An Immune Rat Model of Autism: Does Environmental Enrichment Alter Higher Social Functioning?

By

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Abstract

The neurodevelopmental condition autism spectrum disorder (ASD) is marked by impaired communication, impaired social interaction, and repetitive or restricted behaviours (American Psychiatric Association, 2013). ASD is caused by a combination of genetic and environmental factors, including maternal infection during pregnancy. There are no pharmacological treatment options for ASD, but environmental enrichment (EE) is a promising alternative to rehabilitate or prevent related symptoms. Patients with ASD often have a deficit in higher social abilities, including prosociality, but to date, no rat studies have investigated how EE affects higher social functions in the context of ASD. Therefore, this thesis investigated the effect of EE on higher social functions in an *in vivo* rodent model of ASD. Using a two-by-two factorial design, pregnant Sprague Dawley rats received either saline or the viral mimic polyinosinic-polycytidylic acid (poly I:C) to trigger an immune reaction and were placed in either standard or enriched housing. In the enrichment groups, pups received pre-weaning enrichment sessions while animals in the standard-housing groups were left undisturbed until weaning. I assessed the animals' neonatal communication, general sociability and social novelty behaviour, prosocial tendencies, and locomotor activity. The main hypothesis I investigated was that the maternal immune reaction would disrupt fetal neural development and alter communication and social behaviours in the offspring and that EE would reduce or reverse these deficits.

There was a transient poly I:C effect on neonatal communication early on, but the treatment did not cause long-term social deficits. Poly I:C also affected adult locomotor activity but did neither reduce sociability nor prosociality. As the very weak treatment effects did not result in clear deficits, EE could not lead to improvements. While the findings did not offer insight into the enrichment effect on impaired prosocial behaviours, this was likely due to methodological issues and limitations. Future ASD research should address these concerns to further the understanding of how environmental adjustments can stimulate the impaired social parts of the brain to redirect the atypical developmental trajectory towards a more typical one.

Keywords: autism, rodent model, maternal immune activation, poly I:C, environmental enrichment, prosocial behaviour

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ASD	Autism spectrum disorder, also: autism
ANOVA	Analysis of variance
APA	American Psychiatric Association
APOE	Apolipoprotein E
AVP	Arginine vasopressin
CDC	Center for Disease Control
CSF	Cerebrospinal fluid
DSM	Diagnostic and Statistical Manual of Mental Disorder
EE	Environmental enrichment
EH	Enriched housing
FBZ	Fenbendazole
FDR	False discovery rate
GD	Gestational day
IL	Interleukin
IP	Intraperitoneal
IV	Intravenous
IVC	Individually ventilated cages
LMA	Locomotor activity
LPS	Lipopolysaccharide
MIA	Maternal immune activation
MNS	Mirror neuron system
OC	Open cages
OXT	Oxytocin
PAF	Platelet-activating factor
PAM	Perception–action model (of empathy)
PND	Postnatal day
PIC	Polyinosinic-polycytidylic acid
Poly I:C	Polyinosinic-polycytidylic acid
Q-Q Plot	Quantile-quantile plot
RDoC	Research Domain Criteria
RNA	Ribonucleic acid
SAA	Social approach–avoidance
SAL	Saline
SEM	Standard error of the mean
SERT	Serotonin transporter
SC	Subcutaneous
SD	Sprague Dawley
SH	Standard housing
SSRI	Selective serotonin reuptake inhibitor

TLR	Toll-like receptor
TNF-α	Tumour necrosis factor α
USV	Ultrasonic vocalisations
VPA	Valproic acid
WKY	Wistar-Kyoto
5-HT	5-hydroxytryptamine or serotonin

Chapter 1

General Introduction

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1.1 Thesis Overview

This thesis aims to investigate if environmental enrichment (EE) affects higher social functions in an *in vivo* rodent model of *autism spectrum disorder* (ASD). The first section will define and review ASD, followed by an in-depth assessment of higher social functions. Next, I will look at the aetiology, neurobiology, and treatment of ASD, before considering the role of animal models. I will conclude this chapter with the overall objectives of this thesis.

To date, no known studies have used animal models to investigate how an enriched environment affects higher social functions in the context of ASD, which is what this project aims to change. Rats and mice have been used for pre-clinical research for decades, but most animal studies of social behaviour have focused on the more basic social behaviours, such as play behaviour and social approach–avoidance. Some animal research has explored prosocial behaviours in rats in general (Ben-Ami Bartal et al., 2011; Sato et al., 2015), but not much work has been done in relation to ASD (Fontes-Dutra et al., 2019).

In this thesis, I will mix person-first (e.g., person with ASD) and identity-first (e.g., autistic person) language to refer to individuals with ASD/autistics as per the 7th edition of the American Psychological Association (APA) guidelines on inclusive language (American Psychological Association, 2020). I will also use ASD and autism interchangeable to refer to the condition. Further, I will refer to individuals without a neuropsychiatric condition as neurotypicals.

1.2 Introduction to ASD

1.2.1 Historic Background

The term autism was derived from the Greek word *autos*, meaning self. It was first introduced by the Swiss psychiatrist Eugen Bleuler in 1911 in the context of schizophrenia (Bleuler, 1911/1950). Following this, a psychiatrist from Russia published the first detailed account of several autistic boys and girls in a German journal in 1926 and 1927 but

classified the cases as childhood schizophrenia. Grunya Sukhareva[1] described the hallmark language issues (e.g., odd and stereotypical speech), flat affect with deficient facial expressions, a tendency to withdraw and disinterest in social contacts, as well as repetitive behaviours (Ssucharewa, 1926, 1927). Unfortunately, Sukhareva's contribution to the field went unnoticed until 1996 when it was translated into English (Wolff, 1996). The reasons for this omission are unclear but were possibly a result of the political situation at the time (she was Russian, Jewish, and female).

Almost two decades later, the German child psychiatrist Leo Kanner (1943) from Johns Hopkins hospital in the United States (US) published on the topic of autism as a syndrome. He discussed 11 case studies (8 boys and 3 girls) between the ages of 2 and 10. Besides repetitive and obsessive behaviours, he reported language deficits (e.g., delayed speech, mutism, an inability to use language communicatively), deficient social awareness, an inability to relate themselves to others, and no interest in social engagement but instead a fascination with objects. Of one of the boys discussed he said: "When he had any dealings with persons at all, he treated them, or rather parts of them, as if they were objects" (Kanner, 1943, p. 228). Moreover, several of the cases had previously been misdiagnosed as either feebleminded or schizophrenic.

Independently but simultaneously on the other side of the Atlantic, the Viennese paediatrician Hans Asperger (1944) published a similar paper, presenting four cases of boys with almost identical symptoms as portrayed in the American paper. He also reported hypersensitivity to certain fabrics, water, as well as loud noises. He found *autistic psychopathy,* as he called it, surprisingly contradictory, with its characteristics ranging from being almost brilliantly original to extremely disturbed "imbeciles".

It took over three decades for *infantile autism* to be recognised in the Diagnostic and Statistical Manual version III (DSM-III) as a subcategory of *pervasive development disorder* (PDD) in 1980 (American Psychiatric Association, 1980). Changes to the diagnostic criteria resulted in five distinct autism-type disorders with the fourth edition of the DSM (DSM-IV) in 1994, these being *classical autism*, *Asperger's syndrome*, and PDD not otherwise specified (PDD-NOS), *disintegrative disorder*, and *Rett's syndrome* (American Psychiatric Association, 1994). This was refined in 2013 when the introduction of the new DSM edition 5 (DSM-5) saw the formation of the autism spectrum. Here, several disorders were placed

[1] Her name was transliterated as Ssucharewa by the publishing journal.

under the umbrella ASD. This grouping includes early infantile autism, childhood autism, Kanner's autism, high-functioning autism, atypical autism, pervasive developmental disorder, childhood disintegrative disorder, and Asperger's syndrome (American Psychiatric Association, 2013).

In New Zealand (NZ), the government published the first official autism guidelines for clinicians in 2008 (Ministries of Health and Education, 2016). Recently, the word *Takiwātanga* was added as the Te Reo Māori term for autism; it derived from *tōku/tōna anō takiwā*—in my/his/her own time and space (Bowden et al., 2020).

1.2.2 Prevalence

Large worldwide population-based studies identified a population prevalence of ASD diagnosis between 1.0% and 2.0% (Lai et al., 2014), but the rates vary from 0.1% in Bangladesh to 9.3% in Japan (Chiarotti & Venerosi, 2020). According to the *Center for Disease Control and Prevention* (CDC), prevalence rates depend on factors like sex, ethnicity/racial background, but also region within a country. ASD rates in the US have risen consistently from 0.6% in 2002 to 1.9% in 2016 (Maenner et al., 2020). An Australian study reported even higher prevalence rates of 2.5% (Randall et al., 2015). In New Zealand, the prevalence rate was 0.5% in 2007 and has risen to 2.0% in 2019 (Ministry of Health, 2019). The reasons for the rising numbers across all countries include changes to diagnostic criteria and practice (King & Bearman, 2009), heightened public awareness (Kim et al., 2011), targeted routine health check strategies (Levy et al., 2009), but also rising parental age (Krug et al., 2020; Weintraub, 2011).

The CDC reported a male to female ratio of 4.3:1 in the US (Maenner et al., 2020). Worldwide, however, the ratio is not as large with a ratio between 2:1 and 3:1 (male:female) (Lai et al., 2014). The male to female ratio is even higher when cases with intellectual disability are excluded (Taylor Rivet & Matson, 2011).

1.2.3 Impact on Patient, Society, and Economy

ASD is a lifelong condition and may force patients and their families to live a life of reduced wellbeing and, in the bigger picture, places an enormous burden on society. Globally, ASD is responsible for substantial loss of health throughout a person's life (Baxter et al., 2014).

When looking at the personal lives of people with autism, the outcome strongly depends on symptom severity and cognitive and social abilities; even for people with ASD

who are well-adjusted and functioning at a higher level, the experiences are variable (Chamak & Bonniau, 2016; Howlin, 2000). Some individuals can live independently, have relationships, and are married, but those are in the minority (Howlin, 2000). In a recent study on social outcomes, Farley et al. (2018) found that 75% of autistic participants in their mid-thirties had no experience with dating at all. Most of those in relationships had had one to two romantic relationships throughout their lives. Barendse et al. (2018) reported that autistic adolescents had fewer real friends and, when compared with neurotypical controls, met their friends less frequently (outside of school).

An estimated 50-75% of autistic adults are unemployed and even for those autistics that are considered to be high functioning, the chances of finding a job are substantially lower than for neurotypicals (Hendricks, 2010). Nevertheless, it is a spectrum and there are people with ASD who have adjusted very well, for instance Jessica Benham, who is a wife and mother of three, a politician (Pennsylvania state House representative) (Reynolds, 2020), an award-winning academic (MA in bioethics, Autistic Scholars fellow), and the Director of Development at the *Center for Autistic Advocacy in Pittsburgh* (University of Pittsburgh, 2020).

Unfortunately, people like Jessica are the exception to the rule. A French study reported that even high-functioning adults with university education remained reliant on their families (Chamak & Bonniau, 2016). However, over a five-and-a-half year-period, adults with ASD with a higher level of vocational independence showed reduced autistic symptoms and maladaptive behaviours, as well as increased abilities to be more independent in daily life (Taylor et al., 2014). Howlin (2000) pointed out the scarcity of specialist public services that help navigate the task of finding employment or accommodation, so the autistic has to rely on their family's help to navigate their individual requirements. While recent years have seen great improvements in this area, there are massive differences between and within countries (Rogge & Janssen, 2019). Poland, for instance, has no special education needs strategy specific to autism, whereas countries like Spain, the UK, and NZ have guidelines aimed at improving the prospects in areas such as health, education, and social matters for autistics (Ministries of Health and Education, 2016; Rogge & Janssen, 2019). Jacob et al. (2015) noted that, if governments invested in developing employment services for autistics, it would not only improve the quality of life for the affected but also reduce the cost for life-long government-funded services and allow the—often very talented—potential employees to be contributing members of society.

People with autism frequently rely heavily on their family for ongoing support and care. Järbrink (2007) found that Swedish families of autistic children expended about 1,000 hours annually for the care of their children. When a family has a member with ASD, their lives are often focused on the needs of the autistic child (DeGrace, 2004). Mothers with an autistic child earn less than those with a neurotypical child, it is less likely that both parents work, and the average weekly work hours are five hours less than in families without ASD (Cidav et al., 2012).

When looking at the total annual economic impact of ASD for Sweden (Järbrink, 2007), the United Kingdom (UK), and the US (Buescher et al., 2014), the numbers speak for themselves with $117 million[2], $57 billion, and $264 billion respectively (all adjusted to NZ dollars). This breaks down to an annual societal cost for care per individual of $92,000[3] (Sweden), $60,000 (UK) and $86,000 (US). While no data on New Zealand's economic burden could be found, Horlin et al. (2014) reported that the annual cost per autistic individual in Australia came to approximately $37,000[4]. The study did not report the total annual economic impact, but there were 205,200 people with autism in Australia in 2018 (Australian Bureau of Statistics, 2019), so the total annual cost for ASD in Australia in 2018 accumulated to about $7.6 billion[5]. Such costs include the cost for medical/healthcare services, therapy, education, loss of income for patients and their families, external care, and accommodation (Rogge & Janssen, 2019). With ASD diagnoses on the rise, the projected cost of ASD in 2025 is an estimated $407 billion[6] in the US alone (Leigh & Du, 2015).

ASD has a massive economic impact that is carried by governments, families, and, most importantly, patients across the globe. Besides monetary aspects, however, a diagnosis of ASD usually means difficulties and reductions in quality of life, not only for the individuals but also for their families. The points made in this section highlight the need for a change of focus in terms of treatment options to effectively ameliorate the outcomes for those affected by ASD.

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[2] €66 million (Sweden), £29 billion (UK), and US$175 billion annually (aggregated).

[3] €51,877 (Sweden), £30,712.89 (UK), and US$56,606.64 annually per autistic individual.

[4] AU$34,900 per autistic individual annually.

[5] AU$7.1 billion total annual economic impact.

[6] Average of US$268 billion.

1.2.4 Diagnosis and Symptoms Overview

ASD is a group of neurodevelopmental disorders defined by impaired social communication and interaction, and repetitive or restricted behaviours (RRB) (American Psychiatric Association, 2013). The first domain relates to social aspects and includes three subcategories: (1) Impaired social and emotional reciprocity; (2) deficient nonverbal communicative behaviours for social interaction; and (3) problems with the development, maintenance, and understanding of relationships. The second domain involves restricted and repetitive behaviours (RBB) and comprises four components:
(1) Stereotyped/repetitive motor movement, utilisation of objects or speech; (2) insistence on sameness, clinging to routines, ritualised behaviour patterns—verbal and nonverbal; (3) highly fixated interests; and (4) hyper-/hypo-reactivity to sensory input, and odd interests in sensory environmental aspects.

Symptoms usually first appear during the first three years of a child's life (Levy et al., 2009), but autistic individuals differ vastly in presence and expression of symptoms, cognitive abilities, and social-emotional abilities (Glezerman, 2013). This heterogeneity of symptoms is best illustrated with an example. At one end of the spectrum, patients are severely cognitively impaired, nonresponsive, mute, and incapable of caring for themselves. At the other end, they are highly intelligent—even gifted—and lead successful lives, one example being the well-known autism-rights activist and professor of animal science Temple Grandin who has published several books and over a hundred journal articles (The Editors of Encyclopaedia Britannica, 2020). Another example of the symptom heterogeneity is the reaction to sensory stimuli: Some patients are hypersensitive, while others are hyposensitive (American Psychiatric Association, 2013). What all autistics have in common, however, are the varying degrees of social-emotional and communicative deficits, including a preference for inanimate objects over people (Klin et al., 2002b). ASD often co-occurs with other conditions, ranging from developmental (e.g., intellectual disability, attention deficit hyperactivity disorder, language disorders, motor abnormalities, etc.), to general medical (e.g., sleep conditions, epilepsy, gastrointestinal issues, immune dysregulations, etc.), psychiatric (anxiety, depression, oppositional defiant disorder, etc.), and behavioural abnormalities (e.g., aggression, self-harming, etc.) (Lai et al., 2014).

Symptom expression differs between males and females. When looking at emphasising (the ability to identify and respond to another's emotions/thoughts) and systemising (the drive to analyse/build a rule-based system) in the general population,

males usually have higher systemising scores than females, whereas females score higher on emphasising measure. In the ASD sample, females shift toward the typical male profile, closing the gap between the sexes (Baron-Cohen et al., 2014; Greenberg et al., 2018). Further, Mandy et al. (2012) found that autistic females were more likely to have better fine motor skills than autistic males. Also, when compared with autistic females, autistic males displayed a greater level of repetitive and stereotyped behaviours like lining up toys and large accumulation of factual knowledge (i.e., systemising behaviours) (Mandy et al., 2012). Females appear to be better at blending in, hiding their social-communicative deficits more effectively than boys, possibly due to them being more self-aware and making a greater effort to understand social norms and acquire social skills (Rynkiewicz & Łucka, 2015). Also, adult females with ASD process information faster than ASD males, implying greater proficiency at using explicit cognitive coping mechanisms, which may explain why females cope better in social situations (Lehnhardt et al., 2016).

Autistic children have difficulties integrating multisensory input (Brandwein et al., 2013), which subsequently impacts on social abilities (Klin et al., 2003). This includes difficulties with dividing auditory attention, which also contributes to issues in social situations, where stimuli compete for attention (Kenworthy et al., 2009). Autistics also show unusual face scanning patterns in naturalistic social situations (complex dynamic social task), that is, they focus on the mouth region in an attempt to gather social cues, rather than the eyes, the face region neurotypicals generally focus on (Klin et al., 2002b). Klin et al. (2002a) investigated the reaction to non-verbal cues and found that autistics did not use the non-verbal cue provided (i.e., did not follow the pointing gesture with their eyes) to identify the object in question. When asked after the session whether they understood the meaning of the gesture, the autistic could define what the gesture meant without difficulties, but they were not able to use this knowledge spontaneously during the session. Indeed, it seems there is a disconnect between social-cognitive skills and spontaneous social action (or interaction) in ASD because salience is not given to social stimuli but other physical stimuli (Klin et al., 2003). Autistics often lack some or all of the social-cognitive tools that enable neurotypicals to generate adaptive moment-by-moment social reactions in social situations, so their social behaviour is often less efficient than that of neurotypicals.

Indeed, across the lifespan, social-behavioural deficits are the most persistent symptoms, while restrictive and repetitive behaviours are less prevalent in adulthood (Shattuck et al., 2007). This is especially difficult during adolescence, which is when social

and emotional processing develops and differences between autistics and typically developing peers become more apparent (Crone & Dahl, 2012). Social-behavioural deficits often lead to increased anxiety and loneliness in high-functioning teens and adolescents (Bauminger et al., 2003; White & Roberson-Nay, 2009). Later in this chapter, I will focus on prosocial behaviour in general and how it is affected by ASD.

1.3 Higher Social Functions

While autism symptomology is very heterogenic, every person diagnosed with ASD presents with some level of social deficit. Glezerman (2013) suggested that the understanding of the self (the "I" concept) as separate from others is often deficient in autistics. ASD is marked by a degree of "social blindness" and frequently autistics cannot rely on what for neurotypicals is an inherent ability—to read social cues or emotions from other people's facial expressions (Baron-Cohen, Wheelwright, et al., 1997), a deficit unrelated to overall intelligence (Baron-Cohen, Jolliffe, et al., 1997). Autistics can recognise different basic facial expressions (Baron-Cohen, Jolliffe, et al., 1997; Humphreys et al., 2007) but often struggle to categorise them. Typical autistic behaviours (e.g., low eye contact) are often interpreted as a lack of interest in other people (Klin et al., 2003), causing social isolation. The point that autistic individuals prefer not to engage in eye contact with others (Klin et al., 2002b) is not well understood, but several explanations, including abnormal amygdala activity (Hadjikhani et al., 2017; Pujol et al., 2009), hyperarousal in response to facial stimuli (Kaartinen et al., 2016; Kylliäinen et al., 2012), or a lack of interest in other people (Chevallier et al., 2012) have been presented. However, Jaswal and Akhtar (2018) offered a different explanation, namely that some autistics deliberately chose to avoid eye contact, as it can cause anxiety; some may also struggle to control involuntary repetitive movements.

The ability to understand non-verbal social cues is an important component of empathy—understanding and sharing another person's emotions (separate from one's affective state) (Stueber, 2014). Such abilities enable social interaction between people and their lack creates an invisible barrier of which autistics are all too aware (Verbeke et al., 2005). Often high-functioning autistic adults compensate by studying social behaviour to better understand what other people feel (Verbeke et al., 2005). Furthermore, these social deficits likely contribute to the previously discussed chronic underemployment of otherwise well-functioning autistic adults. In the next section, I will talk about what empathy as a higher social function is, what it entails, and how it works, as one of the key focus of this thesis is the study of prosocial behaviour in ASD.

1.3.1 The Concept of Empathy

Empathy was first introduced into the English language in 1909 by Edward Titchener who translated the German word *Einfühlung*, meaning "to feel into" (Stueber, 2014; Titchener, 1909/2014) and the word itself can be traced back to the ancient Greek word *empatheia* (Frankel, 2017). Preston and de Waal (2002) noted that because empathy is a multidimensional concept, it has many definitions. Indeed, in a review of empathy, Cuff et al. (2016) identified 43 discrete definitions of the term.

Empathy is defined broadly as a person's emotional and cognitive abilities to perceive and share another's emotions and formulate an appropriate response to those shared emotions (de Waal & Preston, 2017; Perry & Shamay-Tsoory, 2013). According to Perry and Shamay-Tsoory (2013), a person's level of empathy is determined by their ability to (a) notice, (b) comprehend, and (c) care about another's emotions. For example, a person meeting with their friend notices that the friend is upset. If they notice, understand, and care, they might act prosocially and, for instance, comfort their friend to alleviate their distress. This indicates a link between empathy and certain prosocial behaviours (Eisenberg et al., 2010; Masten et al., 2011), which will be discussed in greater detail below.

An individual's abilities to notice, understand, and respond to other's affective states are subject to individual differences including sex, intelligence, education, personality, interests, attitudes, and values and are, thus, highly variable. Further, neurological conditions, including ASD and schizophrenia, have been linked to low trait empathy (Chakrabarti & Baron-Cohen, 2013). Dispositional empathy or trait empathy is relatively stable across the lifespan and has a strong genetic basis. However, it is also impacted by experience and the influence of heritability increases with age (Knafo et al., 2008). State empathy describes how empathetic a person feels at a specific moment. State empathy is more fluid, as it is influenced by everyday experiences (i.e., the environment) and expressed through behaviours, feelings, and thoughts (Bennett, 1995).

There are also empathy differences between the sexes. Females are quicker and more precise than males in facial expression recognition and recognition of bodily emotions (particularly neutral or angry body language) and are also more susceptible to emotional contagion than males (Christov-Moore et al., 2014). The next section will explore the specific parts of empathy in more detail.

1.3.2 *Components of Empathy*

Empathy includes two distinctly different systems that generally work together—cognitive empathy and emotional or affective empathy. Cognitive empathy refers to the ability to understand others' affective states and create a theory about its social and affective meaning (i.e., affective theory of mind), followed by taking the other's perspective (Brothers & Ring, 1992; Davis, 2006; Frith & Singer, 2008). Basically, an individual infers what another thinks or feels in a given situation (based on their beliefs) and uses this inference to predict the other's behaviour. The second system—emotional empathy—is a process that involves the various spontaneous emotions experienced in response to observing another's emotions. In addition, there are several related concepts, including emotional contagion, where one automatically "catches" and subsequently experiences someone else's emotional state (Hatfield et al., 1993). Nakahashi and Ohtsuki (2015) called emotional contagion an evolutionary strategy to acquire social learning. It involves mirroring another's behavioural or affective state such as, for example, one crying baby in a nursery usually results in all of them crying, spreading distress (de Waal, 2008). This contagious effect is based on autonomic nervous system activation in response to another's emotional state; the more familiar the individuals are with each other, the bigger the contagious effect (Preston & de Waal, 2002).

Emotional contagion requires the activation of a neural system called the mirror neuron system (MNS), a system consisting of several brain areas that activate when an individual executes an action, but also when one observes someone else executing an action (Rizzolatti & Craighero, 2004; Rizzolatti et al., 1996). It was initially thought to enable motor mimicry only (di Pellegrino et al., 1992), but subsequent research found that it extended beyond the neural motor areas (e.g. Keysers & Gazzola, 2009). The MNS not only plays a fundamental role in imitation learning in humans but is also connected to social perception (Keysers & Gazzola, 2009; Rizzolatti & Craighero, 2004).

According to the influential *perception–action model* (PAM) of empathy, the attended perception of a person's emotional state results in an automatic activation of a representation of the state, situation, and focus (Preston & de Waal, 2002). Further, this activation automatically triggers the appropriate autonomic and somatic responses, unless suppressed. These PAM mechanisms form the centre of empathic behaviours and, like a Russian nesting doll, become more complex towards the outer layers (de Waal & Preston, 2017). At the core lie simple behaviours like emotional contagion and motor mimicry (i.e., state matching), while complex empathy behaviours that require affective self-regulation

and additional cognitive skills, such as perspective-taking and targeted helping, are found in the outer layers of the doll (de Waal, 2012). The key point here is that all empathy-related behaviours are based on the PAM (de Waal, 2012; de Waal & Preston, 2017; Preston & de Waal, 2017).

1.3.3 Empathy and ASD

Empathy is often deficient in ASD (Dziobek et al., 2008; Harmsen, 2019; Mazza et al., 2017; Mazza et al., 2014). The ability to process—recognise and understand—socially relevant information is impaired in ASD (Dziobek et al., 2008; Mazza et al., 2017; Mazza et al., 2014). Also, autistics struggle with perspective-taking (Mazza et al., 2014). Cognitive empathy but not emotional empathy (empathic concern) can be deficient in ASD (Dziobek et al., 2008). The underlying reasons for the empathy deficits found in ASD are not well understood, but Decety and Moriguchi (2007) suggested that children with ASD often struggle to feel and express emotions, preventing empathic engagement with others in social situations. Dawson (2008) suggested that empathy deficits in ASD are the outcome of reduced social motivation during development, resulting in reduced engagement with social stimuli. Also, females tend to be more empathetic than males when considering neurotypicals (Christov-Moore et al., 2014) as well as autistics (Baron-Cohen & Wheelwright, 2004; Schulte-Rüther et al., 2008).

As brain development depends on what the person focuses their attention on (Leppänen & Nelson, 2006), the reduced social focus means that autistic brains get less time to develop the pathways necessary for empathy, resulting in developmental deficit. Further, Cook et al. (2013) connected the presence of *alexithymia*, a condition marked by difficulties with the interpretation of emotional states, to reduced emotional recognition abilities but found that the latter was not correlated with the severity of ASD. Others attributed empathy deficits in ASD to a deficient MNS (Dapretto et al., 2006; Williams, 2008).

The notion of a deficient MNS derives from the idea that for someone to be able to read and interpret others' intentions, one needs a fully functioning MNS (Williams et al., 2001). However, autistics struggle to imitate others, likely as a result of deficits in the MNS (Dapretto et al., 2006; Williams, 2008). Dapretto et al. (2006) found reduced activity in the inferior frontal gyrus, an area connected to understanding another's intention, as well as a negative correlation between neural activity and social abilities. In autism, this can lead to difficulties with understanding social dynamics like reciprocal altruism or shared intent (Constantino et al., 2000; Tomasello et al., 2005). Moreover, the capacity to recognise other

people's emotions by merely studying their faces is also connected to the MNS (Dapretto et al., 2006). Dapretto et al. used a task that involved looking at facial expressions and imitating them and found that, in neurotypical children, the MNS was triggered when they observed and imitated emotional expressions, but children with ASD showed almost no activity in the same region. Thus, the MNS enables the recognition of affective states from the face for neurotypicals but does not work effectively for those diagnosed with ASD.

1.3.4 Prosocial Behaviour

Prosocial behaviours are actions performed by an individual in response to another's distress or need, aiming to benefit this individual by, for instance, helping or comforting (Hay, 1994). As discussed above, according to the PAM model, these targeted prosocial behaviours are the outer layers that rely on additional cognitive abilities and emotional self-regulation. These behaviours develop in the first two years of a person's life (Dunfield et al., 2011), where they are strongly influenced by environmental factors, while genes have a greater effect on prosociality later in life (Knafo et al., 2008).

For someone to respond in a prosocial manner, they must first be able to identify that there is a problem (social cognition), what is causing the problem, and lastly, be driven to help resolve the problem (affective motivation). Such behaviours, in both humans and animals, are generally driven and mediated by empathy and more likely to be extended to kin or known individuals (Decety et al., 2016; Lockwood et al., 2014; Yamamoto, 2016). Reducing the other's distress will also alleviate the helper's tension; plus, the act of helping has been reported to be enjoyable (Decety et al., 2016). This section will focus mainly on prosocial behaviours in response to another person's distress or negative emotions—namely the act of helping.

1.3.5 Prosocial Behaviour and ASD

As noted above, autistics frequently have problems with emotion recognition, suggesting that this group would display fewer prosocial behaviours than neurotypical individuals. However, most research on the topic of prosociality has been conducted with non-autistic participants, and the few studies that have used autistics had disparate results. Charman et al. (1997) found that children aged 20 months at risk of developing ASD expressed reduced facial concern in reaction to another person's distress relative to neurotypical children. Further, Liebal et al. (2008) found that 2- to 5-year-old autistic children tended to provide help less frequently when compared with developmentally

delayed children. They did, however, stress that the ASD group showed both the skills and motivation necessary to help another person. In a recent study, Dunfield et al. (2019) compared three types of prosocial behaviour—helping, sharing, and comforting—between groups of three-year-old children with autism and those without. Interestingly, the ASD group did not differ from the control group in their ability to differentiate between situations with or without need. As the differences between the two groups varied in types of behaviour, the authors suggested that the three behaviour types cannot be grouped into one category, as they have different socio-cognitive requirements (i.e., motivations and skills). There was no significant group difference in helping or comforting behaviour. Autistic children were happy to help when the cost of acting prosocially was low (i.e., comforting the experimenter), but less so when it was higher (i.e., share treats).

Hepach et al. (2020) suggested that autistics may be at a disadvantage because of a deficit with anticipating prosocial behavioural requirements (i.e., the cognitive rather than the emotional aspect of helping). Prosociality increases with age because sociocognitive functions like perspective-taking or social problem-solving also mature (Eisenberg et al., 2007; Fabes et al., 1999). The studies discussed in this section do not offer conclusive evidence, possibly due to methodological differences between studies. In summary, prosocial behaviour is mediated by empathy and people with ASD generally present with various empathy-related impairments, particularly cognitive empathy, that interfere with reading social requirements accurately.

1.4 The Aetiology of ASD

There is no single cause of ASD (Happe et al., 2006). However, high concordance rates between 64% and 91% for monozygotic twins and over 50% for dizygotic twins point at a strong genetic link (Bailey et al., 1995; Colvert et al., 2015; Rosenberg et al., 2009; Tick et al., 2016). In a study on familial reoccurrence rate, the authors found that almost 10% of participating families had more than one child with ASD; indeed, the risk of having a second child with autism was found to be 8.6% (Ritvo et al., 1989). Even the earliest reported ASD cases in the 1920s found that undiagnosed family members often displayed symptoms from social withdrawal to communication issues (Kanner, 1943), all associated with ASD. Later studies corroborated these findings, reporting communication and social impairments, as well as restricted/repetitive and stereotyped behaviours in close relatives of autistic individuals (Bolton et al., 1994; Pickles et al., 2000; Piven et al., 1997). Jeste and Geschwind (2014) pointed out that random structural variations of chromosomes called *de novo* copy number variants (deletions or duplications) played a role in 10-20% of ASD

cases, but only about 1% of those were caused by an individual mutation. These results emphasise autism's high heritability rate, but as the aforementioned twin studies showed concordance rates of under 100%, it is obvious that environmental factors play an important role as well. Genes linked to ASD play a role in neural development, synaptic plasticity, and impact neurotransmitters (Cardoso & Almeida, 2019).

Since genes alone cannot explain the aetiology, another line of research focuses on prenatal environmental factors that cause embryonic malformation (teratogens) linked to ASD, including drugs like, for example, valproic acid (VPA) (Christensen et al., 2013; Lloyd, 2013) or thalidomide (Strömland et al., 1994), pollution (Volk et al., 2014), maternal stress (Kinney et al., 2008; Varcin et al., 2017), and maternal infections (al-Haddad et al., 2019; Atladóttir et al., 2010). In a recent Swedish study with over 1.7 million women, al-Haddad et al. (2019) found that a maternal infection increases the child's risk of developing ASD by a factor of 1.8. Ciaranello and Ciaranello (1995) called prenatal infection one of the biggest risk factors in ASD. The reason for this heightened risk could be linked to maternal fever, as antipyretic medication reduces the ASD-risk (Zerbo et al., 2013). Although a more recent study identified an equal risk for mild infections (e.g., urinary tract infections) that do not usually present with fever (al-Haddad et al., 2019).

1.4.1 Genes and the Environment

In a gene-environment interaction study, Volk et al. (2014) connected prenatal exposure to traffic-related air pollution with an increased ASD risk, if the child also has the *MET* rs1858830 CC genotype, which has been linked to immune dysfunction (Heuer et al., 2011). Due to the high heritability in ASD, some authors suggested that only those with a genetic predisposition would develop ASD following prenatal maternal infection (Patterson, 2009; Smith et al., 2007). Schwartzer et al. (2013) demonstrated that genetic predisposition determined the severity of ASD symptoms following maternal immune activation (MIA) in mice, which may mean that the risk to bear an autistic child is a lot higher than initially assumed in genetically predisposed women (Mazina et al., 2015).

Moreover, evidence connected the activation of the maternal immune system to epigenetic changes in the fetal brain to increased risk for an ASD diagnosis in the offspring (Nardone & Elliott, 2016). Epigenetics involves the study of heritable changes in gene expression that do not affect the DNA sequence (Jirtle & Skinner, 2007). Environmental insults like prenatal exposure to teratogens can change the epigenetic programming, resulting in an increased risk of developing illnesses, including neuropsychiatric ones like

ASD. How then does a temporary maternal immune reaction during gestation have such a major impact on a child's entire life?

1.4.2 *The Role of the Immune System*

The role of the maternal immune system as a risk factor for neurodevelopmental conditions such as autism and schizophrenia has been researched extensively in both humans and animals (Alexopoulou et al., 2001; Lins et al., 2018; Meyer et al., 2006; Patterson, 2009). To explore the role the immune system plays in the aetiology of ASD, we have to first understand how the immune system works. Beck and Habicht (1996) called the mammalian immune system "one of the most complex and wondrous of all evolutionary creations" (p. 60). It is a body's mechanism for defending itself against external harmful invasions. It consists of cells (phagocytic cells, inflammatory mediators, and natural killer cells) and molecules (complement, acute-phase proteins, and cytokines) with specific tasks related to the defence against pathogens (Delves & Roitt, 2000). The immune system's role is twofold: First, to recognise the body's own components, such as cells and tissues, to be able to identify anything foreign present within the body, and second, eliminate these foreign invaders (e.g., viruses, bacteria, fungi, parasites) via the so-called immune response (Beck & Habicht, 1996). There are two types of response: Innate—the first line of defence without immunologic memory—and acquired or adaptive, when the initial response fails; this system improves based on repeated pathogen exposure (Delves & Roitt, 2000).

Vargas et al. (2005) identified chronic neuro-inflammatory processes in various brain regions of autistic individuals, most pronounced in the cerebellum, but also in the cortex and white matter. The authors found that, within the autistics' brains, innate immune responses resulted in increased activation of both microglia and astrocytes. As cytokines are small proteins that are the link between maternal infection and the behavioural pathology common to ASD, they are the focus of the next section.

1.4.3 *Cytokines*

Cytokines are inflammatory modulators and, while some are cell-bound, the majority are soluble molecules (Masi et al., 2017). Cytokines are classified into three groups based on the type of immune response: (1) Adaptive immunity, (2) pro-inflammatory signalling, and (3) anti-inflammatory signalling. There are several cytokine families, including interleukin-1 (IL-1), tumour necrosis factor (TNF), and chemokines (Masi et al., 2017). Cytokines are involved in neural development and their dysregulation

has a detrimental effect on the developing prenatal brain (Patterson, 2009). The activation of the maternal immune system triggers an increase in cytokines in the placenta, amniotic fluid, and the fetal brain (Bauer et al., 2007; Meyer, Nyffeler, Schwendener, et al., 2008). The pro-inflammatory cytokines MCP-1 (Vargas et al., 2005) and TNF-α were found to be elevated within the cerebrospinal fluid (CSF) of autistic children (Chez et al., 2007), indicating activation of pro-inflammatory pathways.

The blood levels of the pro-inflammatory cytokines IL-1β, IL-6, and TNF-α were elevated in children with ASD with and without intellectual disability, as well as some of their non-autistic siblings (Jones et al., 2017; Jyonouchi et al., 2001). Jones et al. (2017) further identified increased levels of IL-1α. Also, an ASD study revealed elevated cytokine levels (including IL-6 and IL-10) in post-mortem brain samples (cerebral cortex and cerebellum) (Vargas et al., 2005). The study included brain tissue samples from ASD patients aged five to 44 years, indicating that the state of activated immunity began early in life and appears to be permanent.

1.5 The Neurobiology of ASD

1.5.1 Neuroanatomy

While the autistic brain shows various structural disparities when compared to a neurotypical one, a diagnosis is made on a behavioural basis only. Regions of interest in ASD research include the amygdala, the frontal cortex, the cerebellum, and the hippocampus (Donovan & Basson, 2017). These structural differences include neuron numbers, density, organisation, and composition, but also altered connectivity within and between these regions.

The amygdala, an almond-shaped structure in the anterior medial temporal lobe is involved in emotional processing, social interaction, as well as facial and emotional recognition—all functions implicated in autism (Donovan & Basson, 2017). Research has linked amygdala enlargement early in life to social communication impairments (Munson et al., 2006; Schumann et al., 2009), as well as increased anxiety (Juranek et al., 2006), though the enlargement could not be detected in adolescence and adulthood (D. G. Amaral et al., 2008; Schumann et al., 2004). By contrast, Murphy et al. (2012) reported that the difference in volume persisted into adulthood. In a post-mortem study that investigated the total number of neurons in adult amygdalae, neuron numbers were significantly lower in the ASD group, although there was no difference in overall amygdala volume (Schumann & Amaral, 2006). These divergent results are very likely

related to the symptom heterogeneity of the autism spectrum and future work should place a focus on the comparison of symptom severity with structural differences.

Another brain region implicated in ASD is the frontal cortex, which is involved in higher cognitive processes such as executive functioning, including inhibitory control, working memory, and cognitive flexibility (e.g., adjusting to new demands or rules) (Diamond, 2013). These skills are essential for successfully navigating the social environment and school or career, which can be a challenge for some autistics. In ASD, the frontal lobes have been found to display irregular growth patterns and cortical thickness, as well as neuronal disorganisation throughout the cortical layers and connections to other neural structures (Donovan & Basson, 2017). Schumann et al. (2010) found that, in toddlers, the frontal cortex was among the brain regions most severely affected by volume increases of both grey and white matter when compared to neurotypicals.

The cerebellum is also associated with autism. It is the brain region with the highest number of neurons, despite having only 10% of the total brain volume, which is why it is also called the "little brain" (Becker & Stoodley, 2013). It is part of the anterior-dorsal hindbrain and has been linked to fine motor control, proprioception, language, affective regulation, and cognitive processing. Cerebellar dysfunction in ASD, including loss of Purkinje cells during early development, is related to abnormal eye movements, motor deficits (fine and gross), and atypical gestures and imitation (e.g., motor imitation, facial expressions, lack of social gestures), which in turn affects social communication (Becker & Stoodley, 2013). D'Mello et al. (2015) found that symptom severity correlated with the degree of reduction in regional and lobular grey matter in different cerebellar regions.

The hippocampal formation is an area in the medial temporal lobes of the brain that is involved in cognitive processing (e.g., memory formation, spatial navigation, etc.), but also inferential reasoning, emotional processing, decision making, and problem-solving (Reinhardt et al., 2020). Deficiencies in many of these functions have been associated with ASD and the hippocampal areas are often enlarged in children and adolescents (Hasan et al., 2012), but not always in adults with ASD (Murphy et al., 2012). Interestingly, an MRI study with a sample size of over 1,000 participants aged between 6 and 36 years found no significant volume differences of either amygdala or hippocampus (Haar et al., 2016). It would have been interesting to segment the data by age group to show the developmental trajectory.

While not specifically for the hippocampus, in a longitudinal study, Courchesne et al. (2011) investigated brain abnormalities in ASD across the lifespan (1-50 years of age).

They reported overgrowth—excess neurogenesis—during the first years of life, after which the growth slowed down in late childhood. By late adulthood, the brain volume had decreased to a below-average level. This early overgrowth was most pronounced in the frontal cortex, but also in the temporal and parietal lobes.

Another area of growing interest in neuroscience in general, and ASD in particular, is connectivity/dysconnectivity. Structural and functional neural connectivity is different in the autistic brain when compared with a neurotypical one (Rudie et al., 2013; Valenti et al., 2020; Vissers et al., 2012; Wass, 2011). Structural connectivity represents the hardwired connections between different neural areas, while functional connectivity is the interaction between activity in different brain regions (Eickhoff & Müller, 2015). In the neurotypical brain, structural and functional connectivity are positively correlated (Damoiseaux & Greicius, 2009), but structural connectivity is not required for neural regions to be connected functionally via indirect connections (Honey et al., 2009).

The *atypical connectivity* theory is based on the idea of abnormal development of connections in areas associated with higher-order functioning (including frontal lobes, amygdala, cerebellum, etc.) in brains affected by ASD (Belmonte et al., 2004; Catani et al., 2016). In a review of over 70 papers on connectivity in ASD, Vissers et al. (2012) reported reduced functional connectivity during task performance, mainly between frontal and parietal brain areas. They also found impaired local connectivity (i.e., within a brain region) but noted that abnormal connectivity patterns (both local and long-range) in autism are rather complex. Another study confirmed the atypical local connectivity, specifically increased connectivity in the cingulate cortex, an area linked to emotional processing, learning, and memory, in the ASD sample when compared with controls (Ball et al., 2017). In a recent review of neuroimaging studies, Sato and Uono (2019) identified atypical structural and functional network patterns in brain regions associated with social behaviour (including the amygdala). The corpus callosum, a brain region that connects the two hemispheres, has consistently been linked to ASD, having not only reduced volume but also abnormal functional and structural connectivity (Valenti et al., 2020). They reported atypical functional activity during tasks for social cognition, executive functions, and working memory. In addition to the connecting pathways, however, chemical messengers, including neurotransmitters and neuropeptides, are required to facilitate communication between and within the various areas of the brain, so these will be discussed next.

1.5.2 Neurotransmitters

The function of several neurotransmitters and neuropeptides is often altered in ASD. Of the neurotransmitters, serotonin (*5-hydroxytryptamine*, short: 5-HT) is the one most often implicated in ASD. 5-HT is involved in social behaviour, emotional regulation, sleep, aggression, anxiety (Cook & Leventhal, 1996), perception, attention, and many more (Berger et al., 2009). Indeed, Berger et al. (2009) noted that "it is difficult to find a human behaviour that is not regulated by serotonin" (p. 356). The first biomarker that connected ASD to 5-HT was elevated blood 5-HT levels, a condition called *hyperserotonemia*, with about 30% higher 5-HT levels in autistics when compared to neurotypical controls (Gabriele et al., 2014). A variant of the gene responsible for elevated 5-HT blood levels (i.e., *ITGB3*) has been associated with ASD (Napolioni et al., 2011). Also, *SLC6A*, a gene that codes for the serotonin transporter (SERT), has been connected to the occurrence of hyperserotonemia in ASD (Coutinho et al., 2004). Hyperserotonemia is a condition consistently linked to and unique to ASD, that is, it does not appear in other neurodevelopmental disorders (Veenstra-VanderWeele & Blakely, 2012), and was first noted in 1961 by Richard Schain and Daniel Freedman during a time when ASD research was in its infancy. Indeed, serotonin was not accepted as a neurotransmitter until the 1980s (Folk & Long, 1988).

Chugani et al. (1999) reported abnormal development of the neural capacity to synthesise 5-HT in autistic children when compared with neurotypical children. Research found that selective serotonin reuptake inhibitors (SSRIs) reduce restrictive and repetitive behaviours associated with ASD (Hollander et al., 2005), but overall, results have been mixed (Williams et al., 2013). SSRIs prevent the reuptake of 5-HT by inhibiting the SERT at the presynaptic axon terminal. SERT binding is lower throughout the autistic brain, resulting in deficits in social cognition (SERT reduction in anterior and posterior cingulate cortices) and repetitive and obsessive behaviours (SERT reduction in the thalamus) (Andersson et al., 2020; Nakamura et al., 2010). Further, Makkonen et al. (2008) found reduced SERT binding in the medial frontal cortex, an area connected to the ability to interpret another's thoughts and intentions (theory of mind) in children and adolescents with ASD. Theory of mind can be defined as a person's ability to attribute mental states to themselves as well as others (Premack & Woodruff, 1978). Other studies found decreased 5-HT_{2A} receptor binding in both children (Goldberg et al., 2009) and adults with ASD (Murphy et al., 2006). In summary, in ASD, the development of the 5-HT system does not follow the same trajectory as in neurotypical children and SERT is less available in the

autistic brain, contributing to autistic symptoms. However, further research is needed to investigate the effectiveness of SSRIs in the treatment of ASD-related symptoms.

