The interactive effects of *Cyfip1* haploinsufficiency and maternal immune activation on pre-weaning social behaviour

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Abstract

CYFIP1 is a gene associated with risk for autism spectrum disorders (ASD) and schizophrenia. The reasons for differing clinical outcomes when possessing *CYFIP1* genetic variant remain unclear. In this current study, a *Cyfip1*$^{+/-}$ mouse model was combined with a model of infection during pregnancy, maternal immune activation (MIA), to investigate potential interactive effects on social behaviour early in development. In Chapter 3 work identifying a sub-threshold dose of poly(I:C) for use in the two-hit model, demonstrated a higher dose (10 mg/kg) produced offspring less likely to show homing behaviour at postnatal day 9 (P9) and reduced social novelty preference at P26. Effects not seen with a lower dose (5 mg/kg), the designated sub-threshold dose. Chapter 4 demonstrated *Cyfip1*$^{+/-}$ interacting with MIA in a sex dependent fashion to reduce social interest in MIA *Cyfip1*$^{+/-}$ males at P9 in a homing test but not in MIA *Cyfip1*$^{+/-}$ females or control *Cyfip1*$^{+/-}$ males. At P28 all *Cyfip1*$^{+/-}$ groups showed reduced social interaction compared to wild-type (WT) littermates in a direct social interaction test. WT stimulus animals at P28 show less interest in MIA exposed WT offspring compared to *Cyfip1*$^{+/-}$ littermates and control mice, an effect not observed using the same dose in a pure WT cohort. Beyond social interaction in early development, Chapter 5 developed a novel social test, the Social Interaction Platform (SIP) which allows assessment of direct interaction between unfamiliar adult male mice. This current work provides direct and indirect evidence of interactive effects between haploinsufficiency of *Cyfip1* and MIA, whilst confirming the importance of considering both experimental and stimulus animals when investigating social interaction. These findings suggest *Cyfip1*$^{+/-}$ is important for development of social behaviour and may interact with MIA, causing an earlier onset of social deficits in males, highlighting the need to interrogate social behaviour models early in development.

Abbreviations

ABI1	Abl interactor 1
ADHD	Attention-deficit hyperactivity disorder
ALSPAC	Avon Longitudinal Study of Parents and Children
AMPAR	α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid receptor
ARP2/3	Actin-related protein 2/3
ASD	Autism spectrum disorders
BP	Breakpoint
°C	Degrees Celsius
C-section	Caesarean-section
Cfyip1	Cytoplasmic fragile X mental retardation 1 interacting protein (CYFIP1)
cm	centimetre
CNV	Copy number variants
DLC	DeepLabCut
ds-RNA	double-stranded RNA
DTI	diffusion tensor imaging
E	Embryonic day
eIF4E	eukaryotic translation initiation factor 4E
FA	fractional anisotropy
FMRP	Fragile-X mental retardation protein
g	grams
GABA	Gamma aminobutyric acid
GWAS	Genome-wide association study
GxE	gene x environment
HPSC300	haematopoietic stem/progenitor cell protein 300
ICC	Intraclass correlation
ID	intellectual disability
IL-6	Interleukin-6
KO	knockout
LM	Linear model
LMM	Linear mixed models
LMW	Low molecular weight

LPS	Lipopolysaccharide
MECP2	Methyl CpG-binding protein 2
mGluR	metabotropic glutamate receptor
MIA	Maternal immune activation
mPFC	medial prefrontal cortex
NCKAP1	Nck-associated protein 1
NIPA	non-imprinted in Prader-Willi/Angelman
Nlgn3	Neuroligin 3
P	Postnatal day
PGS	Polygenic scores
Poly(I:C)	Polyinosinic polycytidylic acid (Poly I:C)
RDoC	Research Domains Criteria
rsfMRI	resting state function magnetic resonance imaging
s	seconds
SCDC	Social and Communication Checklist
SIP	Social interaction platform
SNP	Single nucleotide polymorphisms
TLR3	toll-like recptor-3
TNF-α	Tumour necrosis factor -α
TSC	Tuberous sclerosis complex
TUBGCP5	Tubulin gamma complex-associated protein 5
USV	ultrasonic vocalisations
VBS	Visible Burrow System
VCA	verprolin-homology central acidic region
WRC	WAVE regulatory complex
WT	Wild-type

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Chapter 1 : Introduction

Autism spectrum disorders (ASD), also known as autism are a common group of neurodevelopmental disorders affecting approximately 1-2% of the population, with males at least 3 times more likely to be diagnosed than females (Loomes et al. 2017; Lord et al. 2020). Diagnosis is based on the early life presentation of difficulties in social communication, restricted interests and repetitive behaviour as well as issues around sensory processing (Lai et al. 2014). These broad diagnostic criteria lead to vast differences in ASD presentation with any two individuals displaying very different phenotypic features (Lord et al. 2020). Beyond differences in core diagnostic features, multiple factors contribute to the heterogeneity observed in ASD including IQ, co-occurring conditions, sex and gender (Frazier et al. 2013; Havdahl et al. 2016; Lai and Szatmari 2020; Warrier et al. 2020). The conditions that often co-occur include motor dysfunction, intellectual disability (ID), epilepsy, sleep disorders, anxiety, attention-deficit hyperactivity disorder (ADHD) and depression (Geschwind 2009). How each of these factors modifies the manifestation of ASD challenges the resolution of casual and pathological mechanisms and the design of effective interventions.

Since autism was first described by Kanner, endeavours to resolve the underlying aetiology remain incomplete. Initially autism was thought to be of environmental origin but advances in understanding the influence of genetics on health shifted the focus to heritable risk factors (Rylaarsdam and Guemez-Gamboa 2019a). Advances in technology have facilitated the identification of numerous risk loci, with more than 100 strongly associated with ASD (Chaste et al. 2014; Satterstrom et al. 2020). Risk for ASD does not arise solely from genetic factors, indeed epidemiological studies are highlighting the contribution of environmental risk factors (Lyall et al. 2017). Moving beyond the idea of single causes, it is now understood that ASD manifests following a complex interplay of genetic and environmental factors (Chaste and Leboyer 2012). Following consideration of the genetic and environmental

contribution to ASD risk the way these factors may interaction to yield ASD will be considered.

1.1 Genetic contribution to ASD

Evidence for the genetic contribution to ASD arises from twin and family studies. Monozygotic twins have the greatest concordance rates (70-90%) compared to dizygotic twins (~30%) and siblings in general (3-19%) (Rosenberg et al. 2009; Hallmayer et al. 2011a; Ozonoff et al. 2011; Constantino et al. 2013; Ronald and Hoekstra 2014). Furthermore, it has been shown that risk of developing ASD increases with the amount of the genome shared with an affected parent or sibling (Risch et al. 2014; Sandin et al. 2014). Up to 5% of people with ASD carry structural chromosomal aberrations, as revealed using classical karyotyping techniques (Wiśniowiecka-Kowalnik and Nowakowska 2019). Whilst the causal role of such chromosomal alterations is unclear, some are recurrent. For example, maternally derived 15q11q13 duplication is detected in 1-3% of individuals with ASD and features several genes with important roles in brain function (Hogart et al. 2010). Copy number variants (CNVs) are structural changes in chromosomes undetectable by classic karyotyping techniques. These submicroscopic changes, such as duplications, deletions, translocations and inversions can be as large as several kilobases and span multiple genes (Marshall et al. 2008). CNVs are detected in 7-14% of individuals with idiopathic ASD (Wiśniowiecka-Kowalnik and Nowakowska 2019). Individuals with a diagnosis of sporadic ASD a more likely to possess a rare *de novo* CNV than those with a family history. Sebat et al. (2007)demonstrated this association, revealing 10% of patients from simplex families as carrying rare *de novo* CNVs compared to 3% of patients from multiplex families and 1% of controls. Further work obtained comparable results reporting *de novo* CNVs in 5.8-8.4% of sporadic ASD cases (Marshall et al. 2008; Sanders et al. 2011). Taken together, these reports suggest rare *de novo* mutations convey a significant risk to individuals with no family history of ASD.

