21.601)	129.769 (21.812)	147.444 (35.617)	89.455 (21.318)
	6	94.417 (15.409)	59.538 (9.551)	86.583 (13.876)	56.545 (13.375)	83.727 (18.572)	82.077 (12.355)
	8	84.833 (12.782)	52.923 (9.830)	110.083 (19.609)	54.154 (15.574)	62.769 (9.895)	43.462 (5.167)
Average low call frequency (kHz)	4	61.307 (1.174)	62.328 (1.353)	62.687 (1.153)	63.817 (1.987)	63.193 (0.995)	66.643 (1.63)
	6	66.997 (1.307)	69.022 (1.820)	66.464 (1.540)	70.680 (1.934)	66.421 (1.252)	70.150 (1.606)
	8	66.687 (1.703)	71.587 (2.028)	63.02 (1.779)	73.22 (2.323)	69.446 (1.319)	74.402 (1.464)
Average high call frequency (kHz)	4	71.318 (1.342)	71.302 (1.873)	73.388 (1.344)	74.741 (2.125)	73.426 (1.212)	77.011 (2.054)
	6	76.721 (1.207)	81.102 (2.061)	76.834 (1.829)	82.128 (1.812)	76.985 (1.721)	81.633 (2.224)
	8	77.973 (2.766)	81.58 (2.286)	74.387 (2.644)	84.228 (2.799)	80.269 (1.435)	86.767 (1.623)
Average call frequency (kHz)	4	66.313 (1.219)	66.815 (1.558)	68.037 (1.212)	69.279 (2.031)	68.309 (1.090)	71.827 (1.833)
	6	71.859 (1.201)	75.062 (1.901)	71.649 (1.650)	76.404 (1.784)	71.703 (1.444)	75.892 (1.879)
	8	72.33 (2.222)	76.584 (2.130)	68.704 (2.189)	78.724 (2.512)	74.857 (1.263)	80.584 (1.503)
Average call duration (ms)	4	30.602 (2.950)	26.71 (1.621)	27.268 (2.095)	30.189 (2.183)	26.968 (1.290)	23.631 (1.835)
	6	95.567 (71.904)	24.313 (1.792)	27.69 (2.308)	23.358 (1.750)	25.203 (1.204)	25.689 (1.132)

	8	28.083 (1.268)	23.299 (1.531)	28.575 (1.634)	22.057 (1.523)	23.278 (1.387)	23.053 (1.428)
Total call duration (s)	4	4.136 (0.782)	2.492 (0.485)	3.686 (0.616)	4.379 (0.862)	3.999 (1.038)	2.03 (0.484)
	6	2.208 (0.474)	1.549 (0.291)	2.438 (0.404)	1.258 (0.270)	2.086 (0.421)	2.153 (0.368)
	8	2.338 (0.330)	1.361 (0.332)	3.166 (0.617)	1.396 (0.507)	1.477 (0.236)	1.043 (0.146)

Note. Number of animals vary per measure due to experimental disruption or error.

Table 6

Means (SEM) for the number of isolation USV calls per classification type in neonatal males by MIA treatment and hormone treatment at PND 4 and 6.