Another neurotransmitter that has been connected to ASD is dopamine, which is involved in motivation, learning, attention, and social behaviour (DiCarlo et al., 2019; Paval, 2017; Yamaguchi et al., 2017). Deficient dopamine signalling in the midbrain results in social-behavioural deficits and stereotypical behaviours (Paval, 2017). In his review, Paval reported that dopamine antagonists may improve these symptoms. A single nucleotide polymorphism of the dopamine-3-receptor gene *DRD3* was linked to the repetitive and stereotyped behaviours of ASD (Staal, 2015). Also, Nakamura et al. (2010) reported increased dopamine transporter binding in the ASD group in the orbitofrontal cortex, an area associated with emotional regulation. In this study, the dopamine transporter binding increase in the orbitofrontal cortex was negatively related to the previously mentioned SERT reduction.

The neuropeptides oxytocin (OXT) and arginine vasopressin (AVP) have also been associated with ASD in rodents (Hammock & Young, 2006) and humans (Modahl et al., 1998; Neumann & Landgraf, 2012). Both regulate social behaviours, including social recognition, communication, and bonding. OXT has a prosocial effect and promotes empathy (Panksepp & Panksepp, 2013) and has been found to improve social-cognitive functioning, social skills, and repetitive behaviours in ASD (Ooi et al., 2017). Reduced blood levels of OXT (Green et al., 2001; Modahl et al., 1998) and AVP (Carson et al., 2015) have been found in autistic children. Single nucleotide polymorphisms of the genes for the OXT receptor (*OXTR*) (Damiano et al., 2014; Yamasue, 2013) and the AVP receptor (*AVPR1*) (Wassink et al., 2004) have been linked to dysfunctional reward motivation and social interaction in ASD respectively. As mentioned above, reduced social motivation is connected to the social deficits often found in ASD.

To summarise, both neurotransmitter and neuropeptides are an essential part of neurodevelopment and their imbalance can have detrimental effects on social and cognitive functioning and result in an ASD phenotype. In ASD, the dysregulation of various neurochemicals, including 5-HT, dopamine, OXT and AVP, has been connected to the condition's pathogenesis. The next section will consider available interventions in the treatment of ASD.

1.6 Treatment

At present, there are no pharmacological treatment options for the core ASD symptoms. The only drugs approved for ASD are risperidone and aripiprazole, both for the treatment of irritability (US Food and Drug Administration, 2018). There is evidence that both drugs are effective in alleviating irritability-related symptoms (Stepanova et al., 2017). Further, medication to treat behavioural characteristics (e.g., stereotypy or repetitive behaviours), social and emotional deficits (e.g., emotional recognition or social interaction deficits), or cognitive issues (e.g., poor memory or distractibility) are frequently prescribed, but none of them alleviates all core symptoms (LeClerc & Easley, 2015). As indicated above, SSRIs effectively reduce repetitive behaviours in ASD. However, in a review, Stepanova et al. (2017) could not find evidence that SSRIs actually improve the behavioural deficits.

OXT has promising clinical potential in the treatment of ASD (Benner & Yamasue, 2018) and is currently being explored as a pharmacological treatment option for social symptoms (Ooi et al., 2017); age plays a big role and OXT should be administered as early in life as possible, but, so far, not a lot of research has been conducted with children. Intranasal OXT has been found to improve social behaviour deficits in autistic adolescents—a promising result (LeClerc & Easley, 2015; Parker et al., 2017). The pharmacological investigation into the role of the vasopressin 1a receptor (V1a) in the improvement of social-communicative abilities found that its inhibition improved social deficits and social living skills (Bolognani et al., 2019; Schnider et al., 2020). Balovaptan, an orally administered V1a receptor antagonist is currently undergoing phase 3 clinical trials for the treatment of adults (ClinicalTrials.gov, 2020a) and phase 2 for the treatment of children (ClinicalTrials.gov, 2020b).

If there are no pharmaceutical treatment choices for ASD patients, what other options are there to improve the outcome for autistics? Currently, behavioural interventions are the only available choice and there are many options to address the core ASD symptoms (Green et al., 2006). Many of them are integrated into the day-to-day life and done at home, but it is important that parents are educated and trained, as well as aware of the importance of their own mental health, ensuring they can be an effective partner in the treatment of their autistic children (Tonge et al., 2014). All behavioural interventions should be incorporated into the daily routine as early as possible as they show the greatest long-term effect when applied in the first four years of life (Dawson, 2008; Estes et al., 2015).

As autism is such a heterogeneous condition, the ideal solution to address social deficits would likely be a combination of early behavioural interventions together with a potential pharmacological option. However, more work is required to find pharmacological treatment options that address all the behavioural deficits, including social ones, which is why the development of good ASD animal models on social behaviour is so important in current research. In addition to this, alternative non-pharmaceutical interventions like environmental enrichment have shown great potential and will be discussed in detail in the following section on enrichment.

1.6.1 Environmental Enrichment

A promising direction to rehabilitate or prevent the symptoms of psychiatric illnesses is environmental enrichment (EE) (Aronoff et al., 2016; Ball et al., 2019; Döbrössy & Dunnett, 2001; Kelly & Hannan, 2019). EE involves adapting the environment to create multisensory, cognitive, and motor stimulation, enhancing neural plasticity—the nervous system's ability to adapt in response to environmental changes. Interestingly, sensory deprivation has the opposite effect; neglected human orphans have developed a condition called post-institutional autistic syndrome that presents with similar behavioural and social impairments as those observed in ASD (Hoksbergen et al., 2005).

In laboratory rodents, EE includes creating an interesting and changing environment where the animals can exercise, hide, forage, and play with other animals. Ever since 1947, when Donald Hebb found that enrichment early in life improved problem-solving in adult rats, researchers have been studying how symptoms of neurological conditions can be treated with an enriched environment. In the 1960s, Mark Rosenzweig, David Krech, Edward Bennett, and Marian Diamond described changes such as higher cortical weight in rat brains as the result of a life in an enriched environment (Krech et al., 1960; Rosenzweig et al., 1962).

Since then, several studies have investigated the effects of EE, including changes in expression of genes involved in neuronal plasticity (e.g., *Lis1*), structure, function (e.g. *Apoe*), and neurotransmitters (e.g., *Prep*) in rodents (Rampon et al., 2000) and humans (Rogers et al., 2019). Such changes to genes involved in neuroplasticity and neuro-structure can result in functional and morphological modifications such as greater neurogenesis, higher synaptic numbers, and increased dendritic branching in brain areas like the hippocampus and cerebellum, all changes that have positive effects on learning and memory performance and fine motor skills (Nithianantharajah & Hannan, 2006). The *Lis1* gene encodes for platelet-activating factor (PAF) acetylhydrolase (also known as Lp-

PLA2) and is an important inflammatory mediator within the innate immune system (McIntyre et al., 2009). Cytokines are an example of such inflammatory mediators. PAF also plays a role in long-term potentiation, a process involved in synaptic plasticity, which in turn affects learning and memory (Dorninger et al., 2020). EE has been directly connected to an increase in long-term potentiation (synaptic strengthening) and synaptic density (Lonetti et al., 2010). Another aetiological factor connected to ASD involves the reelin protein—an important factor during neurodevelopment. It binds to the apolipoprotein E (APOE) receptor 2, which has been implicated in autism (Giunco et al., 2009). Apolipoprotein E (APOE) is a protein that transports cholesterol and various other lipids in the central nervous system and APOE hypermethylation has been associated with ASD (Giunco et al., 2009; Hu et al., 2018). Altered expression of genes involved in the regulation of neurotransmitters can also have wide-ranging long-term effects. Take for example prolyl oligopeptidase (also called prolyl endopeptidase), an enzyme encoded by the PREP gene that is involved in the degradation of neuropeptides, including OXT and AVP. Momeni et al. (2005) found that prolyl endopeptidase's activity levels varied greatly in an ASD sample, but not in the control group. This implicates dysregulation of the enzyme in the aetiology of ASD.

Animal models for enrichment will be discussed in the next section, but first, it is vital to consider emerging evidence of treatments involving multisensory stimulation in humans. In fact, between 80% and 90% of people with ASD suffer from sensory abnormalities in auditory, visual, and proximal (e.g., touch, smell, taste), domains; symptoms include under-responsivity, over-responsivity, and sensory-seeking behaviours (Ben-Sasson et al., 2009; Leekam et al., 2007). In line with the previously discussed connectivity issues, Chang et al. (2014) presented evidence that white matter temporal tracts linked to social-emotional processing are impaired in ASD. These tracts have also been associated with auditory processing, social skills, inattention, and working memory.

The most common type of therapy requested by caregivers is sensory intervention—about 60% of autistic children received treatment involving clinic- or home-based sensory intervention therapy (Green et al., 2006). The sensory-rich activities can be clinic- or home-based and are usually adult-directed. Such interventions are intended to ease sensory processing problems and improve behavioural regulation, but they have to be tailored to the child's needs, as often highly stimulative environments can be very stressful for an autistic child (Case-Smith et al., 2015).

Based on the more extensive literature on enrichment in rodents, Woo and Leon (2013) and Woo et al. (2015) designed an intervention plan for autistic children. It utilised sensorimotor exercises such as olfactory stimulation with essential oils, music enrichment, objects to play with (e.g., beads, fishing rods, and toys with different shapes and textures), physical exercises, and a spa treatment exercise with body oils and scented soaps. After 6 months the children showed increased cognitive abilities and decreased ASD-like behaviours. Of the children in the enrichment group, 21% no longer qualified for a diagnosis of autism, while in the control group no changes in diagnosis occurred (Woo et al., 2015). The authors suggested that the improvements could be a result of a combination of improved social skills (including receptive language), cognitive functioning, and attention. They further noted that the children's improved tolerance to sensory stimuli allowed them to be more attentive and participate more during enrichment, which subsequently enabled them to show their true cognitive abilities.

Following these two studies, the group developed an online version of the intervention plan for a home-based subscription-based enrichment regime provided by caregivers of autistic children (Aronoff et al., 2016). They had created an Internet database containing over 400 different sensory exercises to ensure novel experiences. Over 1,000 children between 1 and 18 years of age participated in the sensory enrichment therapy study that combined different sensory and motor tasks to be administered daily for up to 7 months. The exercises were personalised to each participant and included sensory stimulation (distinctive textures like sponges, bubble wrap, aluminium foil, adhesive tape, etc.), object manipulation (e.g., arranging beads, inserting toothpicks into Play-Doh, etc.), and thermal stimulation (e.g., varying water or object temperature), and visual stimulation (e.g., photos, pieces of art, etc.). There was also auditory stimulation through music and sounds, proprioceptive and vestibular stimulation such as walking or ascending/descending stairs with an object on their head, balancing while blindfolded, and olfactory stimulation with pleasant scents. Overall, the personalised online treatment regime showed greater improvements than the two previous studies with standardised exercises. However, there were several limitations, including the varying length of participation, possibly a direct result of the associated cost of participation. Also, because the therapy was home-based, there was no control of consistency in administration. Also, none of the participants had been assessed by a clinician, that is, caregivers provided information on prior diagnoses, as well as progress. However, the children's improvements were consistent with previous studies (Woo et al., 2015; Woo & Leon, 2013), showing great potential for a mainstream, subscription-based enrichment regime in

particular, and sensory enrichment therapy in general. If this online service could be set up to be accessible by clinicians as a prescribed intervention where the cost is subsidised by the government and/or health insurers, more patients could benefit from it, improving the quality of life for the autistic individual and, subsequently, reducing the ASD-related economic burden.

1.7 Animal Models

Animal modelling goes back to the ancient Greek (Ericsson et al., 2013; Fisch, 2007). In the context of neuroscience, animal modelling is a way to investigate the connections between behaviour(s) and the brain in an animal (e.g., an animal like a mouse, rat, fish, fruit fly, etc.) and transfer the gained understanding to a human context (van der Staay, 2006). Pre-clinical ASD research has been utilising animal models to investigate aetiology and treatment approaches, though it is imperative to ensure an animal model has the highest possible translational value for clinical practice. Therefore, when modelling a psychiatric condition in animals, the human aetiology should be mimicked closely (construct validity), its expression should be similar to those in humans (face validity), and treatments should have the same effect in the model and the disorder being modelled (predictive validity) (McKinney & Bunney, 1969; Willner, 1986). Mice and rats are commonly used to model features of neurological disorders, as such models help understand not only aetiology, but also the connection between pathophysiology (e.g., brain structures, neurotransmitters, or genes) and behaviour. In a comprehensive review of the behavioural differences between the two species, Ellenbroek and Youn (2016), have showcased the advantages of using rats over mice for studies of social behaviour and communication, including rats' larger brain size and greater sociability. This thesis will use an ASD animal model of the impact of environmental enrichment on prosocial behaviour, but it does not include a brain analysis component. The next three sections will focus on a review of animal models for ASD, prosocial behaviour, and environmental enrichment respectively.

1.7.1 Modelling Autistic Symptoms in Rodents

One of the key issues with rodent models is that autism is heterogenic at a genetic as well as a behavioural level, making modelling of all biological and behavioural features in one model virtually impossible. Also, there is a considerable overlap of symptoms and aetiology between ASD and schizophrenia, including social behavioural deficits and maternal infection (Patterson, 2009). Therefore, a good approach is to focus on the core

features of the condition (Crawley, 2012). In addition, animal behaviour has to be comparable to human behaviour and this can be achieved using animal models. For example, humans use audible language, that is spoken words to communicate, whereas rodents vocalise in the ultrasonic range. Whilst in humans it is relatively easy to ascertain whether the patient has impaired language skills, there is no way to tell if a sound emitted by a mouse or rat is what it should be. Nevertheless, it is possible to compare quantitative rodent call features like number, duration, and frequency of produced calls (Brudzynski, 2013).

There is a multitude of rodent models simulating the behaviours found in ASD, some based on genetics, and others, like the one utilised in the present project, based on environmental influences (Patterson, 2011; Servadio et al., 2015). Initially, the rat (*Rattus norvegicus*) was used as the primary rodent to model neuropsychiatric conditions, but due to the ease of genetic manipulations, the mouse (*Mus musculus*) became the most widely used rodent in biomedical research. However, in recent years more tools for altering the genome have become available, so rats have become increasingly popular, as their larger brains, lower aggression, and higher sociability are attractive advantages over mice (Ellenbroek & Youn, 2016). Genetic mouse models with an autistic phenotype (e.g., *Nlgn4*, BTBR, BALB, *Foxp2*, etc.) mirror some ASD-like behaviours such as repetitive behaviours and impaired social interaction and communication, but they are often based on single-gene alterations (Silverman et al., 2010). This is problematic, as ASD is not a Mendelian single gene disorder but involves a multitude of genes (Carvalheira et al., 2004). Models based on prenatal environmental risk factors include maternal exposure to the anticonvulsant drug VPA where models showed social-behavioural and communication deficits, as well as repetitive behaviours in the offspring (Campolongo et al., 2018; Moldrich et al., 2013; Nicolini & Fahnestock, 2018; Schneider et al., 2006). Another model based on environmental factors is the *lipopolysaccharide* (LPS) model where prenatal LPS exposure mimics a bacterial infection, triggering a maternal immune reaction that results in autism-like behaviours in the offspring (Kentner et al., 2016; Kirsten & Bernardi, 2017; Kirsten et al., 2012; Kirsten et al., 2010).

In the present study, the prenatal *polyinosinic-polycytidylic* acid (poly I:C) model was used to investigate specific autism-like behaviours, mainly focussing on social behaviour and communication. This model is a well-established and validated ASD rat model based on maternal immune activation (MIA). Poly I:C is a model based on prenatal viral infection, which is a risk factor for both ASD and schizophrenia. This raises the possible

criticism that poly I:C does not create a specific model of ASD, but rather a more general model of the range of symptoms seen across both ASD and schizophrenia. However, a large population-based study recently reported that maternal immune activation puts the offspring at much greater risk of developing ASD than schizophrenia (al-Haddad et al., 2019). This result suggests that poly I:C creates a sufficiently specific model of ASD for the use in the current project.

Poly I:C mimics a viral infection (without the presence of pathogens) and activates the maternal immune system for about 24 hours, making the use of poly I:C in a laboratory environment a very safe option. One injection of the synthetic double-stranded ribonucleic acid (RNA) poly I:C is enough to raise pro-inflammatory cytokine levels (including the above-mentioned IL-6) in the dam (Fortier et al., 2007; Lins et al., 2018; Meyer et al., 2005; Smith et al., 2007) and the fetal brain, resulting in autism-like behaviours in the offspring (Alexopoulou et al., 2001; Lins et al., 2018; Meyer et al., 2006; Patterson, 2009). Specifically, exposure to poly I:C in utero resulted in lower sociability (Lins et al., 2019; Smith et al., 2007; Zhu et al., 2014), increased repetitive behaviours, and reduced vocalisation (Malkova et al., 2012). Schwartzer et al. (2013), however, reported increased vocalisation. Also, Zhu et al. (2014) found that, in addition to reduced sociability, mice prenatally exposed to poly I:C showed significantly elevated microglial activity in the cerebral cortex, hippocampus, and thalamus. In a mouse study, Smith et al. (2007) identified that administration of IL-6 antibodies following a poly I:C injection prevented the expected behavioural and social deficits and averted the associated changes in gene expressions as a result of the maternal immune activation (MIA). Further, IL-6 knockout mice (genetically altered mice that do not produce IL-6) exposed to poly I:C did not develop any behavioural or social deficits, which stresses the principal role of IL-6 in the aetiology of ASD.

1.7.2 Environmental Enrichment

Offering an interesting social and an ever-changing physical environment has been the centre of a fast-growing pre-clinical research field (Reynolds et al., 2010). Environmental enrichment can have positive effects on healthy animals, but also those with neurological defects (van Praag et al., 2000). Most enrichment studies employ a post-weaning enrichment regime where animals are moved into enriched housing once they have been weaned (Papadakakis et al., 2019; Peña et al., 2006; Rae et al., 2018; Varty et al., 2000). Post-weaning enrichment increased general sociability in mice compared to mice in standard housing (Rae et al., 2018; Zheng et al., 2020). Also, musical enrichment following

pre-weaning maternal deprivation in rats reversed the negative effect on sociability (Papadakakis et al., 2019). It is important to note that post-weaning enrichment does not always remedy the effects of early maternal deprivation—deprived animals did not show the expected preference for a novel social stimulus over a familiar one in the social approach–avoidance test, even after living in enriched housing (Kentrop et al., 2018).

The developing brain is a lot more plastic than the adult one and the earlier interventions are employed, the more likely they are to have a lasting effect (Luo & O'Leary, 2005). Only a few studies (e.g. Kohl et al., 2002; Schneider et al., 2006; Venable et al., 1988) included pre-weaning enrichment sessions where the pups were removed from their home cage to receive sensory stimulation-based treatment. Rats exposed to enrichment at this early age showed better problem-solving abilities (Venable et al., 1988) and more exploratory behaviours (Schneider et al., 2006), and an increase in both number and length of cortical dendrites at postnatal day 8 (PND) (Schapiro & Vukovich, 1970) and PND 25 when compared to controls (Venable et al., 1989). Nevertheless, pre-weaning enrichment alone does not seem to have a lasting effect on neurogenesis (the process during which new neurons are born) in the hippocampus in adulthood, even though the animals were less anxious than control mice in adulthood (Kohl et al., 2002). Kohl et al. (2002) assessed neurogenesis at 3 months after the animals had experienced pre-weaning enrichment and found no difference between groups. It would have been interesting to assess neurogenesis and proliferating cell numbers directly after completion of the pre-weaning enrichment sessions, as it is possible that pre-weaning enrichment initially promoted increased neurogenesis and/or proliferating cells, while the following period of standard housing reversed this initial effect. This would be plausible, as continuous long-term exposure to an enriched environment increased neurogenesis in adult mice, but not in mice that lived in enriched housing for only 2 months, followed by 3 months in standard housing (Kempermann & Gage, 1999). While neurogenesis did not differ between groups, Kempermann and Gage (1999) reported that the latter group had more than double the number of proliferating cells than controls.

To my knowledge, no poly I:C autism study that also investigated the effects of pre- and post-weaning enrichment has been conducted. The only rat ASD study that also involved EE utilised VPA to induce ASD (Schneider et al., 2006). Between PND 7 and 21 (corresponding roughly to the period between gestational week 36 and year 2-3 in humans [Semple et al., 2013]) the pups experienced multisensory stimulation, including swimming in warm water and placement on surfaces with different textures and temperatures (e.g.,

paper towels, glass, or wood). Post-weaning, from PND 21 to 35 (corresponding to 2-12 human years [Semple et al., 2013]) the animals were housed in groups of 12 in a large enclosure with wheels, tunnels, shelters, toys, and ladders; the layout was changed every 2 days to increase cognitive demand. At the end of the testing period, the VPA enrichment rats were more engaged socially, increasingly explorative, less anxious, and displayed fewer repetitive behaviours when compared with VPA rats in standard housing. Thus, it seems that, both in humans and animals, EE has beneficial effects on some of the ASD-like deficits. However, particularly in rats, the study of ASD has been limited to relatively simple social and cognitive skills (such as social approach–avoidance behaviour or novel object recognition), which is why the social approach–avoidance paradigm has been included in the present study to allow comparisons with existing research.

Environmental enrichment has the potential to improve the core symptoms of ASD, not only in animal models but also in humans. However, to date, no ASD study has investigated the effects of EE on higher social functions, prosocial behaviour in particular. An animal model showing that EE has the potential to improve prosocial behaviour could pave the way for the development of clinical treatment options for autistic individuals, which, in turn, would offer many advantages, not only for the patient and their whānau but also for society.

1.7.3 Social Behaviour

Like humans, rodents have social ties and can recognise known conspecifics (Kogan et al., 2000). They can also read their conspecifics' emotions and use this knowledge to predict future behaviour (Sotocinal et al., 2011).

Basic Social Behaviours. Basic social behaviours have been researched extensively in rodent models of autism, but other aspects of ASD like higher social functions have not been modelled yet. One of the most investigated social concepts in rodents is the social approach–avoidance paradigm (Moy et al., 2004). It investigates general sociability and social novelty behaviours and has been used widely to model the social-behavioural deficits in various rodent models of ASD (Crawley, 2007). I have included this paradigm in this thesis as doing so enables a direct comparison to previous research.

Prosocial Behaviours. As outlined above, prosociality is frequently deficient in ASD. The multidimensional concept that covers emotional and behavioural self-awareness and understanding and perceiving these in others (American Psychiatric Association, 2013) taps into the empathy construct and there are several rodent models that can be used

to investigate related behaviours. While empathy has traditionally been considered as specific to humans (or at least primates), recent studies have now shown that these behaviours are also observed in a range of other species, including rodents, as outlined below.

One of the first studies to investigate prosocial behaviour in rats showed that the animals that had experienced foot shocks themselves were less likely to press a lever that provided both a food reward to themselves and an electric foot shock to a rat in an adjacent cage compartment (Church, 1959). This suggests that the rats relinquished a treat—acting in a way that benefitted another. A few years later, Rice and Gainer (1962) reported that rats were significantly more likely to activate a mechanism to lower a rat suspended from a harness than a suspended block of foam. More recently, evidence emerged showing that a rat will free a distressed rat trapped in a restrainer (Ben-Ami Bartal et al., 2011) or a pool of water (Sato et al., 2015), more so when the animal in distress is familiar (i.e., a cagemate or sibling) (Ben-Ami Bartal et al., 2011). Even without the prospect of social interaction, the rats continued to free their cage mate; when presented with an empty restrainer though, they did not open the door. The authors described this behaviour as a display of prosociality in response to a conspecific's distress. As noted above, prosociality is empathy-driven behaviour, which makes this assay well suited to investigate higher social functions—or a lack thereof—in rats.

1.8 Overall Aims and Predictions

So far, no rat study has investigated the effects of enrichment on prosociality in ASD, which is why I have designed a research project that will explore this in a poly I:C ASD model. I have chosen poly I:C to induce social-behavioural deficits in Sprague Dawley rats, because prenatal viral infection is one of the biggest non-genetic risk factors of autism. Additionally, enrichment has shown great promise as a non-pharmaceutical treatment option, so the objective of this thesis is the creation of an in vivo rodent model of specific aspects of ASD, these being social behaviour, prosociality in particular, and how EE impacts deficits in this area. The overall hypothesis this project investigates is that poly I:C exposure in utero disrupts neural development and changes communication (an essential factor of social dynamics) and social behaviours and that EE reduces or reverses these deficits.

Chapter 2 summarises all non-behavioural animal manipulations, these being the maternal poly I:C treatment, including its effect on the dams and the offspring, as well as

the pre- and post-weaning enrichment regime. A reliable indicator of treatment success is an increase in body temperature in the first hours after the poly I:C injection and reduced weight gain 24 hours post treatment. Dams that either held their weight or lost weight showed the expected behavioural features (Bronson et al., 2011; Horska et al., 2017; Howland et al., 2012; Missault et al., 2014; Ozawa et al., 2006).

Neonatal ultrasonic vocalisations (USV) are a good way to study abnormal communication in rodents, which is why Chapter 3 explores how prenatal poly I:C exposure impacts the offspring's communication early in development. Pups' USV recorded at PND 7 and 14 were used to compare call numbers and durations between groups. Following this, Chapter 4 investigates the poly I:C effect on basic social functions, specifically, general sociability and the preference for social novelty in adolescence and adulthood. In Chapter 5, the focus turns to prosocial behaviour. It considers how prenatal poly I:C exposure influences the adult offspring's tendency to help a conspecific in need. The last experimental report follows in Chapter 6, where the topic is locomotor activity (LMA) and how prenatal poly I:C exposure and/or enrichment affects its expression in adulthood. LMA is often affected in MIA and enrichment models, so I included this last experiment as a positive control.

Chapter 2

Experimental Manipulations

Chapter 2
Experimental Manipulations

Chapter 2 outlines the poly I:C treatment and the environmental enrichment protocol, both of which are central to the overall experimental design of the thesis. The methodologies for the specific behavioural experiments are described in the subsequent individual chapters.

2.1 Background

The ASD model for rats was based on prenatal viral infection. Poly I:C is a synthetic double-stranded ribonucleic acid (RNA) that activates the toll-like receptor 3 (TLR 3), resulting in elevated cytokine levels in the pregnant rat and fetal brain (Alexopoulou et al., 2001; Lins et al., 2018; Meyer et al., 2006). In the fetal brain, maternal infection can severely disrupt cytokine levels. Meyer et al. (2006) reported that exposure to poly I:C on gestational day 9 (GD) resulted in lower levels of the anti-inflammatory cytokine IL 10 and higher levels of the pro-inflammatory cytokines IL-1β and IL-6 when compared to saline controls. When treated on GD 17, though, the effect was the opposite. One theory is that the cytokinal dysregulation during infection is responsible for subsequent autism-like behaviours in the offspring (Patterson, 2009). There is also evidence that the dysregulation results in defects of serotonergic (IL-6) and dopaminergic neurons (IL-1β, L-6, and TNF-α) (Meyer et al., 2009). Also, neural dopamine levels were increased (Meyer, Engler, et al., 2008; Meyer, Nyffeler, Yee, et al., 2008), whilst serotonin levels were decreased following MIA (Winter et al., 2009). The behaviours resulting from these changes include impaired social communication (i.e., USV) (Yee et al., 2012), social interaction (Malkova et al., 2012; Ratnayake et al., 2014; Smith et al., 2007; Zhu et al., 2014), stereotyped and repetitive behaviours (Patterson, 2011), and anxiety (Chow et al., 2016; Meyer et al., 2005; Shi et al., 2009; Smith et al., 2007). Historically, poly I:C has mainly been used in rat models of schizophrenia. As prenatal infection is one of the biggest risk factors to both schizophrenia and—to a much greater extent—ASD (al-Haddad et al., 2019; Patterson, 2009), poly I:C is becoming an increasingly popular animal model for ASD.

Depending on when during pregnancy the environmental insult occurs, the effect on the developing fetal brain differs, because neural structures develop at varying points during neural development. The development of the deep cerebellar nuclei and Purkinje cells peak around GD 13 in the rat, GD 11.5 in the mouse, and GD 47 in humans; the

amygdala peaks on GD 14.3 in rats, GD 12.5 in mice, and GD 49.2 in humans (Clancy et al., 2001). All of these brain regions are associated with ASD (Donovan & Basson, 2017).

I conducted a review of the poly I:C literature in early 2018. This review informed the doses and time of administration selected for the pilot study. It showed that there is no consistent protocol with doses ranging from 0.75 mg to 20 mg per kilogram of body weight, and time of administration from GD 9 to GD 19 (see Table 2-1 for more details). Only four of the reviewed studies looked at social interaction (Malkova et al., 2012; Ratnayake et al., 2014; Smith et al., 2007; Zhu et al., 2014). Of all reviewed studies, only Malkova et al. (2012) investigated behaviours from all three autism symptom categories—sociability, communication, and repetitive behaviours. This is in line with a recent review on MIA that noted that not many studies have explored how MIA impacts more complex social behaviours (Careaga et al., 2017), a gap this study aims to address.

In terms of the doses used in the four studies that looked at sociability, two used 5 mg/kg and two 20 mg/kg. All four studies reported reduced sociability, so apparently, this deficit is seen across a wide dose-range. Further, all four studies used mice, not rats. Consequently, I conducted a pilot study to determine the optimal dose and time of administration in rats to reliably produce an autistic phenotype. Results of this pilot will help not only the present study but allow the investigation of more complex ASD-like behaviours in subsequent studies.

2.2 Pilot Study

Two different doses at two different time points were selected. As some studies found that higher doses of poly I:C can result in miscarriages (e.g., Meyer et al., 2005), the highest dose was set to be 8 mg/kg, the lowest 5 mg/kg; both doses were administered on PND 10 or PND 15. Four of the five groups had routine pregnancies and produced normal litter sizes, but 8 mg/kg on GD 10 led to increased miscarriage rate and unusually small litter sizes, so this group was excluded from further testing. The behavioural tests included separation-induced neonatal USV, social approach–avoidance (SAA), and prosocial behaviour (all of which are also included in the main study). The dose of 5 mg/kg on GD 15 reliably showed alterations in social behaviour and communication (pilot results not shown here), consistent with an ASD-like phenotype (Patterson, 2011) and was, therefore, used for the main study.

Table 2-1

Studies that Investigated the Effects of Poly I:C

Species [a]	Dose	Administration		Finding [c]	Reference
	mg/kg	Route [b]	GD		
Rat (SD)	0.75, 1	IP	10,11,15 16,18,19	→ Sensorimotor gating	Fortier et al. (2007)
Rat (SD)	4	IV	15	↓ Sensorimotor gating	Dickerson et al. (2010)
Rat (SD)	4	IV	15	↓ Sensorimotor gating	Wolff and Bilkey (2008)
Rat (SD)	8	IP	14	↓ Maternal weight	Bronson et al. (2011)
Rat (W)	4	IV	15,17	↓ Latent inhibition	Zuckerman et al. (2003)
Rat (W)	2, 4, 8	SC	9 15	↓ Maternal weight ↑ Mat. serum IL-1β → Sensorimotor gating → Locomotor activity	Missault et al. (2014)
Rat (W)	4	IV	15	↓ Maternal weight, Latent inhibition, ↓ Reverse learning	Zuckerman and Weiner (2005)
Rat (W)	8	SC	15	↓ Maternal weight	Horska et al. (2017)
Rat (LE)	4	IV	15	↓ Maternal weight ↑ Maternal temperature ↓ Sensorimotor gating ↓ Object-in-place recognition memory	Howland et al. (2012)
Mouse	2.5, 5,10	IV	9	↑ Mat. serum IL-10 → Fetal IL-10 → Locomotor activity ↓ Exploratory beh., Sensorimotor gat. ↓ Latent inhibition, Working memory	Meyer et al. (2005)
Mouse	5	IP	10.5+12.5 +14.5	↓ **Sociability,** Communication ↑ Repetitive behaviour, self-grooming	**Malkova et al. (2012)**
Mouse	5	IP	12-17	↓ Maternal weight ↓ Anxiety, sensorimotor gating ↓ Novel-object recognition memory	Ozawa et al. (2006)
Mouse	5	IV	9,17	↑ IL-1β, IL-6, IL-10, TNF-α ↑, → Perseverative behaviour	Meyer et al. (2006)
Mouse	5	IV	9,17	↓, → Sensorimotor gating ↓ Working Memory	(Meyer, Nyffeler, Yee, et al., 2008)
Mouse	5	SC	20	↓ Maternal weight → **Sociability**, Anxiety → Novel-object recognition memory	**Ratnayake et al. (2014)**
Mouse	20	IP	12.5	↓ **Sociability.** Sensorimotor gating ↑ Anxiety	**Smith et al. (2007)**
Mouse	20	IP	9	↓ **Sociability,** Sensorimotor gating	**Zhu et al. (2014)**
Mouse	20	IP	12.5	↑ IL-6, Anxiety ↓ Sensorimotor gating	Chow et al. (2016)

[a] SD = Sprague Dawley, W = Wistar, LE = Long Evans. [b] IP = intraperitoneal, IV = intravenous, SC = subcutaneous. [c] ↑ = increase, ↓ = decrease, → no difference between poly I:C and control group.

2.3 Ethics Statement

All experiments and procedures outlined in this thesis have been approved by the Victoria University of Wellington (VUW) Animal Ethics Committee (Application ID 25848) and were performed in accordance with the university's Animal Ethics Guidelines.

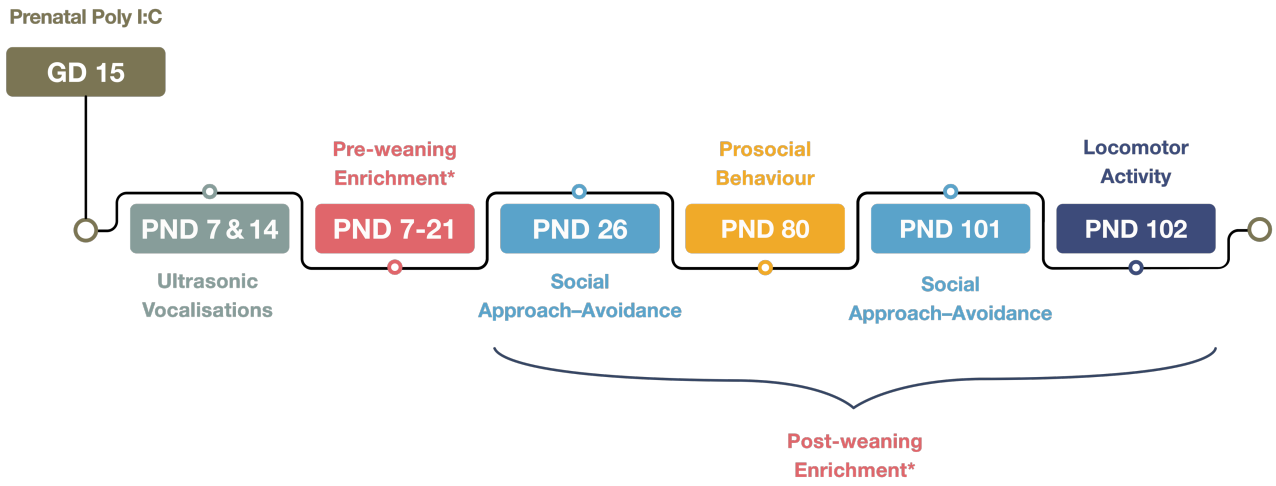
2.4 Method

2.4.1 Experimental Timeline

Figure 2-1 shows the order of experiments, starting with prenatal poly I:C administration on GD 15. While All animals went through all experiments, they may have played different roles. What I mean with this is that as part of the prosocial behaviour paradigm (see Chapter 5), I ran a confidence test with the animals. The more confident animal of the pair subsequently became the free rat, whereas the less confident one became the helper rat. When I ran the first two experiments—USV on PND 7 and PND 14 and SAA—I did not know which animal would become the free and which the helper rat. Similarly, in the SAA paradigm, some juveniles were stimulus animals and others were test animals. However, I used the same animals throughout, but they may not always have been the focal animal. This means that correlation analyses across all five experiments are difficult.

Figure 2-1

Visual Project Timeline



*Enrichment groups only.

2.4.2 *Housing Conditions for All Animals*

The following applies to all animals used in this study. Only Sprague Dawley (SD) rats were used. The animals were kept in the VUW vivarium under a reversed day/night cycle (lights off at 7 am, on at 7 pm). The environment was controlled with humidity levels between 55 and 60% at a temperature of 21°C (±2° C). Cages were cleaned once a week and the animals had access to water and standard rat chow *ad libitum.* All cages were equipped with a chew block made from untreated wood.

Due to several cases of pinworm (*Syphacia obvelata*), the entire colony was treated with feed containing *Fenbendazole* (FBZ) for two cycles of five weeks each (alternating week treatment regime i.e., one week on/one week off). FBZ is an anthelmintic drug commonly used to treat pinworm infestation; it is generally considered safe (Villar et al., 2007). While, overall, findings on FBZ's effect on the immune system are somewhat conflicting, in a comprehensive review on the subject, Villar et al. (2007) concluded that—so far—there is no conclusive evidence that a therapeutic dose of FBZ negatively affects reproduction, development, or behaviour in laboratory animals. One study that investigated how FBZ affects breeding outcomes in SD rats reported smaller litter sizes in FBZ-treated animals (Johnston et al., 2006). The present study, however, did not corroborate these findings (Table 2-2). While Reiss et al. (1987) found that FBZ did not change immune responses following influenza exposure in mice, another study reported that rats treated with FBZ and intrastriatal LPS showed prolonged microglial activity (Hunter et al., 2007). No adverse effects of FBZ in the context of poly I:C have been reported, though. Thus, research to date does not suggest that it is likely that FBZ affected responding on the experimental tasks used in this project, but out of an abundance of caution, I examined each cohort separately to confirm this (see experimental chapters below).

In the current study, pups of cohort 1 were affected by the FBZ treatment for 9 days in utero. Dams and pups of cohort 5 were exposed to FBZ for between 7 and 14 days. Also, all adults in cohort 4 received the FBZ-medicated feed for a total of 35 days.

2.4.3 *Timed Mating*

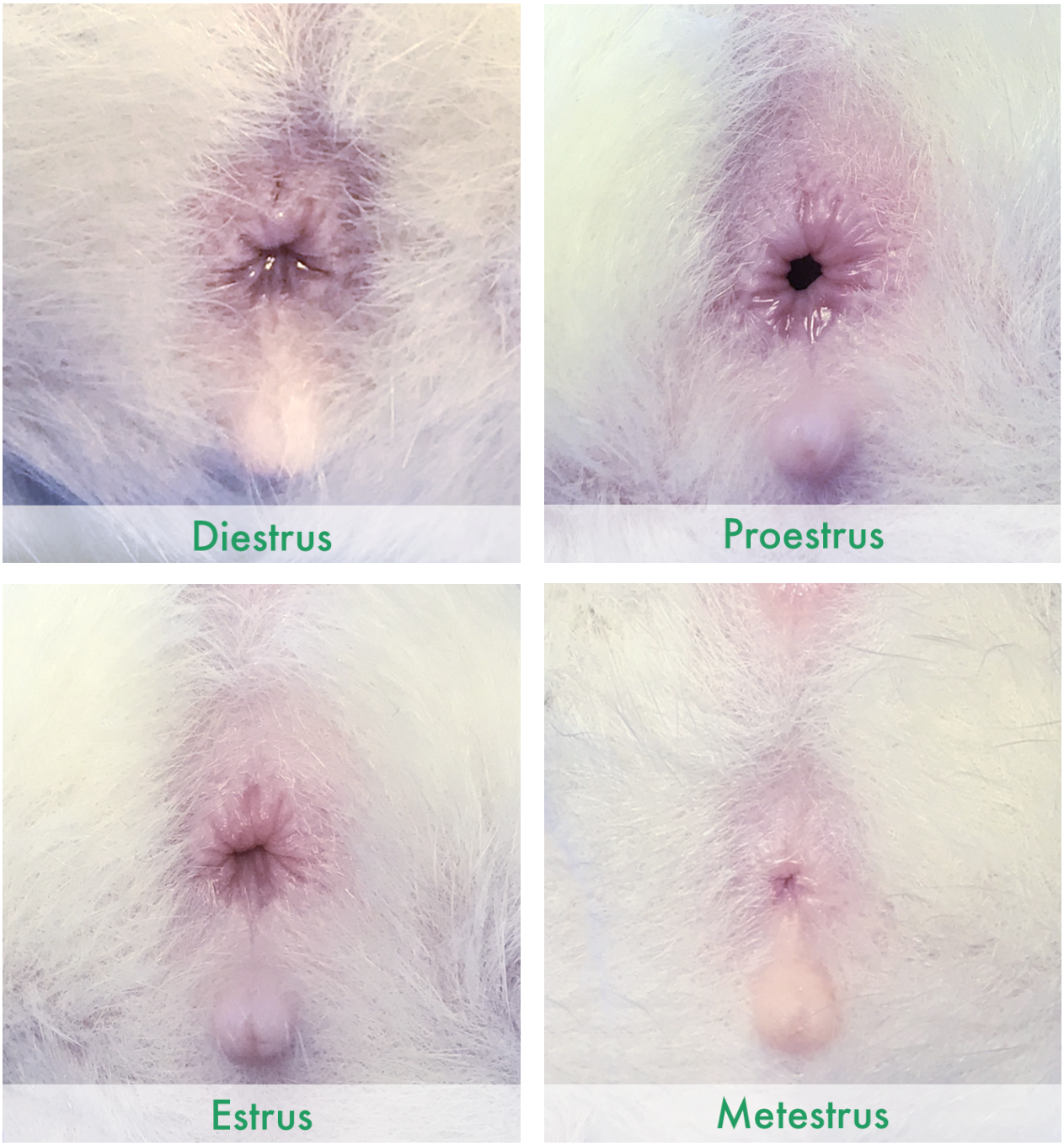
Pairing a female when she is most fertile not only increases the success rate of mating, but it also reduces the duration the animals have to spend in the mating box. For this project, the Behavioural Neurogenetics lab's existing timed-mating protocol was revised to include visual vaginal inspection, a method that allows more reliable

determination of GD 0. Points of observation were colour and swelling of the tissue surrounding the vagina and vaginal dilation (Byers et al., 2012; Champlin et al., 1973). Figure 2-2 shows the four stages of the estrus cycle in the SD rat as observed in our laboratory.

All photos are of the same female. While every care was taken to match the lighting conditions for the different photos, movement of the individual animals during the shooting resulted in subtle variations. All females had been handled for at least 10 minutes on 5 consecutive days before pairing, so the animals were used to the experimenter. This reduced the stress of the vaginal inspection and other routine checks and treatments during gestation. The new pro-estrus/estrus timed-mating protocol required the females to undergo visual vaginal inspection twice daily during the dark phase (7 am to 7 pm) to monitor the estrus cycle. In addition to the visual examination, two behaviours that indicate sexual receptiveness, the presence of a lordosis reflex—a deep arching of the back in response to flank stimulation (Erskine, 1989; Kow et al., 1979), as well as ear wriggling (Erskine, 1989), were used as indicators that the female was nearing the estrus phase of her cycle, which is when the chances of pregnancy are highest.

Figure 2-2

Sprague Dawley Rat Estrus Cycle Stages



Note. **Diestrus:** The tissue is not swollen, more purple than pink, very moist; the opening is somewhat dilated. **Proestrus:** The tissue is swollen, pink, and moist; the opening is very dilated and striations on the lips are prominent. **Estrus:** The tissue is less swollen, less pink, and not as moist as proestrus; striations are prominent, and the opening is slightly dilated. **Metestrus:** The tissue is not swollen and dry; the opening is fully contracted.

As soon as the female displayed these outward signs of sexual receptiveness, she was paired with a randomly selected non-related male and housed in a standard housing cage set up for mating as shown in Figure 2-3.

Figure 2-3

Basic Mating Cage

Note. Metal subfloor to allow vaginal plugs to gather underneath for easy assessment. The cage floor area was 870.9 cm^2.

Twice a day the cages were checked for the presence of vaginal plugs. While such plugs indicate sexual intercourse, they are no guarantee that the animal is pregnant. Generally speaking, the more plugs there are, the higher the chances that the female is pregnant. The plugs form post copulation and consist of dried sperm cells, sealing the vaginal opening. Hence, while plugs usually drop out and gather beneath the metal cage subfloor, it is important to also check the vaginal opening in case the plug has not yet dislodged. If plugs were present, the pair was separated, if not, they were moved to a clean cage and checked again for plugs the following day. If no plugs were found after 5 days, the pair was separated and placed under observation for about 7 to 10 days and, if not pregnant, the female was paired with a different male. The day of successful retrieval of vaginal plugs marked GD 0. At this point, the female was placed in a standard polycarbonate cage like the one shown in Figure 2-7, but with two forms of enrichment—a wooden chew block and a red square polycarbonate tunnel. On GD 20 the cage was

further outfitted with nesting material. After an average gestation period of 22 days, females gave birth, constituting postnatal day 0 (PND).

2.4.4 *Maternal Immune Activation*

Poly I:C Preparation. Poly I:C was procured from Sigma-Aldrich New Zealand (product number P1530, CAS number 42424-50-0) and stored at -18° C until preparation. The powder was dissolved in saline (SAL) at a concentration of 5 mg/ml (ratio of 1 ml to 1 kg rat body weight). To allow reannealing of the double-stranded RNA structure, the solution was heated to 50°C and cooled down to room temperature (22°C). The solution was prepared fresh within a week of injection. Any leftover solution was kept at -18° C.