The majority of CNVs identified in people with ASD are infrequent and nonrecurrent which highlights the genetic heterogeneity underpinning ASD (Shen et al. 2010) . Recurrent CNVs are targets for investigation in attempts to resolve disease mechanisms. One such variant, the 16p11.2 contains 25 genes, many of which are important in the developing nervous system. To-date no single gene from this CNV has been identified as the sole driver of disease with studies supporting different target genes (Wiśniowiecka-Kowalnik and Nowakowska 2019b). Iyer et al. (2018) used RNAi in drosophila to illustrate 24 interactions between genes within the 16p11.2 locus as well as 46 interactions with genes important in neurodevelopment. This finding points to potential mechanisms for the manifestation of complex phenotypes observed in CNV carriers whilst demonstrating the shortfalls of focussing on single genes. Studying the disease mechanisms of individual CNVs is challenging as even the most prevalent CNVs associated with ASD, namely 16p11.2 and 15q11-13 are only identified in approximately 1% of ASD cases (Rylaarsdam and Guemez-Gamboa 2019b). Additionally, no CNVs have complete penetrance, with studies revealing unaffected carriers of an ASD associated CNV or affected siblings without the CNV (Marshall et al. 2008). Even if a carrier is affected, the variability of the phenotype presented is diverse, with ASD-associated CNVs being associated with other neuropsychiatric conditions such as ID, schizophrenia, and ADHD(Girirajan and Eichler 2010). Phenotypic variability can be partially explained by CNV size, shorter or longer CNVs will include less or more genes respectively. Girirajan et al. (2013) reported a positive correlation between the size of a CNV duplication and ASD severity but found no correlation with duplication size and non-verbal IQ. Incomplete penetrance may also arise from the absence of additional “hits”. A “two-hit model” has been put forward based on the observation 10% of patients will possess a second pathogenic CNV (Rylaarsdam and Guemez-Gamboa 2019). CNVs undoubtedly contribute risk for ASD but incomplete penetrance and phenotypic variability highlights a need to interrogate the factors, genetic or otherwise that influence disease outcomes.

ASD may not be caused by a single gene, but it is associated with a number of monogenic syndromes, which occur in 5-10% of ASD patients (Devlin and Scherer 2012). The most common of these syndromes is Fragile X syndrome (FXS) which is diagnosed in 1.5-3% of ASD patients. FXS is caused by mutations in the *FMR1* gene

which encodes the synaptic protein fragile-X mental retardation protein (FMRP), which is a regulator of mRNA and translation within the synapse (Ascano et al. 2012). Tuberous sclerosis complex occurs in 1-4% of individuals with ASD and has two causative genes *TSC1* and *TSC2* which are inhibitors in the mammalian target of the rapamycin (mTOR) signalling cascade (Smalley 1998). Rett syndrome occurs in 1% of female ASD patients. The pathogenic mutation in Rett syndrome is in the *MECP2* gene which encodes the Methyl CpG-binding protein (MeCP2), a regulator of many genes within neurons (Liu and Takumi 2014). Mutations in *MECP2* have been identified in ASD patients with no diagnosis of Rett syndrome with the patients showing a wide degree of phenotypic variability (Wen et al. 2017). The authors suggested the variability may arise due to the influence of genetic modifiers such as skewed X-inactivation. This is feasible but suggests that even syndromes with monogenic causes are susceptible to the influence of interactive effects of other genes or environmental factors, emphasising the challenge of resolving the aetiology of ASD.

Many of the early successes in studying the genetics of ASD highlighted rare, *de novo* mutations within coding regions as key contributors to liability of developing ASD. This contrasts with adult-onset neuropsychiatric disorders which have seen success by investigating common variants via genome-wide association studies (Lord et al. 2020). Attempts to identify common variants such as single nucleotide polymorphisms (SNP) associated with ASD tend to identify markers that are study specific or do not highlight any associated markers (Ronald et al. 2010; Hu et al. 2011; Anney et al. 2012). This is not to say common variation is not a substantial source of risk for ASD. Individual SNPs each convey a small effect on ASD risk, but the use of genetic scores, compiled from numerous common variants can be used to predict risk (Anney et al. 2012).These scores are also used to estimate the heritability assigned to common variants which can be as high 50%, contributing greater liability than rare *de novo* mutations (Klei et al. 2012; Gaugler et al. 2014). This work highlights the importance of common variants in ASD risk but also the challenges faced by studying common variants, namely phenotypic heritability and cohort sizes.

Warrier et al. (2022) looked to interrogate the genetic differences within ~ 13,000 ASD individuals based on core ASD features, co-morbid developmental conditions and sex, all known sources of heterogeneity in ASD presentation. This study used polygenic scores (PGS) to compare common variants with *de novo* variants and found common variants were associated with core features of ASD but *de novo* variants were not. Higher ASD PGS were associated with a lower chance of co-morbid developmental conditions and SNP heritability was greatest in males with ASD and those with ASD but not ID. This work highlights the need for greater phenotypic characterization to facilitate the resolution of how the complex genetic architecture of ASD influences behaviour and co-morbid conditions. Reducing the influence of phenotypic heterogeneity can in part be achieved by investigating specific traits or endophenotypes. Indeed, in the study by Hu et al. (2011) no SNP was associated with ASD, when the entire cohort was considered altogether. However, when the cohort was split into 4 subphenotype groups 18 associated SNPs were identified. Lowe et al. (2015), focussed on a single endophenotype, Social Responsiveness Scale score and via linkage analysis identified two loci on chromosome 8 associated with the score. No common variants were associated but the study demonstrates the value of an endophenotype or trait approach. A recent study also undertook an endophenotype approach and found the score for Stereotyped Behaviours and Restricted Interests was associated with multiple variants in a causal gene for Cohen syndrome, *VPS13B* which itself is a candidate gene for syndromic ASD. This finding requires validation, but this work may also have failed to identify associated SNPs for other endophenotypes due to a modest sample size (Lee et al. 2022).