		Saline		Early MIA		Late MIA	
Call classification	Postnatal day (PND)	Oil PND 4 (n = 12) PND 6 (n = 12)	Testosterone (n = 13) (n = 13)	Oil (n = 13) (n = 12)	Testosterone (n = 13) (n = 11)	Oil (n = 8) (n = 10)	Testosterone (n = 9) (n = 12)
1-frequency-step	4	7.500 (2.707)	8.000 (2.345)	13.538 (3.614)	9.846 (3.053)	13.750 (3.272)	9.667 (2.068)
	6	9.250 (1.793)	9.769 (2.797)	7.667 (2.013)	7.545 (2.163)	6.700 (1.856)	10.083 (2.382)
2-frequency-step	4	0.750 (0.279)	2.000 (0.934)	2.615 (0.903)	0.538 (0.268)	1.625 (0.498)	0.444 (0.294)
	6	2.000 (0.788)	0.846 (0.390)	2.583 (1.221)	1.091 (0.415)	4.500 (2.207)	0.917 (0.229)
Downward	4	20.000 (3.627)	10.000 (2.415)	16.692 (3.628)	23.538 (4.672)	21.125 (3.044)	10.778 (2.332)
	6	8.917 (1.530)	8.615 (2.030)	7.083 (1.756)	10.273 (2.731)	6.800 (1.812)	9.750 (0.854)
Upward	4	0.833 (0.241)	1.538 (0.386)	1.538 (0.351)	1.231 (0.303)	1.625 (0.565)	0.556 (0.242)
	6	1.667 (0.582)	2.308 (0.720)	3.167 (0.683)	2.182 (0.600)	2.200 (0.940)	3.750 (1.038)
Chevron	4	11.000 (2.502)	7.077 (1.778)	12.308 (2.625)	16.923 (4.447)	12.500 (3.100)	5.444 (1.617)
	6	6.583 (1.738)	5.692 (1.434)	7.583 (1.579)	4.000 (0.863)	5.400 (1.543)	5.083 (0.753)
Reverse chevron	4	0 (0)	0.077 (0.077)	0 (0)	0 (0)	0.125 (0.125)	0 (0)
	6	0 (0)	0.077 (0.077)	0.083 (0.083)	0.091 (0.091)	0.200 (0.133)	0.167 (0.112)
Flat	4	12.583 (2.091)	12.077 (2.795)	12.385 (1.169)	15.385 (2.903)	15.875 (2.255)	8.111 (2.721)
	6	9.250 (1.706)	4.231 (0.962)	8.250 (2.000)	11.364 (2.313)	8.600 (2.441)	11.500 (2.621)

Short	4	0.500 (0.151)	0.538 (0.243)	0.692 (0.308)	0.231 (0.166)	1.625 (0.596)	0.556 (0.294)
	6	1.083 (0.514)	0.846 (0.373)	1.500 (0.571)	1.545 (0.888)	1.200 (0.389)	0.583 (0.260)
Complex	4	3.667 (1.010)	2.769 (0.786)	2.385 (0.821)	3.154 (0.741)	4.875 (1.767)	0.889 (0.423)
	6	1.667 (0.678)	2.154 (0.451)	1.750 (0.479)	1.364 (0.388)	1.700 (0.616)	2.250 (0.871)
Unstructured	4	2.250 (1.156)	1.923 (0.923)	2.077 (0.684)	1.462 (0.606)	1.125 (0.666)	0 (0)
	6	1.000 (0.348)	0.923 (0.684)	3.083 (1.351)	1.182 (0.377)	2.000 (0.745)	1.000 (0.326)

Note. Number of animals vary per measure due to experimental disruption or error.

Table 7

Means (SEM) for USV parameters and call classifications in juvenile males interacting with a same- or opposite-sex conspecific by MIA treatment and hormone treatment.

			Saline		Early MIA		Late MIA	
		Stimulus sex	Oil (n = 12)	Testosterone (n = 9)	Oil (n = 11)	Testosterone (n = 11)	Oil (n = 10)	Testosterone (n = 10)
Measure	Number of calls	Male	527.417 (171.468)	75.778 (46.933)	212.300 (69.070)	25.455 (15.019)	72.400 (38.239)	205.000 (101.644)
		Female	958.917 (175.658)	98.556 (56.551)	651.9 (174.804)	95.727 (87.866)	200.400 (106.684)	104.600 (72.768)
	Average low call frequency (kHz)	Male	67.996 (1.797)	55.094 (3.657)	70.148 (1.104)	65.332 (3.429)	65.535 (2.972)	60.750 (6.947)
		Female	65.483 (1.719)	64.012 (3.791)	65.568 (2.367)	53.831 (8.669)	53.118 (9.410)	63.879 (2.190)
	Average high call frequency (kHz)	Male	78.603 (2.075)	61.691 (5.195)	81.100 (1.483)	70.975 (4.107)	71.705 (3.495)	67.630 (7.844)
		Female	77.670 (2.505)	70.753 (4.658)	76.857 (3.583)	58.204 (9.352)	59.394 (10.613)	70.729 (2.935)
	Average call frequency (kHz)	Male	73.300 (1.824)	58.393 (4.405)	75.624 (1.105)	68.153 (3.753)	67.844 (3.500)	64.190 (7.366)