Maternal Poly I:C Treatment. Following the timed-mating protocol and poly I:C preparation process described above, the animals received either saline or poly I:C subcutaneously (SC) at a dose of 5 mg per kg body weight on GD 15. A key predictor of treatment success is maternal weight loss, a known outcome of an immune response to infection. In past studies, it reliably predicated disease features in the offspring (e.g., Bronson et al., 2011; Horska et al., 2017; Howland et al., 2012; Missault et al., 2014; Ozawa et al., 2006). Therefore, the females were weighed, and their temperature was taken at the anterior tail base with a non-contact infrared thermometer (Pro's Kit, MT-4612) at time of exposure to poly I:C, as well as 4 and 24 hours post injection.

The dams were left undisturbed for the remainder of their pregnancy, except for standard animal husbandry tasks such as cage cleaning once a week. The cages were checked for successful delivery once a day from the outside without disturbing the dams. Table 2-2 shows the breeding outcome by cohort; it seemed unaffected by the FBZ treatment.

A maximum of two males and two females per litter were selected for experimental testing to ensure behavioural features were the result of the MIA treatment rather than a litter effect. Once pups were weaned, the dams were euthanised with carbon dioxide. At the end of the behavioural testing, all experimental animals were euthanised with sodium pentobarbital for subsequent brain extractions. The brains were stored at -80° C and will be processed in future studies.

Table 2-2

Main Study Breeding Details

Cohort	Dams (*n*)	Litters			Pre-weaning deaths (*n*)
		Size (*n*)	Total pups (*n*)	*M size* [b]	
#1					
Saline	6	2 [a]	23	12	11
Poly I:C	4	3	41	14	0
#2					
Saline	6	5	69	14	0
Poly I:C	5	3	35	12	0
#3					
Saline	6	4	46	12	1
Poly I:C	9	4	46	12	3
#4					
Saline	5	4	53	13	0
Poly I:C	5	5	67	13	0
#5					
Saline	5	3	47	16	0
Poly I:C	3	2	28	14	0
Total					
Saline	28	18	238	13	12
Poly I:C	26	17	217	13	3

Note. All animals received an injection (SC) on PND 15.

[a] One female failed to nurse her pups and they were found dead on GD 5. [b] Litter sizes were rounded up.

2.4.5 Pup Marking

Due to their size, rat pups cannot be microchipped before weaning age (approximately PND 21). Therefore, the pups selected for treatment received a tattoo marker on either a paw or the tail to ensure identification during pre-weaning procedures. On PND 7, the maternal cage was moved to an experimental room where separation-induced USV were recorded. Following the USV recording, one pup at a time was prepared for marking. The area was swabbed with 70% ethanol, then Vaseline, and, lastly, tattoo ink. The palm (or tail) was secured between two fingers and the skin superficially

penetrated with a three-pronged hand-held tattoo needle to create a mark. The skin was again swabbed with ethanol to remove any excess ink and covered with a thin layer of Vaseline to soothe the skin. The pup was then reunited with its mother.

Pups were weaned after 23 days on average and equipped with a small cylindrical radio-frequency identification transponder chip (size approximately 5 mm in length x 1.5 mm in diameter). Each chip stored a unique ID number that could be read with a hand-held chip reader. The chips were pre-loaded in syringes and subcutaneously implanted at the scruff of the neck. Post implantation the chip was scanned to confirm full functionality.

2.4.6 *Pre-weaning Enrichment*

Background. Enrichment is a promising manipulation to reduce autistic symptoms in rats. Offering an interesting social and an ever-changing physical environment has been the centre of a fast-growing research field. However, most studies provide enrichment post-weaning and, in comparison, only a small amount of research (e.g., Kohl et al., 2002; Schneider et al., 2006; Venable et al., 1988) has focused on pre-weaning enrichment where the pup was removed from the home cage for a brief period to receive sensory stimulation-based treatment. In an autism study, Schneider et al. (2006) found that, in rats, enrichment at such an early age contributed to increased social engagement and exploratory behaviour and decreased anxiety-related behaviours. Noirot (1972) described an experiment where pups that had been separated from the dam for a brief period emitted many long USV when returned to the nest, triggering maternal retrieval, and licking behaviour; non-handled pups produced short sonic calls. The increased maternal attention may be responsible for the behavioural changes discussed above, rather than the pups' experiences during separation (Brouette-Lahlou et al., 1992; Zimmerberg et al., 2003). The period from PND 7 to 21 in rats roughly corresponds to the time from gestational week 36 to approximately 3 years in humans (Semple et al., 2013). The following section describes the pre-weaning enrichment procedure.

Procedure. The pups were divided into four groups: (1) Standard housing (SH)/saline (SAL), (2) SH/poly I:C, (3) enriched housing (EH)/SAL, and (4) EH/poly I:C. The groups were further divided by sex for future analysis. Between PND 7 and PND 21, pups in the EH groups received 10 enrichment sessions as detailed in Table 2-3. Both SH groups remained in the housing room. All sessions were run between 9 am and 6 pm under red light conditions. The dam and littermates remained in the home cage, which was stored at the opposite side of the room during the session. While several litters had

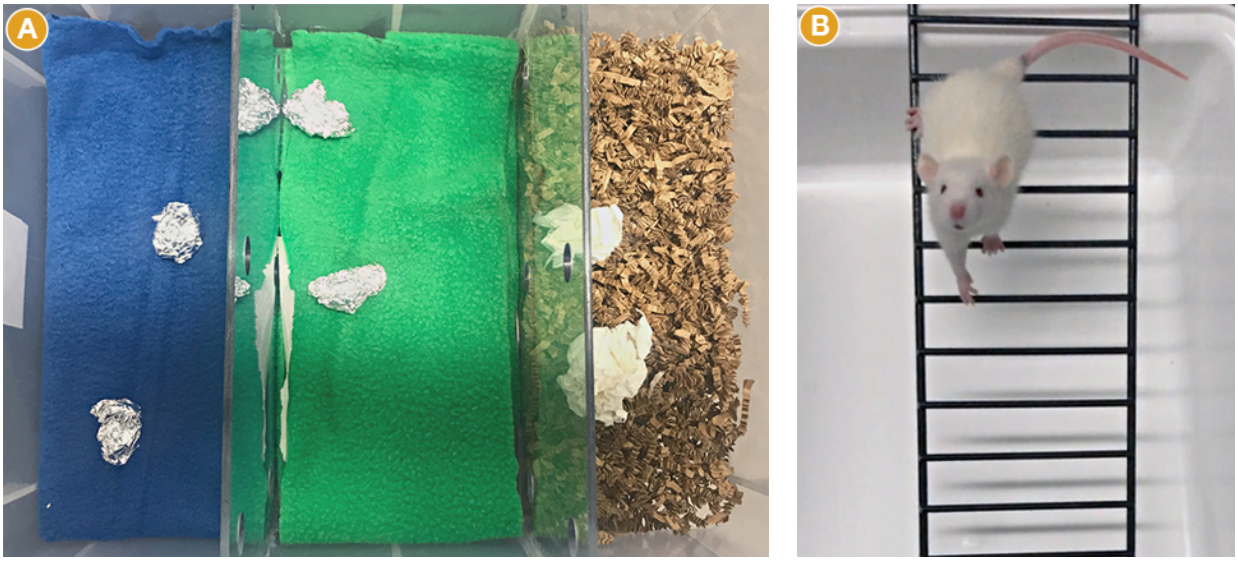
their sessions in the same room at the same time, litters were always kept separate and occupied individual experimental boxes.

The rat pups were removed from the home cage and placed into a polycarbonate box that had been subdivided into three smaller compartments, as can be seen in Figure 2-4. The box was placed on a heated pad to ensure the pups would not get hypothermic. This is important, as rat pups cannot regulate their body temperature until about PND 18 (Noirot, 1972).

The first session ran for 12 minutes, and the duration increased to a maximum of 25 minutes. Pups were gently prompted into motion as required; they were also handled for 2 minutes as part of the treatment, mimicking maternal attention. Each session had sensory components: Tactile: crinkled paper, bubble wrap, fleece, aluminium foil shreds; olfactory: rosemary, lemon, cinnamon; auditory: soft piano music; and a motor component: walking on a slowly rotating rod, walking inside a counter-rotating tube, and bathing in warm water. The motor sessions ran for 60 seconds and concluded each session. After each session, the pups were put back in their home cage and returned to the housing room.

Figure 2-4

Pre-weaning Enrichment Setup



Note. (A) Plastic box with fleece covering and aluminium foil shreds left/centre and crinkled paper on the right. The dividers allowed accommodation of multiple groups. Each compartment measured 28 x 14 x 13 cm. (B) Twenty-one-day-old pup on ladder.

Table 2-3

Pre-weaning Enrichment Protocol

Session	Duration [a]	Tactile	Olfactory [b]	Motor	Auditory [g]
1	12	Crinkled paper	Rosemary	Walking inside slowly counter-rotating tubes [c]	Piano
2	15	Crinkled paper	Rosemary	Walking inside slowly counter-rotating tubes [c]	Piano
3	20	Fleece, aluminium foil shreds	Rosemary	Walking on slowly rotating rod [d]	Piano
4	25	Fleece, aluminium foil shreds	Lemon	Walking inside slowly rotating tube [e]	Piano
5	25	Fleece, paper	Cinnamon	Walking on slowly rotating rod [d]	Piano
6	25	Fleece, paper	Cinnamon	Walking on slowly rotating rod [d]	Piano
7	25	Bubble wrap, crinkled paper	Lemon	Swimming in warm water [f]	Piano
8	25	Bubble wrap, crinkled paper	Lemon	Swimming in warm water [f]	Piano
9	25	Plain play sand, Lego pieces	Cinnamon	Walking up and down a metal ladder at 45° (see Figure 2-4)	Piano
10	25	Plain play sand, Lego pieces	Cinnamon	Walking up and down a metal ladder at 45°	Piano

[a] Minutes. [b] Organic essential oils. [c] 2x 40 mm diameter, cardboard. [d] 25 mm diameter, wood. [e] 80 mm diameter, plastic.

[f] The water temperature: 32-34°C, water level: 5.5 cm. [g] The playlist "Peaceful piano" (Spotify, 2020) was played during all sessions.

2.4.7 *Post-weaning Enrichment*

Following exposure to pre-weaning enrichment, and weaning, the animals were housed in an enriched environment that provided multisensory stimulation. It comprised of (1) a rich social environment through living in groups, (2) motor stimulation through exercise and activity, and (3) experience of novelty through a regularly changing cage outfit, including occasional food treats. The animals remained in this environment until the end of the behavioural testing in adulthood. The period from PND 21 to 60+ spans from about 3 to 20+ years in humans (Semple et al., 2013).

Enriched Housing Environment. After weaning, the pups were still small enough to fit through the bars of the enrichment cages and were, thus, kept in special day-care enrichment boxes for the first week post weaning (separated by treatment and sex).

Figure 2-5

Interim Enrichment Day-care Box Setup

Note. The mezzanine floor (38 x 38 cm) was made transparent in the picture to show the tunnels and toys beneath it.

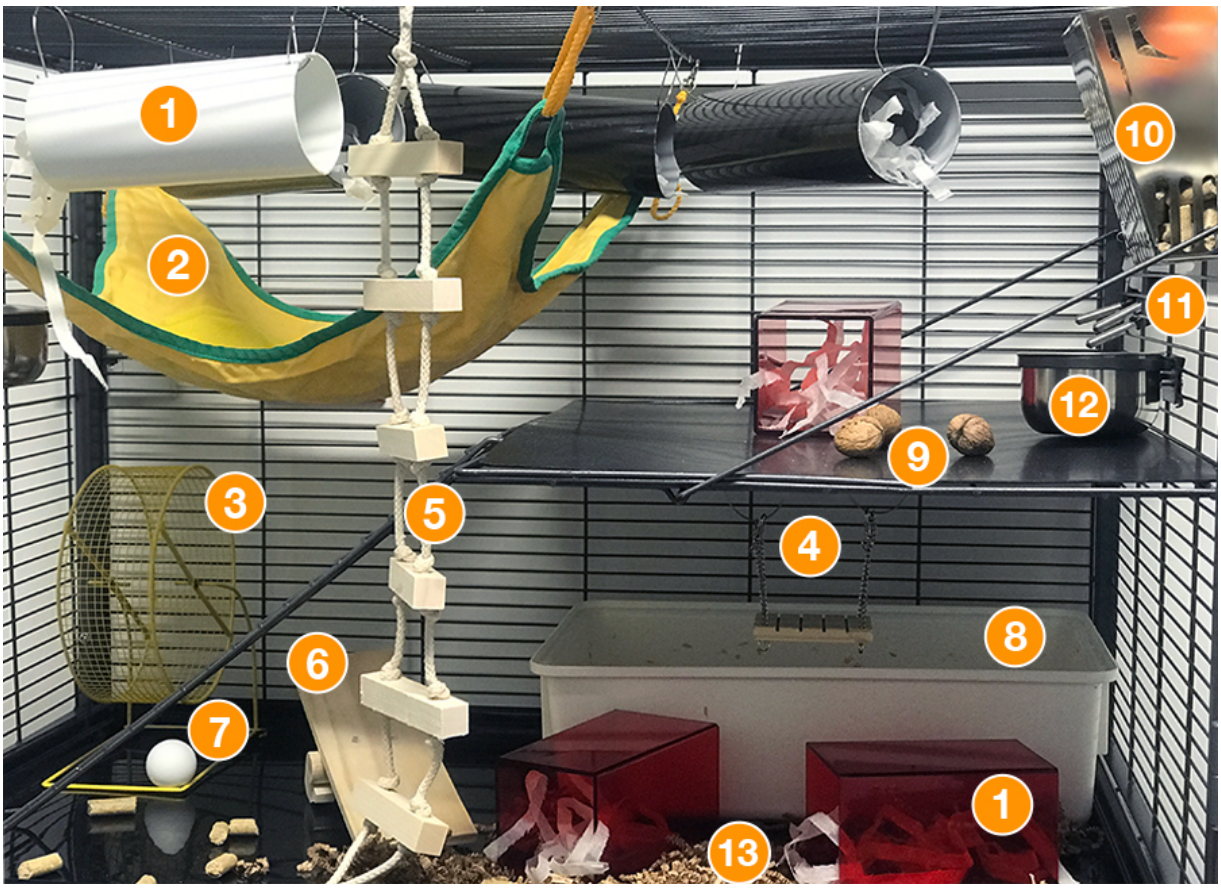
Each morning at 8 am, the pups were moved to their day-care box and returned to the standard housing room for the night at 6 pm. They spent the night in standard housing cages. The polyolefin boxes were 65 cm (length) x 50 cm (width) x 25 cm (height) in size

with a clip-on lid. Each unit was filled with a thick layer of wood shavings and had a mezzanine level, several tunnels, chew blocks, Lego blocks, a running wheel, water bottles, and rat chow pellets buried in the bedding material to encourage foraging. The setup was changed every second day.

From PND 28 on, rats were kept in a housing room physically separate from the standard housing room and no longer returned to standard housing overnight. They occupied two double-unit cages where each unit measured 93.5 cm (length) x 63.5 cm (width) x 60 cm (height). Cages were equipped with 1.2 cm horizontal bars, providing additional climbing space. Each multi-level unit featured the same basic setup as shown in Figure 2-6.

Figure 2-6

Enrichment Cage-outfit



Note. (1) A variety of tunnels, (2) fabric hammock, (3) running wheel(s), (4) swings, (5) a rope ladder for climbing and chewing, (6) a seesaw, (7) toys (e.g., ping-pong ball for chasing), (8) litter tray, (9) walnuts, (10) chow station, (11) water station, (12) water-filled bowl for bathing and playing, and (13) a variety of ground-covering materials (crinkled paper, wood shavings, or nesting material).

Between 6 and 10 animals lived together, separated by sex and treatment. Once a week the mezzanine level was switched to the opposite cage side and the location of the two running wheels changed. Occasionally the animals received one type of additional enrichment—either walnuts (one nut per animal), fruit pieces (apple or pear, individually wrapped in paper towels to provide extra enrichment), or frozen peas in a bowl of water. Every second day, tunnels, hammocks, and water bottles were moved to different locations and new toys were introduced or reintroduced.

Standard Housing Environment. The rats were kept in standard polycarbonate cages (Animal Care Systems, Optirat® GenII) with a floor area of 870.9 cm^2. Post weaning, four pups were housed together for up to 2 weeks, by which time they had become too big and were split up into the final housing arrangement of two rats. Each pair occupied a cage outfitted with two 400 ml water bottles, a stainless-steel feeder, and a wooden chew block (see Figure 2-7). The individually ventilated cages were kept on a rotatable rack (Optirat GenII IVC rack system).

Figure 2-7

Standard Housing Cage

2.5 Results

2.5.1 Statistical Analyses

I conducted all statistical analyses in this thesis with R (R Core Team, 2020) using the editor RStudio (RStudio Team, 2020). The alpha (α) level was set at .05 and a *p*-value lower than that ($p \leq \alpha$) was considered statistically significant. I used jitter plots showing the datapoints by cohort to confirm the absence of FBZ effects. Error bars in the bar charts always show ± standard error of the mean (*SEM*). I checked ANOVA assumptions of normality and homogeneity of variance for each dataset. Non-significant Shapiro Wilk tests (either on residuals or grouped by factor) and visual examination of both a Q-Q plot and a histogram of residuals confirmed normality, and homogeneity of variance compliance was assessed with Levene tests. The data can be treated as independent, as all animals were tested individually.

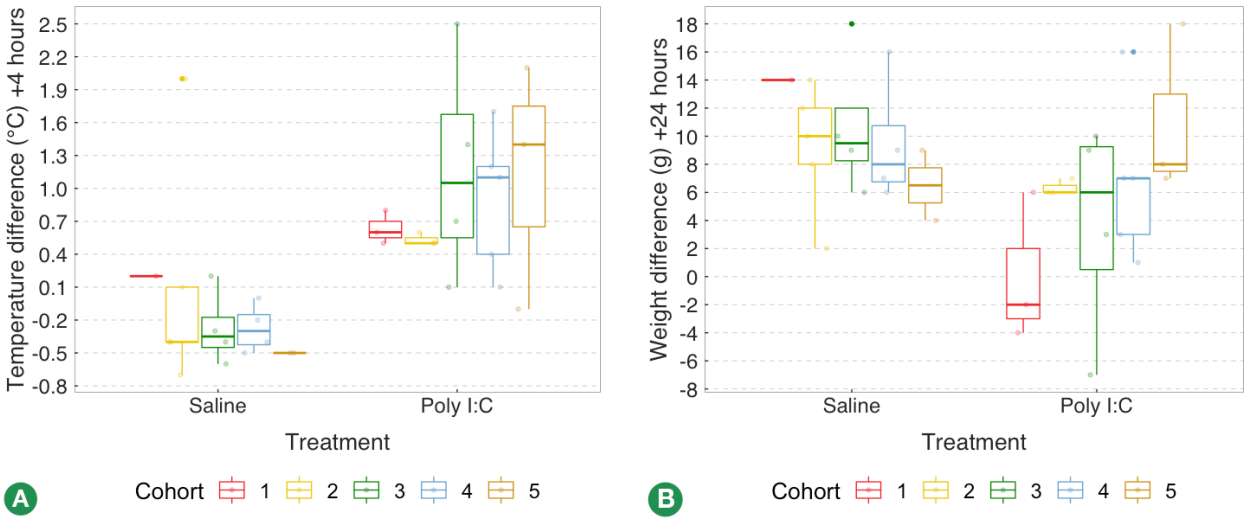
I analysed the maternal weight and temperature dataset with Student's t-tests and reported effect sizes with Cohen's *d*. For the weight-over-time dataset, I computed a two-way ANOVA (treatment x housing) for weight comparisons at weaning and reported effect sizes with eta-squared (η^2). Levene's test for the weight data for PND 80 was significant, indicating unequal variance. Therefore, I proceeded with robust methods, as standard parametric (and non-parametric) analysis options are sensitive to even minor divergence from normality and also susceptible to Type II Error (Field, 2013; Field et al., 2012; Wilcox, 2017). Effect sizes were reported with Wilcox and Tian's explanatory measure of effect size (ξ) for robust ANOVAs ($\hat{\psi}$), where ξ = 0.10, 0.30, and 0.50 stand for small, medium, and large effect sizes respectively (Wilcox & Tian, 2011). To analyse the treatment effect within sex, I ran one-way robust ANOVAs (*F*) using the WRS2 function "t1way" (Mair & Wilcox, 2020).

2.5.2 *Maternal Temperature and Weight*

Figure 2-8 shows a pattern for the poly I:C treatment, though this was more obvious for temperature (A) than weight (B). There was, however, no noticeable cohort effect that could be attributed to the FBZ treatment. There was a lot of within-group variability, potentially because the group sizes were relatively small (see Table 2-2 for sample sizes per cohort).

Figure 2-8

Jitter Plots: Temperature and Weight Differences by Cohort (Dams)

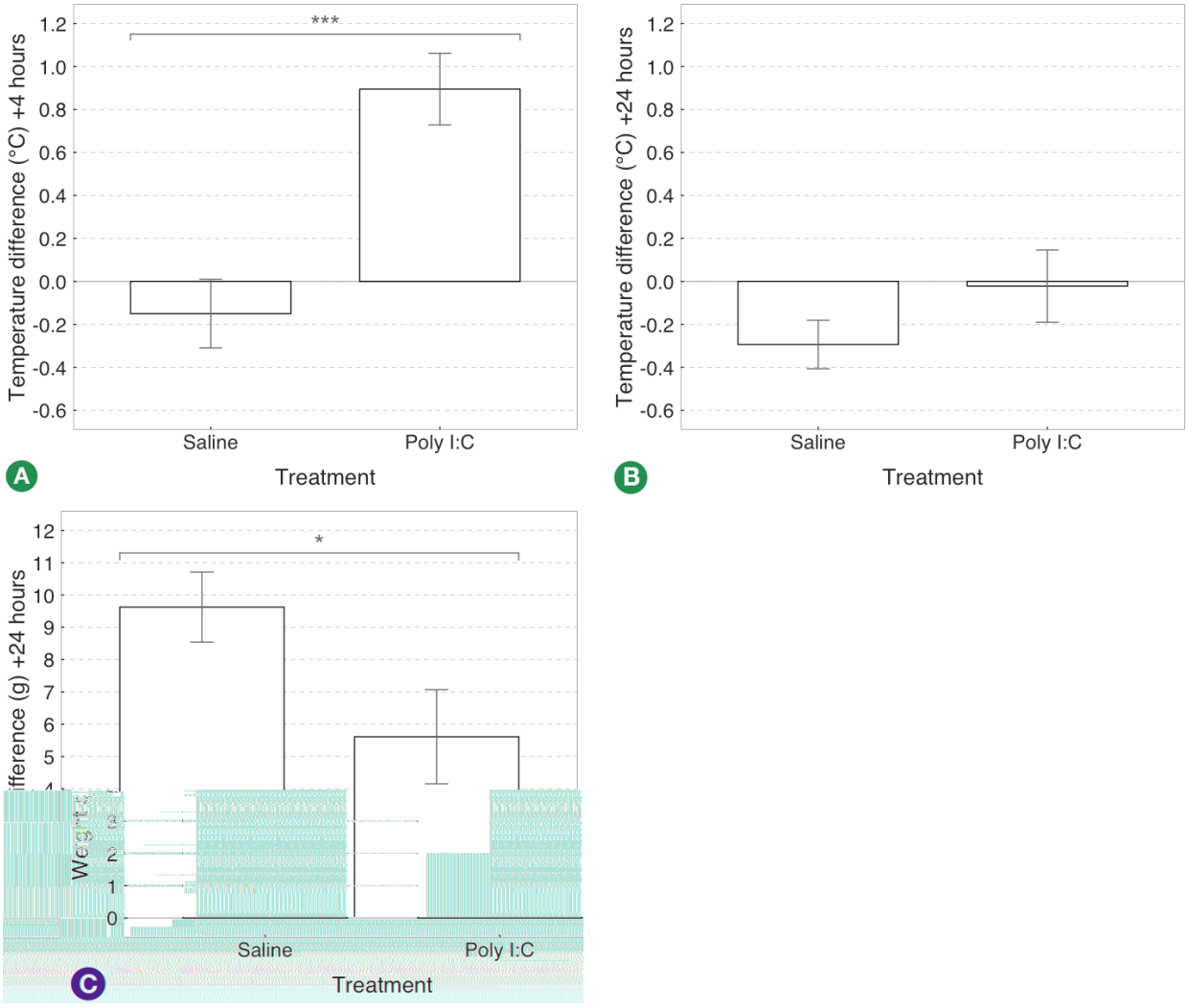


Note. (A) Temperature and (B) weight differences.

As predicted, dams in the poly I:C condition had a significantly greater increase in body temperature than saline dams at the 4-hour post injection check-in, $t(32) = -4.50$, $p < .001$ (see Figure 2-9A and Table 2-4). This constitutes a large effect, $d = -1.55$. Twenty-four hours post injection—at this point the poly I:C effect had subsided—the temperature difference between groups was non-significant, $t(32) = -1.31$, $p = .200$ (Figure 2-9B). Furthermore, dams in the poly I:C group had gained significantly less weight than saline dams 24 hours after their treatment, $t(32) = 2.16$, $p = .040$ (see Figure 2-9C). The effect size was moderate with $d = 0.74$. These results are in line with previous findings (e.g., Bronson et al., 2011) and suggest that poly I:C caused the intended temporary immune reaction resulting in higher body temperature during the immune reaction and less weight gained 24 hours post treatment.

Figure 2-9

Temperature and Weight Differences (Dams)



Note. Error bars show mean ± *SEM*. (A) Temperature increased 4 hours and (B) 24 hours post maternal treatment. (C) Weight difference 24 hours after MIA.

* $p \leq .05$. *** $p \leq .001$.

2.5.3 *Offspring Weight Over Time*

It has been consistently reported that animals living in EH (post-weaning) weigh less than those in SH (Fiala et al., 1977; Peña et al., 2006). Rats in SH exercised less, had more adipose tissue, and less muscle oxidative capacity than enriched animals (Spangenberg et al., 2005). Fiala et al. (1977) reported a higher intake of chow in the standard-housed group and speculated that the rats ate out of boredom, which makes sense, as they had less opportunity to exercise or socialise. Higher physical activity burns more energy and generally results in lower fat deposits and lower body weight. Rats in

enriched housing spent less time eating because they were busy with climbing, chasing, and playing (Spangenberg et al., 2005). Consequently, I predicted that enriched animals in the present study would weigh less (on average) than rats in standard housing. All animals were weighed once a week on a digital bowl scale starting on PND 7.

Table 2-4

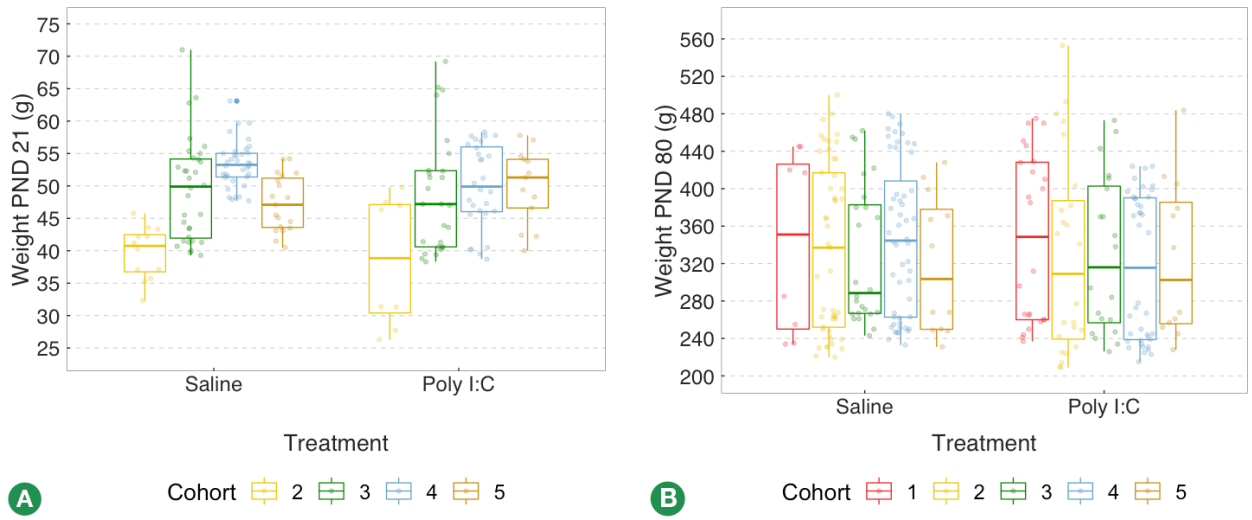
Descriptive Statistics for Maternal Temperature and Weight Differences

Group	*n*	*M*	*SD*	*SE*	95% CI
			Temperature (°C)		
Saline	16	-0.15	0.64	0.16	0.34
Poly I:C	18	0.89	0.71	0.17	0.35
			Weight (g)		
Saline	16	9.63	4.33	1.08	2.31
Poly I:C	18	5.61	6.19	1.46	3.08

Figure 2-10 shows that FBZ did neither affect the animals' weight at weaning nor in adulthood. The animals' weight did not vary systematically between cohorts, despite their different exposures to FBZ.

Figure 2-10

Jitter Plots: Weight Differences by Cohort (Offspring)



Note. (A) Weight at weaning. Cohort 1 weight data at weaning was lost and could not be included. (B) Weight in adulthood.

There was no significant main effect of pre-weaning enrichment, $F(1, 172) = 0.14$, $p = .910$, $\eta^2 < .001$, or treatment, $F(1, 172) = 0.71$, $p = .400$, $\eta^2 < .001$, on weight at time of weaning. Similarly, there was no interaction between the independent variables. Table 2-5 shows mean weights and sample sizes.

Table 2-5

Body Weight by Sex at Weaning and in Adulthood (Offspring)

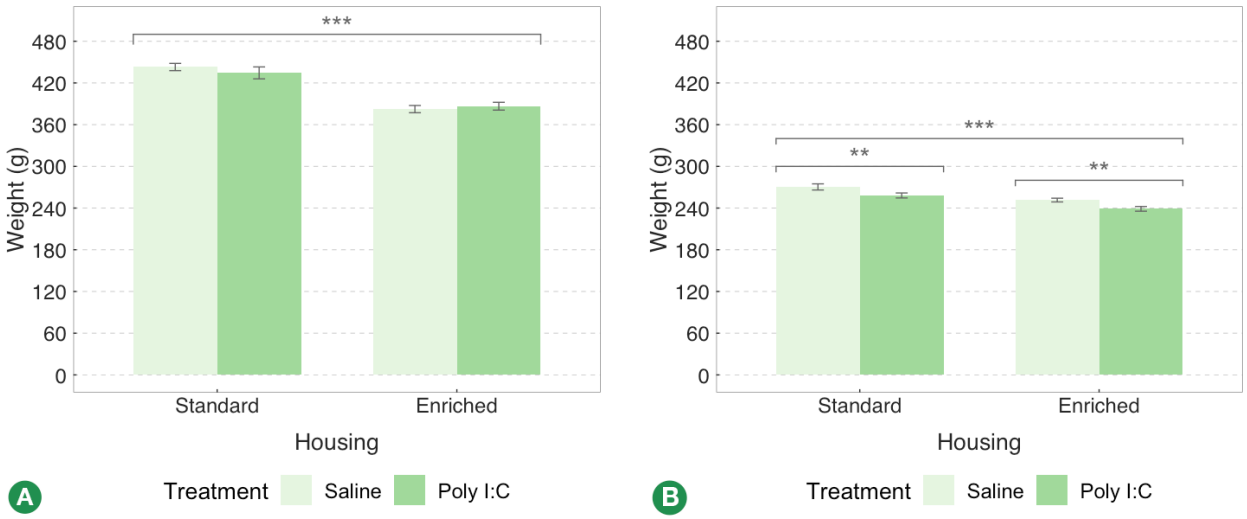
Time point	Sex	Standard housing		Enriched housing	
		Saline (*n*)	Poly I:C (*n*)	Saline (*n*)	Poly I:C (*n*)
Weaning	M	51.6 ± 2.1 (17)	47.6 ± 2.4 (10)	50.0 ± 1.3 (27)	50.0 ± 1.6 (26)
	F	48.3 ± 1.6 (21)	46.9 ± 2.8 (13)	48.2 ± 1.0 (34)	47.6 ± 1.5 (27)
Adulthood	M	442.9 ± 5.2 (34)	434.5 ± 8.6 (26)	382.4 ± 5.2 (41)	386.6 ± 5.6 (34)
	F	270.4 ± 4.4 (33)	258.1 ± 3.6 (30)	251.5 ± 2.7 (37)	238.9 ± 3.4 (30)

Note. Group sizes differ due to missing weight data. Displayed are the mean weights (g) ± *SEM*.

As anticipated, robust ANOVA results showed that, in adulthood (PND 80), animals in the enrichment group weighed significantly less than those in the non-enrichment group, $\hat{\psi}(1, 137) = 98.67$, $p < .001$, $\xi = 0.18$. Animals prenatally exposed to poly I:C weighed significantly less than when exposed to saline, $\hat{\psi}(1, 152) = 6.26$, $p = .015$, $\xi = 0.07$. Also, males weighed significantly more than females, $\hat{\psi}(1, 107) = 1{,}694.90$, $p = .001$, $\xi = 0.99$ (see Figure 2-11 for bar charts), and there was an interaction between housing and sex, $\hat{\psi}(1, 152) = 27.23$, $p = .001$, $\xi = 0.98$ (Figure 2-12). Further investigation of the effect of treatment by sex showed that females prenatally exposed to poly I:C weighed significantly more than females exposed to saline, $F(1, 69) = 7.79$, $p = .006$, $\xi = 0.38$, but there was no significant difference between the two treatment groups for males, $F(1, 79) = 1.18$, $p = .281$, $\xi = 0.16$.

Figure 2-11

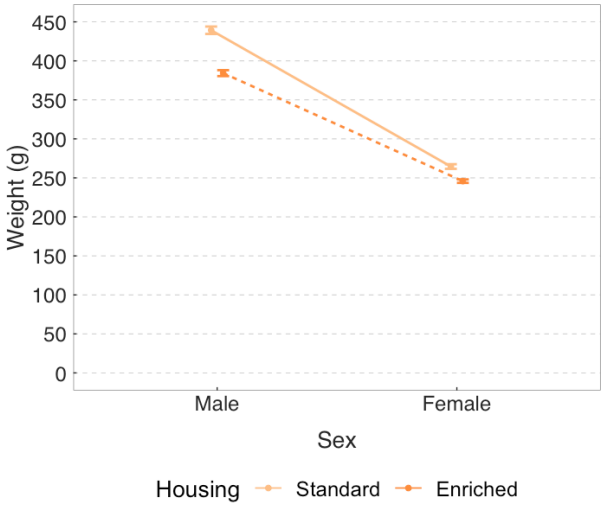
Weight Differences at PND 80



Note. Error bars show mean ± *SEM*. (A) Weight differences for males and (B) females. ** $p \leq .01$. *** $p \leq .001$.

Figure 2-12

Interaction Effect Housing x Sex at PND 80



Note. Error bars show mean ± *SEM*.

2.6 Summary

Chapter 2 started with an outline of poly I:C as a well-accepted option to trigger a maternal immune reaction. Because the literature did not provide a clear path forward in terms of poly I:C dose and administration time, a pilot study was conducted. The pilot study tested two different doses on two different days (5 or 8 mg/kg SC on PND 10 or 15). Based on its results, the optimal dose to produce animals with autism-like symptoms was determined to be 5 mg/kg on GD 15, which I used in the main study reported in this chapter. Besides this, the pilot study also prompted the improvement of the existing timed-mating protocol; the new method utilises visual vaginal examination to determine the fertile window and best time for successful mating. This procedure was subsequently developed and employed in the main study; it resulted in the successful breeding of 440 animals from 34 different litters.

In terms of poly I:C treatment success, the results were in line with past research (Fortier et al., 2004). Pregnant animals had a significantly higher body temperature than control animals four hours after exposure to poly I:C. Twenty-four hours post injection no temperature difference could be detected. Further, animals gained less weight in the 24 hours following poly I:C injection when compared with animals treated with saline. Together, these results show that poly I:C had the anticipated effect in evoking a maternal reaction on PND 15.

Two types of enrichment were used: Pre-weaning, where pups underwent a multisensory stimulation regime from PND 7 to 21, and post-weaning, where rats were housed in an environment providing social, motor, and cognitive stimulation; this included occasional fresh-food treats. Seeing there is a link between ASD and the gut microbiome (Cryan et al., 2020), care was taken to prevent potential confounding effects of these non-standard food treats by giving them very rarely.

Environmental enrichment results in lower overall body weight (Peña et al., 2006), a finding corroborated by the present study. While no weight difference was found at weaning, adult animals in enrichment weighed significantly less than control animals, very likely because they had more exercise. Regardless of living conditions (i.e., enriched vs standard), the offspring of poly I:C treated dams weighed significantly less than their saline counterparts, but housing and treatment did not interact, highlighting that both poly I:C and saline control animals were equally engaged with the enriched environment. The following chapters investigate if and how these differences impact the animals' social behaviour, communication, locomotor activity, as well as anxiety levels.

Chapter 3

Ultrasonic Vocalisations

Chapter 3
Separation-induced Ultrasonic Vocalisations

3.1 Background

This chapter will discuss the effects of prenatal poly I:C exposure on rat pups' communication when separated from their mothers. Communication is one of the key areas affected by ASD (American Psychiatric Association, 2013). This study investigated USV in 7- and 14-day-old rat pups to find out if poly I:C, and pre-weaning enrichment, or a combination of the two, altered communication patterns. While the full scope of rat communication is not yet fully understood, the decade-long substantial research effort into the way they communicate has provided a great many details. For example, rats communicate mostly in the ultrasonic range and the frequency of these USV depends on the environmental context (i.e., danger, food, etc.), affective state, and age (Brudzynski, 2013; Portfors, 2007). Calls in the audible range are usually reserved for pain (Noirot, 1972). Small rodents like mice and rats emit USV during defensive or offensive behaviours, avoidance (e.g., danger or aggressive interactions with other rats), play and social behaviours, as well as sexual interactions (Brudzynski, 2013; Portfors, 2007; Wöhr & Schwarting, 2013).

From the day they are born, rats use USV for social communication and to convey their emotional state to their conspecifics (Brudzynski, 2013; Wöhr & Schwarting, 2013). Juvenile and adult rats make use of two distinct call types to communicate positive and negative states. Calls associated with a positive affective state are higher than those associated with a negative affective; their frequency ranges from 32 kHz to 96 kHz and they are commonly known as 50 kHz calls (Portfors, 2007). These calls are normally short, between 30 ms and 50 ms, whereas negative affect calls, known as 22 kHz calls, are between 300 ms and 4,000 ms long and have a narrower frequency range, from 18 to 32 kHz (Portfors, 2007). Rat pups (PND 1-18), emit USV mainly to communicate distress, including hunger, cold, and separation from their mother and littermates, and trigger maternal care behaviour like nursing, grooming, or retrieval (Brewster & Leon, 1980; Portfors, 2007; Shair, 2018). The response to pup USV in lactating females is so strong that pre-recorded pup USV triggered search behaviour in the females even when no pups were present (Noirot, 1972).

Pup USV are commonly referred to as 40 kHz calls because they range from 35 kHz to 65 kHz (Portfors, 2007). Boulanger-Bertolus et al. (2017) reported an even higher upper

limit with 70 kHz. Some studies found that male rat pups call more (Bowers et al., 2013; Hahn & Lavooy, 2005), but most found no differences between sexes (Hahn & Lavooy, 2005). Delta frequency often referred to as range or bandwidth—the difference between the lowest and highest frequency in per call—increases with age (Brudzynski et al., 1999). Greater bandwidth allows for more varying calls and calls usually differ between individual pups, enabling the dam to not only distinguish her own litter but also individual pups (Brudzynski et al., 1999).

In the context of the present study, available research on the topic of deficient pre-weaning ultrasonic communication in a poly I:C rodent model is very limited. To my knowledge, no published study has reported on the effects of poly I:C on neonatal separation-induced USV and subsequent impact of pre-weaning enrichment on the MIA-related deficits. However, related studies suggest that communication deficits are present but less discernible in older pups. Chou et al. (2015) found reduced call numbers in the offspring of SD rats prenatally exposed to poly I:C on PND 11. Malkova et al. (2012) found that 8-day-old mouse pups prenatally exposed to poly I:C vocalised less than saline controls; the calls were also shorter, contained fewer harmonics, but were overall more complex; by PND 14, the differences had disappeared. In an LPS rat study, which, similarly to poly I:C, triggers a maternal immune reaction, separation-induced pup calls were fewer and shorter on PND 5 in the LPS group when compared with the saline control group (Baharnoori et al., 2012). Moreover, these differences were no longer detected on PND 9 or PND 11.

By contrast, Schwartzer et al. (2013) observed that the offspring of mice treated with poly I:C emitted significantly more calls than controls on PND 8 and PND 10. The findings of these studies differed in terms of direction and it is unclear whether the individual results were driven by the choice of animal strain, administration time, dose of gestational treatment, or a combination of those factors (Jouda et al., 2019). Nevertheless, they show that rodents consistent with an autistic phenotype present with severely impaired communication, highlighting that neonatal USV are a good way to study impaired communication in the laboratory.

3.2 Predictions

Even though there is not enough data available to form a directional hypothesis, delta frequency will be analysed as a developmental marker. In terms of the effect of pre-weaning enrichment on pup communication in the present study, enrichment started on PND 7, so pups would have had about half their pre-weaning enrichment sessions on the second recording day (i.e., PND 14).

Chapter 3 will investigate the following hypotheses:

A. SD rat pups prenatally exposed to poly I:C will produce fewer and shorter calls relative to control animals.
B. The communication deficit will be more prominent on PND 7 than on PND 14.
C. Poly I:C pups that received enrichment will be less affected by the poly I:C effect than poly I:C pups that did not.

3.3 Methods

3.3.1 Animals

The offspring of SD rats treated with 5mg/kg poly I:C on GD 15 were tested in five cohorts and divided into four groups of 32 for a total sample of 108. The groups were (1) standard housing (SH)/saline (SAL), (2) SH/poly I:C, (3) enriched housing (EH)/SAL, and (4) EH/poly I:C (see Table 3-1 for animals by cohort). The offspring originated from 16 litters in the saline and 18 in the poly I:C treatment condition. Because USV were recorded on PND 7 and PND 14, the animals lived with their dam and littermates in the standard housing room.

Table 3-1

Total Number of Tested Animals by Cohort (USV)

Cohort	Treatment	Housing	Males (7/14) *	Females (7/14) *	Total (7/14) *
#1	Saline	Standard	3/3	3/6	6/9
		Enriched	0/0	0/0	0/0
	Poly I:C	Standard	6/6	6/6	12/12
		Enriched	0/0	0/0	0/0
#2	Saline	Standard	4/4	7/6	11/10
		Enriched	4/4	5/4	9/8
	Poly I:C	Standard	2/2	3/2	5/4
		Enriched	3/2	2/2	5/4
#3	Saline	Standard	5/4	4/3	9/7
		Enriched	7/6	4/4	11/10
	Poly I:C	Standard	5/5	5/5	10/10
		Enriched	6/7	6/8	12/15
#4	Saline	Standard	4/3	2/2	6/5
		Enriched	5/5	5/5	10/10
	Poly I:C	Standard	2/2	2/3	4/5
		Enriched	5/6	5/5	10/11
#5	Saline	Standard	0/1	0/0	0/1
		Enriched	0/1	2/3	2/4
	Poly I:C	Standard	1/1	0/0	1/1
		Enriched	2/2	3/0	5/2
Total			64/64	64/64	128/128

* 7/14 stands for recording day i.e., PND 7 or PND 14.

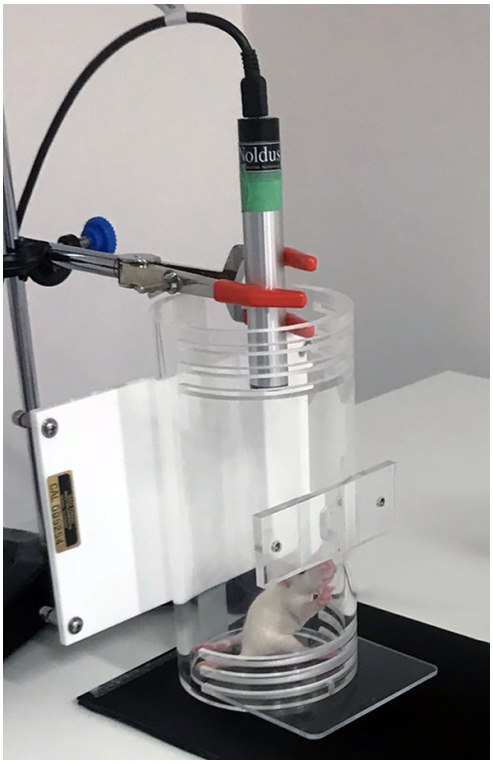
As discussed in section 2.4.2, pups of cohort 1 were affected by the FBZ treatment for 9 days in utero. Dams and pups of cohort 5 were exposed to FBZ for between 7 and 14 days. Also, all adults in cohort 4 received the FBZ-medicated feed for a total of 35 days.