The genetic architecture underpinning the aetiology of ASD is clearly complex with liability attributable to rare inherited and *de novo* variants and common SNPs, as well as combinations of all kinds of variants. Furthermore, there is genetic and phenotypic overlap between ASD and other neuropsychiatric conditions, adding to the challenge of disentangling the genetic aetiology of ASD. For example, CNVs that convey risk for ASD are also associated with schizophrenia with 1q21.1, 7q11.23, and 16p11.2 duplications associated with both disorders. 16p11.2 deletion is also associated with both but more strongly with ASD than schizophrenia whereas the converse is for 1q21.1 deletion (Malhotra and Sebat 2012; Mollon et al. 2023). Genetic overlap also

exists within common variants for ASD and schizophrenia with genetic correlations suggesting ASD shares almost a fifth of its common genetic influences with schizophrenia (Bulik-Sullivan et al. 2015). ASD and schizophrenia overlap phenotypically as well as genetically, with difficulties in social communication a feature of both disorders (Dickinson et al. 2007; Lai et al. 2014). Pourcain et al. (2018), assessed the genetic overlap of common polygenic liability between ASD, schizophrenia and social communication impairment measured in a typically developing cohort. This study revealed genetic overlap for ASD and social communication difficulties was strongest during middle childhood, whereas the overlap for schizophrenia and social communication difficulties was strongest in late adolescence. A finding that reflects the developmental trajectories of the two disorders but points away from shared genetic susceptibility despite phenotypic overlap.

Schizophrenia is just one example of a disorder that overlaps with ASD in terms of genetics and phenotypic presentation. In fact, most genetic risk factors for ASD can be found in members of the population with no neuropsychiatric diagnoses. For example, having a deletion at the 16p11.2 locus is a well-established risk factor for ASD, but most carriers do not meet the diagnostic criteria for ASD (Hanson et al. 2015). Equally, many people will display a level of social impairment that does not cross any diagnostic threshold, which is due to the innate variability within social behaviour (Robinson et al. 2011). Such subthreshold traits are commonly seen in undiagnosed close relatives of people with ASD suggesting typical social behaviour has a heritable component (Murphy et al. 2000). Robinson et al. (2016a), assessed the association of genetic risk for ASD with social behaviour in the general population, as measured using the Social and Communication Checklist (SCDC) in the Avon Longitudinal Study of Parents and Children (ALSPAC) cohort at age 8. This work observed strong genetic correlation between genetic risk for ASD and social and communication difficulties in two separate samples, both exceeding previous estimates of genetic correlation with ASD and schizophrenia, depression and bipolar disorder. Observed correlations suggest approximately one-quarter of genetic influences of ASD, also influence the SCDC. Such findings point to a need to consider neuropsychiatric disorders on a continuum of normality as the behavioural impairments observed in these disorders reflect the extreme tails in the distribution of

typical behaviour. A shift away from binary categorization e.g. yes/no for ASD will help illuminate the genetic aetiology underpinning the affected behavioural traits that can lead to such a diagnosis. This approach will also help address the phenotypic overlap seen between diagnosed disorders. Bralten et al. (2021), constructed an overarching sociability phenotype using responses to 4 questions in the UK Biobank database that address different components of social behaviour: (1) asked about family/friend visits, (2) asked about nature and number of social venues visited, (3) asked about worries regarding social embarrassment and (4) asked about feelings of loneliness. The resulting sociability score ranged from 0-4 with 4 being highly sociable and individuals with depression, bipolar disorder, schizophrenia and ASD displayed significantly lower scores than unaffected individuals. The correlation between genetic influences and sociability scores were significant for depression, schizophrenia and ASD. Interestingly the genetic correlation between ASD and trait was comparable with Robinson et al. (2016) despite the use of a boarder phenotype in the work of Bralten et al. (2021). The Bralten et al. (2021) study identified 18 significant loci and one of the strongest hits from the GWAS was the *DRD2* gene, which encodes the D2 dopamine receptor subtype. *DRD2* is a candidate gene for schizophrenia that has been confirmed by GWAS whilst it has also been associated with depression (35,36). When neuropsychiatric cases were excluded from analysis the *DRD2* association remained significant, highlighting the distribution of disease relevant variants in an unaffected population. This work adds merit to studying traits or endophenotypes on a continuum of normality where disease phenotypes represent the extreme tails of the trait distribution. A limitation of this work is the context of the phenotypic data, which is restricted to a period of 1 year, which will not capture the dynamic nature of sociability, nor will it capture differences in developmental profiles in the way Pourcain et al. (2018) managed. This is particularly relevant to ASD, as signal from the genetic influences may have been missed given the average age of participants was 56 years old. Unpicking the full genetic aetiology is a massive challenge and gaps remain. Focussing on traits/endophenotypes across the general population will undoubtedly help fill in those gaps but the aim is not to merely resolve aetiology but also to shed light on pathological mechanisms so therapeutic interventions can be designed and delivered.

The genetic risk for ASD is evidently multifaceted with rare and common, inherited and *de novo* variants contributing to liability via both additive and non-additive interactions. This presents a massive barrier to disentangling the underlying pathological mechanisms that yield ASD. The study of single gene models of mutations from both syndromic e.g. fragile X syndrome, and idiopathic e.g. *SHANK3,* forms of ASD has produced many insights into the potential pathophysiology (Lord et al. 2020). Translating the observations from model systems to ASD in humans has not been fruitful thus far and is an issue that needs addressing (State and Šestan 2012; Sestan and State 2018). Focussing on a single gene may yield phenotypes that partially recapitulate a human disorder but due to the simplicity of design they lack the heterogeneity of aetiology that produce neuropsychiatric disorders such as ASD. Risk genes associated with ASD often demonstrate pleiotropic effects that will vary in a brain region specific manner, according to developmental stages. Whilst a single gene may confer significant risk for ASD, causal links are not always clear, which is particularly true of risk genes associated with idiopathic ASD (Lord et al. 2020). Bridging the gap between genetic risk and behaviour remains a massive challenge. An approach to bridging this gap is to search for convergence. Numerous risk genes lead to a common diagnosis thus shared pathological mechanisms are likely. Indeed, the concerted efforts in studying the genetics of ASD has revealed risk genes tend to encode proteins that fall into two general categories (1) function and structure of synapses and (2) regulation of gene expression and chromatin remodelling (O'Roak et al. 2012; De Rubeis et al. 2014).

1.2 CYFIP1

Cytoplasmic fragile X mental retardation 1 interacting protein (CYFIP1) is a versatile synaptic protein that through protein-protein interactions influences a range of biological pathways (Bardoni and Abekhoukh 2014).*CYFIP1* is expressed in the cortex and cerebellum throughout development, in addition to many other tissues of the body (Bonaccorso et al. 2015). CYFIP1 has important neuronal development functions(Bonaccorso et al. 2015; Abekhoukh et al. 2017), and has recognized roles in synaptic processes including the formation of dendritic spines, morphology and branching (Pathania et al. 2014; Oguro-Ando et al. 2015). Synaptic dysfunction is associated with an array of neuropsychiatric disorders including ASD(van Spronsen

and Hoogenraad 2010; Lima Caldeira et al. 2019). Following a description of the neuronal function of CYFIP1, this section will review the association of *CYFIP1* with ASD.