		Female	71.577 (2.041)	67.383 (4.189)	71.212 (2.960)	56.018 (8.993)	56.256 (9.996)	67.304 (2.521)
	Average call duration (ms)	Male	21.761 (2.022)	23.613 (2.597)	19.813 (2.446)	14.173 (1.254)	15.614 (1.848)	13.431 (2.776)
		Female	28.073 (2.399)	20.251 (2.700)	23.874 (2.195)	13.734 (2.685)	36.178 (21.910)	19.925 (2.388)
	Total call duration (s)	Male	13.694 (5.000)	1.750 (1.089)	5.434 (2.026)	0.351 (0.207)	1.334 (0.789)	4.945 (2.649)
		Female	27.668 (5.942)	2.104 (1.400)	18.274 (5.739)	2.681 (2.569)	4.751 (2.610)	2.688 (2.072)
Calls per classification type	1-frequency-step	Male	36.167 (13.688)	3.250 (2.136)	15.909 (5.718)	2.273 (1.663)	2.600 (1.536)	12.900 (6.313)
	2-frequency-step	Male	12.167 (5.465)	0.500 (0.378)	3.000 (1.935)	0.455 (0.455)	0.200 (0.200)	3.000 (1.619)
	Downward	Male	23.333 (7.560)	1.375 (1.117)	8.545 (3.088)	0.818 (0.630)	1.500 (0.734)	7.800 (3.915)
	Upward	Male	54.167 (17.930)	15.000 (11.752)	33.727 (11.523)	5.091 (3.553)	6.200 (2.981)	30.700 (15.956)
	Chevron	Male	7.583 (3.213)	0.25 (0.164)	2.727 (1.287)	0.182 (0.122)	0.200 (0.133)	1.200 (0.663)
	Reverse chevron	Male	2.667 (0.987)	0.500 (0.500)	1.182 (0.724)	0.182 (0.182)	0 (0)	0.900 (0.458)

Flat	Male	31.417 (6.546)	5.750 (1.544)	13.455 (3.478)	6.636 (2.643)	13.100 (3.295)	17.300 (7.939)
Short	Male	3.500 (0.917)	0.500 (0.189)	1.636 (0.388)	0.909 (0.495)	1.00 (0.298)	1.700 (0.473)
Complex	Male	7.750 (3.023)	0.250 (0.250)	3.636 (1.884)	0.182 (0.182)	0.300 (0.300)	1.400 (1.293)
Unstructured	Male	10.583 (3.171)	0.750 (0.620)	1.818 (0.736)	2.182 (1.426)	2.000 (0.931)	2.900 (1.552)

Table 8

Means (SEM) for the three-chamber social paradigm in males by MIA treatment and hormone treatment.