3.3.2 Apparatus

Pups in cohort 1 were recorded in a polycarbonate rodent restrainer (8.5 cm x 20 cm) setup with the opening facing up (see Figure 3-1).

Figure 3-1

Initial Pup USV-recording Setup



Note. The microphone was suspended at 15 cm above floor level.

To improve the recording quality, I constructed a recording chamber lined with acoustic foam with an opening to insert the microphone through the lid. Acoustic foam absorbs sound, enhancing recording quality. Figure 3-2 shows the Styrofoam outer and inner layers (23.5 cm x 23.5 cm x 25.5 cm, lid: 2.5 cm, wall thickness: 3.5 cm). The interior recording chamber measured 12 cm x 12 cm x 19 cm. A piece of fleece fabric (not shown here) was put inside the chamber so pups would not accidentally soil the foam. The fleece was replaced when soiled and washed at 60°C at the end of each recording day. The USV

were recorded with an ultrasonic microphone (Ultramic 250K) with the recording software Audacity (Audacity Team, 2019).

Figure 3-2

Final Pup USV-recording Setup

Note. (A) Styrofoam recording chamber with the lid on and microphone inserted through a hole in the lid. (B) Chamber interior where the pup was placed during the recording.

3.3.3 Procedure

I recorded the USV on PND 7 and PND 14, always before the pre-weaning enrichment sessions during the animals' dark cycle under red light. As recording took place before weaning, the home cage, including dam and siblings, was taken to the experimental room 15 minutes before the session. On PND 7, all pups were sexed before the first pup was taken from the home cage, weighed, and placed into the recording chamber. After 5 minutes, the pup was removed from the chamber and received a tattoo marker on its palm (described in Chapter 2); it was returned to the home cage following marking. The tattoos of cohort 1 had faded into invisibility and the same animals could not be recognised with certainty; different animals were likely recorded on PND 14. On PND 14, the pups marked on PND 7 were identified, if possible, weighed, and individually placed into the chamber for USV recording. After 5 minutes, the pup was returned to the home cage.

3.3.4 Data and Statistical Analysis

DeepSqueak is a USV processing application that uses a pre-trained network to automatically analyse USV files, which is why I have used it to analyse the audio files for further analysis (Coffey et al., 2019). Unfortunately, the self-noise generated by the microphone produced a continual noise line at about 60 kHz that interfered with the call analysis. DeepSqueak did not reliably detect pup calls, as these differ from the type of adult rat call the built-in networks were trained to detect, although it is possible that this was related to the noise in the files. I, therefore, manually processed about 100 files and hand-selected each call in the sonogram, which is a visual representation of the calls (see Figure 3-3). I used the resulting selections to train a new call-detection network for DeepSqueak. However, I used Raven (Center for Conservation Bioacoustics, 2014) as a manual control measure to confirm and—where required—correct DeepSqueak's detections, as the latter does not allow manual call selections. Following this, the selection tables were re-imported into DeepSqueak and exported for further analysis with the RStudio editor (RStudio Team, 2020). The alpha (α) level was set at .05 and a p-value lower than that ($p \leq \alpha$) was considered statistically significant.

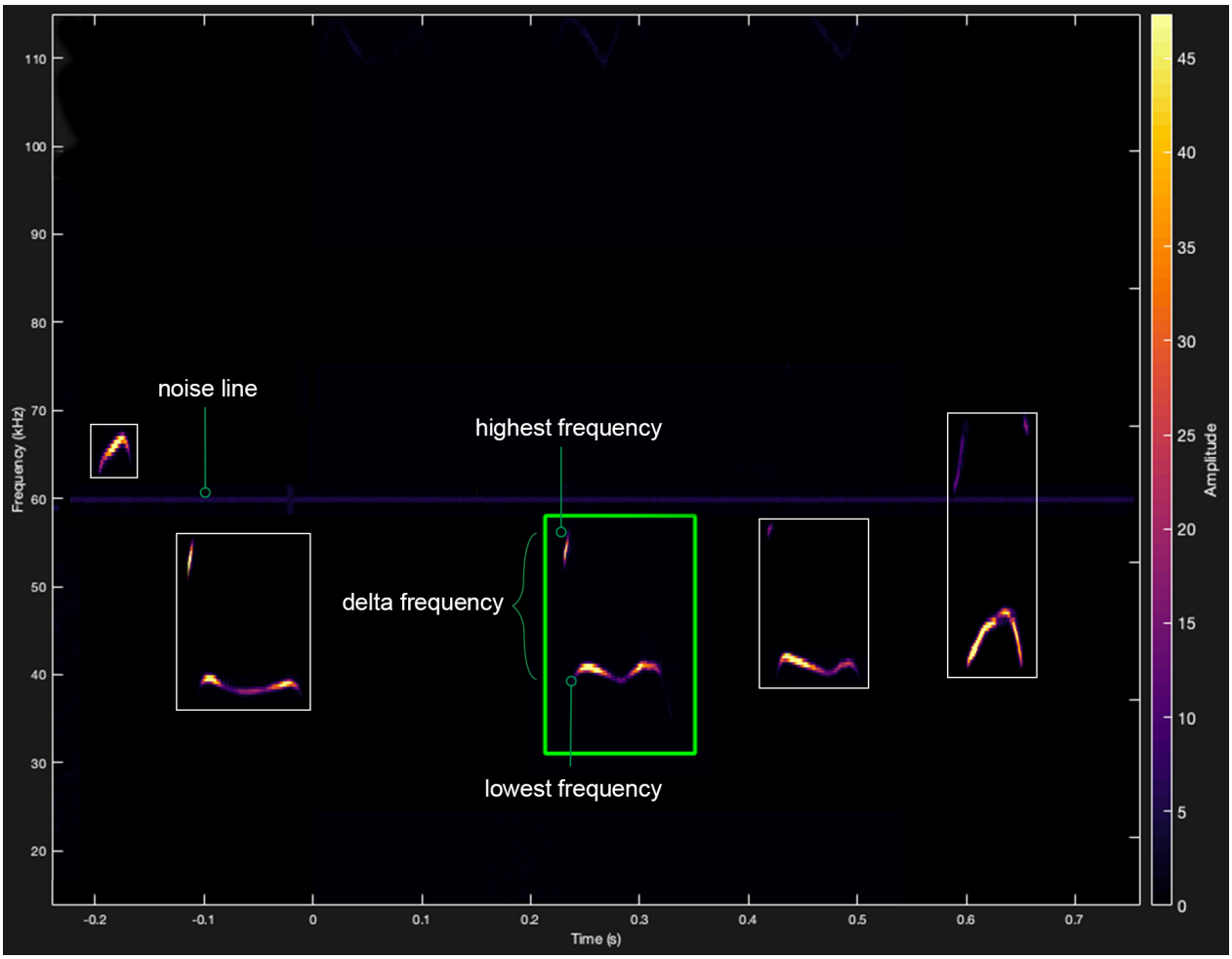
As mentioned in Chapter 1, a lot of animal research has been done with male animals, but as I used both males and females in the present study, I included sex as an independent variable in the models. I followed up significant interaction effects with individual within-group analyses (WRS2 package, function "rmanova") (Mair & Wilcox, 2020). I conducted pairwise comparisons on the individual levels of the treatment variable using the robust function "t1way"; the p-values were adjusted for multiple comparisons using the FDR[7] method.

The independent variables for the PND 7 datasets were treatment (saline, poly I:C) and sex (male, female), and for the PND 14 datasets they were treatment (saline, poly I:C), enrichment (yes/no), and sex (male, female). As not all animals were recorded on both recording days, I created a combined dataset with matched pairs; a total of 91 animals were matched for analysis to compare number of calls, call duration, and delta frequency between recording days. The between-subject variables were treatment (saline, poly I:C) and sex (male, female). This resulted in an unbalanced number of animals per group.

[7] False discovery rate

Figure 3-3

Sonogram of Pup Calls



Note. The visual shows five calls, frequency call features, and the noise line at 60 kHz.

The investigated parameters were the total number of calls, call duration, and delta frequency. As datasets contained outliers and most violated either both or one of the tested assumptions (i.e., normality and/or homogeneity of variance), I proceeded with robust methods, as standard parametric (and non-parametric) analysis options are sensitive to even minor divergence from normality and also susceptible to Type II Error (Field, 2013; Field et al., 2012; Wilcox, 2017). I used two-way and three-way robust ANOVAs ($\hat{\psi}$) for the analyses, all based on a 20% trimmed mean. For the combined PND 7 and PND 14 datasets I also used robust methods, but the downside of this approach is that there is no robust option that can handle two between- and one within-subject variable, so I ran two individual robust two-way mixed repeated-measures ANOVAs, both with PND as the within-subject variable. The dependent variables for the repeated-measures

analyses were treatment (saline/poly I:C) and sex (male/female). Effect sizes were reported with Wilcox and Tian's (2011) explanatory measure of effect size (ξ) for robust ANOVAs ($\hat{\psi}$), where ξ = 0.10, 0.30, and 0.50 stand for small, medium, and large effect sizes.

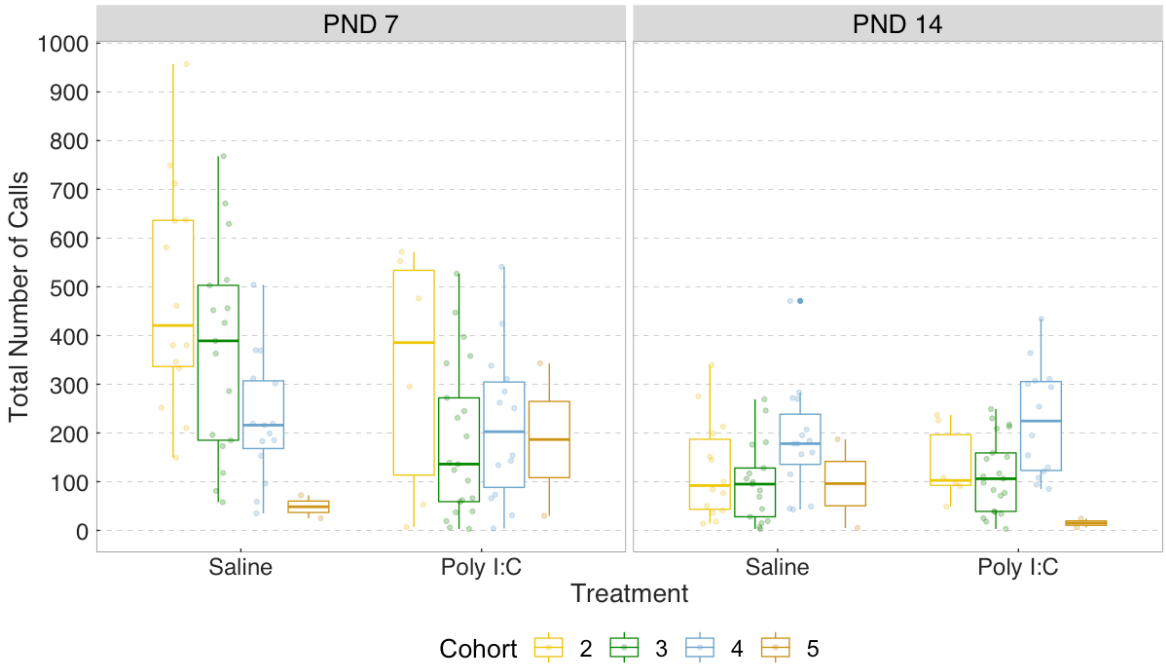
3.4 Results

3.4.1 Number of Calls

The jitter plot in Figure 3-4 shows no clear effect of FBZ on call numbers; there was no difference between the cohorts on either recording day. The spread in cohort 5 is so small because there were only two animals in the saline group on PND 7 and three poly I:C animals on PND 14.

Figure 3-4

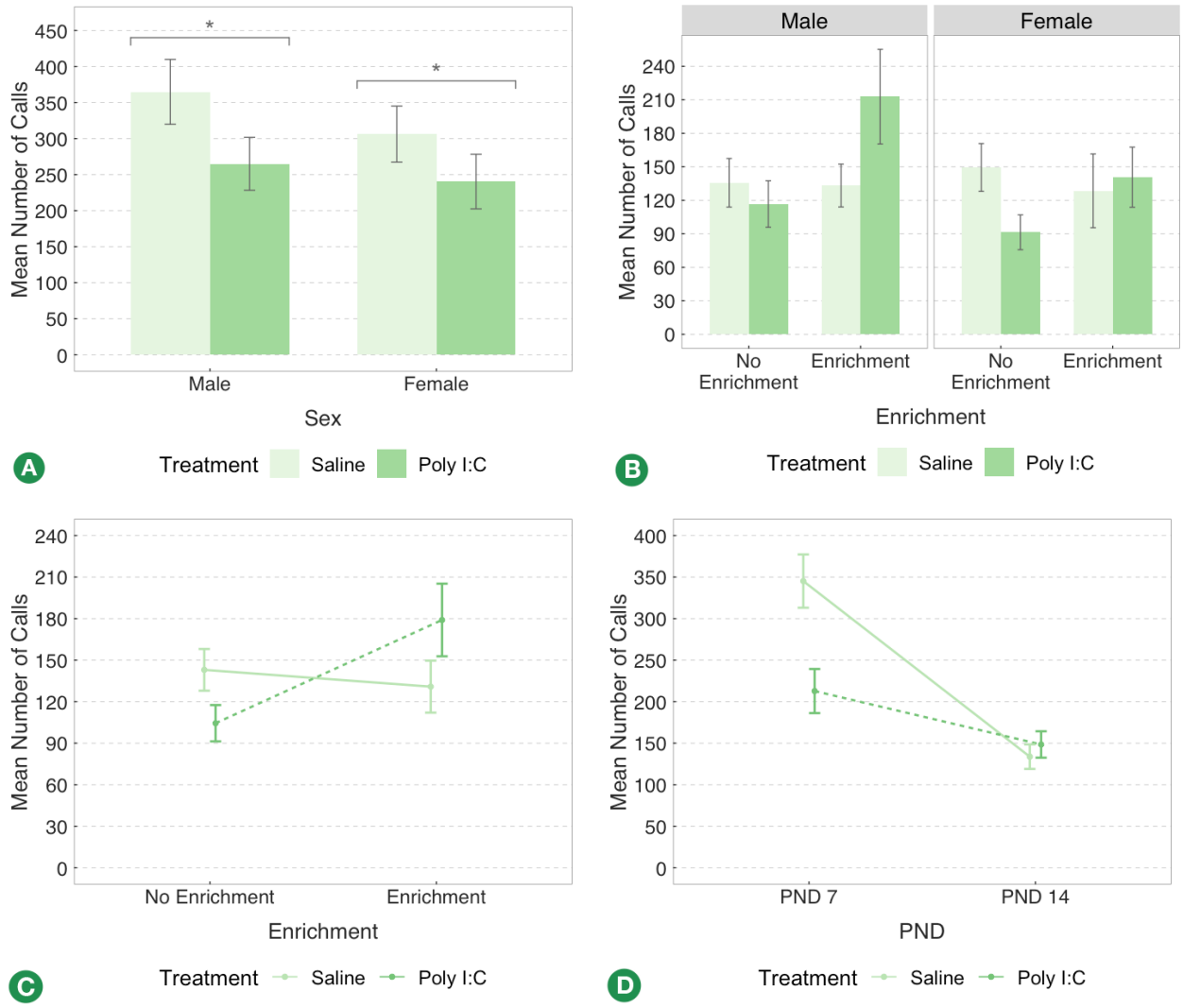
Jitter Plots: Number of Calls by Cohort



PND 7. A robust two-way ANOVA showed that pups prenatally exposed to poly I:C produced significantly fewer calls than pups exposed to saline, $\hat{\psi}(1, 119) = 4.34$, $p = .040$, ξ = 0.25. Sex did not affect call numbers, $\hat{\psi}(1, 121) = 1.10$, $p = .298$, ξ = 0.13. There was no interaction between the two independent variables, $\hat{\psi}(1, 119) = 0.18$, $p = .670$, ξ = 0.17 (Figure 3-5A).

Figure 3-5

Number of Calls



Note. Error bars show mean ± *SEM*. A) PND 7 treatment effect, (B) PND 14, (C) PND 14 treatment x enrichment interaction, and (D) treatment x PND interaction (combined dataset). * $p \leq .05$.

PND 14. A robust three-way ANOVA showed a significant interaction of treatment x enrichment: Poly I:C animals that had received pre-weaning enrichment produced more calls than saline animals but without pre-weaning enrichment, it was the opposite, $\hat{\psi}(1, 105) = 5.10$, $p = .027$, $\xi = 0.34$ (Figure 3-5C). None of the main effects were significant, treatment, $\hat{\psi}(1, 115) = 0.04$, $p = .850$, $\xi = 0.01$, enrichment, $\hat{\psi}(1, 105) = 2.67$, $p = .110$, $\xi = 0.18$, sex, $\hat{\psi}(1, 120) = 1.40$, $p = .241$, $\xi = 0.14$ (Figure 3-5B). There were no other interactions. Descriptive statistics are shown in Appendix A below.

Planned comparisons at each level of treatment showed that poly I:C pups that had received pre-weaning enrichment produced significantly more calls than pups that had not received enrichment, $F(1, 46) = 6.47$, $p = .029$, $\xi = 0.42$. There was no difference between the two enrichment groups in the saline group, $F(1, 59) = 0.25$, $p = .617$, $\xi = 0.09$.

PND 7 vs PND 14. Table 3-2 shows the robust mixed repeated-measures ANOVA results. There was a significant treatment x PND interaction (Figure 3-5D); on PND 7 poly I:C animals produced significantly fewer calls than saline animals, but on PND 14 poly I:C animals produced marginally more calls than saline animals. Also, poly I:C control animals produced significantly fewer calls than saline-exposed animals overall. There were significantly fewer calls on PND 14 than on PND 7.

Table 3-2

Robust ANOVA Results (Number of Calls)

DF	Analysed variable	$\hat{\psi}$	p	ξ
		Treatment x PND		
1, 58	Treatment	5.19	.026 *	0.18
1, 58	PND	21.91	< .001 ***	0.53
1, 58	Treatment x PND	7.49	.008 **	0.40
		Sex x PND		
1, 56	Sex	1.85	.180	0.14
1, 56	PND	19.96	< .001 ***	0.53
1, 56	Sex x PND	0.18	.677	0.16

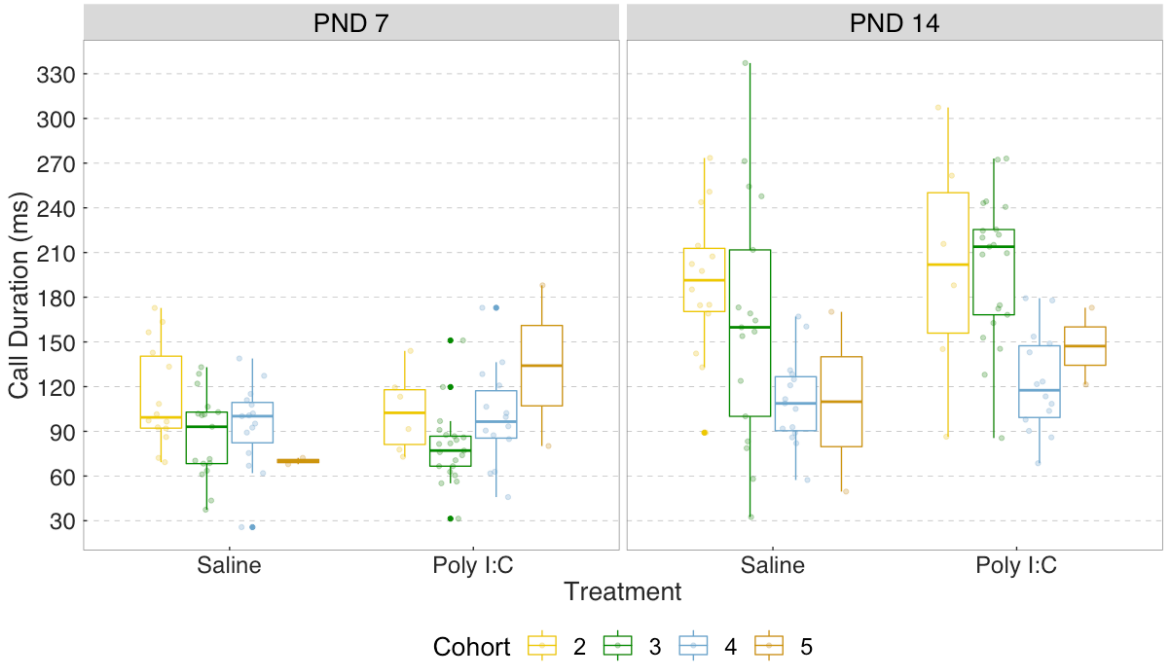
* $p \leq .05$. ** $p \leq .01$. *** $p \leq .001$.

3.4.2 *Call Duration*

There was no obvious effect of FBZ on call duration on either recording day (Figure ***3-6***).

Figure 3-6

Jitter Plots: Call Duration by Cohort



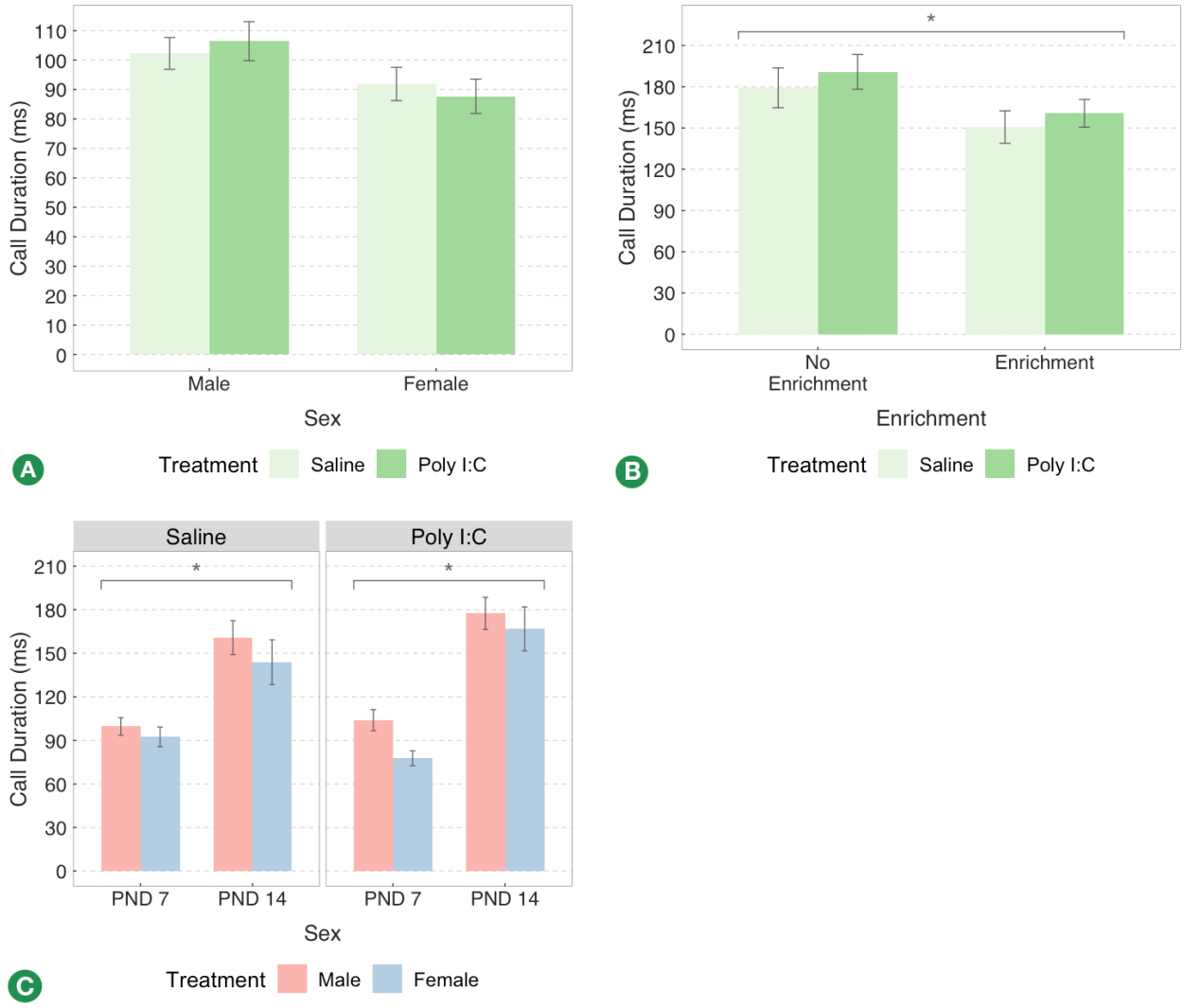
PND 7. A robust two-way ANOVA showed that males produced significantly longer calls than females, $\hat{\psi}(1, 122) = 6.10$, $p = .015$, $\xi = 0.30$, but treatment did not affect call duration, $\hat{\psi}(1, 119) < 0.01$, $p = .997$, $\xi = 0.01$. There was no interaction between the two independent variables, $\hat{\psi}(1, 119) = 0.59$, $p = .480$, $\xi = 0.17$ (Figure 3-7A).

PND 14. A robust three-way ANOVA showed that animals in enrichment produced significantly shorter calls than animals that did not receive pre-weaning enrichment, $\hat{\psi}(1, 113) = 5.70$, $p = .019$, $\xi = 0.30$ (Figure 3-7B). Neither treatment, $\hat{\psi}(1, 119) = 0.73$, $p = .400$, $\xi = 0.11$, nor sex, $\hat{\psi}(1, 118) = 0.60$, $p = .440$, $\xi = 0.10$, affected call duration (Figure 3-7B). There were no interactions. Descriptive statistics are shown in Table A-2.

Planned comparisons at each level of treatment showed no difference between the two enrichment groups for neither poly I:C pups, $F(1, 58) = 3.47$, $p = .133$, $\xi = 0.29$, nor saline pups, $F(1, 60) = 2.32$, $p = .133$, $\xi = 0.26$.

Figure 3-7

Call Duration



Note. Error bars show mean ± *SEM*. (A) PND 07, (B) PND 14, and (C) sex main effect over time. * $p \leq .05$.

PND 7 vs PND 14. Table 3-3 shows the robust mixed repeated-measures ANOVAs. Calls were significantly shorter on PND 7 than on PND 14, but treatment did not affect call duration. Also, male animals produced significantly longer calls than female animals (see Figure 3-7C). There were no interactions between any of the independent variables.

Table 3-3

Robust ANOVA Results (Call Duration)

DF	Analysed variable	$\hat{\psi}$	p	ξ
		Treatment x PND		
1, 58	Treatment	1.16	.286	0.06
1, 58	PND	69.20	< .001 ***	0.82
1, 58	Treatment x PND	3.76	.058	0.17
		Sex x PND		
1, 50	Sex	4.41	.041 *	0.26
1, 53	PND	61.75	< .001 ***	0.82
1, 53	Sex x PND	0.24	.629	0.32

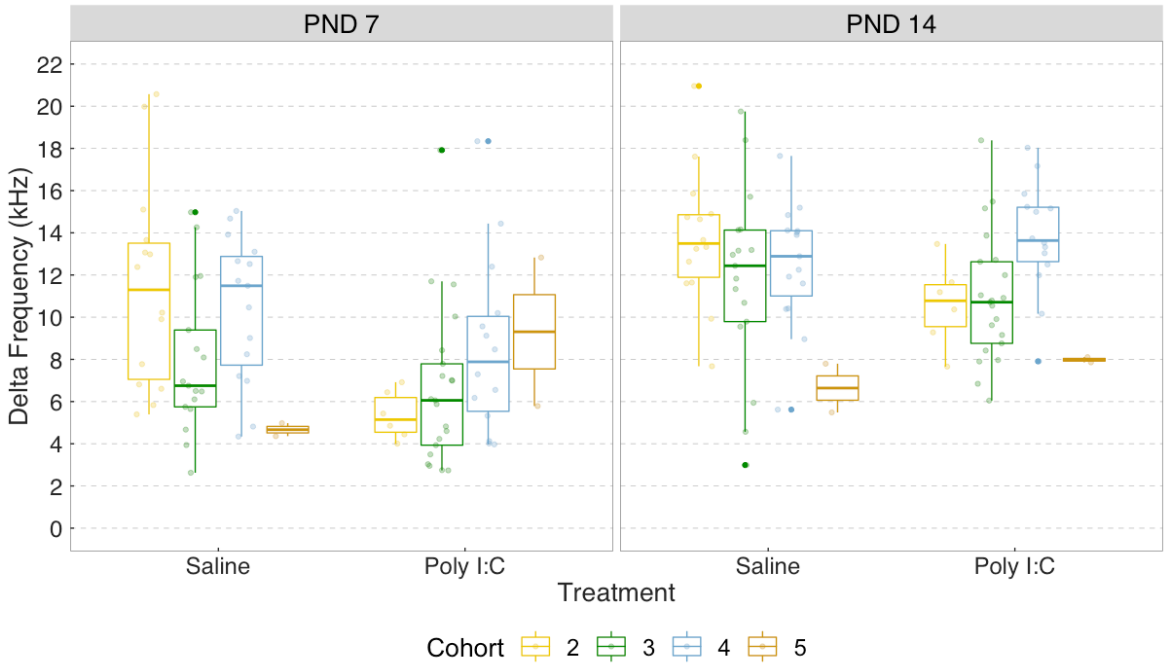
* $p \leq .05$. *** $p \leq .001$.

3.4.3 *Delta Frequency*

There was no clear effect of FBZ on delta frequency on either recording day (Figure ***3-8***). As previously mentioned, cohort 5 consisted of two/three animals on PND 7 and PND 14 respectively, which is why the distribution is so narrow.

Figure 3-8

Jitter Plots: Delta Frequency by Cohort



PND 7. A robust two-way ANOVA showed that saline animals had a significantly greater delta frequency than poly I:C animals, $\hat{\psi}(1, 118) = 8.82$, $p = .004$, $\xi = 0.36$ (Figure ***3-9***A). There was no difference between the two sexes, $\hat{\psi}(1, 121) = 1.36$, $p = .246$, $\xi = 0.15$. There was also no interaction between the two independent variables, $\hat{\psi}(1, 118) = 0.08$, $p = .780$, $\xi = 0.10$.

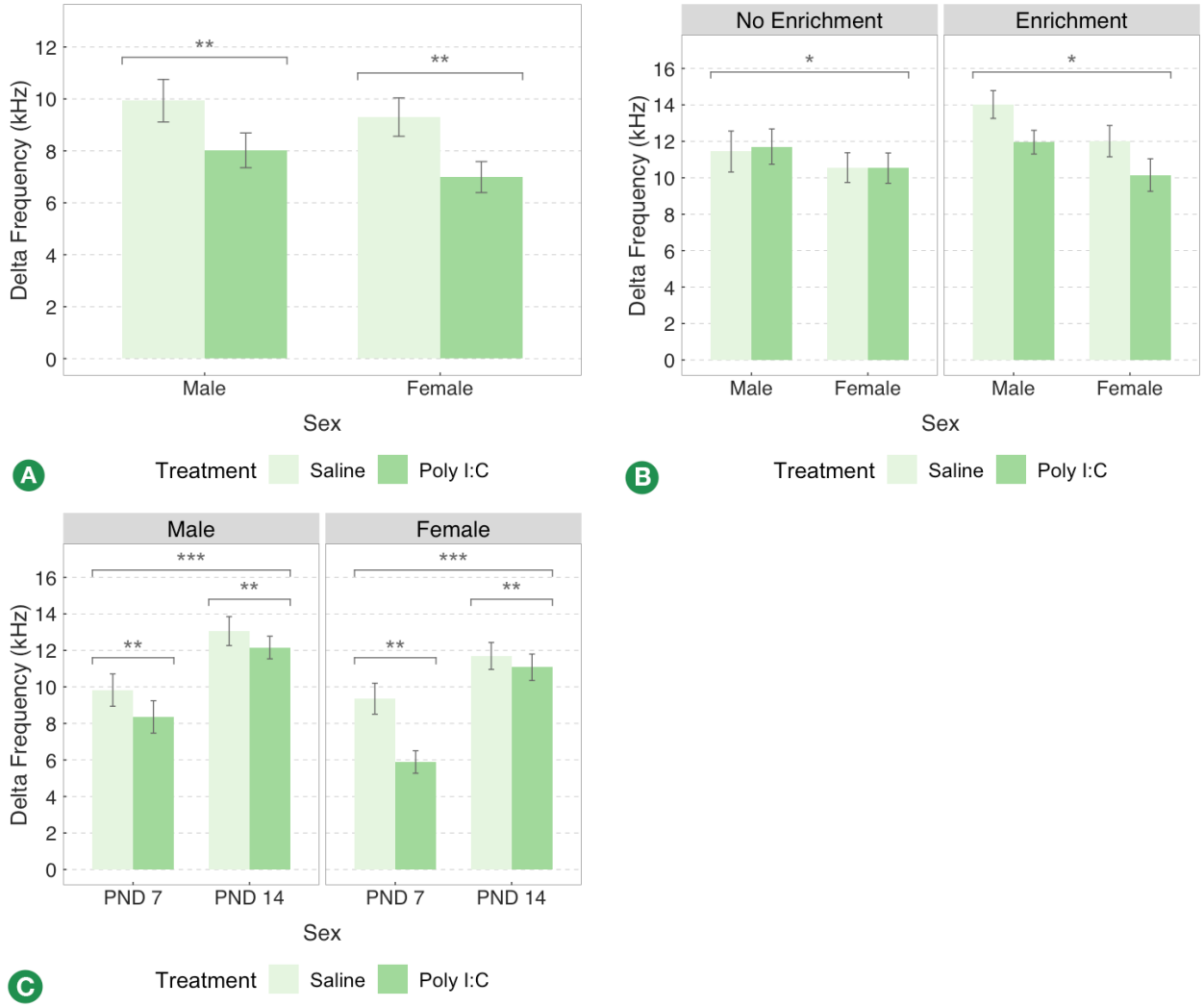
PND 14. A robust three-way ANOVA showed that males had a significantly greater delta frequency than females, $\hat{\psi}(1, 121) = 5.67$, $p = .020$, $\xi = 0.29$ (Figure 3-9B). Neither treatment, $\hat{\psi}(1, 120) = 2.23$, $p = .140$, $\xi = 0.17$, nor enrichment, $\hat{\psi}(1, 119) = 2.49$, $p = .120$, $\xi = 0.20$, affected delta frequency, though. There were no interactions between the independent variables, though treatment x enrichment came close, $\hat{\psi}(1, 119) = 2.85$, $p = .095$, $\xi = 0.20$. Descriptive statistics are shown in Table A-3.

Planned comparisons at each level of treatment showed no difference between the two enrichment groups for poly I:C pups, $F(1, 60) < 0.01$, $p = .971$, $\xi = 0.12$. In the saline group, the difference was no longer significant after adjusting for multiple comparisons, $F(1, 61) = 5.17$, $p = .053$, $\xi = 0.39$.

PND 7 vs PND 14. The robust mixed repeated-measures ANOVAs in Table 3-4 show the analysis results. As expected, delta frequency increased from PND 7 to PND 14, and saline animals had a significantly greater delta frequency than poly I:C-exposed animals (Figure 3-9C). While there were no interactions between the independent variables, treatment x PND came close.

Figure 3-9

Delta Frequency



Note. Error bars show mean ± *SEM*. (A) PND 7 treatment effect, (B) PND 14 sex effect, and (C) PND and treatment effect.

** $p \leq .01$. *** $p \leq .001$.

Table 3-4

Robust ANOVA Results (Delta Frequency)

DF	Analysed variable	$\hat{\psi}$	p	ξ
		Treatment x PND		
1, 58	Treatment	8.48	.005 **	0.28
1, 58	PND	78.55	< .001 ***	0.63
1, 58	Treatment x PND	3.00	.089	0.43
		Sex x PND		
1, 53	Sex	3.00	.089	0.21
1, 55	PND	68.68	< .001 ***	0.63
1, 55	Sex x PND	0.14	.713	0.23

** $p \leq .01$. *** $p \leq .001$.

3.4.4 Results Summary

Table 3-5

Overview of Significant USV Results

Variable	PND	Effect			Significance level	
		Condition #1 [a]	Direction [b]	Condition #2 [a]	Main	X [c]
Number of calls	7	Saline	>	Poly I:C	*	
	14	Saline	=	Poly I:C		*
		No enrichment	=	Enrichment		
	14	PIC/No enrichment	<	PIC/Enrichment	***	
	7, 14	Saline	>	Poly I:C	*	**
		PND 7	>	PND 14	***	
Call duration	7	Males	>	Females	*	
	14	No enrichment	>	Enrichment	*	
	7, 14	Males	>	Females	*	
		PND 7	<	PND 14	***	
Delta frequency	7	Saline	>	Poly I:C	**	
	14	Male	>	Female	*	
	7, 14	Saline	>	Poly I:C	**	
		PND 7	<	PND 14	***	

[a] SAL = saline, PIC = poly I:C. [b] Effect direction is indicated by < (smaller), > (greater), and = (equal). [c] Interaction.

* $p \leq .05$. ** $p \leq .01$. *** $p \leq .001$.

3.5 Discussion

This study investigated the impact of prenatal exposure to the viral mimic poly I:C on rat pups' communication and whether pre-weaning enrichment attenuates the effect. The first hypothesis was that, when compared to saline control animals, pups prenatally exposed to 5 mg/kg poly I:C on PND 15 would produce fewer and shorter calls and spend less time calling overall. Secondly, the communication deficit would be more prominent on PND 7 than on PND 14. The third hypothesis was that pups prenatally exposed to poly I:C that received enrichment would be less affected than those that did not receive enrichment.

Overall, the data supported part of the first hypothesis—pups exposed to poly I:C in utero produced fewer calls than their saline-exposed counterparts on PND 7, which corroborates published results (Chou et al., 2015; Malkova et al., 2012). Contrary to reported findings (Malkova et al., 2012), there was no difference in call duration between treatment groups on either recording day. While all other findings were as anticipated, the lack of significance between treatment groups for call duration was unexpected. It is possible this was a result of outliers and violation of assumptions in the dataset, but as I used robust methods for all analyses (i.e., trimmed means to reduce the impact of outliers), it is not a likely explanation. Another possible explanation is animal type; Malkova et al. (2012) used C57BL/6J mice, while I used SD rats in the present study. Chou et al. (2015) also used SD rat pups, but they did not report total call duration differences. A very recent study that used the same poly I:C treatment (5 mg/kg on GD 15) as was used in the present study also found that poly I:C animals called less but that the call duration did not differ between treatment groups (Potasiewicz et al., 2020). Further, SD rat pups produce fewer calls and spend less time calling than Long Evans and Wistar rats (Schwarting & Wöhr, 2018). Also, in rat pups, the number of calls is more affected by the stress caused by maternal separation than other call features like call duration (Stark et al., 2020). This suggests that call features like call duration might be affected more in some rodent types/strains than others.

The second hypothesis, that the poly I:C-induced deficit would be more prominent on PND 7 than on PND 14 found support in the data as well. While there was a significant treatment effect for the number of calls and delta frequency on PND 7, this effect could not be observed on PND 14 (Figure 3-5D). The reduction of calls in the poly I:C group could be due to reduced emotional response to maternal separation (Malkova et al., 2012). From

PND 14 on, rat pups start exploring their environment more independently and subsequently produce fewer calls (Stark et al., 2020). Environmental influences between PND 7 and PND 14, together with the developmental trajectory might have been the reason for the waning of the poly I:C effect.

Finally, as poly I:C did not show an effect on PND 14, the data did not support the third hypothesis that poly I:C-exposed pups that received pre-weaning enrichment would be less affected by poly I:C than poly I:C pups that did not receive enrichment. Following pre-weaning enrichment, the pups also emitted shorter calls relative to controls, which is interesting, seeing Noirot (1972) reported that upon reunion with their mother, the pups produced several long USV that resulted in maternal attention and licking, whereas the pups that had remained with the dam emitted short audible calls. While little is known about the meaning of rodent communication, this difference in call duration could be the expression of a closer relationship with their mother due to the extra time she spent with them after their enrichment sessions.

In addition to call numbers, call duration, and delta frequency—an acoustic call feature that provides further information about call complexity—was assessed. Delta frequency generally increases with age (Brudzynski et al., 1999), which it also did in the present study. Further, on PND 7, it was greater in the saline group than in the poly I:C condition, indicating less call complexity in the latter group. As pups get older and more independent, they develop more adult-like behaviours, including more adult-like call complexity (Stark et al., 2020).

In terms of sex differences, on PND 7, males' calls were longer than those of females, but there was no difference on PND 14. Call numbers also did not differ between the sexes. Several studies found no difference in call numbers between sexes (Hahn & Lavooy, 2005), but others reported that males called more than females (Bowers et al., 2013). One explanation for this is related to litter-sex ratio. Naito and Tonoue (1987) found that males produced more USV in mixed-sex litters than in predominantly male litters, so sex differences may differ depending on the litter-sex ratio in the examined sample.

In recent years more and more research has looked at the qualitative call features in addition to quantitative parameters like call numbers, duration, and frequency range. In a seminal paper on pup call classification by Brudzynski et al. (1999), they described 10 distinct sonographic call shapes. Further, poly I:C-induced communication deficits include call repertoire differences, including fewer harmonic calls, more short calls, and more complex call shapes than saline controls (Malkova et al., 2012). I intended to identify

qualitative call differences between treatment groups in addition to the standard quantitative call features discussed in this chapter and have undertaken a large amount of call classification and network training work to classify the calls by shape. Unfortunately, due to technical limitations posed by DeepSqueak—mainly the fact that the settings for call classification cannot be adjusted on a per-audio-file-basis—this part of the study had to be deferred until the next big DeepSqueak release.

3.6 Summary

This study showed that poly I:C exposure in utero changed quantitative measures of pup calls following separation from their mother early during development. These animals produced fewer calls and had a narrower frequency bandwidth than their saline conspecifics. This transient poly I:C effect could not be detected on PND 14, though. While enrichment interacted with treatment, we cannot speak of an improvement of poly I:C related deficits, as there was no overall poly I:C effect. Future research could record USV daily up to PND 20 to investigate how pre-weaning enrichment alters the developmental trajectory. Chapter 4 will continue to study the key symptoms of ASD, moving from communication to general sociability and social novelty behaviour.

Chapter 4

Social Approach–Avoidance

Chapter 4
Social Approach–Avoidance Behaviours

4.1 Background

This chapter explores the effect of maternal poly I:C treatment on general sociability and preference for social novelty in the offspring. The study assessed these behaviours in adolescence and adulthood to see if either poly I:C or environmental enrichment—or both—impacted on behavioural expression over time. One of the markers of ASD is atypical social approach behaviour, including abnormal eye contact, facial expressions, body language, social and emotional reciprocity, difficulties with emotional perception, and misreading of social cues. Also, autistic adults tend to struggle with social and environmental novelty (American Psychiatric Association, 2013). One of the most well-known tools to evaluate social interaction in rodents is the Social Approach–Avoidance (SAA) test developed by Moy et al. (2004). It measures general sociability and response to social novelty in rodents.

The test comprises three phases:

1. Habituation: The rat spends 10 minutes alone in an arena with three interconnected chambers.
2. General Sociability: A stranger rat is placed under a wire-mesh cylinder on one side of the arena, while an identical unoccupied cylinder—a novel non-social object—is located at the other end. Through the wire-mesh, the animals can see, hear, and smell each other but not physically interact (Crawley, 2004). The test animal can freely explore each of the three chambers for 10 minutes. In untreated animals, research has repeatedly demonstrated a strong preference for a social stimulus over a non-social one (Moy et al., 2004; Moy et al., 2008).
3. Social Novelty: A new stranger rat is placed under a cylinder on one side, while the now-familiar stimulus rat from phase #2 is located on the other side. The test rat can move freely in the arena for another 10 minutes. Untreated animals will typically spend more time with the novel compared to the known social stimulus (Moy et al., 2004; Moy et al., 2008).

The SAA paradigm is highly validated, as it has been utilised extensively in rodent research using the poly I:C model to display the deficient social interaction characteristic of autism (Crawley, 2007). Several studies used the SAA model with rodents prenatally

exposed to poly I:C and found that these animals spent less time with the social stimulus than did control animals (Chen et al., 2019; Lins et al., 2018; Lins et al., 2019; Malkova et al., 2012; Smith et al., 2007). The only study that also included social novelty behaviour reported that the poly I:C group did not prefer the novel over the familiar rat (Chen et al., 2019). The poly I:C effect was relatively robust across differing factors such as poly I:C dose, gestational administration day, rodent type (i.e., rats or mice), sex (i.e., males and females), as well as animal age (i.e., adolescents and adults).

Various authors have explored how rodent social interaction is affected by environmental enrichment, but this has not yet been researched in the context of ASD. An interesting experiment showed that pre-weaning maternal deprivation impacted negatively on sociability and that exposure to classical music for 12 hours per day reversed this effect (Papadakakis et al., 2019). The maternally deprived rats in the music condition spent significantly more time with the social stimulus than animals in the ambient noise group, highlighting the positive effect auditory enrichment had on general sociability. The study did not include phase #3 (social novelty behaviour), but Kentrop et al. (2018) found that enrichment did not improve the negative effects resulting from pre-weaning maternal deprivation.

Further, Rae et al. (2018) and Zheng et al. (2020) reported that mice that lived in an enriched environment spent more time with the social stimulus than did control animals, showing that enrichment can lead to improved sociability. By contrast, Hendershott et al. (2016) did not find an effect on preference for the social over the non-social stimulus in mice housed in enrichment post weaning. However, because their enrichment protocol consisted of common laboratory items like pipette tips and sample tubes rather than a commonly used multisensory stimulatory setup with running wheels, tunnels, and toys, the environment may not have been enriched enough to instigate behavioural changes.