1.2.1 Molecular function of CYFIP1

At the synapse CYFIP1 is a negative regulator of protein translation, directly binding FMRP (Schenck et al. 2001; Schenck et al. 2003) forming the CYFIP1-FMRP complex which in turn behaves as a binding protein for the eukaryotic translation initiation factor 4E (eIF4E) The coupling of the eIF4E-CYFIP1-FMRP complex with target mRNAs supresses translation within the synapse until activation via tropomyosin receptor kinase B or group I mGluRs releases eIF4E allowing translation of previously bound mRNAs (Napoli et al. 2008). Reduced levels of CYFIP1 leads to increased protein levels of FRMP targets supporting the role of CYFIP1 as a negative regulator of protein translation (Napoli et al. 2008). Beyond regulating protein translation CYFIP1 inhibits actin polymerisation as part of the WAVE regulatory complex (WRC), which also features WAVE 1/2/3, abl interactor - 1/2 (ABI 1/2), Nck-associated protein 1 (NCKAP1), and haematopoietic stem/progenitor cell protein 300 (HPSC300) (Takenawa and Suetsugu 2007). CYFIP1 can inhibit the verprolin-homology central acidic region (VCA) motif of WAVE1. In the absence of CYFIP1 the WRC can bind and activate actin-related protein 2/3 (Arp 2/3), promoting actin polymerisation (Eden et al. 2002; Kim et al. 2006; Chen et al. 2010). The WRC is maintained in an inhibited state by CYFIP1 until RAC1 binds triggering the dissociation of CYFIP1 facilitating the remodelling of the actin cytoskeleton through the action of Arp 2/3 (De Rubeis et al. 2013). CYFIP1 is unable to form complexes with FMRP and WRC simultaneously and under basal conditions is more likely to be associated with WRC (De Rubeis et al. 2013). Synaptic activity induces a conformational change in CYFIP1, from globular to planar which pushes the distribution more towards the WRC rather than FMRP (Chen et al. 2010; De Rubeis et al. 2013; Di Marino et al. 2015). Translation of mRNA and cytoskeleton dynamics are crucial processes for synaptic plasticity, highlighting the importance of intact CYFIP1 in synaptic structure and function (Klann and Dever 2004; Costa-Mattioli et al. 2009; Gal-Ben-Ari et al. 2012). Further to mediating these two essential processes at synapses, other roles for CYFIP1 are emerging.

Hsiao et al. (2016) demonstrated a presynaptic function of CYFIP1 in the developing hippocampus of mice. At postnatal day (P) 10 mice possessing a single copy of *Cyfip1* display larger presynaptic terminals and a higher vesicle release probability than WT mice, effects no longer present at P21. Dysregulation of WRC was established as the driver of the observed alterations given the effects were reversed by expression of mutant *Cyfip1* with preserved actin regulatory function or with use of Rac1 inhibitors. Impaired presynaptic neurotransmission in the corpus callosum was observed in adult mice with *Cyfip1* haploinsufficiency, along with decreased myelination of callosal axons (Domínguez-Iturza et al. 2019). Previous work in *Cyfip1* mutant fly models observed altered actin polymerisation in presynaptic terminals adding further support to the presynaptic role of CYFIP1. Evidence for non-neuronal roles of CYFIP1 is emerging. In mouse microglia, conditional knockout (KO) of *Cyfip1* altered morphology, with cells displaying an increased activation state. (Drew et al. 2020). Similarly, haploinsufficiency of *Cyfip1* in rats impacts the morphological features of microglia through the dysregulation of actin polymerisation which in turn affects the phagocytic capacity of microglia (Correa-da-Silva et al. 2024). In human iPSC-derived microglia-like cells lacking *CYFIP1* display reduced phagocytosis of synaptosomes as well as altered morphology and motility (Sheridan et al. 2024). Taken together these works highlight the importance of CYFIP1 in the regulation of cytoskeletal dynamics in microglia function. Recent work, has illuminated a key role for CYFIP1 early in development, protecting cortical neurogenesis (De La Fuente et al. 2024). Using stem cell models, gene dosage of *CYFIP1* was shown to impact neurogenesis with reduced CYFIP1 expression causing premature neuronal differentiation whereas increased CYFIP1 expression promotes maintenance of neural progenitors. This work identified CYFIP1 as a regulator of cholesterol metabolism, which when disrupted impacted oxysterol-liver X receptor (LXR) signalling, the mechanism responsible for the premature neuronal differentiation observed with reduced *CYFIP1* (De La Fuente et al. 2024). Such findings reaffirm the pleiotropy of *CYFIP1* and reinforces the need to study CYFIP1 in a developmental context. CYFIP1 as a regulator of both protein translation and cytoskeletal dynamics is essential to many biological processes, at the synapse and beyond. How CYFIP1 contributes to the aetiology of ASD will be reviewed considering clinical and pre-clinical findings.

1.2.2 CYFIP1 in neuropsychiatric disorders

Within the proximal long arm of human chromosome 15 deletions and duplications generate CNVs that arise at 5 common breakpoints (BP1-BP5) (Cox and Butler 2015). Prader-Willi syndrome and Angelman syndrome are neurodevelopmental disorder resulting from deletions of paternal or maternal origin respectively. Type I deletions are large, occurring between BP1 and BP3 with type II deletions being smaller, between BP2 and BP3. *CYFIP1* is located between BP1 and BP2, the 15q11.2 interval along 3 other genes: non-imprinted in Prader-Willi/Angelman 1 (*NIPA1*) and 2 (*NIPA2*) and tubulin gamma complex-associated protein (*TUBGCP5*) (Chai et al. 2003). The 15q11.2 locus was first associated neurodevelopmental psychiatric disorders following the observation of more severe behavioural phenotypes in Prader-Willi syndrome or Angelman syndrome patients with type I deletions compared to those with type II deletions, where the 15q11.2 interval is intact (Butler et al. 2004; Bittel et al. 2006). Subsequent investigations identified patients with deletions and duplications that flanked the 15q11.2 interval (Cox and Butler 2015). CNVs in the 15q11.2 interval are estimated to be present in 1 in 100 individuals who are genetically screened, though prevalence in the general population is estimated to be around 1 in 500 (Butler 2017). Individuals with CNVs in the 15q11.2 region, present with motor development delays, behavioural issues, seizures and ASD (Burnside et al. 2011; Vanlerberghe et al. 2015; Woo et al. 2019). Deletions in the region, referred to as Burnside-Butler syndrome, are associated with ASD in 30% of individuals but the biggest impact appears to be on cognition(Burnside et al. 2011; Cox and Butler 2015; Butler 2017). The penetrance of 15q11.2 deletions is low with the risk of presenting with phenotypic abnormalities estimated at around 10% (Kirov et al. 2014). Most carriers of 15q11.2 deletions maybe be classed as unaffected, but it is likely that carriers present with subthreshold phenotypes that do not meet current diagnostic criteria. The low penetrance of 15q11.2 deletion is reinforced by estimates that suggest it is inherited from an unaffected parent more often than an affected parent. Whilst the deletion

may arise *de novo* up to a fifth of the time (Doornbos et al. 2009; Cox and Butler 2015).