		Saline		Early MIA		Late MIA	
Phase	Behaviour	Oil (n = 12)	Testosterone (n = 13)	Oil (n = 12)	Testosterone (n = 13)	Oil (n = 12)	Testosterone (n = 12)
Sociability	Duration in social chamber (s)	238.45 (13.085)	169.392 (24.647)	216.292 (16.919)	235.377 (30.870)	262.858 (19.269)	221.275 (15.526)
	Duration in social interaction zone (s)	133.45 (13.543)	110.085 (17.276)	126.508 (18.976)	84.223 (8.352)	109.592 (7.375)	117.525 (14.877)
	Duration in non-social chamber (s)	111.158 (11.209)	163.862 (26.65)	128.342 (15.26)	156.623 (28.93)	96.375 (13.817)	124.258 (13.749)
	Duration in non-social interaction zone (s)	34.492 (6.431)	33.908 (5.715)	28.200 (4.055)	33.062 (5.326)	25.95 (6.365)	43.125 (12.542)
	Entries into social chamber	89.500 (8.514)	56.538 (6.407)	95.417 (7.767)	64.154 (6.113)	70.583 (5.721)	79.083 (8.066)
	Entries into social interaction zone	73.833 (8.231)	41.846 (6.416)	70.167 (6.591)	52.308 (5.541)	55.667 (5.246)	66.083 (7.844)
	Entries into non-social chamber	47.250 (4.356)	46.231 (4.817)	43.333 (3.964)	34.692 (3.525)	34.000 (5.063)	36.333 (2.740)
	Entries into non-social interaction zone	30.083 (3.130)	28.615 (4.044)	26.750 (2.939)	23.385 (3.0850)	17.500 (2.169)	23.917 (1.549)
	Sociability index	0.431 (0.059)	0.127 (0.130)	0.377 (0.066)	0.231 (0.124)	0.503 (0.076)	0.333 (0.091)

		(n = 12)	(n = 13)	(n = 11)*	(n = 13)	(n = 12)	(n = 12)
Social novelty	Duration in familiar stimulus chamber (s)	149.283 (28.142)	98.969 (24.799)	123.455 (15.432)	135.862 (22.901)	180.742 (38.798)	127.367 (25.136)
	Duration in familiar stimulus interaction zone (s)	64.725 (9.475)	43.762 (7.803)	74.164 (6.796)	48.108 (9.315)	69.658 (14.496)	63.050 (9.021)
	Duration in novel stimulus chamber (s)	190.742 (23.761)	253.892 (39.434)	189.064 (19.761)	253.415 (39.634)	163.625 (33.846)	200.650 (23.017)
	Duration in novel stimulus interaction zone (s)	125.525 (15.188)	105.777 (15.523)	111.236 (14.211)	73.392 (11.245)	84.225 (18.496)	112.058 (23.025)
	Entries into familiar stimulus chamber	61.667 (4.764)	37.308 (5.172)	53.727 (5.401)	39.385 (4.758)	51.500 (6.854)	42.750 (4.337)
	Entries into familiar stimulus interaction zone	41.000 (5.256)	26.538 (4.483)	40.545 (4.796)	28.615 (3.672)	35.167 (5.137)	33.333 (2.994)
	Entries into novel stimulus chamber	84.333 (8.895)	57.077 (8.819)	68.636 (6.848)	51.077 (7.237)	53.833 (10.743)	67.667 (9.245)
	Entries into novel stimulus interaction zone	65.750 (9.181)	36.231 (8.294)	49.273 (6.209)	40.231 (6.217)	37.917 (10.427)	52.833 (8.571)
	Social novelty index	0.185 (0.118)	0.419 (0.104)	0.207 (0.095)	0.268 (0.111)	-0.011 (0.184)	0.240 (0.096)

* *Note.* One animal excluded due to technical issues.

Table 9

Means (SEM) of juvenile female somatic measures by MIA treatment and hormone treatment.

	Saline		Early MIA		Late MIA	
Measure	Oil (n = 11)	Testosterone (n = 11)	Oil (n = 12)	Testosterone (n = 12)	Oil (n = 11)	Testosterone (n = 10)
Body weight (g)	17.364 (0.622)	19.000 (0.588)	16.417 (0.645)	18.583 (0.793)	17.364 (0.453)	17.700 (0.396)
Gonadal weight (g)	0.051 (0.008)	0.144 (0.034)	0.052 (0.012)	0.094 (0.024)	0.053 (0.009)	0.041 (0.005)
Gonadal weight / body weight	0.003 (0)	0.008 (0.002)	0.003 (0.001)	0.005 (0.001)	0.003 (0)	0.002 (0)

Table 10

Means (SEM) for the righting reflex in neonatal females by MIA treatment hormone treatment across PND 4-16.