4.2 Predictions

While the SAA paradigm has been used with rodents in the context of poly I:C as well as environmental enrichment individually, to date, no study has explored the effect of environmental enrichment on general sociability and response to social novelty in a poly I:C rat model. Therefore, this study will investigate the following hypotheses:

1. Standard-housed poly I:C animals will show less general sociability and a lower preference for social novelty than control animals. When compared to control animals, they will spend less time with the social stimulus in phase #2 and with the novel social stimulus in phase #3.
2. Enrichment will reverse this effect. Poly I:C animals in enriched housing will spend more time exploring the social stimulus in phase #2 and the novel social stimulus in phase #3, showing higher sociability and a greater preference for social novelty than poly I:C animals in standard housing.

4.3 Methods

4.3.1 Animals

All animals used in this experiment were handled at least five times before assessment; they were tested in five cohorts and divided into four groups: (1) SH/SAL, (2) SH/poly I:C, (3) EH/ saline, and (4) EH/poly I:C. Table 4-1 shows the sample size for each group. The offspring came from 16 litters in the saline and 18 in the poly I:C treatment group. As discussed in section 2.4.2, pups of cohort 1 were affected by the FBZ treatment for 9 days in utero. Dams and pups of cohort 5 were exposed to FBZ for between 7 and 14 days. Also, all adults in cohort 4 received the FBZ-medicated feed for a total of 35 days.

Table 4-1

Total Number of Tested Animals by Cohort (SAA)

Cohort	Treatment	Housing	Males	Females	Total
			Pups *		
#1	Saline	Standard	1	1	2
		Enriched	0	0	0
	Poly I:C	Standard	3	3	6
		Enriched	0	0	0
#2	Saline	Standard	4	4	8
		Enriched	1	1	2
	Poly I:C	Standard	1	0	1
		Enriched	3	3	6
#3	Saline	Standard	4	5	9
		Enriched	5	5	10
	Poly I:C	Standard	3	4	7
		Enriched	5	6	11
#4	Saline	Standard	4	3	7
		Enriched	4	4	8
	Poly I:C	Standard	6	6	12
		Enriched	3	2	5
#5	Saline	Standard	0	0	0
		Enriched	3	3	6
	Poly I:C	Standard	0	0	0
		Enriched	2	2	4
Total			52 (13/39)	52 (12/40)	104 (25/79)
			Adults		
#1	Saline	Standard	2	2	4
		Enriched	0	0	0
	Poly I:C	Standard	6	6	12
		Enriched	0	0	0
#2	Saline	Standard	5	5	10
		Enriched	5	5	10
	Poly I:C	Standard	3	3	6
		Enriched	3	3	6
#3	Saline	Standard	6	6	12
		Enriched	5	5	10
	Poly I:C	Standard	4	4	8
		Enriched	5	5	10
#4	Saline	Standard	0	0	0
		Enriched	3	3	6
	Poly I:C	Standard	0	0	0
		Enriched	5	5	10
Total			52	52	104

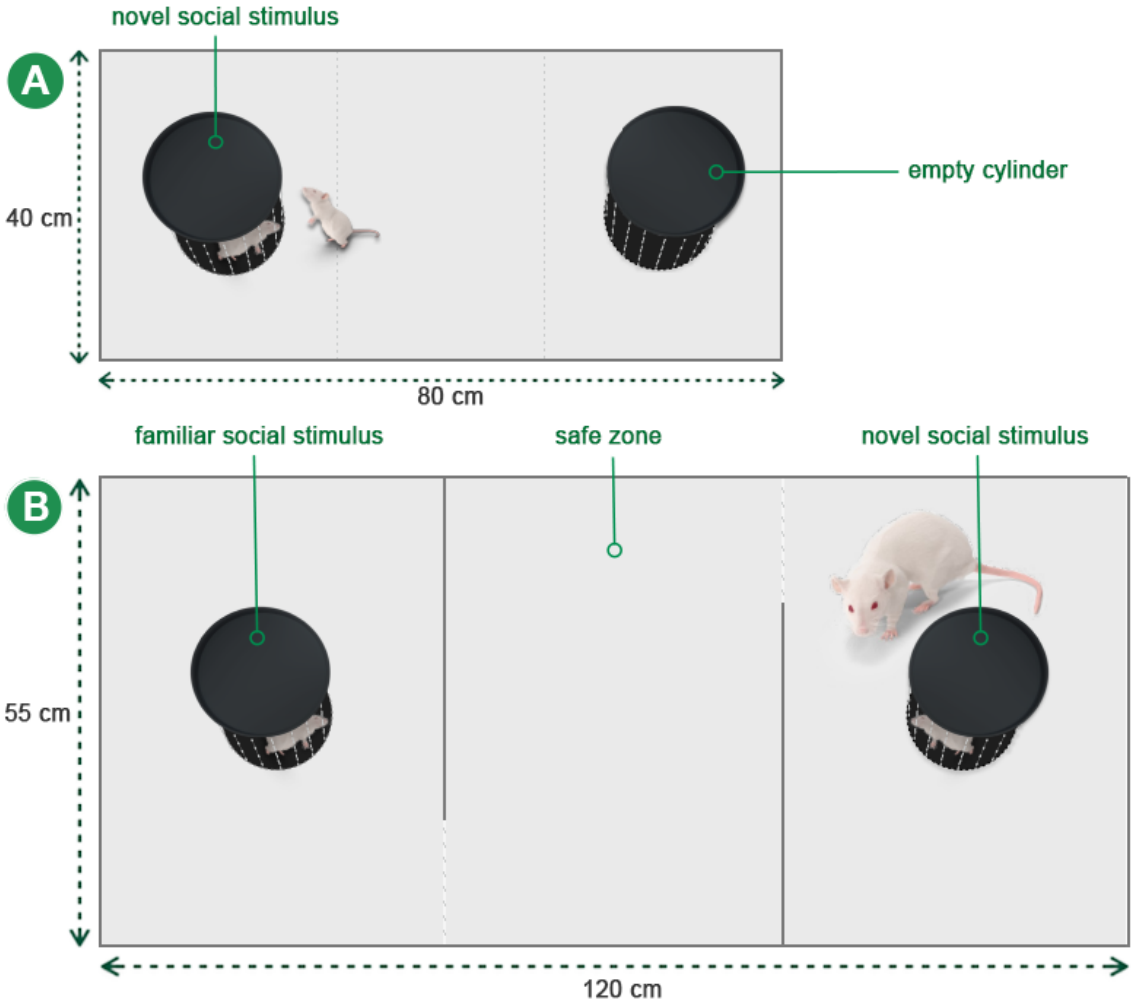
* The arena setup was changed after cohort 2 (see Figure 4-1). Numbers in brackets show the group size for cohorts 1-2/3-5.

4.3.2 *Apparatus*

The pups in the first two cohorts were tested in a smaller arena without internal chamber dividers, as shown in Figure 4-1A. It measured 80 cm (length) x 40 cm (width) x 40 cm (height). Unpublished research from our laboratory group found that a lack of individual chambers made the choice for the social vs non-social side less deliberate and also removed the option of a "safe zone" in the centre chamber. Therefore, I changed the setup to a three-chamber arrangement that was used for pups of cohorts 3 to 5 and all adults (see Figure 4-1B).

Figure 4-1

Social Approach–Avoidance Testing Arenas



Note. (A) The smaller arena used with pups of cohorts 1 and 2. (B) The larger arena used for all other animals.

The larger arena measured 120 cm (length) x 55 cm (width) x 40 cm (height) and featured black internal polycarbonate dividers (40 cm x 40 cm), creating three distinct chambers. This forced the experimental animal to deliberately choose which side to visit. Two identical wire mesh cylinders (diameter = 10 cm, height = 12.5 cm) were placed on each side and weighed down to prevent accidental movement.

The testing arena was setup on a black rubber mat above which a high-definition camera (Logitech C930) was mounted to a ceiling rig. Some adult animals jumped out of the arena in which case the arena was covered with multiple perforated transparent polycarbonate sheets (50 cm x 50 cm). Phase #2 and #3 were recorded with Ethovision (XT9) (Noldus et al., 2001). Between sessions, both the arena and the wire cylinders were wiped with F10 disinfectant and disposable paper towels. Sex- and strain-matched juvenile stimulus animals aged between 21 and 28 days were selected from different unrelated litters.

4.3.3 Procedure

All sessions were run during the animals' dark phase under red light. On each testing day, 12 animals were tested in four sets of three rats (2 hours per block, 8 hours per day, 138 hours of testing in total). The pups were tested between PND 23 and PND 28 and the adults between PND 83 and PND 111. Test and stimulus animals were transported to the experimental room in their home cages approximately 10 minutes before the test session. Each test phase ran for 10 minutes (a total of 30 minutes per animal). After the initial environmental habituation period, phase #1—arena habituation—commenced where the test animal explored the arena alone to get to know the testing space.

At the end of the arena habituation, the animal was returned to its home cage while a juvenile stimulus animal (rat 1) was placed under one of the wire cylinders in preparation for phase #2 (general sociability). The side of the social stimulus was randomly assigned and varied between test animals (random numbers were generated with Microsoft Excel 365). This ensured that experimental animals did not develop a preference for a particular side. Phase #2 preparation took an average of 2 minutes. The test rat was placed in the centre of the arena and allowed to explore the two sides (phase #2). Figure 2-3A shows phase #2 with the rat on the left side and an empty cup on the right.

Following the general sociability phase #2, the experimental animal was returned to its home cage for a 30-minute inter-trial interval. Phase #3 involved testing the response to

social novelty. The now-familiar social stimulus from phase #2 was placed under the same cylinder as before and a novel social stimulus (rat 2) went under the cylinder at the opposite side (see Figure 2-3B). Once again, the experimental animal was returned to the arena where it could interact with both the familiar and novel social stimulus for 10 minutes. After phase #3 all animals were returned to the housing room in their respective home cages.

4.3.4 Data and Statistical Analysis

I used the latest version of Ethovision (XT15) to analyse the video files (Noldus et al., 2001) and the RStudio editor for data analysis (RStudio Team, 2020). The alpha (α) level was set at .05 and a *p*-value lower than that ($p \leq \alpha$) considered statistically significant.

As mentioned in the general introduction in Chapter 1, a lot of animal research has been done with male animals (Clayton & Collins, 2014), but I used both males and females in the present study. To allow comparability with existing research, I analysed males and females separately. I used jitter plots showing the datapoints by cohort to confirm the absence of FBZ effects. The data can be treated as independent, as all animals were tested individually.

Error bars in the bar charts always show ± standard error of the mean (*SEM*). The ANOVA assumptions of normality and homogeneity of variance were checked for each of the datasets. Normality was confirmed through a non-significant Shapiro Wilk test and visual examination of both a Q-Q plot and histogram of residuals, homogeneity of variance was confirmed with a non-significant Levene test.

To investigate the level of general sociability and social novelty behaviour, I used a preference ratio calculated as (time $_{\text{social}}$/[time $_{\text{social}}$ + time $_{\text{non-social}}$] x 100) for phase #2, and (time $_{\text{novel social}}$/[time $_{\text{novel social}}$ + time $_{\text{familiar social}}$] x 100) for phase #3. To ensure compliance with the assumption of independence of the observations, which Brodkin et al. (2004) questioned for the SAA paradigm, the third area—the centre—was not included in the analysis. Effect sizes were reported with Wilcox and Tian's (2011) explanatory measure of effect size (ξ) for robust ANOVAs ($\hat{\psi}$), where ξ = 0.10, 0.30, and 0.50 stand for small, medium, and large effect sizes, and eta squared (η^2) for *F*-tests, where η^2 = 0.01, 0.06, and 0.14 indicate small, medium, and large effect sizes.

I analysed the side-preference datasets with two-way ANOVAs (*F*), except, where assumptions were violated, I used robust two-way ANOVAs ($\hat{\psi}$). The independent

variables were treatment and housing. I used one-sample Wilcoxon signed-rank tests (R function "wilcox.test" from the "stats" package) on the preference ratio with a population mean *mu* = 50 to investigate whether the respective groups displayed a preference for the social/novel social stimulus over the non-social/familiar stimulus (phase #2/3) respectively. I chose this test because some of the datasets were heteroscedastic and non-normally distributed.

For the pups' locomotor activity (LMA) analysis, I separated the pups' dataset for analysis, as comparison of the two datasets showed that cohorts 1 and 2 (dataset 1) covered less distance on average than cohorts 3 to 5 (dataset 2), which is not surprising as they were tested in smaller experimental chambers. The sample size of the two datasets was small and further subdividing by sex was likely to affect experimental power. Therefore, I did not separate the two data-subsets by sex. As the group sizes were small in cohorts 1 and 2 (Table 4-1) and these two cohorts were tested in a smaller experimental arena, cohorts 1 and 2 have been excluded from further analysis. I analysed dataset 2 and the adults' datasets with robust two-way ANOVAs ($\hat{\psi}$) whenever assumptions had been violated.

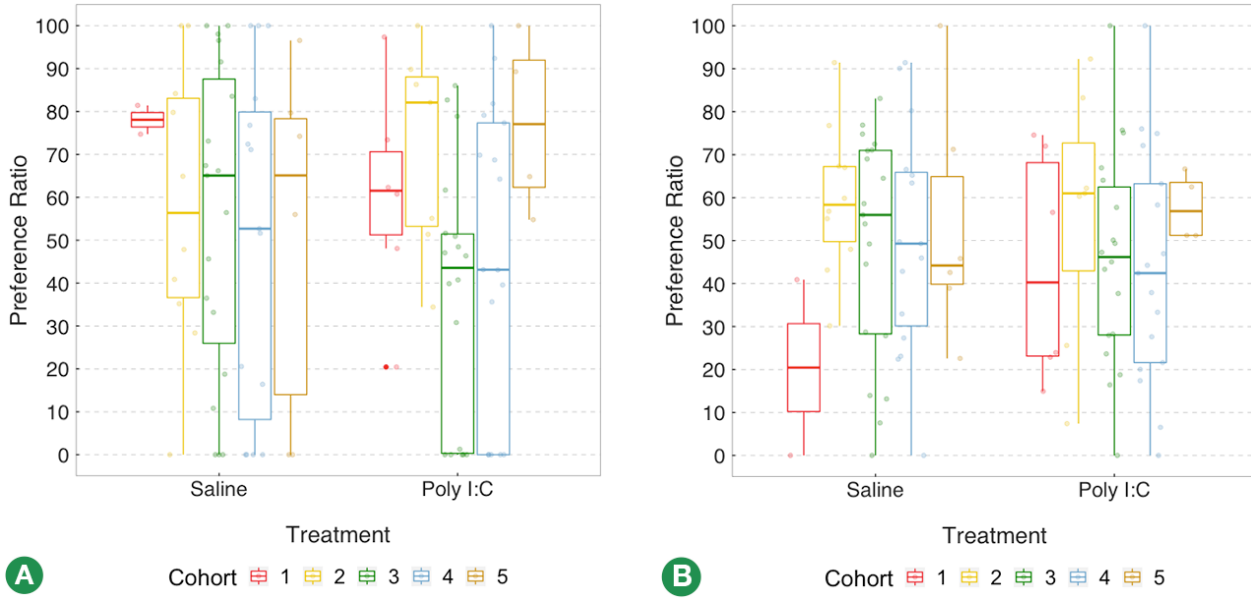
4.4 Results: General Sociability and Social Novelty

4.4.1 Pups

Figure 4-2 shows that FBZ did not affect the preference ratio in pups. It also shows that the change of arenas after cohort 2 did not affect the preference ratio results, which is why I analysed the pups as one dataset. Also, the saline group in cohort 1 comprised of only two animals and such a small group is unlikely to reveal a true effect. Another point to make is that the jitter plots show that arena size made no difference in terms of preference.

Figure 4-2

Jitter Plots: Preference Ratio by Cohort (Pups)



Note. (A) Phase #2 and (B) phase #3.

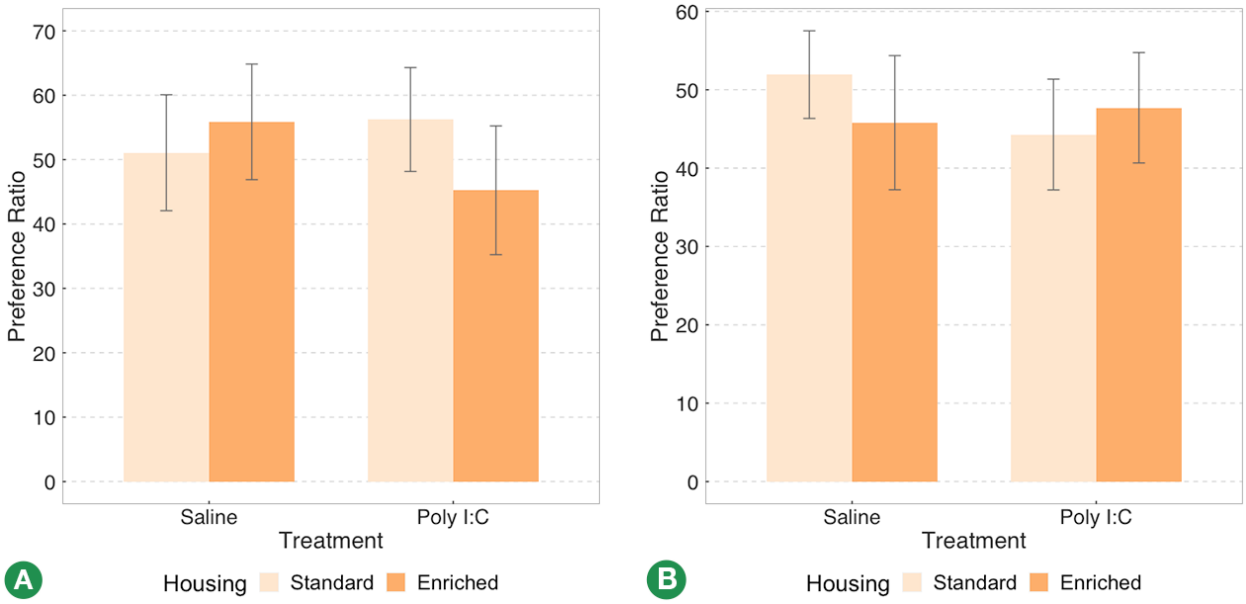
Surprisingly, Wilcoxon signed-rank tests showed that none of the groups preferred the social stimulus in phase #2 or the novel social stimulus in phase #3 (Table 4-2). This makes interpretation of the results difficult, as the control group usually shows a preference for the social/novel social stimulus over the non-social/familiar social stimulus.

Males Phase #2. A robust two-way ANOVA found no significant effect of treatment, $\hat{\psi}(1, 29) = 0.18$, $p = .673$, $\xi = 0.05$, or housing, $\hat{\psi}(1, 26) = 0.06$, $p = .813$, $\xi = 0.02$, on preference ratio (see Figure 4-3A). There was also no significant interaction between the independent variables (treatment x housing), $\hat{\psi}(1, 26) = 0.71$, $p = .407$, $\xi = 0.10$.

Males Phase #3. The two-way ANOVA found no significant effect of treatment, $F(1, 48) = 0.36$, $p = .554$, $\eta^2 = 0.01$, or housing, $F(1, 48) = 0.83$, $p = .366$, $\eta^2 = 0.02$, on preference ratio for males, as can be seen in see Figure 4-3B. There was no interaction between the independent variables, $F(1, 48) = 0.45$, $p = .507$, $\eta^2 = 0.01$. Descriptive statistics for both phases are shown in Table B-1.

Figure 4-3

Mean Preference Ratio (Male Pups)



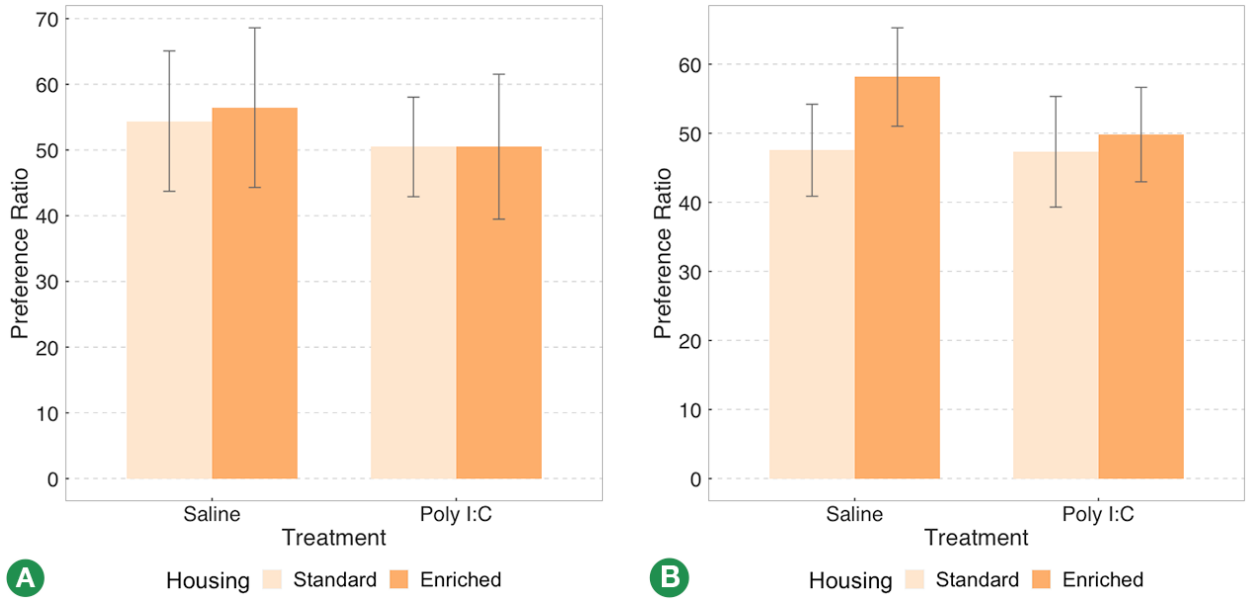
Note. Error bars show mean ± *SEM*. (A) phase #2 and (B) phase #3.

Females Phase #2. A robust two-way ANOVA found no significant effect of treatment, $\hat{\psi}(1, 28) = 0.13$, $p = .719$, $\xi = 0.08$, or housing, $\hat{\psi}(1, 24) < 0.01$, $p = .977$, $\xi = 0.02$, on preference ratio for females. There was also no significant interaction between the independent variables (treatment x housing), $\hat{\psi}(1, 24) = 0.06$, $p = .814$, $\xi = 0.03$ (see Figure **4-4**A).

Females Phase #3. The two-way ANOVA found no significant effect of treatment, $F(1, 48) = 0.16$, $p = .690$, $\eta^2 < 0.01$, or housing, $F(1, 48) = 0.04$, $p = .850$, $\eta^2 < 0.01$, on preference ratio for females, as can be seen in Figure 4-4B. There was no interaction between the independent variables (treatment x housing), $F(1, 48) = 0.32$, $p = .575$, $\eta^2 = 0.01$. Descriptive statistics for both phases are shown in Table B-1.

Figure 4-4

Mean Preference Ratio (Female Pups)



Note. Error bars show mean ± *SEM*. (A) phase #2 and (B) phase #3.

Table 4-2

Wilcoxon Signed-rank Test Results for Preference Ratio (Pups)

Phase	Treatment	Housing [a]	*V* [b]	*p*	*Med* [c]	95% *CI*
			Males			
2	Saline	Standard	48.00	.889	52.15	30.78, 72.92
		Enriched	56.00	.484	59.38	33.29, 75.82
	Poly I:C	Standard	57.00	.455	56.61	35.68, 74.15
		Enriched	37.00	.575	43.15	19.94, 70.54
3	Saline	Standard	47.00	.946	50.53	38.75, 64.95
		Enriched	38.00	.625	45.71	24.61, 67.00
	Poly I:C	Standard	36.00	.542	45.46	28.23, 61.06
		Enriched	43.00	.893	49.11	33.82, 62.18
			Females			
2	Saline	Standard	51.00	.725	51.77	30.71, 82.49
		Enriched	50.50	.751	53.69	32.45, 89.85
	Poly I:C	Standard	51.00	.727	52.37	30.84, 67.53
		Enriched	43.00	.888	50.00	24.23, 75.69
3	Saline	Standard	41.00	.787	49.30	31.43, 63.20
		Enriched	60.00	.340	59.61	42.59, 71.98
	Poly I:C	Standard	39.00	.685	47.27	27.48, 66.67
		Enriched	44.00	.946	49.24	34.46, 66.67

Note. The alternative hypothesis was that the true location differs from 50, which would imply a preference for the social/novel social stimulus over the non-social/familiar social stimulus.
[a] $n = 13$ for each group. [b] Preference ratio. [c] Pseudo median provided by the R function "wilcox.test".

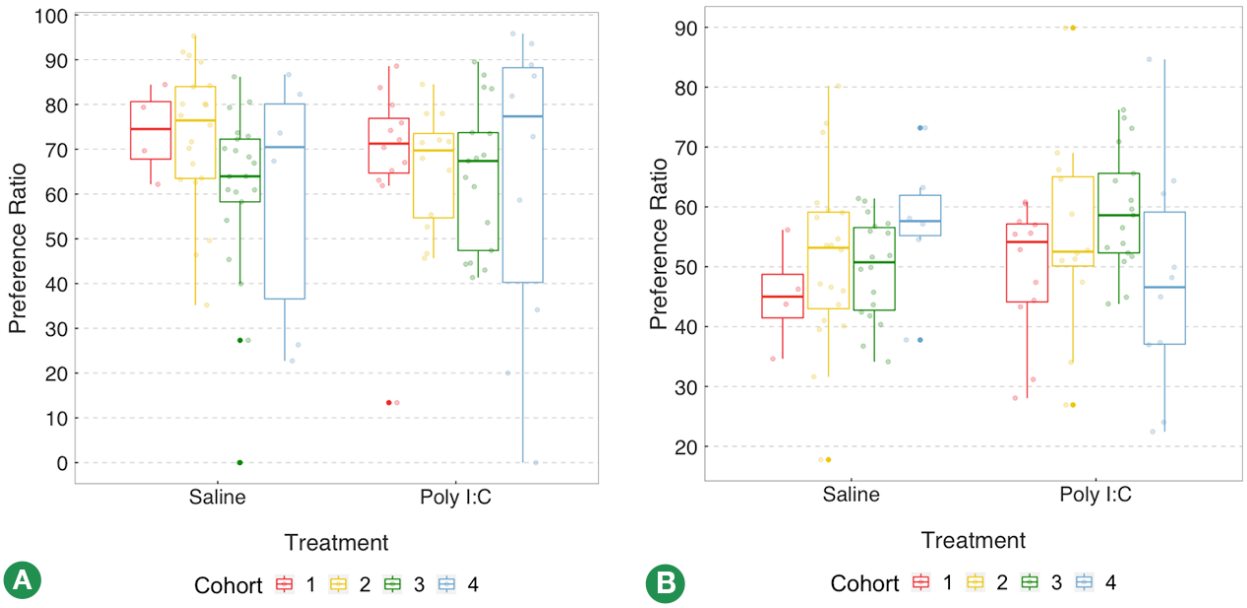
4.4.2 *Adults*

A total of seven rats from the enriched housing condition were excluded, as they climbed the sidewalls and showed no interest in the stimuli. This left 102 and 99 animals in phase #2/#3 respectively for statistical analysis.

While there was a lot of within-group variability, visual examination of the graphs in Figure 4-5 shows that there was no difference between the groups. This confirms that FBZ did not affect the preference ratio in adult animals.

Figure 4-5

Jitter Plots: Preference Ratio by Cohort (Adults)



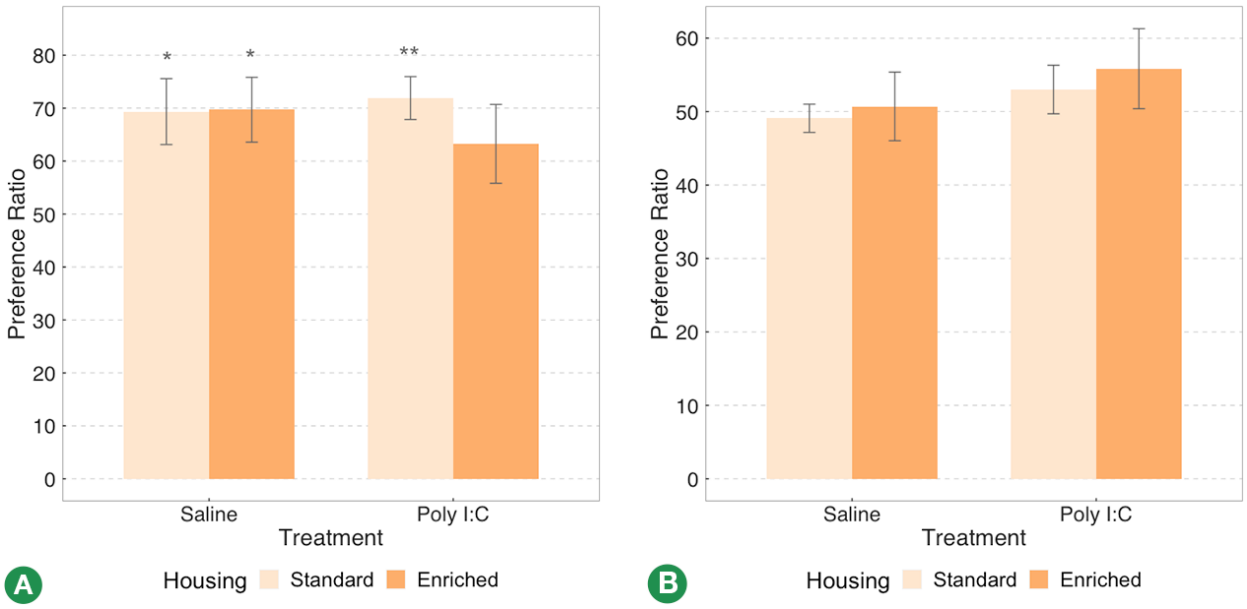
Note. (A) Phase #2 and (B) phase #3.

Males Phase #2. A robust two-way ANOVA found no significant effect of treatment, $\hat{\psi}(1, 26) = 0.55$, $p = .468$, $\xi = 0.10$, or housing, $\hat{\psi}(1, 23) = 0.41$, $p = .526$, $\xi = 0.14$, on preference ratio (see Figure 4-6A). There was also no significant interaction between the independent variables, $\hat{\psi}(1, 23) = 0.33$, $p = .572$, $\xi = 0.05$. A Wilcoxon signed-rank test found a significant preference for the social stimulus for control animals in standard ($V = 78.00$, $p = .022$) and enriched housing ($V = 78.00$, $p = .022$), as well as for animals prenatally exposed to poly I:C in standard housing ($V = 88.00$, $p = .001$). Table 4-3 shows the results for all groups.

Males Phase #3. A robust two-way ANOVA found no significant effect of treatment, $\hat{\psi}(1, 27) = 0.52$, $p = .480$, $\xi = 0.18$, or housing, $\hat{\psi}(1, 19) < 0.01$, $p = .982$, $\xi = 0.03$, on preference ratio (Figure 4-6B). There was also no significant interaction between the independent variables (treatment x housing), $\hat{\psi}(1, 19) = 0.32$, $p = .581$, $\xi = 0.50$. None of the groups showed a significant preference for either of the stimuli, as the Wilcoxon signed-rank test results in Table 4-3 show. Descriptive statistics for both phases are shown in Table B-2.

Figure 4-6

Mean Preference Ratio (Male Adults)



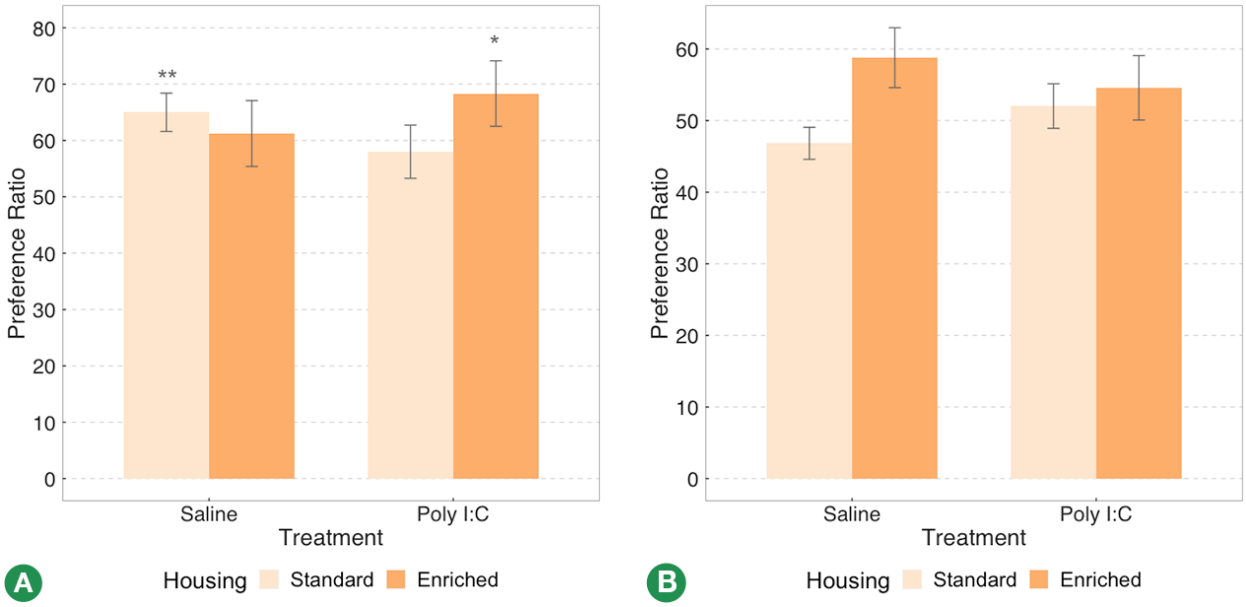
Note. Error bars show mean ± *SEM*. (A) Phase #2 and (B) phase #3.
* $p \leq .05$. ** $p \leq .01$.

Females Phase #2. A robust two-way ANOVA found no significant effect of treatment, $\hat{\psi}(1, 28) = 0.18$, $p = .673$, $\xi = 0.04$, or housing, $\hat{\psi}(1, 27) = 0.46$, $p = .504$, $\xi = 0.14$, on preference ratio (see Figure 4-7A). There was also no significant interaction between the independent variables, $\hat{\psi}(1, 27) = 2.10$, $p = .161$, $\xi = 0.22$. A Wilcoxon signed-rank test found a significant preference for the social stimulus for control animals in standard housing ($V = 87.00$, $p = .002$) and poly I:C animals in enriched housing ($V = 69.00$, $p = .016$) (Table 4-3).

Females Phase #3. The two-way ANOVA found no significant effect of treatment, $F(1, 46) = 0.04$, $p = .846$, $\eta^2 < 0.01$, or housing, $F(1, 46) = 3.86$, $p = .055$, $\eta^2 = 0.08$, on preference ratio for females (see Figure 4-7B). There was no interaction between the independent variables, $F(1, 46) = 1.72$, $p = .196$, $\eta^2 = 0.04$. None of the groups showed a significant preference for either of the stimuli, but saline animals in enriched housing almost reached statistical significance ($V = 55.00$, $p = .054$). The Wilcoxon signed-rank test results are shown in Table 4-3 and descriptive statistics for both phases in Table B-2.

Figure 4-7

Mean Preference Ratio (Female Adults)



Note. Error bars show mean ± *SEM*. (A) Phase #2 and (B) phase #3.
* $p \leq .05$. ** $p \leq .01$.

Table 4-3

Wilcoxon Signed-rank Test Results for Preference Ratio for Adults

Phase	Treatment	Housing	*n*	*V* [b]	*p*	*Med* [a]	95% CI
			Males				
2	Saline	Standard	13	78.00	.022 *	73.74	64.54, 80.72
		Enriched	13	78.00	.022 *	72.67	56.50, 82.68
	Poly I:C	Standard	13	88.00	.001 **	72.67	63.26, 81.82
		Enriched	13	69.00	.110	65.80	45.98, 80.27
3	Saline	Standard	13	42.00	.839	49.57	44.84, 53.55
		Enriched	11	38.00	.700	51.12	39.22, 61.40
	Poly I:C	Standard	13	63.00	.244	55.61	44.36, 60.13
		Enriched	12	48.00	.519	53.38	42.76, 69.02
			Females				
2	Saline	Standard	13	87.00	.002 **	66.45	57.54, 72.57
		Enriched	12	61.00	.092	62.57	46.72, 75.78
	Poly I:C	Standard	13	72.00	.068	59.40	46.18, 68.63
		Enriched	12	69.00	.016 *	70.99	54.82, 80.78
3	Saline	Standard	13	30.00	.305	46.26	41.66, 51.50
		Enriched	11	55.00	.054	58.35	49.63, 67.90
	Poly I:C	Standard	13	57.00	.455	51.79	47.18, 58.05
		Enriched	13	62.00	.273	56.93	43.40, 64.35

Note. The alternative hypothesis was that the true location differed from 50, implying a preference for the social/novel social stimulus over the non-social/familiar social stimulus.

[a] Pseudo median provided by the r function wilcox.test. [b] Preference ratio.

* $p \leq .05$. ** $p \leq .01$.

4.5 Results: Locomotor Activity

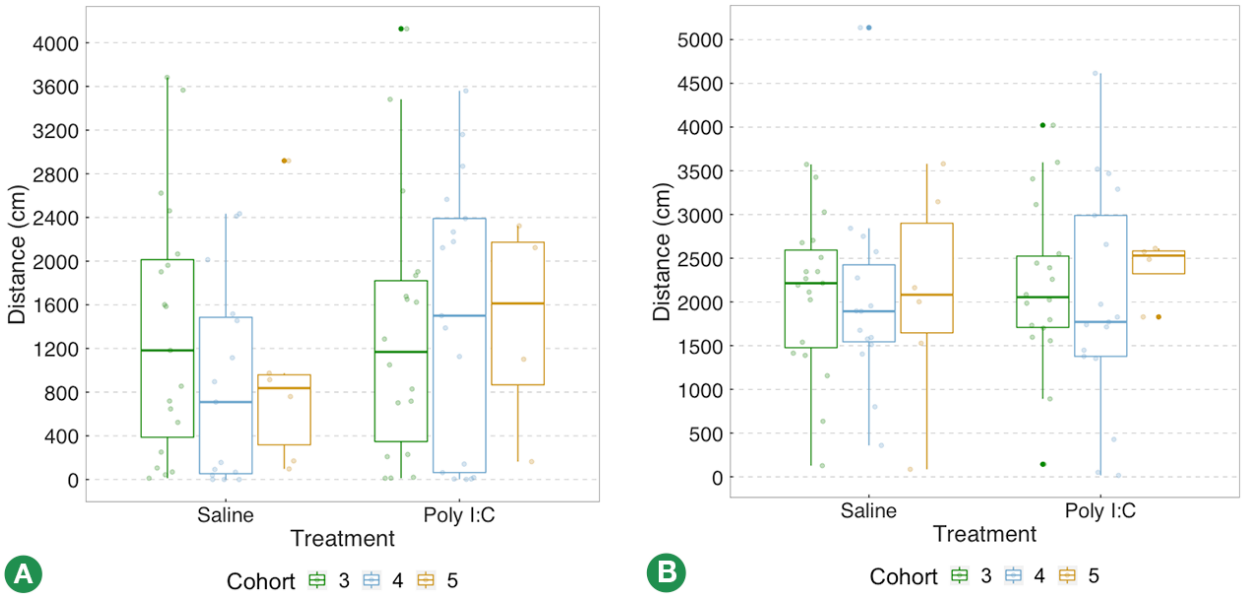
Ethovision recorded general LMA automatically. While other researchers usually do not report the distance covered by their animals during SAA testing, the measure is relevant in the context of enrichment and was, therefore, included in this chapter.

4.5.1 Pups Cohorts 3 to 5

Again, there was a lot of within-group variability, but the visual examination of the graphs in Figure 4-8 showed that there was no difference between the groups. This confirms that FBZ did not affect LMA in these pups.

Figure 4-8

Jitter Plots: Distance by Cohort (Pups)



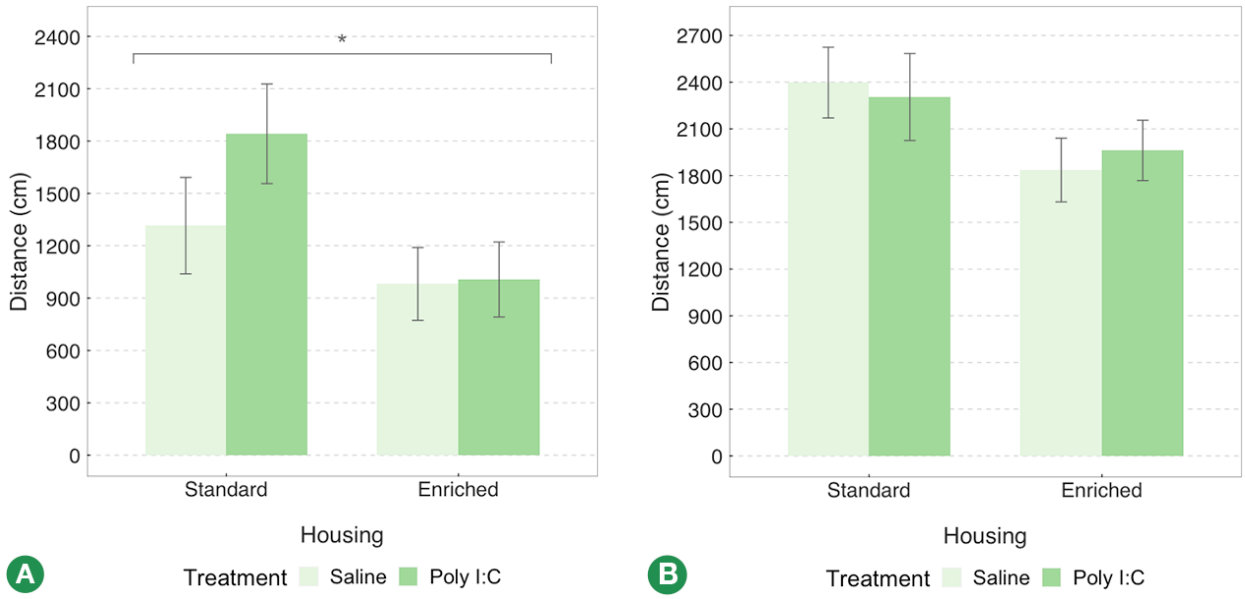
Note. (A) Phase #2 and (B) phase #3.

Phase #2. A robust two-way ANOVA showed that animals in standard housing covered significantly more distance than animals in enriched housing, $\hat{\psi}(1, 39) = 4.98$, $p = .032$, $\xi = 0.38$. There was no significant difference between the treatment groups, $\hat{\psi}(1, 47) = 1.16$, $p = .290$, $\xi = 0.18$. There was also no interaction between the independent variables, $\hat{\psi}(1, 39) = 0.58$, $p = .454$, $\xi = 0.28$ (Figure 4-9A).

Phase #3. A two-way ANOVA showed that treatment did not affect LMA, $F(1, 75) = 0.02$, $p = .899$, $\eta^2 < 0.01$. While close to the α-level of $p = .05$, there was no significant effect of housing on LMA either, as Figure 4-9B shows, $F(1, 75) = 3.87$, $p = .053$, $\eta^2 = 0.05$, and there was no interaction between the independent variables, $F(1, 75) = 0.23$, $p = .636$, $\eta^2 < 0.01$. Descriptive statistics are shown in Table B-3.

Figure 4-9

Mean Distance (Pups)



Note. Error bars show mean ± *SEM*. (A) Phase #2 and (B) phase #3.

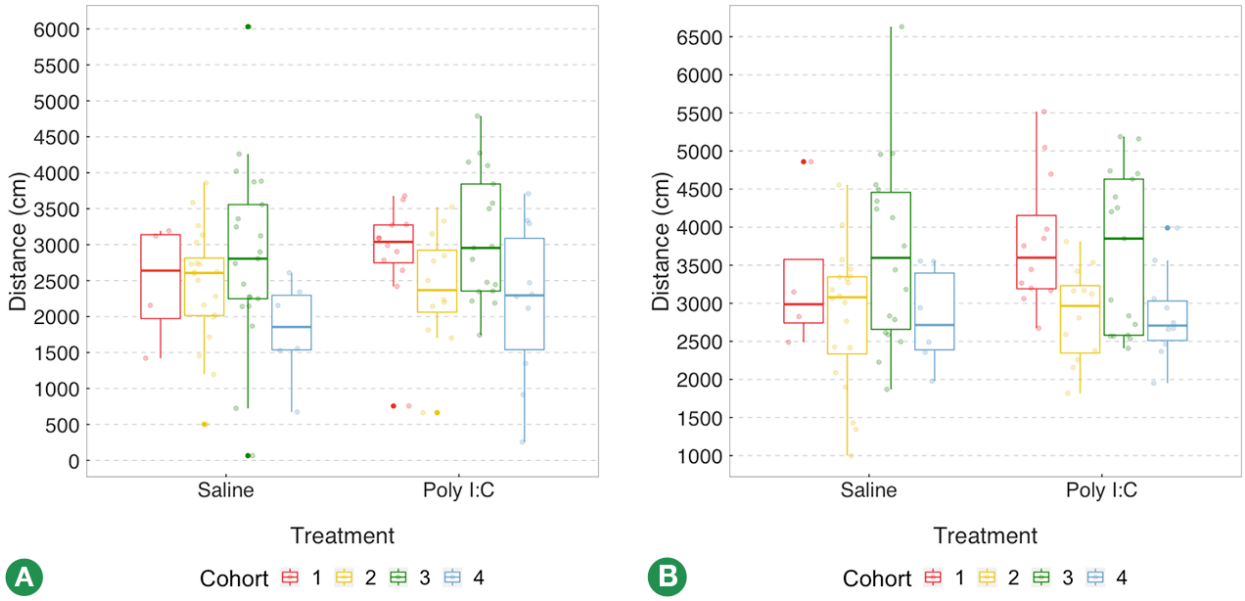
* $p \leq .05$.