Of the four genes located at the 15q11.2 interval, *CYFIP1* is deemed the prime candidate for conferring the biological and behavioural manifestations observed with 15q11.2 BP1-BP2 CNVs (Bozdagi et al. 2012). This candidacy was in-part born from the functional association between CYFIP1 and FMRP which is linked to the neurodevelopmental fragile X syndrome. Direct evidence supporting the role of *CYFIP1* in pathological mechanisms underpinning 15q11.2 CNVs comes from patients carrying deletions, who display reduced expression of CYFIP1 and other proteins of the WRC (Abekhoukh et al. 2017). Furthermore, in stem cell models, both 15q11.2 deletion and CYFIP1 loss of function yield the same phenotype, premature neuronal differentiation (De La Fuente et al. 2024). The biological consequences of altered *Cyfip1* dosage have been illuminated by the study of preclinical models. In mouse models heterozygous for functional *Cyfip1* alterations were observed in dendritic and spine morphology (De Rubeis et al. 2013; Pathania et al. 2014). Similar changes were also observed in a conditional knockout (cKO) model whereas overexpression of *Cyfip1* also impacts the morphology of dendrites and spines (Pathania et al. 2014; Oguro-Ando et al. 2015). Dendritic spine instability was demonstrated in a haploinsufficient *Cyfip1* model (Bachmann et al. 2019). Bozdagi et al. (2012), examined synaptic plasticity in a haploinsufficient *Cyfip1* mouse model which revealed increased levels of mGluR-mediated depression. Reduction of *Cyfip1* in CA1 hippocampal neurons yielded increased inhibitory gamma aminobutyric acid (GABA)ergic transmission coupled with increased expression of GABA receptors point to a shift towards greater inhibition in terms of excitation/ inhibition balance. Conversely, when *Cyfip1* was overexpressed in CA1 hippocampal neurons a potential shift towards greater excitation was observed, as excitatory neurotransmission increased whilst GABAergic neurotransmission decreased (Davenport et al. 2019). This finding was not reproduced in a haploinsufficient *Cyfip1* mouse model which observed unaltered GABAergic signally in the hippocampal dentate gyrus (Trent et al. 2019). In another comparison of *Cyfip1* dosage, complete loss of *Cyfip1* caused an increase in the expression of NMDAR subunits, as well as associated proteins SHANK2 and PSD95 in synaptosomes whereas overexpression saw a reduction in these same proteins. This altered molecular composition in

synapses of the dentate gyrus impacted NMDAR function with cKO mice showing an increased NMDA/AMPA ratio in evocation of excitatory postsynaptic currents compared to WTs whereas overexpression led to a decreased NMDA/AMPA ratio (Kim et al. 2022). These observations point to regulation of postsynaptic proteins being CYFIP1 dosage dependent and along with the other findings described here highlight the importance of CYFIP1 in maintaining the structure and function of synapses.

Beyond the synapse, alterations in *Cyfip1* dosage can have consequences for brain connectivity and white matter structure (Domínguez-Iturza et al. 2019). Examination via resting state function magnetic resonance imaging (rsfMRI) revealed reductions in bilateral connectivity in multiple brain regions of *Cyfip1* heterozygote KO mice. Domínguez-Iturza et al. (2019), observed alterations in white matter structure as measured by diffusion tensor imaging (DTI) which recorded decreased fractional anisotropy (FA) in the *Cyfip1* heterozygotes. These findings were supported by a separate study that observed white matter phenotypes and decreased FA in a *Cyfip1* haploinsufficient rat model (Silva et al. 2019a). However, contrary to the mouse and rat models 15q11.2 deletion patients display increased FA (Silva et al. 2019b). The reason for the opposing observations in patients and pre-clinical models is unclear but may be because the other genes at the 15q11.2 interval influence this phenotype thus further investigation is required.

Rodent models investigating the biological impact of varied *Cyfip1* dosage continue to reveal profound phenotypes that relate to synaptic structure and function (Clifton et al. 2020). However, the behavioural phenotypes observed in these models are often mild. In the first assessment of the *Cyfip1* haploinsufficient mouse model, a rapid loss of extinction memory was identified via assessment in the inhibitory avoidance paradigm, but other aspects of fear learning and memory appeared unaffected (Bozdagi et al. 2012). *Cyfip1* heterozygous KO mice and rats display deficits in motor learning (Bachmann et al. 2019; Domínguez-Iturza et al. 2019), behavioural flexibility (Silva et al. 2019a), and sensorimotor gating (Domínguez-Iturza et al. 2019). These behavioural phenotypes are relevant to the disorders the models are trying to recapitulate but the lack of a profound behavioural phenotype suggests additional insults, genetic and/or environmental are required to elicit more robust behavioural outcomes in pre-clinical models. This may reflect what occurs in

humans, given the low penetrance of 15q11.2 deletions and duplications. Additional challenges or timing of specific environmental insults may determine the clinical outcome e.g. ASD versus schizophrenia. Alternatively, in the context of neurodevelopmental disorders, existing models may miss behavioural phenotypes because they are transient, like the presynaptic phenotype observed by Hsiao et al. (2016) was present at P10 but not after P21. Such transient phenotypes would likely require additional challenges, particularly environmental insults to reinforce the behavioural phenotype making it permanent.

Studying the genetics of clinical populations has revealed CNVs at the 15q11.2 locus are associated with increased risk for a range of neuropsychiatric disorders such as ASD (van der Zwaag et al. 2010; Burnside et al. 2011), attention deficit hyperactivity disorder (ADHD) (Gudmundsson et al. 2019), developmental delay and ID (Cooper et al. 2011; von der Lippe et al. 2011; Chaste et al. 2014), schizophrenia (Stefansson et al. 2008; Kirov et al. 2009) and major depression (Zhang et al. 2019). Common variants in *CYFIP1* have also been associated with increased risk of ASD (Waltes et al. 2014; Wang et al. 2015). In a small exploratory study of children with an ADHD diagnosis, the ASD associated SNP *CYFIP1*-rs3693 was associated with an interaction with acute life events influencing ADHD diagnosis. The same SNP was associated with an interaction with prenatal alcohol consumption influencing risk for comorbid disruptive disorders (Waltes et al. 2019). This is suggestive evidence for *CYFIP1* variants being influenced by environmental factors to yield different phenotypic outcomes.

CYFIP1 is relevant to the study of ASD as it has a well-defined role in synaptic structure and function a key target in the search for convergent pathways of ASD risk genes. This is reinforced because it interacts with other synaptic proteins linked to increase risk for ASD. These include, FMRP, associated with syndromic ASD due to its role in fragile X syndrome (Napoli et al. 2008b; Bagni and Zukin 2019). It has also been demonstrated to interact with the postsynaptic cell-adhesion molecule Neuroligin-3 which is associated with idiopathic ASD (Jamain et al. 2003; Bachmann et al. 2019). Additionally, reduction of *Cyfip1* leads to reduced expression of Neuroligin-3 at inhibitory synapses in the hippocampus (Davenport et al. 2019).

Combination of *CYFIP1* with other risk factors in models of ASD may help elucidate convergent biological mechanisms in ASD pathology.