	Saline		Early MIA		Late MIA	
PND	Oil (n = 11)	Testosterone (n = 11)	Oil (n = 12)	Testosterone (n = 12)	Oil (n = 11)	Testosterone (n = 10)
4	54.912 (5.088)	49.033 (5.924)	46.62 (6.652)	56.102 (3.898)	(n = 7) 60.000 (0)	(n = 8) 53.941 (5.499)
6	18.256 (7.026)	11.138 (5.167)	18.678 (7.258)	(n = 10) 33.208 (9.058)	(n = 7) 43.509 (8.477)	(n = 8) 27.047 (9.941)
8	2.225 (0.260)	2.273 (0.463)	4.497 (1.962)	6.825 (4.838)	(n = 9) 2.682 (0.527)	2.197 (0.344)
9	2.238 (0.497)	1.758 (0.410)	2.007 (0.244)	2.047 (0.268)	2.188 (0.424)	1.679 (0.217)
10	2.626 (0.706)	1.313 (0.204)	2.042 (0.500)	2.006 (0.522)	3.115 (1.237)	3.098 (1.294)
11	1.840 (0.586)	0.914 (0.105)	1.388 (0.257)	1.271 (0.306)	1.333 (0.196)	1.866 (0.473)
12	0.941 (0.091)	0.746 (0.078)	0.878 (0.102)	1.085 (0.189)	0.864 (0.076)	0.864 (0.114)
13	0.605 (0.053)	0.580 (0.042)	0.593 (0.040)	0.645 (0.049)	0.671 (0.092)	0.762 (0.120)
14	0.526 (0.026)	0.509 (0.009)	0.521 (0.021)	0.649 (0.065)	0.579 (0.044)	0.644 (0.085)
15	0.500 (0)	0.500 (0)	0.500 (0)	0.500 (0)	0.500 (0)	0.569 (0.069)
16	0.500 (0)	0.500 (0)	0.500 (0)	0.500 (0)	0.500 (0)	0.500 (0)

Note. Number of animals vary per measure due to experimental disruption or error.

Table 11

Means (SEM) for eye-opening in neonatal females by MIA treatment and hormone treatment across PND 11-17.

PND	Saline		Early MIA		Late MIA	
	Oil (n = 11)	Testosterone (n = 11)	Oil (n = 12)	Testosterone (n = 12)	Oil (n = 11)	Testosterone (n = 10)
11	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)
12	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)
13	0.818 (0.296)	0.636 (0.244)	0.500 (0.230)	0.417 (0.193)	0.545 (0.207)	0.100 (0.100)
14	1.636 (0.203)	1.455 (0.247)	1.917 (0.083)	1.667 (0.188)	1.545 (0.157)	1.600 (0.163)
15	1.909 (0.091)	2.000 (0)	2.000 (0)	1.917 (0.083)	1.909 (0.091)	2.000 (0)
16	1.909 (0.091)	2.000 (0)	2.000 (0)	2.000 (0)	1.909 (0.091)	2.000 (0)
17	2.000 (0)	2.000 (0)	2.000 (0)	2.000 (0)	2.000 (0)	2.000 (0)

Note. No eyes open = 0; one eye open = 1; both eyes open = 2.

Table 12

Means (SEM) for the marble burying task in juvenile females by MIA treatment and hormone treatment.

	Saline		Early MIA		Late MIA	
Timepoint (mins)	Oil (n = 11)	Testosterone (n = 11)	Oil (n = 12)	Testosterone (n = 12)	Oil (n = 11)	Testosterone (n = 10)
5	0.091 (0.091)	0.273 (0.195)	0 (0)	0.167 (0.112)	0.727 (0.428)	0.400 (0.221)
10	0.818 (0.325)	2.000 (0.603)	0.333 (0.188)	1.000 (0.348)	2.273 (0.740)	1.000 (0.394)
15	2.455 (0.545)	3.000 (0.831)	1.500 (0.723)	1.917 (0.434)	3.364 (0.907)	1.600 (0.618)
20	3.727 (0.752)	4.091 (0.803)	2.167 (0.968)	3.000 (0.603)	4.455 (1.147)	2.900 (1.215)
25	4.091 (0.889)	4.909 (0.825)	3.000 (1.044)	3.583 (0.723)	5.273 (0.905)	3.900 (1.080)
30	3.727 (0.589)	5.364 (0.897)	3.083 (1.041)	5.000 (1.030)	7.182 (1.285)	4.000 (1.125)

Table 13

Means (SEM) for isolation USV parameters in neonatal females by MIA treatment and hormone treatment at PND 4, 6 and 8.