4.5.2 *Adults*

Figure 4-10 shows that there was no obvious FBZ-effect on the adults' LMA. As was the case with previous datasets, there was a lot of within-group variability, but visual examination of the graphs showed that there was no difference between the groups. This confirms that FBZ did not affect LMA in adults.

Figure 4-10

Jitter Plots: Distance by Cohort (Adults)



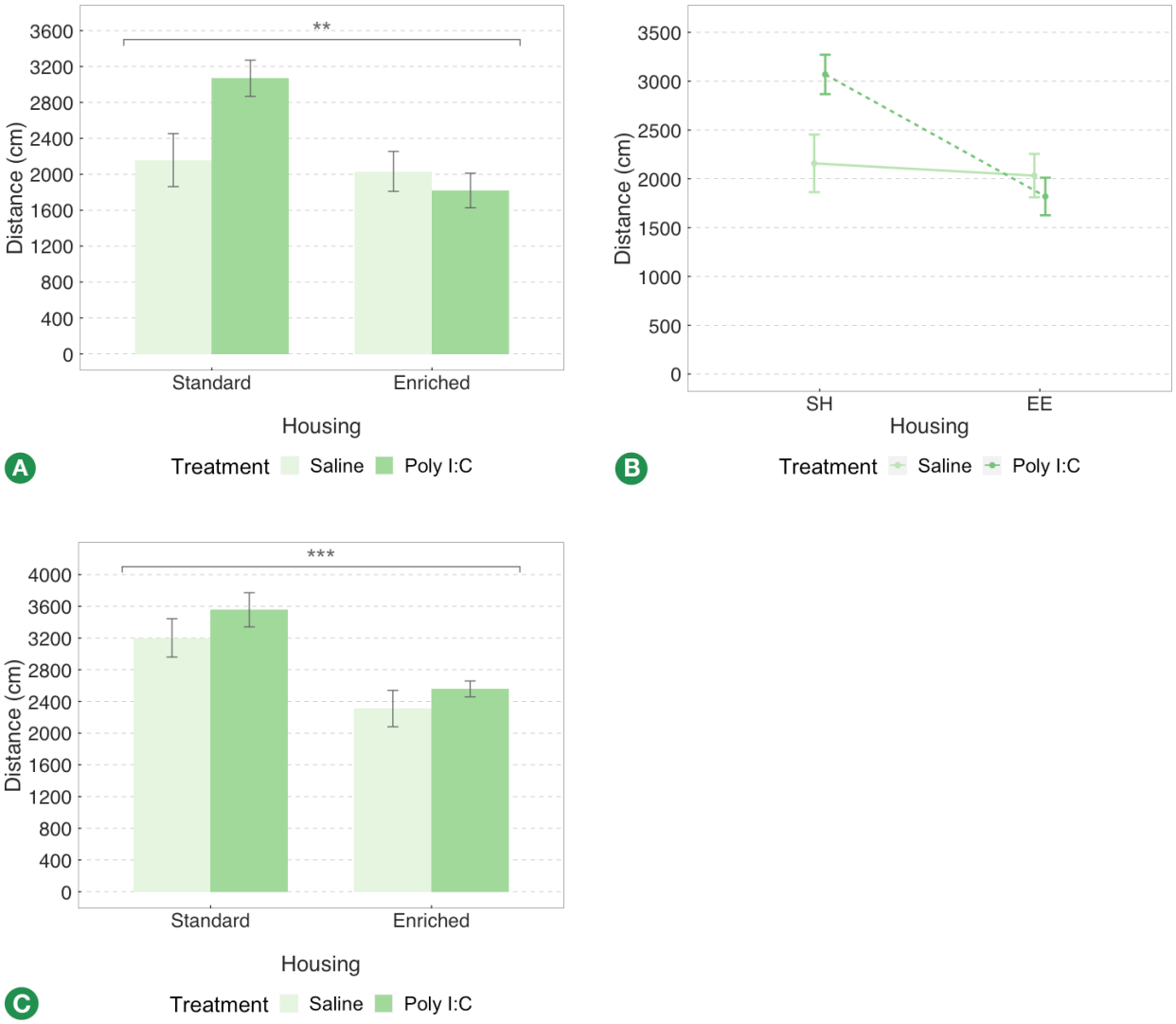
Note. (A) Phase #2 and (B) phase #3.

Males Phase #2. Figure 4-11B shows the significant interaction between treatment and housing, $F(1, 48) = 5.90$, $p = .019$, $\eta^2 = 0.11$. In standard housing, poly I:C animals covered more distance than saline animals, but there was no difference in enrichment. Further, animals in standard housing covered significantly more distance than animals in enriched housing, $F(1, 48) = 8.83$, $p = .005$, $\eta^2 = 0.16$ (a large effect). Treatment did not affect LMA, $F(1, 48) = 2.27$, $p = .139$, $\eta^2 = 0.05$) (see Figure 4-11A).

Males Phase #3. A two-way ANOVA found that animals in standard housing covered significantly more distance than animals in enriched housing, $F(1, 45) = 20.95$, $p < .001$, $\eta^2 = 0.32$. Treatment did not affect LMA, $F(1, 45) = 2.17$, $p = .147$, $\eta^2 = 0.05$ (see Figure 4-11C). There was no interaction between the independent variables, $F(1, 45) = 0.07$, $p = .799$, $\eta^2 < 0.01$.

Figure 4-11

Mean Distance (Male Adults)



Note. Error bars show mean ± *SEM*. (A) Phase #2 bar chart and (B) phase #2 interaction plot. (C) Phase #3.

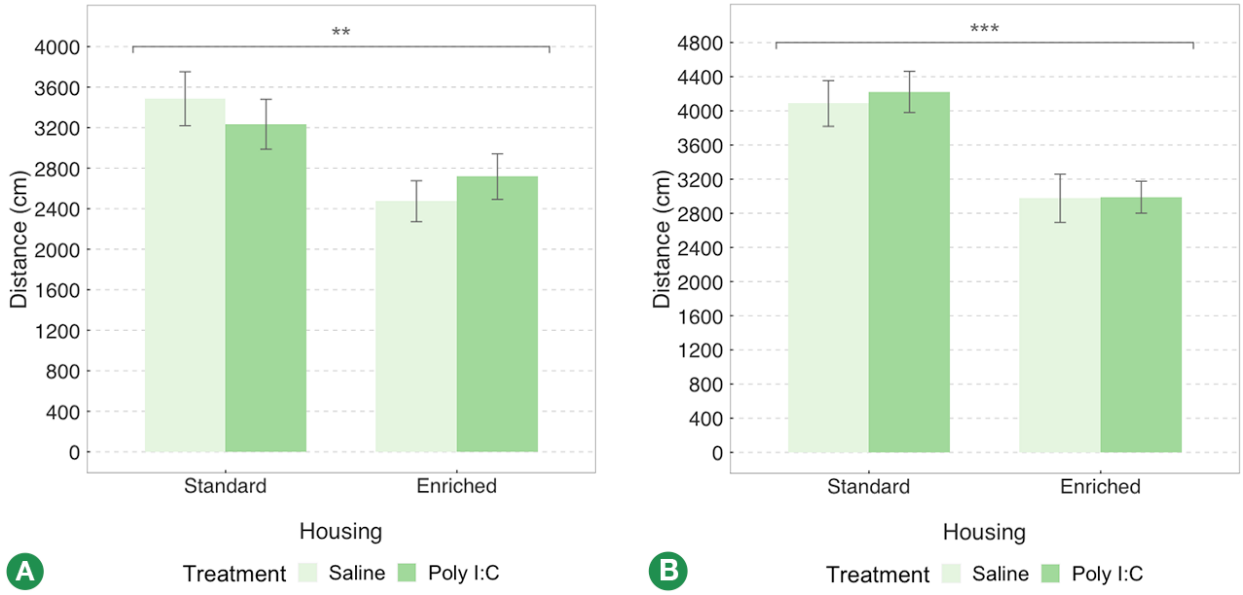
** $p \leq .01$. *** $\leq .001$.

Females Phase #2. A two-way ANOVA found that animals in standard housing covered significantly more distance than animals in enriched housing, $F(1, 46) = 10.27$, $p = .002$, $\eta^2 = 0.18$. Treatment did not affect LMA, $F(1, 46)$, $\eta^2 < 0.01$, $p = .952$, $\eta^2 < 0.01$ (see Figure 4-12A). There was no interaction between treatment and housing, $F(1, 46) = 1.08$, $p = .304$, $\eta^2 = 0.02$.

Females Phase #3. A two-way ANOVA found that animals in standard housing covered significantly more distance than animals in enriched housing, $F(1, 46) = 22.87$, $p < .001$, $\eta^2 = 0.33$. Treatment did not affect LMA, $F(1, 46) = 0.10$, $p = .755$, $\eta^2 < 0.01$ (see Figure 4-12B). There was no interaction between treatment and housing, $F(1, 46) = 0.06$, $p = .805$, $\eta^2 < 0.01$. Descriptive statistics for all groups are shown in Table B-4.

Figure 4-12

Mean Distance (Female Adults)



Note. Error bars show mean ± *SEM*. (A) Phase #2 and (B) phase #3.
** p ≤ .01. *** ≤ .001.

4.5.3 Results Summary

Table 4-4

Overview of Significant SAA Results Including LMA

Sex	Independent Variable	Effect			Pups	Adults	
		Condition #1	Direction [c]	Condition #2	3-5 [d]		
Males	General Sociability [a]	Non-social	<	Social	NA	Saline/SH *	
						Saline/EH *	
						Poly I:C/SH **	
Females	General Sociability [a]	Non-social	<	Social	NA	Saline/SH **	
						Poly I:C/EH *	
Males	Phase #2 Locomotor [b]	Saline	<	Poly I:C			x *
		Standard	>	Enriched	*	**	
	Phase #3 Locomotor [b]	Saline	=	Poly I:C			
		Standard	>	Enriched		***	
Females	Phase #2 Locomotor [b]	Saline	=	Poly I:C			
		Standard	>	Enriched	*	**	
	Phase #3 Locomotor [b]	Saline	=	Poly I:C			
		Standard	>	Enriched		***	

[a] Preference ratio. [b] Distance in cm. [c] Effect direction is indicated by < (smaller than) > (greater than). [d] The dataset was not segregated by sex, so results were for the combined dataset.

* $p \leq .05$. ** $p \leq .01$. *** $p \leq .001$. x = interaction.

4.6 Discussion

This study investigated the impact of MIA following exposure to poly I:C during gestation on general sociability and social novelty behaviour in the offspring; it also explored whether pre- and post-weaning enrichment reverses these behavioural effects. More specifically, I hypothesised that standard-housed juvenile and adult progenies of female SD rats prenatally exposed to 5 mg/kg poly I:C on PND 15 would spend less time with the social stimulus in phase #2 and the novel social stimulus in phase #3 when compared with saline controls. I further hypothesised that enrichment would reverse these effects. The data did not support the first hypothesis—there was neither a preference difference for the social stimulus between the juvenile groups in phase #2, nor for the novel social stimulus in phase #3. Neither treatment nor pre-weaning enrichment affected the animals' behaviour.

4.6.1 Lack of Preference for Social and Novel Social Stimuli in Pups

None of the juvenile groups showed a preference for the social/novel social stimulus (phase #2/3) at all. The absence of social preference in the pups' control group makes result interpretation challenging, especially as several studies have established a clear preference for the social stimulus in phase #2 (Chen et al., 2019; Lins et al., 2018; Lins et al., 2019; Malkova et al., 2012; Smith et al., 2007) and novel social stimulus in phase #3 (Chen et al., 2019). However, all aforementioned studies used adult animals and, to my knowledge, none of the published poly I:C sociability and social novelty behaviour studies used juvenile animals.

The reasons for the absence of social preference in phase #2 are not clear, though arena size can be ruled out, as there was no significant difference between cohorts. It is possible that a period of social isolation before testing would set a better baseline for sociability testing in pups. In particular, as a recent rat study investigating social enrichment found that single-housed animals spent more time with the social stimulus than group-housed animals (Templer et al., 2018). The pups in the present study were tested a few days after weaning, before which they lived with up to 15 other pups and their mother. Between weaning and testing, the standard-housed pups lived in groups of four and the enriched-housed pups in groups of six to 10, so it is conceivable that the pups' social appetite was satiated, and the rewarding effect of an unknown conspecific was not strong enough to show any treatment-related differences. Neither of the previously-mentioned studies that found a social preference had their animals in social

isolation before testing, so the idea that social satiation masked potential treatment effects sounds like a logical explanation.

The lack of preference for the novel social stimulus in phase #3 could additionally or alternatively be related to the 30-minute inter-trial interval between the two phases, which was perhaps too long for juveniles. While the social memory of adult rats is longer than 30 minutes (Engelmann et al., 1995), juveniles did not recognise a previously encountered conspecific after 10 minutes (Thor & Holloway, 1982). With this in mind, we can deduce that the juveniles treated both social stimuli as novel because their social recognition memory had faded.

4.6.2 *Preference for the Social Stimulus in Adults*

As adults, saline males in standard housing preferred the social over the non-social stimulus, creating a baseline for comparison. Surprisingly, males prenatally exposed to poly I:C in standard housing showed a greater preference for the social stimulus than any other group. Of the two groups in enriched housing, saline males preferred the social stimulus, but poly I:C males did not show a social preference. While the one-sample Wilcoxon tests showed a social preference in three of the four groups, the between-group differences of preference ratio were not strong enough to be significant. In phase #3, none of the groups showed a preference for the novel social stimulus and there were also no significant differences between the groups.

The females' results partially supported the hypotheses: Like males, saline females in standard housing also preferred the social over the non-social stimulus, while poly I:C females showed no social preference. Of the two enrichment groups, saline females had no preference for the social stimulus, but females in the poly I:C group did prefer the social stimulus. Like in the male dataset, between-group comparisons did not find a significant preference difference between the groups. In phase #3, none of the groups showed a preference for the novel social stimulus and there were neither significant differences between the female groups nor any interactions. While there was a clear social preference in the majority of adults—particularly females showed the expected poly I:C effect—the between-group differences were not strong enough to be significant.

The adults' phase #2 results corroborate findings from a recent mouse study on enrichment; Hendershott et al. (2016) found that the animals had a social preference, but there was no significant difference between enrichment groups or sexes. Unfortunately, they did not incorporate phase #3 into their study. It is important to note that their animals

were reared in standard housing and moved to an enriched environment once weaned, whereas in the present study, the animals received 10 enrichment sessions before weaning. While, generally speaking, pre-weaning enrichment contributes to a greater increase in social engagement and exploratory behaviours than post-weaning enrichment alone (Schneider et al., 2006), it is possible that in relation to the social approach–avoidance paradigm, it may not have the same impact.

When considering phase #3 results, the complete absence of social novelty preference in any of the groups as well as a lack of between-group differences was unexpected. While for the juveniles a likely explanation was that the too-long inter-trial interval of 30 minutes resulted in the loss of social memory and subsequent lack of recognition of the familiar stimulus, adult rats are generally able to recognise a conspecific for about an hour post initial exposure (Engelmann et al., 1995; Thor & Holloway, 1982). It is possible that the adults' social recognition memory faded faster than in previous studies, but this appears an unsatisfying interpretation, in particular, as group-housed mice recognise conspecifics weeks after the initial encounter (Kogan et al., 2000). This brings us back to the social housing explanation, which would make sense even without taking into account the faster dissipation of social recognition memory in adults. If social housing dulled the rewarding effect of novel conspecifics (as observed by Templer et al., 2018), then pair housing in standard-housed animals potentially reduced the preference for social novelty in phase #3, in particular since the animals were exposed to a novel conspecific in phase #2.

While there was no effect of housing, it is important to note that animals that lived in enriched housing seemed less interested in any of the offered stimuli but rather attempted to climb the sidewalls. If they succeeded, they would circle the arena, walking along the top edge of the sidewalls. Notably, this also offers support to the idea of social satiation. Due to the problem with containment during test sessions, saline and poly I:C animals in enrichment were the only groups where testing was conducted in a top-covered arena.

While some studies ran several habituation sessions with the stimulus mice before testing (Chen et al., 2019; Crawley, 2004; Moy et al., 2008; Nadler et al., 2004), the stimulus animals in the present study had not been habituated to the wire cups. However, other studies (Lins et al., 2018; Lins et al., 2019; Malkova et al., 2012) did not habituate the stimulus animals and yet, they found a social preference.

Where the present study differed from previous studies was that their stimulus animals were age-matched rather than juvenile. Whether a study tested adult mice (Crawley, 2004; Hendershott et al., 2016; Malkova et al., 2012; Moy et al., 2004; Moy et al., 2008; Nadler et al., 2004; Ratnayake et al., 2014) or juveniles (Brodkin et al., 2004), the stimuli were always age-matched. I, however, used sex- and strain-matched juveniles for both, pups and adults, following standard lab-practice. This procedure is based on animal size—juveniles can be contained under a standard wire cup, whereas adult Sprague Dawley rats would require a larger anchored container, which in turn would need a larger arena.

Analysis of sniffing behaviour in the vicinity of the wire cups is an indicator of social exploration (Crawley, 2004; Moy et al., 2008), but due to time constraints, it was not analysed as part of the present study. Future SAA studies should include sniffing as a response variable as it provides an additional measure of social interest. Also, for the pups, it is likely that social isolation before test sessions and no inter-trial interval would result in the expected outcome of a clear preference for the social/novel social stimulus in control animals, thus, creating a solid comparison group and show treatment-related between-group differences. For the adults, in addition to pre-testing isolation and removal of the inter-trial interval, gender-, strain-, and age-matched stimuli are likely to identify differences in social preference/social novelty behaviour.

4.6.3 Locomotor Activity

The LMA during SAA testing is often not reported; researchers tend to run a separate locomotor experiment, often in combination with stimulant administration (e.g., amphetamines or MK-801) (e.g., Lins et al., 2018; Lins et al., 2019). A separate locomotor study without additional stimulants is reported in Chapter 6. Because LMA has relevance for the enrichment condition, I will discuss it briefly in this chapter.

In the present study, standard-housed pups covered significantly more distance than those that received pre-weaning enrichment. In standard housing, poly I:C animals covered significantly more distance than saline animals, but there was no difference in enrichment. In addition, male poly I:C animals in standard housing covered significantly more distance than male saline animals, but this did not apply in enrichment. Further, both male and female adults in standard housing were significantly more active than in enriched housing in both SAA phases. This is similar to findings by Rae et al. (2018) who reported that mice in enriched housing displayed lower LMA than controls. Several studies have also found that enrichment resulted in reduced LMA in an open field test in

mice (O. B. Amaral et al., 2008; Zheng et al., 2020) and rats (Varty et al., 2000). Zheng et al. (2020) reported that all mice moved at the same velocity for the first 4 of the 10 minutes, but animals living in enrichment moved significantly more slowly than standard-housed animals after that. This was likely due to greater novel-environment-exploration efficiency and faster information-processing following enrichment (Elliott & Grunberg, 2005). These behavioural changes can be attributed to greater birth and maturation of neurons in the dentate gyrus, as well as increased neuronal and synaptic connectivity (Nithianantharajah & Hannan, 2006). The results highlight that continuous enrichment from a very young age can have a long-term effect on cognitive abilities in rats.

4.7 Summary

This study did not show a significant poly I:C or enrichment effect on general sociability or novelty-seeking behaviour in juvenile rats. This was likely due to methodological issues like social satiation (phase #2) and a too-long inter-trial interval between the two phases, which resulted in dissipation of social recognition memory. In adults, the poly I:C effect on social preference was clearly present—particularly in females—but there were no between-group treatment effects. Further, enriched adult animals consistently showed hypolocomotion, which will be explored further in Chapter 6. The next chapter will turn to poly I:C's effect on prosociality and a potential rescuing effect of environmental enrichment.

Chapter 5

Prosocial Behaviour

Chapter 5
Prosocial Behaviour

5.1 Background

Chapter 5 describes the effect of maternal poly I:C treatment on the offspring's prosocial behaviours. The present study measured how often the animals helped a conspecific in need to gauge the impact of poly I:C and/or environmental enrichment on empathy-like behaviours.

While empathy-like behaviours have traditionally been considered exclusive to primates, past studies have shown that these behaviours can be observed in a range of other species, including rodents. In one of the first studies that attributed prosocial behaviour—behaviours intentionally executed to benefit another (Jensen, 2016)—to rats, Rice and Gainer (1962) suspended either a Styrofoam block or a distressed rat from the ceiling of a double-storey cage. They noted that the "… animal typically squealed and wriggled satisfactorily while suspended, and if it did not, it was prodded with a sharp pencil until it exhibited signs of discomfort" (Rice & Gainer, 1962, p. 123). At ground-level, a free rat could press a lever to lower the block/rat down to ground-level, which they did more than 15 times more often for the distressed rat than for the block.

More recently, Ben-Ami Bartal et al. (2011) presented a prosocial rodent behaviour study where a rat freed a conspecific from a rodent restrainer without prior training or experimenter-provided rewards. The authors interpreted the rat's behaviour as (1) recognition of the trapped rat's emotional state, conveyed via emotional contagion, and (2) an intentional act where it searched for a way to open the restrainer door. The rats did not open the restrainer when it was empty or occupied by a plush toy rat. To test whether the prospect of social reward drove the prosocial behaviour, Ben-Ami Bartal et al. (2014) changed the experimental setup so that the liberated rat was led into a different cage once the door was opened. Even without social interaction (i.e., social reward), the rats continued to free their cagemate; when the restrainer was empty, though, they did not open the door.

Sato et al. (2015) adapted the social-release paradigm by replacing the restrainer with a pool of water where the helper rat had to open a door to enable the soaked rat in the pool to climb out. They found that most rats would open the door when a conspecific was trapped in the pool, but not when it was unrestrained in a dry compartment,

highlighting the importance of distress in the subsequent rescue action. Further, when the roles were reversed, rats that had previously been trapped in the pool opened the door to free a trapped conspecific a lot faster than rats without prior experience. The free animals' behaviour strongly suggests a level of cognitive abilities, where the animals grasped the specific situational requirement (opening a door to free their conspecific) to alleviate the trapped animal's distress. It appears that the animals had some level of comprehension that the source of the emotion was external rather than originating from within themselves. What differentiates emotional contagion from emotional empathy is self-awareness and the ability to separate between one's own emotional state and that of others (de Waal, 2008; Panksepp & Panksepp, 2017). However, it is not possible to ascertain whether the animals actually empathised or if they merely wanted the noise (i.e., distress calls) to stop. Lavery and Foley (1963) reported that rats were more likely to turn off white noise than recorded pain squeals from a rat exposed to foot shocks. The white noise must have been more aversive than the squeals, but if both noise sources were just that—noise—why did the animals not turn it off at an equal rate? Perhaps because the rats tried to identify the source of the distress calls so they could help their conspecific, which suggests that such calls are more than just a source of noise but trigger an emotional response.

Other authors have interpreted the rats' behaviour differently. Rather than attributing it to empathy, in their view, it was due to a preference for water (Schwartz et al., 2017) or social reinforcement (Silberberg et al., 2013). Because the latter is in direct conflict with the control condition that showed that rats release another, even without social reward (Ben-Ami Bartal et al., 2014), Silberberg et al. (2013) argued that the rats continued to open the door because they had a history of social reward. They showed that rats that had experienced social contact following door opening opened the door faster in subsequent trials where the trapped rat is released into a separate compartment than naïve rats (i.e., rats that had not experienced direct social contact following door opening). However, the issue with this explanation is that the shorter opening latencies could be due to a learning effect, which their study did not control for. Also, the opening behaviour was quickly extinguished when the rats were presented with an empty restrainer (Ben-Ami Bartal et al., 2011).

In their study, Schwartz et al. (2017) used a T-maze with a chamber at each end of the maze. Each chamber contained one rat and one tub, one tub was filled with water, while the other tub was dry. The free rats preferred socialising with the wet rat over the

dry rat. In a control condition, the animals preferred an empty wet tub over an empty dry tub, reinforcing the authors' interpretation of a preference for water. When the empty chamber doors were closed (i.e., no access to the respective tubs), the rats showed no side preference at all. The authors' rejected prosocial behaviour as the driving force behind the behaviour and instead proposed that it was based on a preference for water. This is a valid explanation, in particular as rats were found to be very good swimmers with a natural habitat that often involves water (Traweger et al., 2006; Whishaw & Tomie, 1996). Conversely, Sato et al. (2015) examined water preference and found that rats avoided contact with water as much as possible. However, animals in the Schwartz et al. (2017) study could leave the tubs and as soon as the free rat came within 3 cm of the chamber door, which opened automatically, allowing the stimulus rat to exit the chamber and mingle with the free rat—the latter did not have to intentionally free the stimulus rat. This calls into question whether the rats inside the chambers were in distress, and if they indeed were, it is unlikely that their distress levels were high enough to trigger a prosocial rescue attempt.

In a follow-up study, Ben-Ami Bartal et al. (2016) showed that free rats that received an anxiolytic to disrupt social-affective processing would not open the restrainer door to free a trapped cagemate, but they did open the door to access chocolate. This reinforces the involvement of distress—expressed and received.

The same lab group involved in the previously discussed T-maze study set up a critical replication of the Ben-Ami Bartal et al. (2011) trapped-rat paradigm using an identical T maze as described above (Hachiga et al., 2018). This time, their design comprised three conditions where the free rats could choose between either (1) a rat trapped in a restrainer or an empty chamber, (2) a rat trapped in a restrainer or a chamber with a rat in an open restrainer, and (3) a chamber with a rat in an open restrainer or an empty chamber. In conditions 1 and 3, rats were more likely to approach the side with the rat than the other side. In condition 2, the rats did not show a preference for the side with the trapped rat. The researchers argued that this was because there was a social reward on each side, and, therefore, that helping behaviour is motivated by accessing a social reward rather than empathy triggered by the distress of the trapped rat. Similar to before, there were several issues with the employed procedure. Firstly, the rats did not have to free the trapped rat; the restrainer was opened as soon as the free rat came within 3 cm of the chamber door, which opened automatically when approached from the outside, so no intentional action to free the inside-animal was required. Second, the free animals were

not allowed to investigate both sides; as soon as they neared a chamber door, it opened, providing access to the rat within the chamber. With a social stimulus on each side, this is an important point to make, because, if one side was empty, the free rat would be drawn to the side containing another rat. With both sides occupied and no way to gather clues about the state of the animals within each chamber (beyond ultrasonic vocalisations), it is not surprising that there was no side preference. Also, it is conceivable that the free rat decided to get reinforcement from the rat that did not emit distress signals to combine forces and find a way to rescue the one that did emit distress signals (i.e., strength in numbers). However, the study's design did not allow for this to happen.

At face value, these studies (Ben-Ami Bartal et al., 2011; Ben-Ami Bartal et al., 2014; Sato et al., 2015) demonstrated a display of prosociality in response to another rat's distress, tapping into the empathy paradigm. None of the subsequent systematic replications (Schwartz et al., 2017; Silberberg et al., 2013) offered a convincing alternative explanation for the rats' behaviour, thus making the rodent social-release paradigm well suited to investigate empathic abilities—or a lack thereof—in rats.

Few studies have been published on rodent ASD models of prosocial behaviour. A very recent study employed the social release paradigm in a VPA model of autism (Fontes-Dutra et al., 2019). They found that prenatal exposure to VPA delayed the first day of opening the restrainer door and resulted in less frequent openings overall. The social-release paradigm has not yet been used to investigate prosocial behaviour in either a poly I:C rat model of ASD, an environmental enrichment study, or a combination of the two, making the present study the first of its kind.

5.2 Predictions

The aim of the current study was the investigation of the following three hypotheses:

A. Adult standard-housed SD rats prenatally exposed to poly I:C will show reduced prosociality by freeing a trapped conspecific less frequently and with higher opening latencies than control rats.
B. Enrichment will reverse this effect. Poly I:C-exposed rats that lived in enriched housing will free the trapped rat more frequently and open the door faster than poly I:C-exposed rats in standard housing.
C. Females will show a higher level of prosociality than males.

5.3 Methods

5.3.1 Animals

All animals used in this experiment were handled at least five times before assessment; they were tested in five cohorts (Table 5-1) and divided into four groups: (1) SH/SAL, (2) SH/poly I:C, (3) EH/SAL, and (4) EH/poly I:C. Each group consisted of 26 animals, 13 males and 13 females, that originated from 16 litters in the saline condition and 18 litters in the poly I:C treatment condition.

Table 5-1

Total Number of Tested Animals by Cohort (Prosocial Behaviour)

Cohort	Treatment	Housing	Males	Females	Total
#1	Saline	Standard	2	2	4
		Enriched	0	0	0
	Poly I:C	Standard	6	6	12
		Enriched	0	0	0
#2	Saline	Standard	2	2	4
		Enriched	0	0	0
	Poly I:C	Standard	2	0	2
		Enriched	0	0	0
#3	Saline	Standard	5	5	10
		Enriched	5	5	10
	Poly I:C	Standard	4	4	8
		Enriched	5	5	10
#4	Saline	Standard	4	4	8
		Enriched	5	5	10
	Poly I:C	Standard	1	3	4
		Enriched	5	5	10
#5	Saline	Standard	0	0	0
		Enriched	3	3	6
	Poly I:C	Standard	0	0	0
		Enriched	3	3	6
Total			52	52	104

Some of the animals had been used for USV and SAA testing (as presented in Chapter 3 and Chapter 4). As discussed in section 2.4.2, pups of cohort 1 were affected by the FBZ treatment for 9 days in utero. Dams and pups of cohort 5 were exposed to FBZ for between 7 and 14 days. Also, all adults in cohort 4 received the FBZ-medicated feed for a total of 35 days. Animals that became openers were analysed separately (see group numbers in Table 5-2).

Table 5-2

Number of Animals that Became Openers

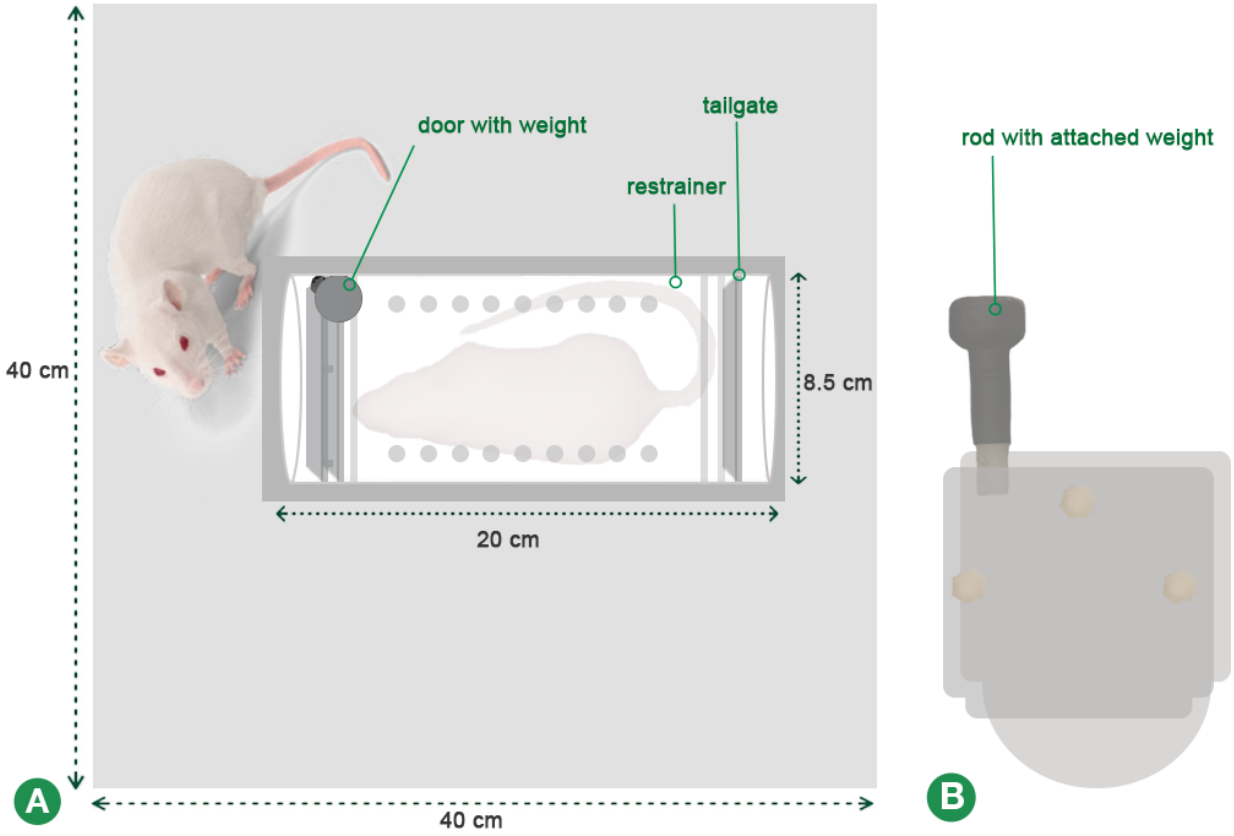
Treatment	Housing	*n*
	Males	
Saline	Standard	9
	Enriched	4
Poly I:C	Standard	7
	Enriched	3
	Females	
Saline	Standard	10
	Enriched	9
Poly I:C	Standard	4
	Enriched	7

5.3.2 Apparatus

The animals were tested in a 40 cm (length) x 40 cm (width) x 40 cm (height) arena constructed from black polycarbonate sheets. Figure 5-1A shows the square arena with a schematic of a rodent restrainer (20 cm x 8.5 cm). The transparent polycarbonate restrainer had openings to ensure olfactory and auditory communication between the animals within the arena. One side of the restrainer was blocked with a transparent tailgate, the other with a transparent door that could only be removed from the outside. The door was constructed from two polycarbonate pieces connected with plastic screws (Figure 5-1B). The shorter piece at the front allowed the animal to nudge and lift the door with its head; the weights atop the rod ensured that the door falls to the side rather than the front, to prevent animals from getting startled/frightened by the door falling onto them. The weight was covered with rubber to muffle the sound at contact with the ground.

Figure 5-1

Prosocial Behaviour Arena



Note. (A) Arena setup with rat trapped in the restrainer. (B) Restrainer door with weighted rod on one side.

Four testing arenas sat on a black rubber mat, each of which was covered with a perforated transparent polycarbonate sheet (50 cm x 50 cm) to prevent climbing. A high-definition camera (Logitech C930) was mounted to a ceiling rig to record the sessions from above and an ultrasonic microphone (Ultramic 250K) was placed centred between the four arenas. I used the software Logitech Capture to record video, and Audacity (Audacity Team, 2019) to record USV. Between sessions, the arenas and restrainers were wiped with F10 disinfectant and disposable paper towels.

5.3.3 Procedure

The procedure was adapted from Ben-Ami Bartal et al. (2011). As familiarity increases the expression of prosocial behaviour (Ben-Ami Bartal et al., 2014), the rats used in this study were cage mates. Before testing, the animals were handled for 5 minutes each day to get comfortable with the experimenter. Four pairs were tested simultaneously, and

animals were aged between 84 and 120 days and not experimentally naïve. Each gender-matched pair comprised a free and a restrained rat that either shared a standard housing cage or lived in the same enrichment cage. All sessions were run during the animals' dark phase under red light from Monday to Friday between 8 am and 6 pm. Approximately 10 minutes before the test session the animals were transported to the experimental room in their home cage.

Confidence Measure. As Ben-Ami Bartal et al. (2011) found that more timid animals were less likely to become openers, the more confident rat from each pair was selected to be the free animal. Confidence was determined by measuring the cage-ledge approach-latency at the beginning of each of the four habituation days. The cage lid was removed and the animal that placed both paws on the ledge first was deemed the more confident of the pair.

Habituation (Days 1 to 4). To habituate the animals to the experimental room and the testing arena, the rat pair was placed in the empty arena and left to explore for 30 minutes after which the animals were returned to their respective housing rooms.

Empty Condition (Days 5 to 8). This was added as a control condition to confirm that animals did not open the restrainer door per se. The free animal spent 30 minutes in the arena with an empty restrainer with the door in place, preventing the animal from entering the restrainer.

Trapped Condition (Days 9 to 15). The trapped rat was placed inside the restrainer, which was closed off with a tailgate on one side and the door on the other. Once the restrainer was in place in the centre of the arena, the free animal was placed in the arena. If it had not opened the restrainer door after 25 minutes, the door was lifted to halfway and rested at a 45° angle at which point either animal could open the door. The animals were left together in the arena for five minutes post door opening and were returned to their home cage afterwards. Some rats learned to open the door or tailgate from the inside. In this case, they were returned to the restrainer with a polycarbonate block in place, which was used for all subsequent sessions. Once the free rat had opened the door, the block was removed, allowing the trapped rat to exit the restrainer tube.

Each pair had one session per day; they were tested for a total of 15 days. Due to technical issues, data from the empty condition was not available for analysis for cohort 1 and two files containing recordings from session 1 from eight cohort 1 trapped sessions were missing from the servers. I recorded USV with one microphone that was set up

between the four arenas. Unfortunately, it was not possible to isolate individual calls, so the recordings were not analysed further.

5.3.4 Data and Statistical Analysis

I used the latest version of Ethovision (XT15) to analyse the video files from the empty condition to obtain the covered distance during the empty sessions (Noldus et al., 2001). Unfortunately, the simultaneous presence of two animals in the arena caused interference such that Ethovision could not be used to reliably analyse the free animals' activity during the trapped condition. I utilised the RStudio editor for data analysis (RStudio Team, 2020). The alpha (α) level was set at .05 and a *p*-value lower than that ($p \leq \alpha$) considered statistically significant.

The between-subject variables for all analyses were treatment (saline vs poly I:C), housing (standard vs enriched), and sex (male vs female). The response variables were opening frequency, opening latency, and mean distance (m). I also included session as a repeated measure to investigate the potential effect of session on housing for both opening frequency and latency. Effect sizes were reported with Wilcox and Tian's (2011) explanatory measure of effect size (ξ) for robust ANOVAs ($\hat{\psi}$), where ξ = 0.10, 0.30, and 0.50 stand for small, medium, and large effect sizes, and eta squared (η^2) for *F*-tests, where η^2 = 0.01, 0.06, and 0.14 indicate small, medium, and large effect sizes. Effect sizes for t-tests were reported with Cohen's *d*, where *d* = 0.20, 0.50, and 0.80 indicate small, moderate, and large effects. For animals that did not open the door, the opening latency was set to 25 minutes. Post hoc tests were conducted on significant main effects (R function "t1way" by sex) (Mair & Wilcox, 2020). When assumptions were violated, I used robust methods, which was the case for all but one dataset—the trapped condition for openers only. In the trapped condition, significant main effects were followed up with Tukey post hoc tests.

Empty Condition. Of the 114 animals, only seven opened the door (3x twice, 4x once). Because of the low numbers, no analysis was run.

Trapped Condition. For each condition, I first analysed the full dataset with robust ANOVAs ($\hat{\psi}$). As previous studies (Ben-Ami Bartal et al., 2011; Sato et al., 2015) excluded animals that did not open the restrainer door, I also analysed the trapped dataset for opening frequency and latency with openers only. Both datasets (frequency and latency) were normality distributed and their variance was equal, so I used standard three-way

ANOVAs (*F*) for the analysis. I computed the planned comparisons with the R function "emmeans" (Tukey method) and reported the effect sizes with Cohen's *d*.

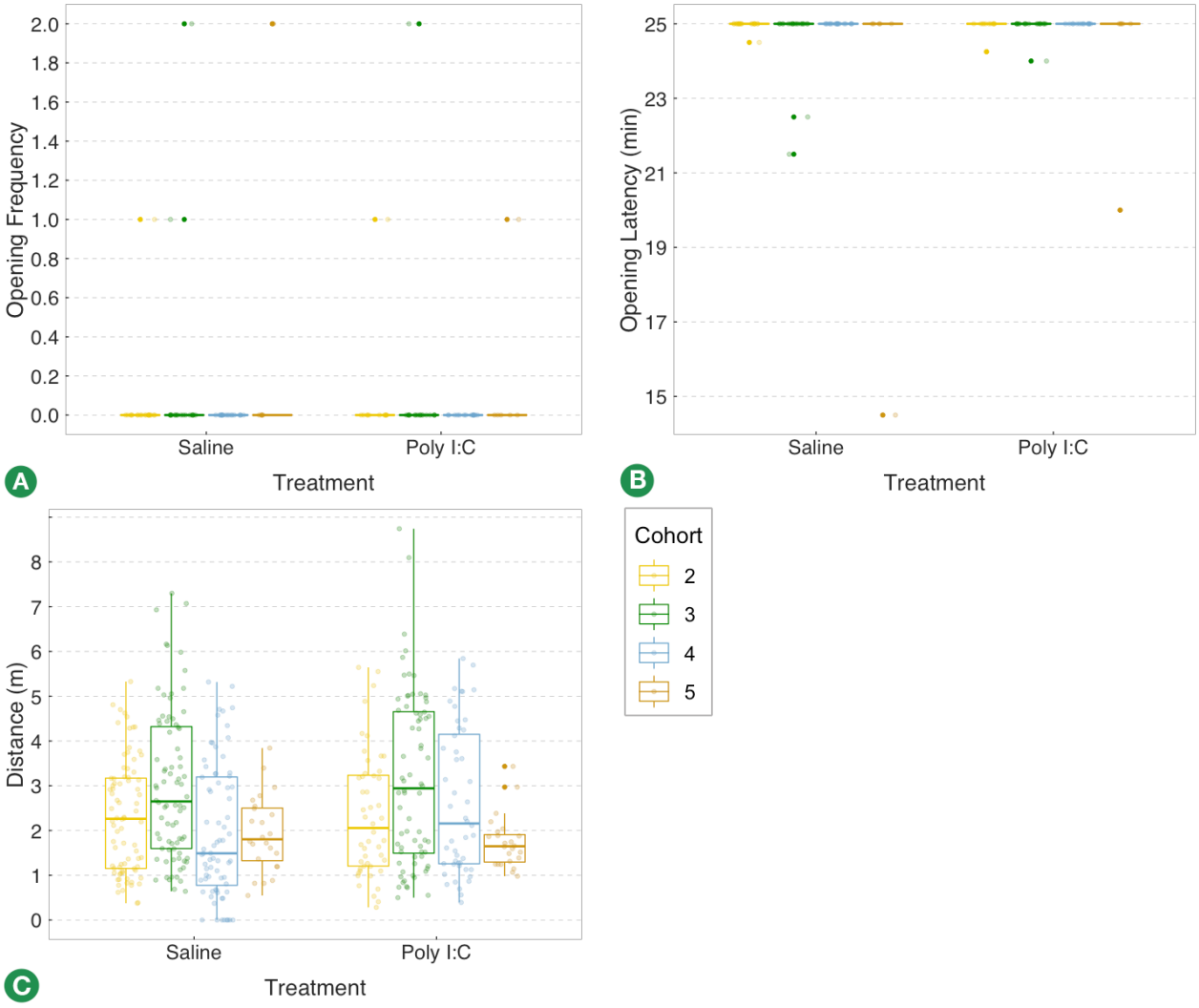
5.4 Results

5.4.1 Empty Condition

Figure 5-2 shows that FBZ did not affect opening frequency, latency, or distance covered. Most of the animals did not open the door which is why the distributions in A and B are so narrow.

Figure 5-2

Jitter Plots by Cohort (Empty Condition)

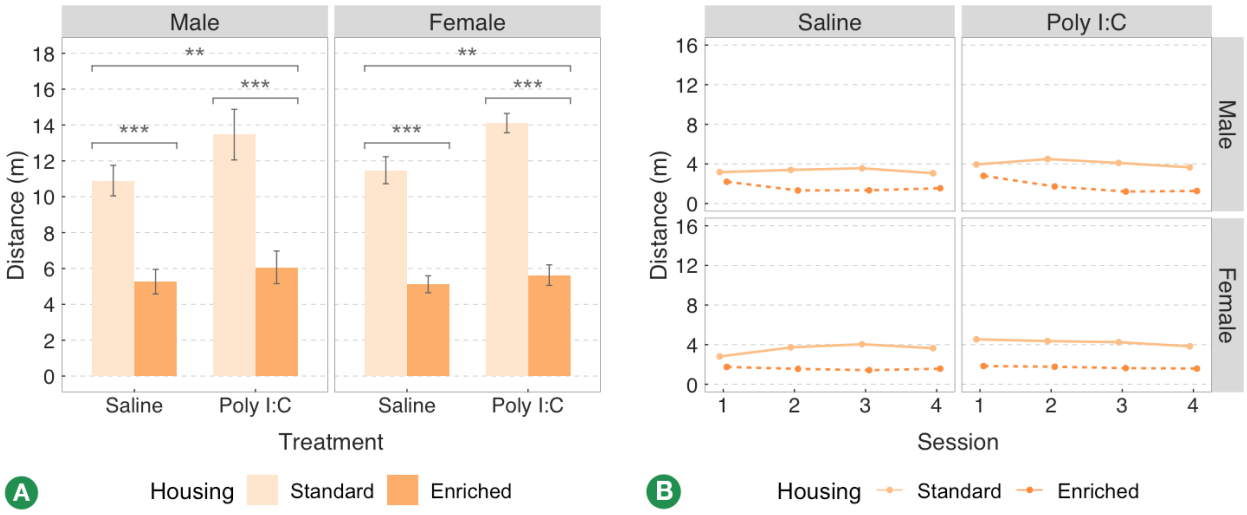


Note. (A) Opening frequency, (B) opening latency in minutes, and (C) distance in metres.