1.3 Environmental risk factors for ASD.

There is a strong genetic component to the aetiology of ASD with twin and family studies highlighting the heritability of the disorder. However, these studies also implicate environmental factors, and some suggest a larger environmental than genetic risk for ASD (Hallmayer et al. 2011b; Risch et al. 2014; Kim and Leventhal 2015). Genetic risk for ASD comes from multiple loci and it is similar for environmental challenges. Epidemiological studies consistently implicated numerous factors in the aetiology of ASD. These include advanced parental age, mothers being overweight, selective serotonin reuptake inhibitor (SSRI) use during pregnancy, chemical exposure, pregnancy complications e.g. pre-eclampsia and maternal infection (Li et al. 2016; Lyall et al. 2017; Y Kim et al. 2019; Cheroni et al. 2020). This list is by no means exhaustive and is intended to illustrate the diversity of environmental influences that may contribute to risk for ASD. These factors as with genetic factors can stack additively to increase risk (Dodds et al. 2011; Lyall et al. 2012). Epidemiological studies are crucial as they facilitate direct examination of human populations, but an innate limitation is the inability to illuminate pathological mechanisms thus require feedback from pre-clinical models to validate associations. Of the environmental challenges list above, maternal infection has well established animal models that recapitulate features of neuropsychiatric disorders (Brown and Meyer 2018). The links between maternal immune activation and ASD will be described below, considering observations from epidemiological and pre-clinical studies.

1.3.1 Maternal immune activation association with ASD

Epidemiological studies have linked maternal immune activation via infections and inflammation with ASD (Abdallah et al. 2012; Brown et al. 2014; Jiang et al. 2016; Jones et al. 2017; Kim et al. 2019c). There is some disagreement, as some studies only associate infection with ASD where the mother was hospitalised (Lee et al.

2015; Zerbo et al. 2015). In an umbrella review that categorised the level of evidence for various ASD associated environmental factors, classed the evidence for infection requiring hospitalisation and risk of ASD as suggestive. The evidence for an association of infections that did not require hospitalisation with ASD was classed as weak (Y Kim et al. 2019). Contrary reports also exist regarding maternal inflammation. Brown et al. (2014) demonstrated an increased risk of ASD when mothers had elevated levels of C-reactive protein, a marker of systemic inflammation, at a mid-gestational timepoint. Another study found elevated C-reactive protein at a similar timepoint to be associated with decreased risk for ASD (Zerbo et al. 2016). A third study found no association but examined an earlier timepoint that the first two studies (Koks et al. 2016). Increased maternal cytokines and chemokines were linked to increase risk of ASD (Goines et al. 2011; Jones et al. 2017) In a Danish cohort amniotic fluid samples were analysed and revealed links to ASD, including tumour necrosis factor (TNF)-α (Abdallah et al. 2012). This finding was supported in a Norwegian cohort. Analysis of maternal mid-gestation plasma samples and cord blood revealed elevated levels of immune biomarkers for boys and girls at both timepoints including TNF-α (Che et al. 2022). Strikingly girls with ASD had a higher number of associated markers than boys which is in keeping with the idea of a female protective effect that suggests females need a higher burden to produce the same phenotype in males, though this is typical discussed in a genetic context. No immune/inflammatory markers were associated with ASD in a Swedish cohort that analysed maternal serum samples early in pregnancy. Brynge et al. (2022), did observe associations for TNF-α with ASD but only when comorbid with ADHD and several cytokines were associated with increased risk of ASD when co-occurring with ID. The conflicting reports referred to here point to mid-gestation as an important timepoint in the context of maternal immune activation and risk of ASD. This may not be unique to ASD as maternal immune activation is associated with a number of neuropsychiatric conditions and there are no clear differences between timing of maternal activation and diagnostic outcomes. Association with multiple phenotypic outcomes is seen in genetic risk loci for neuropsychiatric disorders. Given the phenotypic overlap between disorders e.g. social dysfunction there is potential for epidemiological studies to pursue a symptom-based approach. Is maternal immune activation associated with social dysfunction? Rather than

condition A or B. Robinson et al. (2016b) demonstrated a strong correlation between ASD risk and social behaviour in the general population so the question could be Is maternal immune activation associated with social behavioural outcomes in the general population? The phenotypic heterogeneity i.e. different disease outcomes, associated with maternal immune activation suggests additional, interactive factors can influence phenotypic trajectories. It is likely, maternal immune activation is itself one of those factors. This is reinforced by the fact immune activation in pregnancy is far more common than ASD prevalence, suggesting the need for additional hits. This highlights a key advantage for pre-clinical models, the ability to remove additional hits, human life is inherently messy and hard to control for all influences that have the potential to confound epidemiological studies. Animal models can be used to probe causal mechanisms that underpin the association of maternal immune activation neuropsychiatric outcomes.

Animal models of maternal immune activation fall into three classes. The first class of models utilise live pathogens such as influenza which are excellent for interrogating causal mechanisms including severity thresholds (Antonson et al. 2021; Otero et al. 2024). The second class uses non-infectious immune stimulating agents, which can be used to profile maternal and fetal cytokine imbalances to investigate how prenatal challenges can elicit postnatal neurobiological and behavioural changes (Brown and Meyer 2018) . The third class are specific to immune processes implicated in neuropsychiatric disease such as allergies and asthma (Schwartzer et al. 2015; Schwartzer et al. 2017; Vogel Ciernia et al. 2018). Most maternal immune activation models are based on the second class, using immune activating agents such as the viral mimetic poly(I:C). These models do not fully recapitulate maternal infection, but they offer a high degree of experimental control allowing the examination of dosage and gestational timing effects on offspring brain development and behavioural phenotypes (Meyer et al. 2006). Models utilising poly(I:C)-induced immune activation consistently report aberrant behavioural phenotypes that are relevant to the core symptomology of ASD (Soumiya et al. 2011; Malkova et al. 2012; Coiro et al. 2015; Ikezu et al. 2021; Zhao et al. 2021). Maternal immune activation via poly(I:C) can also yield biological phenotypes relevant to potential ASD pathology such as morphological alterations in dendritic spines (Soumiya et al. 2011; Coiro et al. 2015; Ikezu et al. 2021), abnormal cerebellar development (Shi et al. 2009) and

alterations in synaptic transmission (Nakagawa et al. 2020). These findings support the association of maternal immune activation with ASD without differentiating this association from the different phenotypic outcomes observed following maternal immune activation.

1.4 Gene x Environment interactions

The genetic and phenotypic heterogeneity of ASD coupled with shared risk and symptomology with other neuropsychiatric disorders points to a multifaceted aetiology arising from complex interaction of genetic and environmental factors (Chaste and Leboyer 2012). Disentangling these interactions presents a massive challenge not least because individual risk factors are shared across diagnostic boundaries. To study gene x environment interactions robustly requires large sample sizes, with rich genetic data and phenotypic data spanning development into adulthood. The lack of large cohorts with genotype and high-quality exposure data has limited attempts to probe these interactions. To date there have been a few reports of gene x environment interactions in the context of ASD. These include and interactions between a *MET* risk gene and prenatal exposed to air pollutants e.g. NO_2 (Volk et al. 2014), increased risk of ASD in children with *COMT* variants where mothers did not take prenatal vitamins (Schmidt et al. 2011) and interactions between maternal infection and CNVs associated with ASD. CNVs were reported to interact with maternal infection to increase severity of core ASD symptoms, but did not impact cognition or adaptive functioning (Mazina et al. 2015). These findings need to be replicated as they are limited by the focus on candidate loci or CNV burden rather than a full genome wide analysis which would require much greater sample sizes than utilised in the studies here. A genome-wide study surveying potential interactions between SNPs and maternal cytomegalovirus (CMV) in the aetiology of schizophrenia replicated previous associations but also identified a novel association (Børglum et al. 2014). This finding demonstrates the value of large-scale gene x environment studies and highlights the potential for maternal immune activation to interact with common variants. A complimentary strategy to epidemiological studies would be the generation of animal models combining genetic and environmental risk insults.