		Saline		Early MIA		Late MIA	
Measure	PND	Oil PND 4 (*n* = 13) PND 6 (*n* = 12) PND 8 (*n* = 12)	Testosterone PND 4 (*n* = 11) PND 6 (*n* = 11) PND 8 (*n* = 11)	Oil PND 4 (*n* = 11) PND 6 (*n* = 11) PND 8 (*n* = 12)	Testosterone PND 4 (*n* = 11) PND 6 (*n* = 10) PND 8 (*n* = 12)	Oil PND 4 (*n* = 6) PND 6 (*n* = 8) PND 8 (*n* = 10)	Testosterone PND 4 (*n* = 8) PND 6 (*n* = 10) PND 8 (*n* = 9)
Number of calls	4	97.231 (18.141)	141.818 (30.867)	117.273 (24.765)	107.273 (18.304)	105.833 (21.200)	119.250 (52.418)
	6	83.917 (11.394)	82.636 (14.490)	73.091 (12.533)	77.000 (21.620)	74.000 (17.343)	79.8 (23.877)
	8	86.250 (24.971)	58.545 (11.760)	95.917 (20.598)	53.500 (15.427)	57.000 (11.173)	71.556 (13.083)
Average low call frequency (kHz)	4	61.454 (1.236)	66.211 (0.966)	61.582 (1.077)	63.305 (1.678)	59.653 (1.229)	61.841 (2.035)
	6	62.496 (1.474)	68.099 (2.280)	65.743 (1.516)	65.480 (7.795)	62.102 (1.820)	67.282 (1.697)
	8	66.741 (1.743)	72.358 (1.647)	67.841 (1.530)	71.297 (2.603)	69.338 (1.004)	69.476 (2.314)
Average high call frequency (kHz)	4	71.922 (1.007)	78.050 (1.228)	71.761 (1.084)	75.134 (1.647)	70.155 (1.500)	72.945 (2.793)
	6	72.728 (2.066)	80.218 (3.107)	78.497 (1.867)	75.483 (8.677)	74.080 (2.281)	79.460 (2.367)
	8	77.830 (2.132)	85.304 (2.287)	80.314 (1.479)	83.188 (3.128)	81.406 (1.747)	82.451 (3.157)
Average call frequency (kHz)	4	66.688 (1.103)	72.130 (1.065)	66.672 (1.008)	69.22 (1.649)	64.904 (1.239)	67.393 (2.370)
	6	67.612 (1.753)	74.159 (2.680)	72.12 (1.667)	70.481 (8.206)	68.091 (1.970)	73.371 (2.004)
	8	72.285 (1.886)	78.831 (1.909)	74.077 (1.447)	77.242 (2.817)	75.372 (1.267)	75.963 2.690)

Average call duration (ms)	4	30.602 (2.472)	26.710 (0.925)	27.268 (2.29)	30.189 (2.050)	26.968 (3.533)	23.631 (2.905)
	6	27.231 (1.894)	28.930 (1.904)	25.358 (1.555)	21.806 (3.683)	34.146 (2.653)	28.384 (1.739)
	8	26.178 (2.235)	26.138 (2.106)	25.708 (1.654)	25.756 (2.579)	22.413 (2.121)	24.909 (2.407)
Total call duration (s)	4	3.238 (0.662)	3.715 (0.849)	3.629 (0.912)	3.509 (0.649)	3.178 (0.761)	3.428 (1.485)
	6	2.369 (0.382)	2.437 (0.441)	1.997 (0.382)	2.177 (0.635)	2.427 (0.565)	2.074 (0.531)
	8	2.442 (0.793)	1.588 (0.348)	2.680 (0.614)	1.543 (0.483)	1.396 (0.342)	1.796 (0.340)

Note. Number of animals vary per measure due to experimental disruption or error.