Locomotor Activity. The robust three-way ANOVA showed that animals prenatally exposed to poly I:C covered significantly more distance that those that were exposed to saline, $\hat{\psi}(1, 90) = 7.81$, $p = .008$, $\xi = 0.12$. Furthermore, standard housing animals covered significantly more distance than those in enriched housing, $\hat{\psi}(1, 85) = 143.40$, $p < .001$, $\xi = 0.95$ (see Figure 5-3). Sex, however, did not affect LMA, $\hat{\psi}(1, 108) = 0.08$, $p = .784$, $\xi = 0.05$. There were no interactions between the independent variables. Descriptive statistics are shown in Table C-3 below.

Figure 5-3

Distance (Empty Condition)



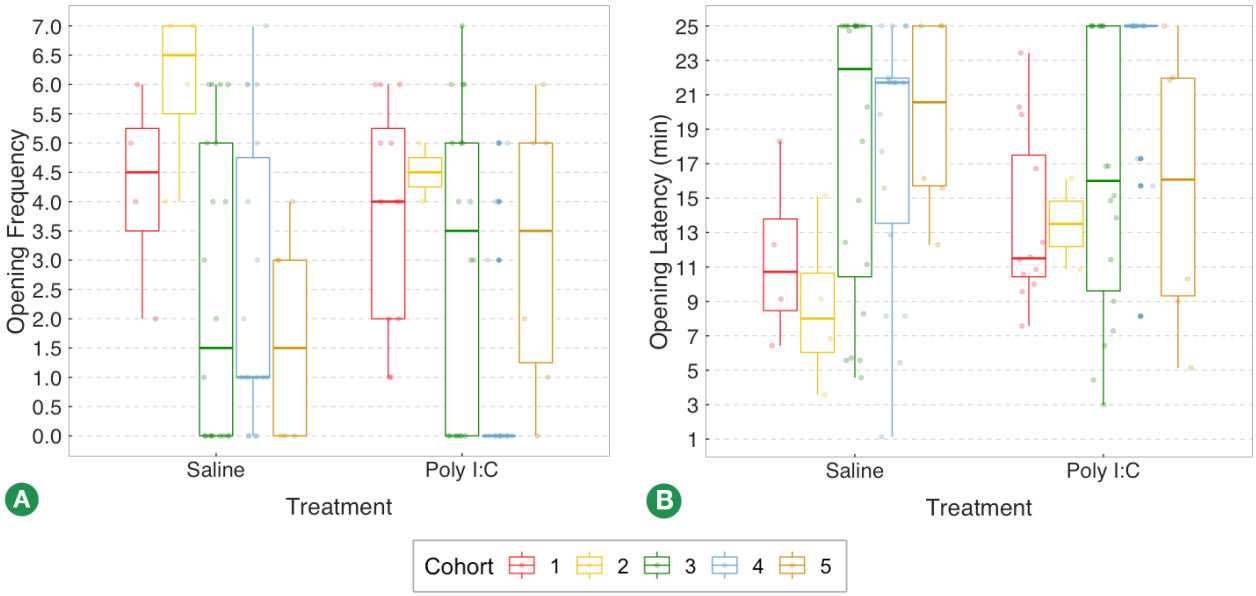
Note. Error bars show mean ± *SEM*. (A) Bar chart showing LMA between treatment and housing groups by sex. (B) Time series showing latency by session.
** $p \leq .01$. *** $p \leq .001$.

5.4.2 *Trapped Condition (All Animals)*

Figure 5-4 shows that FBZ did neither affect opening frequency nor latency in the trapped condition. In the saline condition (A), cohort 2 seems to somewhat differ from the rest, but this particular cohort was not exposed to FBZ at all, so the difference cannot be attributed to FBZ.

Figure 5-4

Jitter Plots by Cohort (Trapped Condition)



Note. (A) Opening frequency and (B) opening latency in minutes.

Opening Frequency. A robust three-way ANOVA showed that animals in standard housing were more likely to free their trapped conspecific than animals in enriched housing, $\hat{\psi}(1, 98) = 39.32$, $p < .001$, $\xi = 0.78$. In addition, females opened the door more frequently than males, $\hat{\psi}(1, 97) = 4.24$, $p = .043$, $\xi = 0.24$ (Figure 5-5). There was neither a significant main effect of treatment, $\hat{\psi}(1, 98) = 0.04$, $p = .860$, $\xi < 0.01$, nor any interactions. Results for the planned comparisons for the individual housing groups are displayed in Table 5-3 and descriptive statistics in Appendix C below. A robust mixed repeated-measures ANOVA showed a significant interaction between housing and sex, $\hat{\psi}(6, 39) = 10.67$, $p < .001$, as well as that animals in standard housing opened the restrainer significantly more frequently than those in enrichment, $\hat{\psi}(1, 57) = 49.96$, $p < .001$, $\xi = 0.57$ (Figure 5-5B). There was also a significant effect of session, $\hat{\psi}(6, 39) = 12.69$, $p < .001$.

Table 5-3

Planned Comparisons Mean Opening Frequency Housing (Trapped Condition)

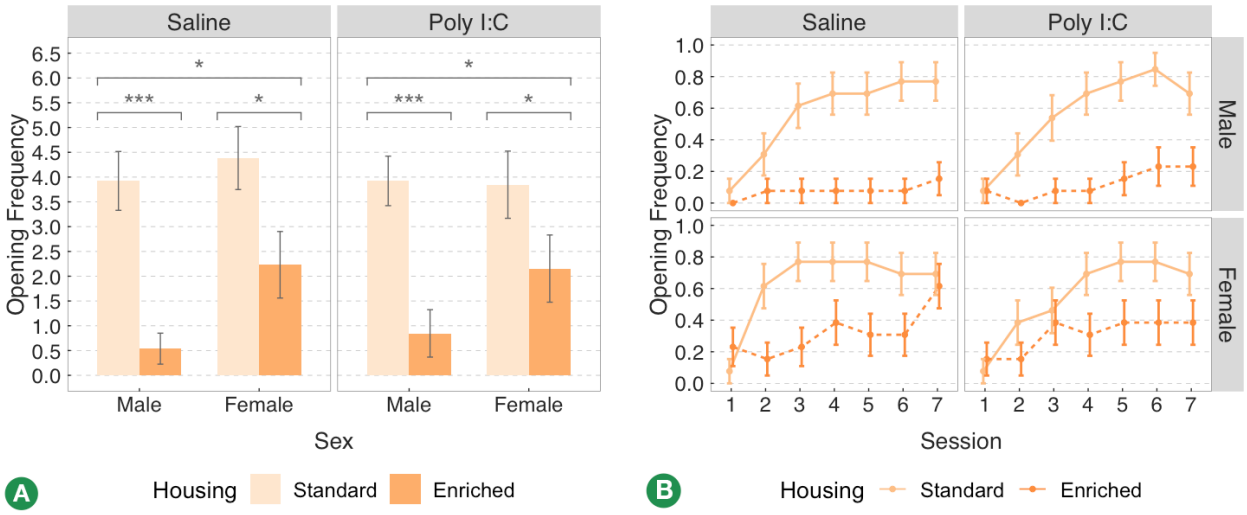
DF	Comparison	Group	$\hat{\psi}$	p	d
1, 96	Standard x Enriched	Saline, Male	4.12	< .001 ***	1.45
		Poly I:C, Male	3.74	< .001 ***	0.65
		Saline, Female	2.62	.010 *	1.04
		Poly I:C, Female	2.06	.042 *	0.57

Note. Also see Figure 5-5.

* $p \leq .05$. *** $p \leq .001$.

Figure 5-5

Opening Frequency (Trapped Condition)



Note. Error bars show mean ± *SEM*. A) Bar chart opening frequency. (B) Time series showing openings by session.

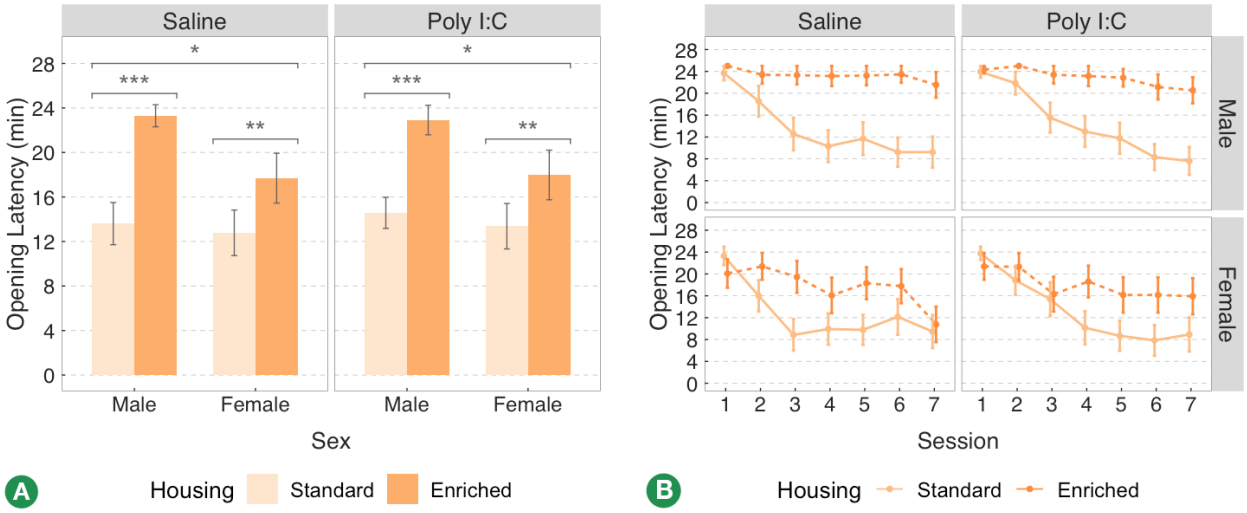
* $p \leq .05$. *** $p \leq .001$.

Opening Latency. The robust two-way ANOVA showed that animals in standard housing opened the door faster than those in enriched housing, $\hat{\psi}(1, 98) = 28.49$, $p < .001$, $\xi = 0.68$ (Figure 5-6). Furthermore, females opened the door at shorter latencies than males, $\hat{\psi}(1, 97) = 5.92$, $p = .018$, $\xi = 0.29$. Treatment, however, did not impact on opening latencies, $\hat{\psi}(1, 98) = 0.08$, $p = .780$, $\xi = 0.01$. There were no interactions, though housing x sex came close to being significant, $\hat{\psi}(1, 97) = 2.72$, $p = .103$. Further investigation of the opening

latency on the significant main effects showed that the housing effect was larger in males, $F(1, 18) = 58.88$, $p < .001$, $\xi = 0.89$, than in females, $F(1, 29) = 8.89$, $p = .006$, $\xi = 0.49$ (see Figure 5-6A). Descriptive statistics are shown in Appendix B below. A robust mixed repeated-measures ANOVA showed a significant interaction of housing and session, $\hat{\psi}(6, 34) = 8.44$, $p < .001$, as well as that animals in standard housing had significantly greater opening latencies than those in enrichment, $\hat{\psi}(1, 52) = 41.99$, $p < .001$, $\xi = 0.58$ (Figure 5-6B). There was also a significant effect of session, $\hat{\psi}(6, 34) = 11.43$, $p < .001$, $\xi = 0.48$.

Figure 5-6

Opening Latency (Trapped Condition)



Note. Error bars show mean ± *SEM*. A) Bar chart opening latency. (B) Time series showing latency by session.

* $p \leq .05$. ** $p \leq .01$. *** $p \leq .001$.

5.4.3 Trapped Condition (Openers)

To investigate if non-openers (i.e., animals that never opened) obscured a treatment effect, I removed all non-openers and ran three-way ANOVAs with the same variables as before. Descriptive statistics are shown in Table C-6 below.

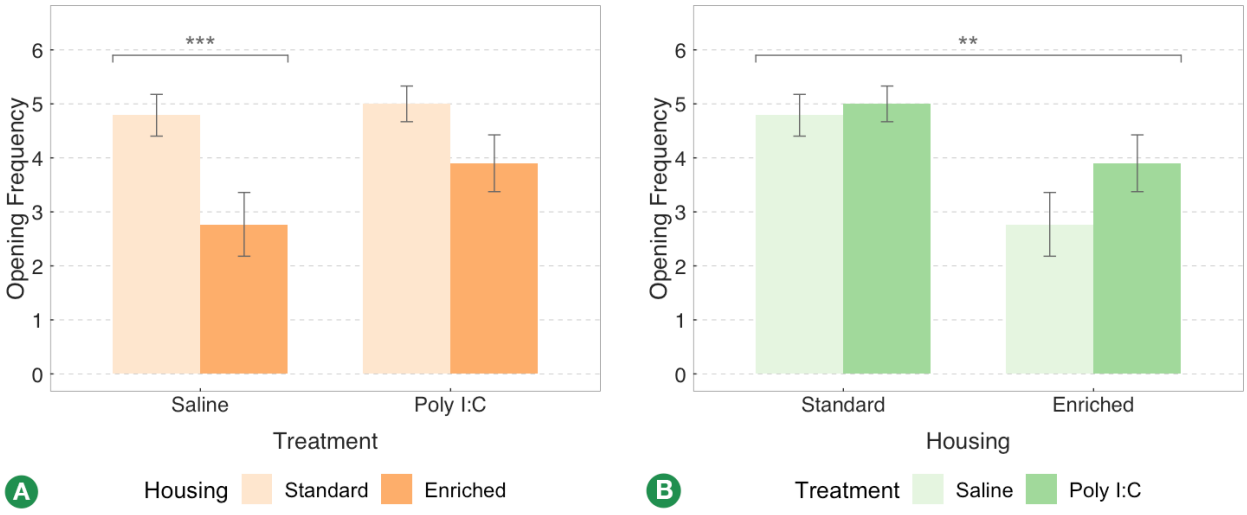
Opening Frequency. Again, animals in standard housing freed their cagemate significantly more frequently than animals in the enriched housing condition, $F(1, 45) = 12.88$, $p < .001$, $\eta^2 = 0.22$. However, there was no significant effect of treatment on opening frequency, $F(1, 45) = 1.75$, $p = .193$, $\eta^2 = 0.04$. Removing the non-openers also

removed the sex effect, $F(1, 45) = 1.30$, $p = .261$, $\eta^2 = 0.03$. This was likely the result of unequal group sizes, that is, 30 females were openers compared to only 23 males (see group sizes in Table 5-2 for reference). There were no interactions between the independent variables.

To investigate if enrichment affected opening frequency at the individual treatment levels, I computed planned comparisons at each level of the treatment variable. In the saline group, animals in standard housing opened the restrainer door significantly more often than animals in enrichment, $t(48) = 3.43$, $p = .001$, $d = 1.08$. In the poly I:C group, however, there was no difference between the two housing groups, $t(48) = 1.70$, $p = .096$, $d = 0.79$ (see Figure 5-7A). When looking at the effect of housing at each level of treatment as part of the planned comparisons, the effect of treatment was negligible in the standard housing group, $t(48) = -0.47$, $p = .643$, $d = -0.14$, but moderate in the enriched housing group, $t(48) = -1.58$, $p = .121$, $d = -0.58$, though it failed to reach significance (see Figure 5-7B).

Figure 5-7

Opening Frequency (Trapped Condition: Openers)



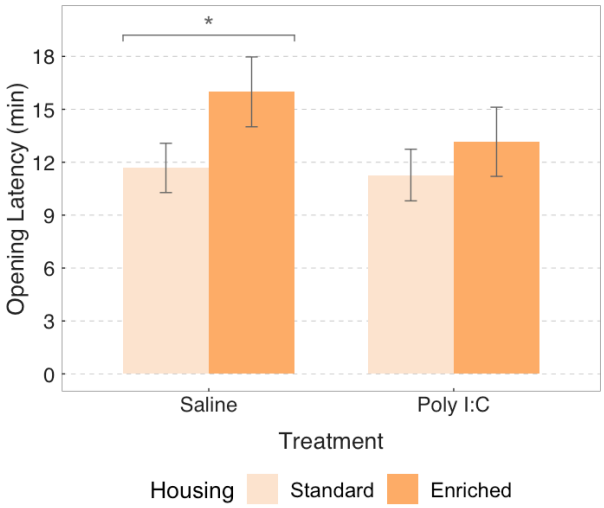
Note. Error bars show mean ± *SEM*. (A) and (B) show the housing effect on opening frequency.
** $p \leq .01$

Opening Latency. Similarly, animals in standard housing freed their cagemate significantly faster than animals in the enriched housing condition, even after the non-openers were removed, $F(1, 45) = 5.40$, $p = .026$, $\eta^2 = 0.11$. However, there was no significant effect of treatment on opening latency, $F(1, 45) = 0.85$, $p = .362$, $\eta^2 = 0.02$. Removing the non-

openers also removed the sex effect, $F(1, 45) = 2.27$, $p = .139$, $\eta^2 = 0.05$. To investigate if enrichment affected opening latency at the individual treatment levels, I computed planned comparisons of the housing effect at each level of the treatment variable. In the saline group, animals in standard housing opened the restrainer door significantly more often than animals in enrichment, $t(48) = -2.15$, $p = .036$, $d = -0.66$. In the poly I:C group, however, there was no difference between the two housing groups, $t(48) = -1.02$, $p = .312$, $d = -0.34$ (see Figure 5-8).

Figure 5-8

Opening Latency (Trapped Condition: Openers)



Note. Error bars show mean ± *SEM*. Bart chart shows the significant housing effect in the standard housing group.

* $p \leq .05$.

5.4.4 Results Summary

Table 5-4

Overview of Significant Prosocial Behaviour Results

Variable	Effect			Significance level	
	Condition #1 [a]	Direction [b]	Condition #2 [a]	Main	X [c]
Empty Condition					
Locomotor Activity	Saline	<	Poly I:C	**	
	Standard	>	Enriched	***	
Trapped Condition (all animals)					
Opening Frequency	Standard	>	Enriched	***	
	Males	<	Females	*	
	SAL/M/Standard	>	SAL/M/Enriched	***	
	PIC/M/Standard	>	PIC/M/Enriched	***	
	SAL/F/Standard	>	SAL/F/Enriched	*	
	PIC/F/Standard	>	PIC/F/Enriched	*	
Opening Latency	Standard	>	Enriched	***	
	Males	<	Females	*	
Trapped Condition (openers only)					
Opening Frequency	Standard	>	Enriched	***	
	SAL/Standard	>	SAL/Enriched	**	
Opening Latency	Standard	>	Enriched	*	
	SAL/Standard	>	SAL/Enriched	*	

[a] Standard = standard housing, Enriched = enriched housing; SAL = saline, PIC = poly I:C; M = male, F = female. [b] Effect direction is indicated by < (smaller), > (greater), and = (equal). [c] Interaction.

* $p \leq .05$. ** $p \leq .01$. *** $p \leq .001$.

5.5 Discussion

The present study explored the effect of poly I:C exposure during gestation on prosociality in the offspring and if/how environmental enrichment alleviates this effect. The investigation tested three hypotheses: First, adult rats prenatally exposed to poly I:C would demonstrate a lower level of prosocial behaviour by freeing their trapped cagemate less frequently than rats exposed to saline; they would also open the door more slowly. Second, an enriched environment would mitigate this effect. Poly I:C-exposed rats in enriched housing would free their cagemate more frequently than poly I:C-exposed rats in standard housing. Finally, females would display a higher level of prosocial behaviour and open the door more frequently and faster than males.

5.5.1 Females are More Prosocial than Males

The findings supported the hypothesis that females are more prosocial than males; females opened the door more frequently than males to free their trapped cagemate, corroborating previous findings (Ben-Ami Bartal et al., 2011). Taylor et al. (2000) theorised that, from an evolutionary perspective, females respond to stress with increased empathy-related behaviours towards known individuals (though less towards strangers). When looking at rodent empathy studies in general, female mice, but not males, approached a familiar mouse in pain more frequently than one that was not in pain (Langford et al., 2010). In contrast, in a recent rat prosocial choice study where rats could press a lever to dispense a sucrose pallet either to themselves only, or to themselves and a conspecific, males were more likely (than females) to share the food (Kentrop et al., 2020). The most obvious difference between the two studies was the presence of distress, which did not play a role in the prosocial choice task that involved food rewards, highlighting the complexity of empathy as a concept and the importance of underlying drivers.

5.5.2 Empty vs Trapped

Like Ben-Ami Bartal et al.'s study in 2011, hardly any of the animals opened the door when the restrainer was empty. As soon as their cagemate was trapped, though, the opening frequency increased drastically, suggesting that door-opening behaviour was connected to the social stimulus. Due to the extremely low opening frequency in the empty condition (7/114 animals) and the high possibility of accidental openings, this result will not be interpreted further.

5.5.3 *Treatment did not Affect Opening Behaviour*

VPA models and poly I:C models do not always produce the same results, which was the case with the present experiment. Surprisingly, in the current study, neither door opening frequency nor latency differed between the two treatment groups. This result was different from recent findings from a VPA autism study that utilised the social-release paradigm to investigate prosocial behaviours. Male rats prenatally exposed to VPA showed delayed helping behaviour; it took them on average 3 days longer than control animals to free the trapped rat, which the authors put down to impaired social communication and a subsequent inability to read social cues accurately (Fontes-Dutra et al., 2019). However, there were slight methodological differences between the Fontes-Dutra et al. (2019) study and the current one. They randomly assigned the trapped rat before each session, so the rat pairs differed between sessions and the trapped rats were chosen based on their body size (10% smaller than experimental animal) rather than their confidence level. Even with differences that would seemingly reduce opening behaviours, the authors demonstrated a clear VPA effect, indicating that there was no detectable poly I:C effect in adulthood the present study.

When considering data from animals that became openers, animals in the saline group freed the trapped rat significantly more often and faster in the standard housing condition than in the enriched housing condition. When studying the poly I:C group, though, there was no difference between the two housing groups (Figure 5-7A and C). Overall, there was no significant effect of treatment. There are no identical studies to compare these findings to, so the reasons for this are not clear. While the maternal poly I:C treatment clearly affected the dams (i.e., higher temperature and less weight gain) and offspring (i.e., lower overall body weight), it is possible that the effect was not strong enough to be detectable with the trapped rat paradigm. Also, it is possible that the poly I:C effect fades over time, perhaps due to facility-specific effects (Weber-Stadlbauer & Meyer, 2019).

5.5.4 *Enrichment Decreased Opening Frequency*

Standard-housed rats opened the restrainer door significantly more frequently than rats in the enriched housing condition. Housing did not interact with treatment, suggesting that the hypothesis that enrichment reduces poly I:C's effect on prosocial behaviour cannot be sustained. However, the treatment effect in the enrichment group was nearing significance with a moderate effect, whereas in standard housing, the effect was negligible. Enrichment reduced opening frequency, but the effect was not significant

in the poly I:C group, only in the saline group. Why then did enrichment reduce prosocial behaviour—the opposite of what was expected—and why did it affect control animals more than poly I:C animals?

As previous studies have demonstrated that the opening behaviour is linked to the trapped animal's distress levels (Ben-Ami Bartal et al., 2016; Sato et al., 2015), we could speculate that trapped rats that lived in enriched housing were less distressed to be in a restrainer than rats in standard housing, as the former were used to being inside tubes and tunnels. They would then not have signalled any distress to the free rat, which in turn did not find itself in a situation that required intentional action. Future studies on the enrichment effect on prosociality could either refrain from using tunnels in enriched housing or use the soaked rat paradigm introduced by Sato et al. (2015) to create a sufficiently novel situation.

In terms of treatment effect, poly I:C-exposed animals were not as affected by the enriched environment as control animals were. Their opening frequency did not drop as dramatically, perhaps because the trapped rat was more anxious in the poly I:C condition (Patterson, 2009; Shi et al., 2003; Smith et al., 2007). This line of reasoning indicates that the social-release paradigm where a rat is trapped in a restrainer may not measure prosocial behaviour when used with animals that live in enrichment, as being in the restrainer does not stress the animal enough to trigger prosocial behaviour (i.e., release from the restrainer) in the free rat.

In terms of distress levels, ideally, USV would have provided a more definite answer, but due to hardware and process issues, the recorded USV could not reliably be attributed to individual animals and, thus, could not be included in the present study. It is difficult to record USV with more than one animal in the arena, as individual calls cannot automatically be assigned to a specific animal. To date, microphones cannot be attached to individual animals. If, however, each arena could be equipped with a microphone to record calls per arena, call volume and type could potentially allow matching calls to the free vs the trapped animal. This, in turn, would provide information on the emotional state of each of the animals.

5.5.5 Locomotor Activity

When looking at LMA during the empty condition, the results are in accordance with Chapter 4 in that animals in standard housing showed significantly higher activity rates that animals in enriched housing (further discussed in Chapter 4 and Chapter 6).

Intriguingly, saline control animals covered significantly less distance than poly I:C-exposed animals, possibly due to heightened anxiety in the poly I:C group (Patterson, 2009). In addition, it would have been interesting to compare LMA during the empty condition with the trapped condition, as hyperlocomotion is usually a response to an unfamiliar environment and the animals would have been habituated to the room and apparatus by this time. As noted above, Ethovision was not able to reliably distinguish between the trapped and the free animal, so automatic tracking was not possible as part of the present experiment.

5.6 Summary

This study showed that environmental enrichment significantly reduced opening frequency and latency. It further supported the hypothesis that females are more prosocial than males. It did not, however, show a significant poly I:C effect on prosociality, nor did enrichment improve poly I:C's effect on prosocial behaviour. Animals housed in an enriched environment are used to tubes and tunnels, so the rat within the restrainer was likely not distressed enough. As the social release paradigm relies on the expression of distress by the trapped rat, a lack of distress likely will result in less intentional release behaviour by the free rat, thus, rendering the model unsuitable in this context. Similar to Chapter 4, animals reared in an enriched environment displayed hypolocomotion; the next chapter will explore this enrichment effect, as well as the poly I:C effect on LMA and exploratory behaviour in more detail.

Chapter 6

Locomotor Activity

Chapter 6
Locomotor Activity

6.1 Background

This chapter will discuss how exposure to poly I:C in utero, along with pre- and post-weaning enrichment affected spontaneous LMA in adulthood.

LMA in a novel environment is an expression of explorative behaviour in rodents. The introductory point to make is that the results of LMA in rodents prenatally exposed to poly I:C differ between the reviewed studies. Prenatal poly I:C exposure increased LMA in a novel open field in Long Evans rats; females were more active than males, and poly I:C animals more so than saline controls (Howland et al., 2012). A recent study confirmed this effect in females Wistar rats but found that poly I:C treatment had the opposite effect on males—they covered significantly less distance than saline controls (Meehan et al., 2017). Another study found that prenatal poly I:C treatment reduced LMA in SD rats overall but that it did not differ between sexes (Van den Eynde et al., 2014). Meyer et al. (2005) found no difference in distance covered between the poly I:C and saline groups in C57BL6/J mice, but animals in the poly I:C group showed significantly less exploratory behaviour in an open field test (measured by the number of entries into the centre area). Further, neither Bronson et al. (2011) nor Ratnayake et al. (2014) found a poly I:C treatment effect on LMA in rats and spiny mice respectively. However, after MK-801 exposure, the offspring of dams that had lost weight following poly I:C treatment showed reduced LMA compared to controls, while the offspring of dams that had gained weight showed increased LMA (Bronson et al., 2011), showing how strongly individual differences in the maternal immune response can influence the offspring's behavioural outcome. Route of administration does not seem to account for these mixed patterns. Following IV administration of poly I:C, Howland et al. (2012) found a general increase in LMA, Van den Eynde et al. (2014) reported an increase overall, Meehan et al. (2017) found an increase in females, but a decrease in males, while Meyer, Murray, et al. (2008) found no difference. Similarly, dose also does not appear to be responsible, as Howland et al., Meehan et al., and Van den Eynde et al. all used 4 mg/kg, and all had different patterns. Likewise, time of administration also produced mixed patterns; administration on GD 15 can result in an overall LMA increase (Howland et al., 2012) or a decrease (Van den Eynde et al., 2014), while administration on GD 9 may not produce an effect (Meyer, Murray, et al., 2008). Meehan et al. chose GD 10 and GD 19 and reported an increase for females and a decrease

for males, but day of administration was not significant. What the studies had in common was that often poly I:C-exposed animals displayed significantly altered LMA when compared with saline controls.

Animals housed in an enriched environment are generally less active in the open field test (O. B. Amaral et al., 2008; Van Waas & Soffié, 1996; Zheng et al., 2020). Environmental enrichment also affects explorative behaviours, mainly in that it results in faster processing of environmental clues (Elliott & Grunberg, 2005; Varty et al., 2000). As mentioned in Chapter 4, enrichment reduced LMA in an open field test in mice (O. B. Amaral et al., 2008; Zheng et al., 2020) and rats (Varty et al., 2000). Zheng et al. (2020) reported that all mice moved at the same velocity for the first 4 of the 10 minutes, but animals living in enrichment moved significantly more slowly than standard-housed animals after that, suggesting faster habituation to a novel environment. This is likely a result of the difference in information processing speed between the housing conditions. They further reported that enrichment animals were less anxious and showed improved learning and memory performance relative to standard-housed animals. From a neuro-structural point of view, these behaviours have been connected to increased neurogenesis and maturation of neurons in the dentate gyrus within the hippocampal formation, a region connected to, among others, novel environment exploratory behaviour (Nithianantharajah & Hannan, 2006; Ohline & Abraham, 2019; van Praag et al., 2000). Further, enrichment increases synaptic strength (Foster et al., 1996).

6.2 Predictions

The studies most similar to mine in dose and day of administration (i.e., Howland et al. and Van den Eynde et al.) reported opposing patterns. Because of this, it is hard to predict how LMA will be affected by the poly I:C treatment in the present study. Therefore, this chapter will investigate the following hypotheses:

A. Animals housed in enrichment will be less active than standard-housed animals.
B. In line with the predictions from the previous chapter, I again predict that poly I:C will affect animals in standard housing more than animals in enriched housing—the treatment effect will be more pronounced in standard housing.

6.3 Methods

6.3.1 Animals

All animals used in this experiment had been part of the experiments described in Chapters 3 to 5. They were tested in five cohorts (Table 6-1) and divided into four groups: (1) SH/SAL, (2) SH/poly I:C, (3) EH/SAL, and (4) EH/poly I:C. Each group consisted of 48 animals, 24 males and 24 females, that originated from 15 litters per treatment condition. As discussed in Chapter 2, pups of cohort 1 were exposed to FBZ in utero for 9 days. Also, all adults in cohort 4 received medicated chow for a total of 35 days.

Table 6-1

Total Number of Tested Animals by Cohort (LMA)

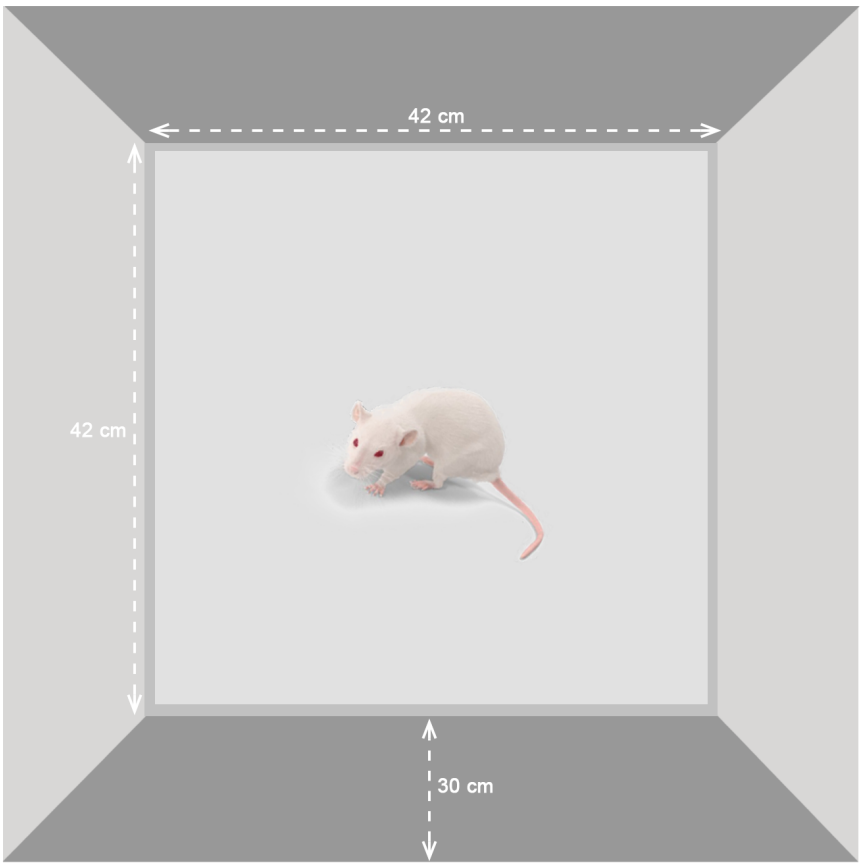
Cohort	Treatment	Housing	Males	Females	Total
#1	Saline	Standard	4	4	8
		Enriched	0	0	0
	Poly I:C	Standard	12	12	24
		Enriched	0	0	0
#2	Saline	Standard	10	10	20
		Enriched	10	10	20
	Poly I:C	Standard	6	6	12
		Enriched	6	6	12
#3	Saline	Standard	10	10	20
		Enriched	8	8	16
	Poly I:C	Standard	6	6	12
		Enriched	8	8	16
#4	Saline	Standard	0	0	0
		Enriched	6	6	12
	Poly I:C	Standard	0	0	0
		Enriched	10	10	20
Total			96	96	192

6.3.2 *Apparatus*

The animals were tested in eight transparent plexiglass open field chambers (Med Associates Inc., USA, model ENV-515) set in sound-attenuating boxes (Model OFA-017). Each chamber measured 42 cm (length) x 42 cm (width) x 30 cm (height) and was equipped with 16 evenly spaced infrared transmitters and receivers on the *x* and *y* axes to track the animals' location within the chamber. The software (Activity Monitor 5.93, Med Associates Inc., USA) used these location coordinates to compute LMA and animal location within the chamber (see Figure 6-1). Each chamber was covered with a transparent perforated plexiglass cover.

Figure 6-1

Open Field Arena



6.3.3 Procedure

The animals were transported to the experimental room in their home cages 30 minutes prior to testing. Before the session, each chamber was checked and confirmed to be functional. One animal at a time was placed inside a testing chamber, which was then covered with the plexiglass cover and the sound-attenuating box door was closed. Each animal was tested once for 60 minutes, and eight animals were tested simultaneously. The chamber started collecting data as soon as the animal had entered the open field. Between sessions, the chambers were cleaned with Virkon disinfectant. The animals were tested under red light during the animals' dark cycle between 8 am and 6 pm. After the session, the animals were returned to their respective housing rooms.

6.3.4 Data and Statistical Analysis

I exported the Activity Monitor (Med Associates Inc., USA) raw data with Visual Basic (Microsoft, Version 7.1) for subsequent statistical analysis with the RStudio editor (RStudio Team, 2020). The alpha (α) level was set at .05 and a p-value lower than that ($p \leq \alpha$) considered statistically significant.

The dependent variable was distance covered in metres. The independent variables were treatment (saline, poly I:C), housing (standard, enriched), and sex (male, female). The distance dataset violated both the assumption of normality (residuals and by-group analysis) and that of equal variance and also contained outliers, so I used robust three-way ANOVAs ($\hat{\psi}$) for analysis. I conducted planned pairwise comparisons on the individual levels of the housing variable using the robust function "t1way"; the p-values were adjusted with the FDR method.

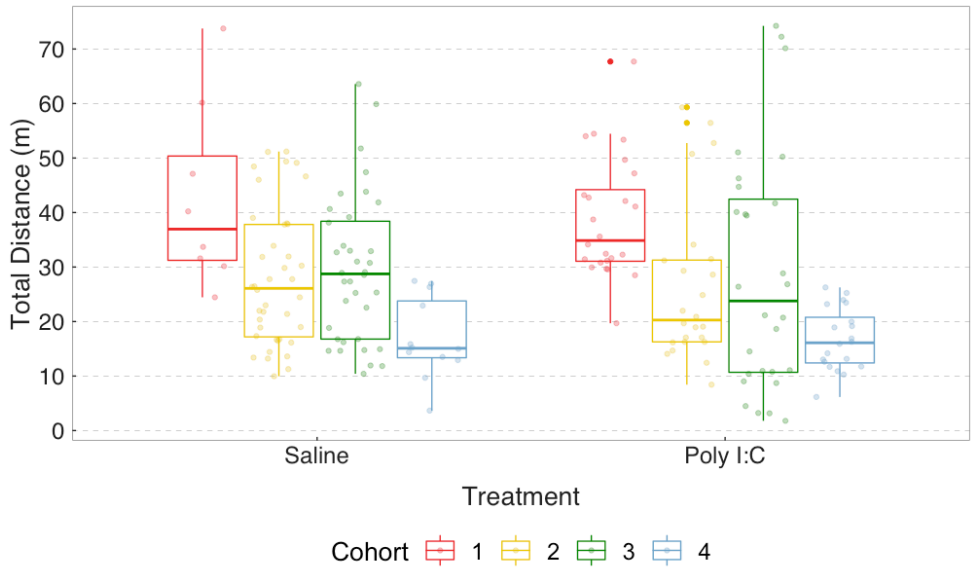
6.4 Results

6.4.1 *Total Distance*

The jitter plot in Figure 6-2 shows no effect of FBZ on total distance covered.

Figure 6-2

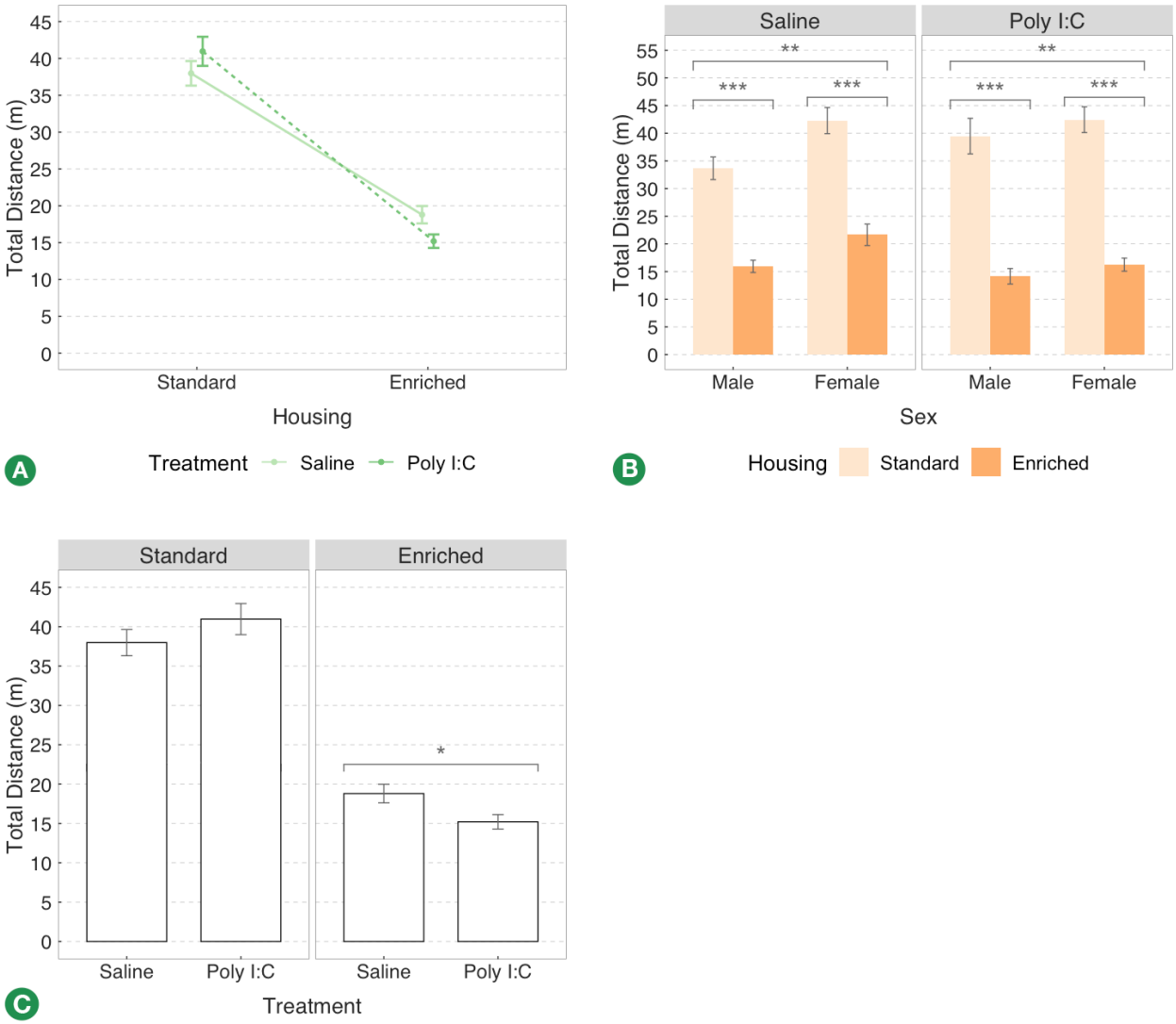
Jitter Plot: Distance by Cohort



Total Distance. A robust three-way ANOVA showed a significant interaction of treatment x housing, $\hat{\psi}(1, 146) = 5.16$, $p = .026$ (Figure 6-3A). Also, animals in enrichment covered significantly less distance than those in standard housing, $\hat{\psi}(1, 146) = 238.69$, $p < .001$, $\xi = 0.74$, and females covered significantly more distance than males, $\hat{\psi}(1, 186) = 11.11$, $p = .002$, $\xi = 0.22$ (Figure 6-3B). There was no significant main effect of treatment, $\hat{\psi}(1, 178) = 0.04$, $p = .840$, $\xi = 0.01$, nor were there any further interactions. A planned comparison at each level of the housing variable showed that, in enrichment, poly I:C-exposed animals covered significantly less distance than saline-exposed animals, $F(1, 89) = 5.77$, $p = .037$, $\xi = 0.35$ (Figure 6-3C). There was no significant difference between treatment groups in standard housing, $F(1, 91) = 1.34$, $p = .251$, $\xi = 0.16$. Descriptive statistics are shown in Table D-1 in Appendix D.

Figure 6-3

Distance Plots



Note. Error bars show mean ± *SEM*. A) Treatment x housing interaction. (B) Main effects. (C) Significant treatment effect in enrichment.

* $p \leq .05$. ** $p \leq .01$. *** $p \leq .001$.

6.4.2 Results Summary

Table 6-2

Overview of Significant LMA Results

Variable	Effect			Significance	
	Condition #1	Direction [b]	Condition #2	Main	X [c]
Distance (Total)	Saline	=	Poly I:C		*
	Standard	>	Enriched	***	
	Male	<	Female	**	
	EH/Saline [a]	>	EH/Poly I:C	*	

Note. [a] EH = Enriched housing. [b] Effect direction: < (smaller), > (greater), and = (equal). [c] Interaction.
* $p \leq .05$. ** $p \leq .01$. *** $p \leq .001$.

6.5 Discussion

This study investigated the impact of exposure to the viral mimic poly I:C during gestation and environmental enrichment on LMA in adulthood. The first hypothesis stated that enriched-housed animals would be less active than standard-housed animals. The second prediction was that poly I:C would affect animals in standard housing more than animals in enrichment.

6.5.1 Animals in Enrichment are Less Active

The result supported the first hypothesis that animals in enrichment would cover less distance than animals in standard housing. Animals reared in enriched-housing were significantly less active than standard-housed animals, which corroborated previous findings (O. B. Amaral et al., 2008; Zheng et al., 2020). This indicates that enrichment promotes faster habituation, as habituation can be defined as reduced activity (Brenes et al., 2016; Elliott & Grunberg, 2005). While the present study differed from theirs in that group size in social enrichment was three to six, whereas it was six to 10 in the current study, the effect seems robust to changes in group size.

6.5.2 Poly I:C's Effect is More Pronounced in Standard-housed Animals

The interaction between treatment and housing lent support to the second hypothesis; poly I:C affected standard-housed animals more than animals in enrichment. Poly I:C animals in standard housing covered more distance than saline animals, but in enrichment they covered less, a finding consistent with that of the SAA study in Chapter 4 where poly I:C-exposed male pups that had received enrichment covered significantly less distance than any of the other groups. The present results strengthen the suggestion that enrichment improved the deficient behaviours resulting from the prenatal poly I:C treatment, at least in the context of LMA. This makes sense, as enrichment is known to strengthen synaptic connections (Foster et al., 1996), which, in turn, results in more efficient processing of environmental cues (Elliott & Grunberg, 2005; Varty et al., 2000).