1.4.1 Animal models of gene x environment interactions

Combining environmental insults with genetic models can demonstrate interactive effects providing insight into phenotypic heterogeneity seen within and across neuropsychiatric disorders. Studies displaying additive effects of neuropsychiatric risk loci and environmental challenges have been reported. Peripubertal exposure to stress in a mouse model of 15q13.3 microdeletion interacted to impair sensorimotor gating, though this interaction was temporally sensitive and exposure to stress in adulthood did not produce this interactive effect (Giovanoli et al. 2019). *CHRNA7* is a risk gene located at the 15q13.3 locus and has been shown to interact with maternal immune activation with *Chrna7*$^{+/-}$ displaying more pronounced behavioural deficits (Wu et al. 2015). Maternal immune activation was shown to interact with *Nrg1* with offspring born to MIA dams displaying intact sociability at P35 but impaired sociability at P90 (O'Leary et al. 2014). In a fragile X model, stress was shown modulate performance of male *Fmr1* KOs in the Y-maze. Stress also demonstrated interactive effects in social behaviour, increasing the sociability of male and female WTs but not in *Fmr1* mutants (Petroni et al. 2022). Poly(I:C)-induced MIA was shown to interact additively to increase social impairment in a *Shank3* model (Atanasova et al. 2023), and in males in a *Cntnap2* model (Schaafsma et al. 2017). Interactive effects aren't always additive as demonstrated by (Kim et al. 2019). In a gene x environment model that combined *Cntnap2* KO with prenatal exposure to valproic acid it was observed that combining these factors rescued the social behaviour deficit observed in mice exposed to just one of these challenges. These findings highlight the ability of different environmental challenges to produce interactive effects in genetic models. Maternal immune activation appears to interact with a range of risk genes. Even at subthreshold dose, poly(I:C) was shown to interact with *DISC1* mutations to impact social behaviour in adult offspring (Abazyan et al. 2010; Lipina et al. 2013). Maternal immune activation is a particularly attractive candidate for combining with genetic risk factors because of the control over intensity and timing of immune response. When combined with genes that are associated with risk for multiple disorders such as *CYFIP1* it has the potential and versatility to probe the mechanisms that underpin phenotypic heterogeneity.

1.5 Behavioural phenotypes in mouse models of ASD

The heterogeneity in ASD presentation is a massive challenge for developing pre-clinical models that recapitulate the symptomology of ASD that in turn produce outcomes that translate accurately back to humans (Silverman et al. 2010). Despite the challenge a range of mouse models utilising genetic and environmental insults associated with ASD risk have been generated and phenotyped with high degrees of face validity.

1.5.1 Models of ASD or Models of social impairment?

To address the heterogeneity of ASD presentation, arguments are made advocating the development of mouse models that are extensively phenotyped, investigating more than the core features of ASD (Silverman et al. 2022). The rationale is logical, if a model is tested in one single task, assessing sociability, any identified deficit may be driven by non-social processes such as impaired motor function or altered sensory perception. However, to-date focussing on replicating ASD in animals has failed to yield robust markers. This is not unique to the study of ASD and affects the research all neuropsychiatric conditions, where disease specific, translatable outcomes from animal models have been lacking, pointing to a need to shift focus from nosological entities to symptoms/endophenotypes (Anderzhanova et al. 2017). The Research Domains Criteria (RDoC) framework was developed as an alternative system to categorize neuropsychiatric disorders, focussing on endophenotypes rather than human diseases (Insel et al. 2010). The RDoC framework currently consists of 6 behavioural domains: (1) negative valence (2) positive valence (3) cognitive systems (4) systems for social processes (5) arousal and regulatory systems (6) sensorimotor systems. Within each domain sits various constructs that can be viewed as endophenotypes that can be studied at all biological levels, from genes to behaviour (Shemesh and Chen 2023). This biological deterministic approach is hoped to produce more translatable outcomes entities (Anderzhanova et al. 2017).

1.5.2 Studying social behaviour endophenotypes

Impairment in social behaviour is a common feature of many neuropsychiatric conditions (Shemesh and Chen 2023). For example, people with ASD, PTSD, anxiety, depression and schizophrenia can display “social withdrawal” thus if an animal model demonstrates a social withdrawal phenotype what condition is it a model for? Observing an endophenotype approach, it is a model of social withdrawal. Therefore, an endophenotype approach can be more objective in unpicking the neurobiological mechanism that underpin normal function as well as pathology. Granted social withdrawal can look different in each of the conditions it is a feature of, but this can be true when comparing any two individuals with the same diagnosis and represents the inherent complexity and heterogeneity of social behaviour.

Human disorders diagnosed based on behavioural output may not map well onto animals, which also applies to specific endophenotypes, particularly those as complex as social behaviour. Therefore, animal models examining social behaviour need to interpret readouts in the context of the model organism. Observed social behaviours share common features across species but the meaning of what is to be social will differ species to species (Goodson 2013). Aggression, for example is a part of social behaviour and in mice it is an important aspect of typical social behaviour (Jabarin et al. 2022) yet aggression in herded farm animals is undesirable where passive tolerance is key (Estevez et al. 2007). Despite the complexity of social behaviour and differences across species, brain circuitry and molecular mechanisms that regulate the expression of social behaviour appear to be well conserved. For example, oxytocin influences many aspects of social behaviour both prosocial and agonistic across species (Anacker and Beery 2013; Grinevich and Neumann 2021). Therefore, even when species differences exist for the outward expression of social behaviour, common neurobiology exists. A challenge, particularly in the context of profiling social behaviour in animal models is identifying analogous behavioural traits in humans (Czéh et al. 2016). Part of this challenge arises from the limitations of behavioural tasks used to assess social behaviour. For a start, no single test of social behaviour has the has the resolution to confirm an animal model is socially impaired or not (Jabarin et al. 2022).

1.5.2.1 Multiple tests

Even if a single test demonstrates a deficit in social behaviour, it may signify a deficit in merely one aspect of social behaviour or equally, if the test does not show a deficit, it may be interpreted that the model does not recapitulate the endophenotype being investigated. The *Cyfip1*$^{+/-}$ mouse model did not demonstrate a sociability deficit (Bozdagi et al. 2012), males did not show lower levels of interaction with a female (Sledziowska et al. 2020b) yet displayed lower levels of interest in social odours (Bachmann et al. 2019). In another model, the 16p11.2$^{+/-}$ mouse line has several reports of intact social preference (Portmann et al. 2014; Brunner et al. 2015; Stoppel et al. 2018) but has also displayed deficits in social recognition and habituation tasks (Portmann et al. 2014; Brunner et al. 2015) as well as altered direct social interaction (Wang et al. 2018). The tests used in both these examples can be used to model the same endophenotype, Affiliation and Attachment (a construct within the System for Social Processes domain), thus demonstrating the value in utilising several social behavioural paradigms. This is of relevance when studying symptoms of neurodevelopment disorders such as ASD as identifying the presence or absence of social deficits at defined timepoints can improve translatability by adding context to any findings. There may also be additional value in using the same test multiple times, as the dynamic nature of social behaviour allow it to be influenced by numerous factors such as the emotional state of subject and stimulus (Jabarin et al. 2022), experimental settings (Kim et al. 2019a) housing conditions (Ricceri et al. 2007) and social rank (Peleh et al. 2019). These factors are often described as confounds but would be better considered as sources of biological variability in measure variables (Voelkl and Würbel 2016).