Table 14

Means (SEM) for USV parameters in juvenile females interacting with a same- or opposite-sex conspecific by MIA treatment and hormone treatment.

		Saline		Early MIA		Late MIA	
Measure	Stimulus sex	Oil (n = 11)	Testosterone (n = 10)	Oil (n = 10)	Testosterone (n = 10)	Oil (n = 11)	Testosterone (n = 6)
Call number	Male	253.091 (120.389)	236.900 (108.142)	98.100 (26.028)	83.500 (41.033)	48.636 (31.163)	26.833 (16.622)
	Female	154.818 (61.328)	94.300 (74.989)	34.200 (22.492)	58.700 (26.690)	92.364 (71.495)	82.500 (65.060)
Average low call frequency (kHz)	Male	65.060 (2.231)	66.695 (1.370)	66.055 (1.472)	65.937 (1.773)	60.838 (1.801)	67.521 (3.932)
	Female	67.346 (2.719)	52.470 (6.580)	62.494 (3.004)	56.617 (6.770)	53.351 (6.260)	63.205 (3.938)
Average high call frequency (kHz)	Male	74.190 (3.346)	75.280 (2.602)	75.117 (2.358)	72.616 (2.443)	66.619 (2.684)	73.078 (4.190)
	Female	75.089 (3.196)	57.973 (7.481)	68.523 (3.501)	62.696 (7.699)	58.495 (6.969)	69.679 (4.678)
Average call frequency (kHz)	Male	69.625 (2.749)	64.523 (6.376)	70.586 (1.816)	69.277 (2.085)	63.729 (2.219)	70.299 (4.022)
	Female	71.218 (2.935)	55.222 (7.020)	65.508 (3.216)	59.657 (7.229)	55.923 (6.595)	66.442 (4.291)
Average call duration (ms)	Male	20.560 (2.101)	19.033 (1.702)	18.178 (2.158)	16.031 (1.272)	18.916 (2.607)	18.625 (2.945)
	Female	21.753 (1.528)	19.506 (3.698)	15.952 (2.595)	17.048 (2.817)	23.296 (7.403)	20.285 (3.274)
Total call duration (s)	Male	6.845 (4.027)	5.608 (3.020)	2.219 (0.692)	1.733 (0.986)	1.092 (0.783)	0.474 (0.319)
	Female	3.506 (1.462)	2.579 (2.263)	1.024 (0.823)	1.238 (0.625)	2.745 (2.346)	2.159 (1.861)

Table 15

Means (SEM) for the three-chamber social paradigm in females by MIA treatment and hormone treatment.

		Saline		Early MIA		Late MIA	
Phase	Behaviour	Oil (n = 11)	Testosterone (n = 11)	Oil (n = 12)	Testosterone (n = 12)	Oil (n = 11)	Testosterone (n = 10)
Sociability	Duration social chamber (s)	228.827 (16.413)	202.300 (34.003)	208.855 (22.190)	195.527 (31.967)	163.636 (28.217)	231.020 (18.952)
	Duration social interaction zone (s)	126.745 (11.321)	97.430 (15.775)	106.709 (10.319)	118.900 (19.685)	85.355 (15.439)	121.350 (15.972)
	Duration non-social chamber (s)	125.136 (20.282)	177.660 (41.630)	127.600 (15.514)	145.800 (34.456)	185.791 (41.197)	109.100 (17.42)
	Duration non-social interaction zone (s)	28.309 (5.246)	32.190 (4.831)	29.236 (1.884)	31.591 (7.017)	31.236 (4.059)	43.710 (5.808)
	Entries social chamber	76.182 (8.877)	52.000 (8.460)	68.545 (9.674)	66.364 (10.855)	57.455 (8.389)	73.900 (11.492)
	Entries social interaction zone	63.000 (9.020)	42.800 (7.975)	51.818 (6.452)	53.545 (9.836)	43.636 (7.925)	56.200 (10.992)
	Entries non-social chamber	43.818 (4.009)	42.100 (6.428)	43.909 (2.226)	31.909 (3.925)	41.000 (4.421)	41.300 (3.712)
	Entries non-social interaction zone	27.091 (3.965)	28.300 (4.171)	27.909 (2.016)	22.182 (3.744)	24.818 (3.230)	26.200 (2.756)
	Sociability index	0.399 (0.075)	0.177 (0.155)	0.325 (0.073)	0.207 (0.172)	0.093 (0.161)	0.383 (0.087)