In terms of distance travelled, there was no significant difference between the treatment groups. This overall lack of treatment effect is consistent with results from Meyer et al. (2005) and Bronson et al. (2011), but Van den Eynde et al. (2014) found that poly I:C animals were less active than saline animals.

6.5.3 *General Sex Differences*

An additional finding was that females were more active than males—overall, they travelled a significantly greater distance than males, corroborating previous findings (Elliott & Grunberg, 2005; Howland et al., 2012). There was no treatment x sex interaction in the present study, but others reported that males exposed to poly I:C covered significantly less distance than saline control males, but it was the opposite for females. (Meehan et al., 2017). Elliott and Grunberg (2005) found that enrichment had a stronger effect on males than on females, but the present study did not find an interaction between housing and sex. To my knowledge, no other study has investigated the influence of enrichment on sex differences in an open field, so this could be an attractive future research direction.

When looking at spatial navigation, a study found that males performed better than females overall, but there was no performance difference between the sexes in rats brought up in enrichment (Peña et al., 2009; Seymoure et al., 1996). Seymoure et al. (1996) suggested that the reason for the sex differences might be due to sex-specific anatomical differences in the prefrontal cortex and hippocampus. Further, females have a more active hypothalamic–pituitary–adrenal axis (HPA axis), which means they perceive and respond to identical environmental stimuli differently (e.g., hyperlocomotion) (Simpson & Kelly, 2012).

There seems to be a great deal of variability in effects of treatment and sex on LMA in rodents. The reasons for this are unclear, but it is very likely due to differences in time, dose, and route of poly I:C administration. The present study used 5 mg/kg SC on PND 15, but others used 4 mg/kg IV on PND 10 or PND 19 (Meehan et al., 2017), or PND 15 (Howland et al., 2012; Van den Eynde et al., 2014). Yet others administered 8 mg/kg IP on PND 15. Also refer to the differences in treatment outcomes across different paradigms discussed in detail in Chapter 2.

6.6 Summary

The present study highlighted that enrichment had a significant effect on LMA in adult SD rats. Animals that received pre- and post-weaning enrichment were significantly less active than standard housed animal. It also identified an interaction between treatment and enrichment, establishing that the enriched environment attenuated the poly I:C effect. It would be interesting to investigate the contribution of pre-weaning

enrichment to these improvements, something a future study on the topic could do to pinpoint when exactly during development enrichment has the greatest effect.

This concludes the reports of behavioural experiments conducted with animals exposed to poly I:C in utero and housed in two different environments—standard and enriched. What follows is the general discussion where I will connect the various findings presented in the individual chapters so far.

Chapter 7

General Discussion

Chapter 7
General Discussion

7.1 Thesis Aims and Predictions

The experiments discussed in this thesis aimed to investigate the impact of environmental enrichment on prosociality using a well-established rodent autism model: prenatal poly I:C administration. The main prediction was that prenatal exposure to poly I:C would result in lower levels of prosocial behaviour and that pre- and post-weaning enrichment would mitigate this deficit. Specifically, I hypothesised that animals prenatally exposed to poly I:C would free a trapped cagemate from a restrainer less frequently and with greater opening latency, and that females would be more prosocial than males (Chapter 5). As ASD's social-behavioural domain also includes communication deficits, I predicted that, following in utero poly I:C exposure, pups would emit fewer and shorter neonatal USV, and that this deficit would be more pronounced early on (Chapter 3). Also, I expected that pre-weaning enrichment would reduce this effect. I further anticipated that poly I:C would negatively impact the expression of basic social functions such as general sociability and social novelty preference in adolescence and adulthood and that pre- and post-weaning enrichment would reverse these deficits (Chapter 4). Lastly, as LMA is often affected in MIA models of autism as well as models of enrichment, I included this paradigm as a positive control. I hypothesised that enrichment would reduce LMA overall and that the poly I:C effect would be stronger in standard-housed animals (Chapter 6).

7.2 Summary of Findings

Table E-1 below provides an overview of all hypotheses and the respective results. When considering the overarching patterns, there were only transient effects of poly I:C on communication very early during development. In the context of the main thesis objective, the picture that emerges is one of little to no change in social behaviours following the in utero poly I:C treatment. It appears that the employed poly I:C treatment protocol only impacted the animals mildly very early during development but did not cause long-term impairments in the social realm. Basic social behaviours were unaffected by enrichment, while prosociality was affected, but not in the expected direction, which was likely the result of methodological issues. Across all experiments, the poly I:C group showed the same variability as the control group, which is one of the themes I will discuss

in detail, as this variability is possibly the underlying reason for the lack of treatment effect on social behaviours.

In general, there are three possible ways to understand these results with respect to the effects of poly I:C: First, poly I:C did not have the expected systemic effect due to procedural issues. Secondly, perhaps poly I:C affected some behaviours (i.e., neonatal communication, LMA), but not others (i.e., social behaviours). Thirdly, the poly I:C effect faded over time and the more time elapsed between treatment and experimental testing, the smaller the effect became. Given the very weak effects of poly I:C, the lack of improvement of enrichment may not be surprising, as there were no clear deficits to improve.

7.3 The Challenge of Variability in Animal Models of ASD

7.3.1 Behavioural Outcomes

Basic social behaviours like general sociability are among the few that show little variability across MIA studies (Kentner et al., 2019). The effect of poly I:C on general sociability and social novelty behaviours is generally robust to variation in dose, administration time, species, sex, or animal age (Chen et al., 2019; Lins et al., 2018; Lins et al., 2019; Malkova et al., 2012; Smith et al., 2007). However, the present study did not find a treatment-related effect on general sociability and social novelty preference as has been discussed in detail in section 4.6. To reiterate, while most adult animals showed a preference for the social stimulus over the non-social stimulus, there was no detectable treatment effect. There was no preference for the novel social stimulus over the familiar one, which was unexpected—at least in the saline control group—as the SAA paradigm is a well-replicated test.

When considering rodent USV following poly I:C treatment, it becomes apparent that here too the results are rather variable. Studies found that poly I:C-treated pups produced either more (Schwartzer et al., 2013) or fewer calls (Chou et al., 2015; Malkova et al., 2012; present study). Calls might be shorter (Malkova et al., 2012) or similar in duration when compared to saline controls, like they were in Chapter 3. The only other MIA study that reported fewer and shorter calls early on used LPS rather than poly I:C (Baharnoori et al., 2012). Delta frequency can be considered a measure of call complexity and in the current study, poly I:C animals presented with lower delta frequency. To date, no other poly I:C studies reported on this call feature, so the study described in Chapter 3 contributed novel knowledge to the literature.

Similarly, poly I:C has a variable effect on LMA, as could be seen in the LMA measures across the studies discussed in this thesis. In the empty condition of the prosocial behaviour study, the poly I:C rats presented with hyperlocomotion, showing a clear treatment effect, but no poly I:C effect on LMA could be detected in either the social approach–avoidance study or the open field test. Based on the previously mentioned inconclusive findings on the poly I:C effect on LMA in rodents, this did not come as a surprise. Rodents showed either hypolocomotion (Meehan et al., 2017; Van den Eynde et al., 2014), hyperlocomotion (Howland et al., 2012), or no difference when compared to control animals (Bronson et al., 2011; Meyer et al., 2005; Ratnayake et al., 2014). In isolation, this could be taken as the ineffectiveness of the poly I:C treatment in the present study, but similar to Meyer et al. (2005), my animals showed less exploratory behaviour than saline controls without significant differences in LMA overall, indicating that poly I:C only affected certain behaviours, rather than resulting in a systemic effect.

Taken together, none of the above-discussed factors offers a reasonable explanation for the variable effects observed across the various studies included in this thesis. Why then did poly I:C affect some behaviours, but not others? The next section will consider conceivable reasons for the observed findings.

7.3.2 Variability in the Poly I:C Effect

Possibly the biggest concern when using poly I:C as an agent to induce immune activation is related to potential differences in the length of the double-stranded poly I:C itself. Poly I:C is a double-stranded RNA polymer consisting of a poly-inosinic and a poly-cytidylic acid strand. Mueller et al. (2019) tested different batches of poly I:C from the same vendor (Sigma Aldrich) and found that they varied in terms of the length of the strands, likely leading to differences in the strength of the resulting immune response and spontaneous abortion rates in mice. In line with this, Careaga et al. found that poly I:C with high molecular weight (i.e., longer strands) resulted in a great increase of cytokine activity and sickness behaviour, while poly I:C with low molecular weight did not have this effect on the animals. For the poly I:C used in the present project, there was no information on molecular weight or composition available, but it is certainly conceivable that the lack of a clear treatment effect was related to the composition of the poly I:C product.

Related to this is the severity of the maternal immune response, which is largely driven by the poly I:C dose (Meyer et al., 2005). As the dose differed vastly between the reviewed studies (see Chapter 2 for details), I conducted a pilot study where I tested the

effects of two doses (5 mg/kg and 8 mg/kg) on social behaviours and communication. Based on the pilot study outcome where a dose of 5 mg/kg on PND 15 of poly I:C showed deficient basic social behaviours using the same paradigms described in this thesis (not reported here), the dose for the main study was set to 5 mg/kg accordingly. While it is important to note that I used the same poly I:C batch for both pilot and main study, the pilot was done on only a few litters, which may explain why some effects of the pilot could not be replicated in the main study.

Most mouse studies used doses ranging from 2.5 mg/kg (Meyer et al., 2005) to 20 mg/kg (Smith et al., 2007; Zhu et al., 2014), though only the higher doses produced abnormal social behaviours. On the other hand, rat studies used doses between 0.75 mg/kg (Fortier et al., 2007) and 8 mg/kg (Bronson et al., 2011); the majority set a dose at 4 mg/kg and reliably produced behavioural deficits (Howland et al., 2012; Lins et al., 2018; Lins et al., 2019; Meehan et al., 2017). It is possible that the dose used in the current study, combined with the length of the poly I:C fragments only induced a very mild immune response. As a result of this, the long-term consequences were very small and the initial deficits (i.e., in USV) faded over time.

Another point to make is the importance of timing in the administration of poly I:C during fetal development (Meyer et al., 2006). Depending on when during gestation an environmental insult occurs, neurodevelopment will be disrupted at a different stage, affecting the behavioural outcome. In rodents, the gestational period is relatively short when compared to humans—approximately 21 days vs 280 days. In humans, a viral infection during trimester 1 resulted in an almost three-fold increase of autism in the offspring, while a bacterial infection during trimester 2 increased the risk by only about one-and-a-half (Atladóttir et al., 2010). Some rodent studies have reported subtle but important behavioural differences depending on timing. Intraperitoneal poly I:C administration on GD 9 resulted in reduced sociability in mice at a dose of 20 mg/kg (Zhu et al., 2014), as well as on PND 12.5 (Ratnayake et al., 2014). However, the first study reported differences in number of contacts with the social stimulus, while the second study reported on preference for social chamber rather than between-group differences. Shi et al. (2009) found a reduction of cerebellar Purkinje cells following poly I:C injection on GD 12.5 in mice, resulting in deficient social communication. Timing of the environmental insult is an important factor in MIA models, but the window of opportunity to interfere with the neural development spans over several days and the interference does not have to occur at the developmental peak, which explains why

studies with different administration timings reported social-behavioural deficits. Derived from the outcome of the pilot study, I chose to administer poly I:C on GD 15. Exposure at this time point usually leads to reduced sociability (Kentner et al., 2019; Lins et al., 2018; Lins et al., 2019) and altered LMA in rats (Howland et al., 2012; Van den Eynde et al., 2014), though not all studies found an effect on LMA (Bronson et al., 2011). Based on the results of the present project, the selected time of administration was appropriate to induce deficits in neonatal USV, but perhaps not ideal to affect prosocial behaviour.

7.3.3 Measures of Treatment Success

While there was no way to determine the composition of the poly I:C used to trigger the maternal immune system, there are other indicators of treatment success like maternal weight loss and heightened temperature following treatment (Cunningham et al., 2007; Fortier et al., 2004). The results presented in Chapter 2 showed elevated temperature and less body weight gain in the poly I:C treated dams after MIA treatment. However, even in the measures where there was a significant effect at group level, variability was still great at the individual level.

Bronson et al. (2011) found that offspring of dams that lost weight showed decreased LMA, but if the dams had gained weight, the offspring showed increased LMA relative to controls. Others had incongruous results where maternal weight gain/loss was not related to altered LMA. Intriguingly, the older the dam, the greater the weight loss in the 24 hours post poly I:C treatment (Bronson et al., 2011). This latter effect could not be investigated in my sample, as the dams were all about the same age at the time of mating. Like Bronson et al. (2011), I found that the maternal weight difference was rather irregular between animals and, while the poly I:C-treated dams gained significantly less weight than the saline controls, only a few of them actually lost weight, which is at odds with past research where all dams lost weight in the 24 hours following poly I:C treatment (Cunningham et al., 2007; Zuckerman et al., 2003). In line with the arguments above, the fact that my animals did not lose weight is indicative of a mild infection in the present sample, whereas actual weight loss would point at a stronger immune response and more severe infection. In the poly I:C group, when comparing the behavioural outcome of pups born to individual dams that lost weight to pups of dams that gained weight, there were no detectable patterns, tentatively indicating that there was no relationship between maternal weight loss and pup behavioural deficits.

When considering the offspring's weight, poly I:C animals weighed significantly less than saline animals in adulthood, though others found that only females exposed to

poly I:C in utero displayed this effect (Bronson et al., 2011; Vorhees et al., 2012). Bronson et al. (2011) further reported that dams that had gained weight in the 24 hours post poly I:C treatment had offspring that weighed the same as saline controls. Others did not find a significant weight difference between saline and poly I:C animals in adulthood (Malkova et al., 2012). The fact that the poly I:C group weighed less than the saline group shows that poly I:C affected the animals long-term, which was also apparent when considering the reduced LMA of adult poly I:C animals in enriched housing. However, this effect was not apparent when considering social behaviour. This indicates that there was a treatment effect on some behaviours, just not social behaviours. Furthermore, it also shows a poly I:C effect in adults, so if the effect indeed fades with time, it does not do so completely.

7.3.4 Environmental Sources of Variability in the Effects of Poly I:C

An important point to consider is a-posterior variability in MIA models. Factors that are facility- and cage-related (e.g., hygiene standards, enrichment, social hierarchies, etc.) can affect experimental control and alter the behavioural outcome of the MIA treatment (Weber-Stadlbauer & Meyer, 2019). In the present study, one such factor was the FBZ treatment some of the animals experienced. FBZ was administered as some animals in the colony had pinworms. Pinworm infestation may affect research outcomes, as it can increase the immune response to antigenic stimuli (Pritchett & Johnston, 2002). It is unclear how many of the animals were affected, but it may have increased the variability in the behavioural responses. Other factors like social hierarchies within a cage are not as easily controlled for, at least not in enrichment, as the social groups are much larger than in standard housing. Less dominant animals might be stressed by the more dominant animal and withdraw socially. In an enriched environment, the more dominant animal(s) might "commandeer" resources and the less dominant animal subsequently misses out on an essential part of the environmental resources (Howard, 2002). This can lead to increased stress in the subordinate animal and result in greater variation in behavioural assays (Weber-Stadlbauer & Meyer, 2019). This also applies to animals in standard housing, as there are also resources (i.e., shelter, chew block) that can be blocked, though to a lesser degree than in enriched housing. This could explain why there was no difference in variability between treatment and control groups in the studies included in this thesis and stresses the importance of constant observations of the individual cages and, where required, changes to social pairings. However, in large enrichment cages with up to 10 animals, it would be difficult to identify such social hierarchies.

While not directly related to variability within a study, an aspect that impacts on reproducibility between labs is different caging systems. Individually ventilated cages (IVC) can change the behavioural effects of genetic mouse models, for instance, mice with a mutation on the schizophrenia risk gene *Nrg-1* did not show deficient prepulse inhibition if they had been raised in IVC, but the deficit was observable in mice raised in open cages (OC) (Logge et al., 2014). Further, male mice reared in IVC showed reduced LMA and increased anxiety (Kallnik et al., 2007), altered sensitivity to MK-801, and increased social interaction in an open field social interaction test in both sexes (Logge et al., 2013). Mueller et al. (2018) observed greater spontaneous abortion rates in mice after administration of 5 mg/kg poly I:C on GD 9 in IVC when compared with OC. Mueller et al. (2018) also found that animals in IVC had significantly increased cytokine activity, as well as higher blood levels of the stress hormone cortisol when compared to animals in OC, indicating that animals in IVC are often more stressed than those in OC (David et al., 2013). This points at a connection between the impact of stress on the immune system and the detrimental effect stress during early development can have on the pregnancy outcome. In the present study, the standard-housed animals lived in IVC, so the effect of poly I:C should have been amplified as a result.

7.3.5 The Impact of Environmental Enrichment on Poly I:C-related Deficits

Like the poly I:C treatment, enrichment did not change general sociability behaviour, similar to past findings in a mouse study (Hendershott et al., 2016), though others found that enrichment did improve sociability behaviour in mice (Rae et al., 2018; Zheng et al., 2020). However, due to the differences in social structures between rats and mice, comparisons should be made with caution. None of the reviewed studies investigated social novelty preference, but this was unaffected by enrichment as outlined in Chapter 3.

The effect of enrichment on the deficits caused by the prenatal poly I:C exposure showed only very early on when investigating neonatal USV. After the animals had experienced about half of the pre-weaning enrichment sessions (on PND 14), enrichment interacted with the poly I:C treatment. Pups in the treatment group that had received enrichment produced more calls, but no such effect was observed in the saline group, nor was there an overall effect of poly I:C at this point. It is possible that the early enrichment triggered neural plasticity in an attempt to compensate for the deficits resulting from the poly I:C exposure.

Plasticity is the brains' adaptive capability that enables improvements of, for example, learning and memory, but it is also the underlying process of repairs following damage from injury or neurodevelopmental disruption. During the early developmental period, the number of neurons, dendritic spines, and axon connections increases immensely (Luo & O'Leary, 2005). Throughout puberty and adolescence, a process called pruning shapes the axonal connections until they reach the precise functional connectivity found in the adult brain. At this developmental stage, early enrichment has a massive impact and can, therefore, have long-term effects on abilities like problem-solving abilities (Venable et al., 1988). In the context of ASD models, early enrichment shows effects like better social engagement and more exploratory behaviours (Schneider et al., 2006).

While there are no other studies that investigated the effect of pre-weaning enrichment on neonatal USV, in adult animals, physical enrichment can negatively impact on social communication in both sender and receiver, while social enrichment has the opposite effect (Brenes et al., 2016). Brenes et al. concluded that social enrichment increased the rats' motivation to engage in social contact and that it compensated for the negative effects following physical enrichment (i.e., a focus on environmental objects rather than conspecifics). It is conceivable that the combination of physical and social enrichment neutralised the respective effects on social behaviours in the enrichment group, resulting in a lack of group differences in the SAA test.

This notion that one requires social interaction to improve one's social skills is reminiscent of the social motivation hypothesis that suggests that the primary driver for social-behavioural deficits in ASD is reduced motivation to engage socially. Reduced motivation leads to less exposure to social situations, which in turn means that the neural underpinnings of social behaviour cannot develop as they would have, had the individual "practiced" their social skills. Therefore, in humans, early intervention with a focus on social interaction practice can improve social abilities long-term (Dawson, 2008).

7.4 Developing an Animal Models for Autism

One of the inherent limitations of ASD animal models is that there is not one model that replicates all core symptoms of the condition (Ruhela et al., 2015), rather they usually model specific disease aspects that may apply to more than once condition. The poly I:C model, for instance, is a model for both ASD and schizophrenia (Patterson, 2009). This overlap between syndromes has prompted the National Institute of Mental Health to launch an initiative called the *Research Domain Criteria* (RDoC) project. RDoC is a research

framework that funds multi-dimensional research (e.g., behavioural, self-reports, genetics, etc.), investigating the psychopathology of neurological conditions like ASD to create new diagnostic systems, screening tools, and treatments. The framework aims to develop a deeper understanding of the underlying physiological, biological, and behavioural constituents of neurological disorders and their developmental trajectory (Insel et al., 2010). RDoC takes an approach where research is based on individual symptoms rather than syndrome-specific symptom categories. One of the key strengths of this approach is that it reveals the immense overlap of symptoms and phenotypes between psychiatric conditions. While not without criticism (e.g., Ross & Margolis, 2019), RDoC offers a different perspective that helps translate pre-clinical findings to specific human functioning without the need for a strict syndrome-to-animal model concordance (Anderzhanova et al., 2017). This is important when considering that often animal models show only certain aspects of the condition, resulting in reduced face validity of the overall model in relation to a syndrome as outlined in the DSM-5.

Also, disease features like language deficits are not as apparent in rodents as they are in humans. They are usually based on quantitative call features (e.g., count, duration, frequency, etc.), or call shape, but the technology to study such qualitative features is still in its infancy. However, it is a promising research field, for instance, a research group has categorised adult 50 kHz calls into 15 different call categories that were affected by social context, while quantitative call features were stable across conditions (Wright et al., 2010). The analysis of call shape repertoires will provide more insight into the social-communicative world of rodents, which will increase the translational value of USV studies immensely. Until reliable analysis methods for qualitative call features are available, quantitative analysis of separation-induced USV seems to be a good way to investigate early communication deficits, as was shown in the present project.

As enrichment is a very promising direction in the search for effective treatment options for psychiatric conditions like ASD, it is important to develop standardised systems to increase reproducibility between different lab groups, but also between studies within a lab. The methodologies for both pre- and post-weaning enrichment differ vastly between studies and lab groups in term of cage system, toys, activities, and cage modification schedules. In most labs, standard housing is standardised, but enriched housing has greater methodological variability. As noted above, the balance between physical and social enrichment can affect social-behavioural paradigms, which stresses the importance of standardised enrichment regimes.

Additionally, when developing animal models, one element to consider is sex. Historically, a lot of pre-clinical research was conducted in males. The main reason for this appears to be that researchers perceived that the female estrus cycle increases variability, but a meta-analysis found that throughout the estrus cycle of female mice there was no more variability in females than in males (Prendergast et al., 2014). Convention, rather than fundamental scientific reasoning, was noted as another potential reason for the exclusion of females in animal models (Clayton & Collins, 2014). Because sex is often a very important factor in neuropsychiatric research, in 2014 the National Institute of Health in the US has developed policies that require grant applications for pre-clinical research to include both male and female animals (Clayton & Collins, 2014).

In the context of the present work, female rats are commonly more prosocial than males (Ben-Ami Bartal et al., 2011). This result was corroborated by the significantly higher opening frequency of females in the prosocial experiment in Chapter 5. These sex differences are similar to findings from human ASD studies. As noted above, there is a male preponderance in ASD—approximately 4:1 (Maenner et al., 2020) and ASD affects males and females in different ways (Hull et al., 2017). Males are often more affected by repetitive and stereotyped behaviours (Mandy et al., 2012), while females are better at blending in, enabling them to cope more effectively in social situations (Lehnhardt et al., 2016). In my thesis, I have included both sexes, as sex is an important factor when it comes to prosocial behaviour, so neglecting to investigate sex-related differences would obscure potential findings.

The last point to make relates to genetic variability. Using outbred rodent strains with high inherent genetic variability generally results in highly variable behavioural outcomes, often at the expense of power. Also, high genetic variability makes study replication by other research groups more difficult. The SD rats used in the present study are such an outbred strain. While the genetic diversity reflects the human population well, the downsides are the high variability in behavioural measures and often low power, resulting in a requirement for a larger sample size (Brekke et al., 2018). This need for a bigger sample also increases the resource cost. While the present study's sample size was not unusually small for a rodent study, it may not have been large enough to detect a true effect. However, while this might have contributed to the present results, it is unlikely that it was the only reason for the lack of treatment effect on measures of social behaviour.

7.5 Limitations

A limitation connected to the point I made above on poly I:C product composition is that the present study did not include blood analyses to determine treatment success. Poly I:C with high, but not low molecular weight resulted in heightened cytokine levels of IL-6, IL-1β, and TNF-α, among others (Careaga et al., 2018). All three of these have previously been connected to ASD: IL-6 (Kutuk et al., 2020), IL-1β, IL-4 (Krakowiak et al., 2017), IL-6, and TNF-α (Jones et al., 2017; Jyonouchi et al., 2001). Therefore, cytokine profiling would have been a way to determine whether the treatment was successful and, if not, the poly I:C treatment could have been repeated. Implementing such a process would provide information of treatment success straight away, so future studies should aim to include cytokine profile analysis to provide additional assurance of treatment success.

Methodological issues with the enrichment regime interfered with the prosocial behaviour paradigm. What was striking in Chapter 5 was that, rather than increased prosociality, enriched animals showed no interest in freeing their trapped cagemate—regardless of treatment. As research showed, it is important that the trapped animal emits distress signals to trigger the rescue behaviour in the free animal (Ben-Ami Bartal et al., 2016). As animals in enrichment were used to spending time inside tunnels and tubes, being inside a rodent restrainer may not have been particularly stressful for them, which explains the consistent lack of opening behaviour in this group. The combination of the restrainer-based social release paradigm and enrichment protocol used in the present study was not ideal. A more appropriate social release paradigm in connection with an enrichment study would be the soaked rat model (Sato et al., 2015). Further, recording of the behaviour and USV of the trapped rat may provide useful further information.

As briefly mentioned in the discussion of Chapter 3, categorisation of USV by sonographic features—call shape—is a way to provide additional information on communication differences between treatment groups based on qualitative call features. Calls can be classified manually, but the sheer number of calls (over 55,000) in the present study rendered such an endeavour out of scope. While DeepSqueak, a more recent addition to the available sound detection softwares, is capable of processing large file numbers automatically (Coffey et al., 2019), the underlying parameters used for the categorisation cannot be adjusted on a per-file or per-call basis. Due to the duration of the study (1.5 years), animals were recorded in different rooms with differing recording

equipment, resulting in audio files requiring different tonality settings, often on a per-call basis. Based on these technical limitations, call categories were not included in this thesis.

7.6 The Future of Animal Modelling and Beyond

ASD is a condition with an aetiology based on complex interactions between genes and the environment. Since autism appeared for the first time in scientific journals almost 100 years ago, we have connected many symptoms to the underlying neurobiology, but the exact mechanisms are still somewhat unclear. Animal models are crucial in our quest to develop effective pharmacological and alternative interventions, including environmental enrichment therapy. The focus should be on specific aspects of the condition, as we must first understand the neural and genetic foundations of, for example, prosociality and how deficiencies are reduced/removed in its entirety by, say, enrichment. This can only happen with a well-developed solid animal model that is replicable across lab borders.

A key area future studies could address is the need for standardisation of poly I:C products. At least part of the reason for the high variability found in the treatment group was likely the result of the poly I:C product used in this study. Product composition that is so variable that researchers cannot rely on a constant effect is a major concern (Mueller et al., 2019). Harvey and Boksa (2012) reported an interaction of dose and batch in a sample including three independently obtained poly I:C samples, which makes the need for greater product testing and/or reporting clear.

Should neither standardisation nor lab-based poly I:C product testing be possible, an option to avoid issues is to change the model type from environmental to genetic. Inbred strains have the big advantage that fewer animals per experimental group are needed to achieve greater power because of their genetic similarity. Whereas there are several inbred mouse strains available for ASD research, not many ASD rat models are based on inbred strains, as such strains are not readily available. However, one lab group found that Wistar-Kyoto (WKY) rats, in particular, the WKY/NCrl substrain show potential as an ASD model, as these animals are less sociable, communicate less, are more anxious than SD rats, and their genome contains high levels of autism-relevant genes (Zhang-James et al., 2014). Using a rat model like the WKY/NCrl could be a good way of simplification to observe the effects of environmental enrichment on social behaviours. The first step here would be to pilot a social release paradigm comparing WKY/NCrl to SD controls. Once a prosocial behaviour rat model has been established, environmental

enrichment following a standardised protocol like the one used in the present study will show if and how it affects prosociality. This approach would require a smaller sample size, which has the advantage that it follows the principle of reduction in the 3Rs (these being Replacement, Reduction, and Refinement) (Russell & Burch, 1992).

As noted above, DeepSqueak's technological constraints meant calls could not be automatically categorised by sonographic features. Goffinet et al. (2019) presented a method that, at face value, appeared to be able to process such complex multifaceted datasets, but the provided software package called AVA[8] does not include a user interface but instead requires advanced Python language skills. In its current state, this software does not offer the usability required to be a viable DeepSqueak alternative. DeepSqueak utilises deep learning algorithms based on artificial neural networks for call categorisation. It extracts contours from the files, but accurate extraction relies on the correct tonality settings, which is what cannot be done on a per-call/per-file basis with the current version of DeepSqueak. However, this feature may be included in future versions of the software, which would allow the analysis of large complex datasets on a per-file/per-call basis and subsequent rapid automated call classification by shape.

Another important aspect related to pre-clinical MIA models is the investigation of the behavioural effects of MIA in human samples. Brown et al. (2009) compared patients with schizophrenia with MIA and without MIA and found clear group differences in executive functioning in general, but also related to prosociality. A clinical study with four groups (neurotypical controls with and without MIA, ASD with and without MIA) could provide a lot of insight into the mechanisms of MIA in ASD.

7.7 Conclusion

This thesis was designed to determine the effects of environmental enrichment on prosocial behaviour in an in vivo ASD rat model. There were only transient effects of poly I:C in the pups, and basic social and prosocial behaviours seemed unimpaired. Overall, the results somewhat strengthen the idea that pre-weaning enrichment weakens the effect of prenatal exposure to poly I:C, but more research on the topic is required. Due to methodological issues and the limitations considered above, the findings did not provide insights into the question of the enrichment effect on deficient social behaviours. Mainly

[8] Autoencoded Vocal Analysis

because the treatment did not result in social-behavioural deficits, so there was nothing for environmental enrichment to improve. Additional work is needed to improve the paradigms to further the understanding of how modification of the environment can stimulate the social parts of the brain to redirect the atypical developmental trajectory towards a more typical one. In closing, this thesis put down the foundation for an ASD rat model of prosocial behaviour that will aid future researchers in the quest of improving interventions for autism, as well as other psychiatric conditions.

Appendix A. Chapter 3

A1. Descriptive Statistics

A1.1 Number of Calls

Table A-1

Descriptive Statistics by PND (Number of Calls)

Sex	Treatment	Enrichment	*n*	*M* *	*SD*	*SE*	95% CI
			PND 7				
Males	Saline	No Enrichment	32	369.81	257.16	45.46	97.72
	Poly I:C		32	301.16	215.11	38.03	77.56
Females	Saline	No Enrichment	32	314.66	229.75	40.61	82.83
	Poly I:C		32	190.72	169.26	29.92	61.63
			PND 14				
Males	Saline	No Enrichment	15	135.73	84.26	21.76	46.66
		Enrichment	16	133.25	76.75	19.19	40.89
	Poly I:C	No Enrichment	16	116.69	83.32	20.83	44.40
		Enrichment	17	212.82	174.89	42.42	89.92
Females	Saline	No Enrichment	17	149.41	88.13	21.38	45.31
		Enrichment	16	128.50	132.14	33.03	70.41
	Poly I:C	No Enrichment	15	91.40	60.27	15.56	33.37
		Enrichment	15	140.73	104.10	26.88	57.65

* Mean number of calls.

A1.2 Call Duration

Table A-2

Descriptive Statistics by PND (Call Duration)

Sex	Treatment	Enrichment	*n*	*M* *	*SD*	*SE*	95% CI
			PND 7				
Males	Saline	No Enrichment	32	102.28	30.56	5.40	11.02
	Poly I:C		32	106.43	98.46	6.62	13.51
Females	Saline	No Enrichment	32	91.90	31.97	5.65	11.53
	Poly I:C		32	87.70	32.99	5.83	11.89
			PND 14				
Males	Saline	No Enrichment	15	178.88	87.71	22.65	48.57
		Enrichment	16	168.65	45.31	11.33	24.14
	Poly I:C	No Enrichment	16	183.16	67.22	16.80	35.82
		Enrichment	17	169.68	58.41	14.17	30.03
Females	Saline	No Enrichment	17	179.49	79.57	19.30	40.91
		Enrichment	16	132.70	80.61	20.15	42.95
	Poly I:C	No Enrichment	15	199.04	75.39	19.47	41.75
		Enrichment	15	150.41	55.82	14.41	30.91

* Call duration in ms.

A1.3 Delta Frequency

Table A-3

Descriptive Statistics by PND (Delta Frequency)

Sex	Treatment	Enrichment	*n*	*M* *	*SD*	*SE*	95% CI
PND 7							
Males	Saline	No Enrichment	32	9.93	4.63	0.82	1.67
	Poly I:C		32	8.02	3.78	0.67	1.36
Females	Saline	No Enrichment	32	9.30	4.19	0.74	1.51
	Poly I:C		32	6.99	3.38	0.60	1.22
PND 14							
Males	Saline	No Enrichment	15	11.44	4.35	1.12	2.41
		Enrichment	16	14.02	3.05	0.76	1.63
	Poly I:C	No Enrichment	16	11.71	3.88	0.97	2.06
		Enrichment	17	11.95	2.68	0.65	1.38
Females	Saline	No Enrichment	17	10.56	3.37	0.82	1.73
		Enrichment	16	12.01	3.44	0.86	1.83
	Poly I:C	No Enrichment	15	10.53	3.22	0.83	1.79
		Enrichment	15	10.15	3.46	0.89	1.91

* Delta frequency in kHz.

Appendix B. Chapter 4

B1. Descriptive Statistics

B1.1 Preference Ratio

Table B-1

Descriptive Statistics for Preference Ratio (Pups)

Phase	Treatment	Housing [a]	*M* [b]	*SD*	*SE*	95% CI
			Males			
2	Saline	Standard	51.08	32.46	9.00	19.61
		Enriched	55.86	32.36	8.98	19.56
	Poly I:C	Standard	56.23	29.09	8.07	17.58
		Enriched	45.23	36.06	10.00	21.79
3	Saline	Standard	51.93	20.15	5.59	12.18
		Enriched	45.80	30.85	8.56	18.64
	Poly I:C	Standard	44.29	25.48	7.07	15.40
		Enriched	47.70	25.40	7.04	15.35
			Females			
2	Saline	Standard	54.40	38.52	10.68	23.28
		Enriched	56.45	43.79	12.14	26.46
	Poly I:C	Standard	50.47	27.31	7.57	16.50
		Enriched	50.50	39.77	11.03	24.03
3	Saline	Standard	47.54	24.01	6.66	14.51
		Enriched	58.14	25.68	7.12	15.52
	Poly I:C	Standard	47.32	28.89	8.01	17.46
		Enriched	49.81	24.65	6.84	14.89

[a] $n = 13$ for each group. [b] Preference ratio.

Table B-2

Descriptive Statistics for Preference Ratio (Adults)

Phase	Treatment	Housing	*n*	*M* [a]	*SD*	*SE*	95% CI
			Males				
2	Saline	Standard	13	69.35	22.43	6.22	13.56
		Enriched	13	69.70	22.07	6.12	13.34
	Poly I:C	Standard	13	71.91	14.61	4.05	8.83
		Enriched	13	63.26	26.83	7.44	16.22
3	Saline	Standard	13	49.09	6.94	1.92	4.19
		Enriched	11	50.71	15.50	4.67	10.41
	Poly I:C	Standard	13	53.00	11.92	3.31	7.21
		Enriched	12	55.84	18.86	5.44	11.98
			Females				
2	Saline	Standard	13	65.00	12.25	3.40	7.40
		Enriched	12	61.24	20.26	5.85	12.87
	Poly I:C	Standard	13	58.01	17.01	4.72	10.28
		Enriched	12	68.33	20.15	5.82	12.80
3	Saline	Standard	13	46.83	8.07	2.24	4.88
		Enriched	11	58.79	13.85	4.18	9.30
	Poly I:C	Standard	13	52.03	11.21	3.11	6.77
		Enriched	13	54.58	16.23	4.50	9.81

[a] Preference ratio.

B1.2 Locomotor Activity

Table B-3

Descriptive Statistics for SAA Locomotor Activity (Pups)

Housing	Treatment	*n*	*M* *	*SD*	*SE*	95% CI
			Phase #2			
Standard	Saline	16	1,315.35	1,105.14	276.29	588.89
	Poly I:C	19	1,841.48	1,245.48	285.73	600.30
Enriched	Saline	24	981.24	1,022.84	208.79	431.91
	Poly I:C	20	1,006.54	961.43	214.98	449.96
			Phase #3			
Standard	Saline	16	2,397.33	908.71	227.18	484.22
	Poly I:C	19	2,304.90	1,216.24	279.03	586.21
Enriched	Saline	24	1,835.99	1,001.73	204.48	422.99
	Poly I:C	20	1,961.79	867.42	193.96	405.96

* Distance in cm.

Table B-4

Descriptive Statistics SAA Locomotor Activity (Adults)

Phase	Housing	Treatment	*n*	*M* *	*SD*	*SE*	95% CI
			Males				
2	Standard	Saline	13	2,157.99	1,063.75	295.03	642.82
		Poly I:C	13	3,068.86	728.00	201.91	439.93
	Enriched	Saline	13	2,032.56	801.55	222.31	484.37
		Poly I:C	13	1,819.03	694.19	192.53	419.49
3	Standard	Saline	13	3,201.86	871.43	241.69	526.60
		Poly I:C	13	3,555.77	775.73	215.15	468.77
	Enriched	Saline	11	2,310.30	761.19	229.51	511.37
		Poly I:C	12	2,558.12	346.12	99.92	219.91
			Females				
2	Standard	Saline	13	3,485.23	959.49	266.12	579.82
		Poly I:C	13	3,233.20	889.28	246.64	537.39
	Enriched	Saline	12	2,473.67	700.63	202.25	445.16
		Poly I:C	12	2,716.76	779.56	225.04	495.31
3	Standard	Saline	13	4,086.11	966.86	268.16	584.27
		Poly I:C	13	4,221.39	869.58	241.18	525.48
	Enriched	Saline	11	2,975.57	936.89	282.48	629.41
		Poly I:C	13	2,988.92	675.58	187.37	408.25

* Distance in cm.

Appendix C. Chapter 5

C1. Descriptive Statistics

C1.1 Empty Condition

Table C-1

Descriptive Statistics for Opening Frequency (Empty Condition)

Treatment	Housing	*n*	*M* [a]	*SD*	*SE*	95% CI
			Males			
Saline	Standard	14	0.00	0.00	0.00	0.00
	Enriched	18	0.11	0.47	0.11	0.23
Poly I:C	Standard	8	0.00	0.00	0.00	0.00
	Enriched	16	0.00	0.00	0.00	0.00
			Females			
Saline	Standard	14	0.29	0.61	0.16	0.35
	Enriched	18	0.00	0.00	0.00	0.00
Poly I:C	Standard	10	0.30	0.67	0.21	0.48
	Enriched	16	0.06	0.25	0.06	0.13

[a] Opening frequency (count).

Table C-2

Descriptive Statistics for Opening Latency (Empty Condition)

Treatment	Housing	*n*	*M* [a]	*SD*	*SE*	95% CI
			Males			
Saline	Standard	14	25.00	0.00	0.00	0.00
	Enriched	18	24.42	2.47	0.58	1.23
Poly I:C	Standard	8	25.00	0.00	0.00	0.00
	Enriched	16	25.00	0.00	0.00	0.00
			Females			
Saline	Standard	14	24.54	1.10	0.29	0.64
	Enriched	18	25.00	0.00	0.00	0.00
Poly I:C	Standard	10	24.83	0.37	0.12	0.27
	Enriched	16	24.69	1.25	0.31	0.67

[a] Opening latency in min.

Table C-3

Descriptive Statistics for LMA (Empty Condition)

Treatment	Housing	*n*	*M* [a]	*SD*	*SE*	95% CI
			Males			
Saline	Standard	14	3.30	1.36	0.18	0.36
	Enriched	18	1.61	1.10	0.13	0.26
Poly I:C	Standard	8	4.05	1.49	0.26	0.54
	Enriched	14	1.76	1.44	0.19	0.39
			Females			
Saline	Standard	14	14	3.55	1.49	0.20
	Enriched	18	18	1.57	0.78	0.09
Poly I:C	Standard	10	10	4.25	0.98	0.16
	Enriched	16	16	1.70	0.79	0.10

[a] Distance in m averaged over sessions.

C1.2 Trapped Condition (All Animals)

Table C-4

Descriptive Statistics for Opening Frequency (Trapped Condition)

Treatment	Housing [a]	*M* [b]	*SD*	*SE*	95% CI
		Males			
Saline	Standard	3.92	2.14	0.59	1.29
	Enriched	0.54	1.13	0.31	0.68
Poly I:C	Standard	3.92	1.80	0.50	1.09
	Enriched	0.85	1.72	0.48	1.04
		Females			
Saline	Standard	4.38	2.29	0.64	1.39
	Enriched	2.23	2.42	0.67	1.46
Poly I:C	Standard	3.85	2.44	0.68	1.48
	Enriched	2.15	2.44	0.68	1.48

[a] $n = 13$ for each group. [b] Opening frequency (count).

Table C-5

Descriptive Statistics for Opening Latency (Trapped Condition)

Treatment	Housing [a]	*M* [b]	*SD*	*SE*	95% CI
		Males			
Saline	Standard	13.60	6.83	1.89	4.13
	Enriched	23.30	3.58	0.99	2.16
Poly I:C	Standard	14.57	5.04	1.40	3.04
	Enriched	22.91	4.78	1.33	2.89
		Females			
Saline	Standard	12.78	7.39	2.05	4.46
	Enriched	17.69	8.07	2.24	4.88
Poly I:C	Standard	13.37	7.36	2.04	4.45
	Enriched	17.98	8.04	2.23	4.86

[a] $n = 13$ for each group. [b] Opening latency in min.

C1.3 Trapped Condition (Openers)

Table C-6

Descriptive Statistics for Opening Frequency (Trapped Condition: Openers)

Treatment	Housing	*n*	*M* [a]	*SD*	*SE*	95% CI
			Males			
Saline	Standard	9	4.67	1.50	0.50	1.15
	Enriched	4	1.75	1.50	0.75	2.39
Poly I:C	Standard	7	4.86	0.69	0.26	0.64
	Enriched	3	3.67	1.53	0.88	3.79
			Females			
Saline	Standard	10	4.90	1.91	0.60	1.37
	Enriched	9	3.22	2.28	0.76	1.75
Poly I:C	Standard	4	5.25	1.71	0.85	2.72
	Enriched	7	4.00	1.83	0.69	1.69

[a] Opening frequency (count).

Table C-7

Descriptive Statistics for Opening Latency (Trapped Condition: Openers)

Treatment	Housing	*n*	*M* [a]	*SD*	*SE*	95% CI
			Males			
Saline	Standard	9	11.71	5.55	1.85	4.27
	Enriched	4	19.47	4.78	2.39	7.61
Poly I:C	Standard	7	12.67	4.01	1.52	3.71
	Enriched	3	15.95	6.55	3.78	16.26
			Females			
Saline	Standard	10	11.64	6.84	2.16	4.90
	Enriched	9	14.44	7.69	2.56	5.91
Poly I:C	Standard	4	8.82	5.81	2.91	9.25
	Enriched	7	11.96	6.15	2.32	5.69

[a] Opening latency in min.

Appendix D. Chapter 6

D1. Descriptive Statistics

D1.1 Distance Covered

Table D-1

Descriptive Statistics Distance

Housing	Treatment [a]	*M* [b]	*SD*	*SE*	95% CI
		Males			
Standard	Saline	33.67	10.00	2.04	4.22
	Poly I:C	39.48	15.76	3.22	6.65
Enriched	Saline	15.95	5.43	1.11	2.29
	Poly I:C	14.15	6.83	1.40	2.89
		Females			
Standard	Saline	42.29	11.57	2.36	4.89
	Poly I:C	42.45	11.42	2.33	4.82
Enriched	Saline	21.65	9.51	1.94	4.02
	Poly I:C	16.26	5.79	1.18	2.44

[a] $n = 24$ for each group. [b] Distance in m.

Appendix E. Chapter 7

Table E-1

Overview Hypotheses and Outcomes

Study	Hypothesis	Confirmed
USV (Chapter 3)	1. Sprague Dawley rat pups prenatally exposed to poly I:C will produce fewer and shorter calls relative to controls.	Partially *
	2. The communication deficit will be more prominent on PND 7 than on PND 14.	Yes
	3. Poly I:C pups that received enrichment will be less affected by the poly I:C effect than poly I:C pups that did not.	Yes
SAA (Chapter 4)	1. Standard-housed poly I:C animals will show less general sociability and a lower preference for social novelty than controls. When compared to control animals, they will spend less time with the social stimulus in phase #2/the novel social stimulus in phase #3.	No
	2. Enrichment will reverse this effect: Poly I:C animals in enriched housing will spend more time exploring the social stimulus in phase #2 and the novel social stimulus in phase #3, showing higher sociability and a greater preference for social novelty than poly I:C animals in standard housing.	No
Prosocial behaviour (Chapter 5)	1. Standard-housed adult Sprague Dawley (SD) rats prenatally exposed to poly I:C will show reduced prosociality by freeing a trapped conspecific less frequently and with higher opening latencies than control rats.	No
	2. Enrichment will reverse this effect. Poly I:C-exposed rats that lived in enriched housing will free the trapped rat more frequently and open the door faster than poly I:C-exposed rats in standard housing.	No
	3. Females will show a higher level of pro-sociality than males.	Yes
LMA (Chapter 6)	1. Animals housed in enrichment will be less active than standard-housed animals.	Yes
	2. Poly I:C will affect animals in standard housing more than animals in enriched housing.	Yes

* Fewer calls but same call duration.

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