Social novelty	Duration familiar stimulus chamber (s)	183.109 (28.142)	136.718 (24.799)	147.417 (15.432)	142.342 (19.556)	192.727 (38.798)	154.200 (25.136)
	Duration familiar stimulus interaction zone (s)	63.373 (9.475)	45.418 (7.803)	49.817 (6.796)	58.467 (11.017)	60.045 (14.496)	61.100 (9.021)
	Duration novel stimulus chamber (s)	170.645 (23.761)	255.073 (39.434)	202.917 (19.761)	162.275 (19.397)	140.427 (33.846)	168.880 (23.017)
	Duration novel stimulus interaction zone (s)	101.355 (15.188)	88.318 (15.523)	105.900 (14.211)	106.450 (16.852)	72.691 (18.496)	138.920 (23.025)
	Entries familiar stimulus chamber	50.636 (4.764)	38.455 (5.172)	46.583 (5.401)	38.167 (5.803)	42.182 (6.854)	41.500 (4.337)
	Entries familiar stimulus interaction zone	36.545 (5.256)	28.455 (4.483)	35.333 (4.796)	27.667 (4.886)	27.364 (5.137)	28.900 (2.994)
	Entries novel stimulus chamber	60.00 (8.895)	52.636 (8.819)	54.667 (6.848)	49.500 (9.110)	42.273 (10.743)	61.500 (9.245)
	Entries novel stimulus interaction zone	47.091 (9.181)	43.455 (8.294)	43.500 (6.209)	37.667 (8.111)	33.364 (10.427)	45.900 (8.571)
	Social novelty index	0.037 (0.118)	0.294 (0.104)	0.198 (0.095)	0.066 (0.113)	-0.033 (0.184)	0.172 (0.096)

Table 16

Means (SEM) for steroid hormone concentrations in neonatal (PND 1) male serum by MIA treatment and hormone treatment.

	Saline		Early MIA		Late MIA	
Hormone (ng/mL)	Oil (n = 3)	Testosterone (n = 3)	Oil (n = 3)	Testosterone (n = 3)	Oil (n = 3)	Testosterone (n = 3)
Cortisol	5.237 (0.607)	12.263 (1.467)	6.503 (1.372)	6.347 (4.409)	7.643 (0.476)	5.550 (0.357)
Progesterone	1.017 (0.119)	1.497 (0.417)	1.050 (0.106)	1.003 (0.541)	1.390 (0.137)	0.760 (0.139)
Estradiol	0.040 (0.030)	0.057 (0.012)	0.063 (0.009)	0.087 (0.032)	0.070 (0.047)	0.060 (0.021)
Testosterone	0.240 (0.047)	0.307 (0.058)	0.377 (0.070)	0.417 (0.088)	0.260 (0.146)	0.267 (0.045)
T3	0.373 (0.041)	0.370 (0.021)	0.407 (0.041)	0.407 (0.097)	0.497 (0.138)	0.480 (0.075)
T4	1.617 (0.816)	1.733 (0.640)	1.190 (0.769)	0.983 (0.983)	1.620 (0.539)	1.597 (0.816)

Note. Samples consist of pooled serum from male offspring from 1-2 litters to achieve the required volume for analysis (≥ 100 μL).