1.5.2.2 Environmental influences

One such source of biological variability for measures of social behaviour is the animal's social environment, specifically the genotype of littermates. The inbred mouse strain BTBR, known for low levels of sociability demonstrated a normalisation of social interest when housed with C57BL/6 mice rather than BTBR mice (Yang et al. 2011a). In a study investigating the behaviour of estrogen receptor α (ERα) knockouts, mice were housed with genotyped-matched conspecifics (WT-WT, KO-

KO) or a mixture of genotypes before weaning, then the mice were only housed with genotype-matched conspecifics. Interesting, the behaviour of WT males from mixed genotype was impacted, displaying significantly more aggression when encountering an unfamiliar mouse (Crews et al. 2009). In another study, using mixed genotype litters the presence of mice with a *Nlgn3* deletion was shown to influence the social behaviour of WT littermates, with WT males showing lower interest in social odours and time spent interacting with a female (Kalbassi et al. 2017). These examples suggest the presence of mutants in a litter influence the social development of WT littermates. This may depend on the model and in the 16p11.2 deletion model, mutants displayed a deficit in courtship specific vocalisation when housed with WTs but not when housed with genotype-matched conspecifics (Yang et al. 2015a). This demonstrates that WTs can also influence the behaviour of mutants in mixed genotype litters. Taken together these examples highlight the role of co-housing different genotypes in modulating social behaviour of WTs and mutants alike. Conceptually, this makes sense, if a gene is important for social behaviour and a mutation in said gene causes social impairments i.e. a gene dosage effect, this may apply to the whole social group even individuals with no mutation. The higher the proportion of mutants in a litter, the higher the chance of WTs experiencing aberrant interactions thus the underlying circuits of social behaviour, though initially intact are updated incorrectly which impacts outward expression of social behaviour in adulthood. For mutants, if they are part of a litter with a high proportion of WTs, these individuals will be faced with a higher number of typical social interactions, which are appetitive stimuli in WTs but could be aversive in the mutant thus producing a deficit not present in the complete absence of WTs. Ergo, rather than treating mixed genotypes as a source of confound it is important to address this in experimental design and analysis as it may help illuminate the importance of a model for in social behaviour.

In summation, rather than try and produce a mouse with autism, it is better to produce models of social impairment, especially when the genetic and environmental insults being examined are associated with multiple disorders than can impact social behaviour e.g. *Cyfip1*$^{+/-}$ and infection during pregnancy. Different aspects of an endophenotype should be assayed at different time points, or the same test used several times to interrogate the developmental and dynamic nature of social

behaviour. Furthermore, biological sources of variability should be addressed as opposed to controlled for. An organism's environment will influence its behaviour and whilst heterogeneity presents a challenge, it may be time to embrace said challenge.

1.5.3 Sex differences

The sex bias observed in ASD has historically been reflected in experimental design, with investigators choosing to study only male animals. The rationale typically cited is that the hormonal cycle of females introduces variability, however the variability in females is reported as being no different from the variability in male animals (Beery 2018). In the context of ASD sex is a contributing factor to the heterogeneity of phenotypic presentation. When considering ASD with ID the sex bias is less evident, but ASD alone sees more male cases than females (Lord et al. 2020). A proposed explanation for this is the female protective effect, to elicit the same phenotype in females a large risk burden is required (Jacquemont et al. 2014). This is evidenced by the observation females display a higher burden for common risk variants compared to males when considering ASD without ID (Warrier et al. 2022; Wigdor et al. 2022). Perhaps more pertinent in the context of modelling endophenotypes is the evidence that ASD may present differently in females, with social difficulties more likely to emerge in adolescence(Lai and Szatmari 2020). Modelling social processes on a continuum of normality acknowledges the variability that exists within social behaviour, and this extends to the variability arising from sex differences. Humans and rodents display sexually dimorphism in social behaviour thus models of social endophenotypes warrant the inclusion of males and females (Yamasue et al. 2009; Zilkha et al. 2021)

1.6 Aims and Objectives

Typically, the pathophysiology of ASD has been interrogated through the study of single risk factors, genetic or environmental (Varghese et al. 2017). Given that no single factor explains all cases of ASD, genetic and environmental risk factors likely combine to produce the characteristic symptomology of ASD (Chaste and Leboyer 2012). The current understanding of how gene-environment interactions lead to ASD

remains limited. To this end, the primary goal of this project is to develop a gene-environment interaction model of affiliation and attachment, examining the combined impact of maternal immune activation and *Cyfip1* haploinsufficiency on the social behaviour in the early life of mice i.e. pre-weaning (Chapters 3 and 4). Social behaviour is complex and multi-faceted, with interactions producing mutual feedback for involved parties (Jabarin et al. 2022). However, tests of social behaviour in pre-clinical models tend to treat social interactions unilaterally, solely focussing on the readout from the experimental animal. Therefore, the secondary aim of this project is to assess a new social behaviour assay that was designed to capture the dyadic nature of social interaction in the context of affiliation and attachment (Chapter 5).

Chapter 3 optimized a well-established MIA protocol to identify a dose of poly(I:C) that stimulated the immune system of the dams without yielding aberrant behavioural phenotypes in the offspring. This sub-threshold dose was selected for use in the gene-environment model of Chapter 4. To achieve this a dose response experiment was undertaken, whereby pregnant mice received an IP injection of saline or poly(I:C), 5, 10 or 20 mg/kg at E12.5. Immune activation was validated via non-invasive methods including infra-red thermometry. To assess outcomes in the offspring and building on previous works identifying social deficits in poly(I:C) MIA models this work focussed on the neurodevelopmental nature of ASD and its associated social impairment. I hypothesised that any social deficits would emerge at early developmental timepoints, thus male and female offspring were assayed with the homing test at P9, social novelty test at P26 and 27 as well as direct social interaction at P28. At the lowest dose where offspring behaviour was affected a 2nd independent cohort was assayed but only in one test, the P28 direct social interaction test to examine if early testing is a potential unintentional hit, adding a source of variability to the MIA protocol used.

Chapter 4 investigated the impact of combining MIA and *Cyfip1* haploinsufficiency on pre-weaning social behaviour of male and female mice. Utilizing the established protocol of Chapter 3, dams were administered the sub-threshold dose of poly(I:C) and offspring were tested in the P9 homing test and P28 direct social interaction test. The impact of social environment was examined to see how the proportion of *Cyfip1*$^{+/-}$ animals in a litter impacted the social behaviour of WT littermates.

Chapter 5 evaluated the Social Interaction Platform (SIP) test as an assay of social behaviour. The behaviour of adult male mice in the SIP in the presence of another mouse was compared to behaviour in the absence of mice and performance in other social behaviour tests such as the three-chamber tests. This study used the SIP to test mice against cagemates before establishing dominance status within the homecage to explore the relationship between social interaction and hierarchy. In an independent cohort, a longitudinal design will investigate how behaviour in the SIP varies with age. Finally, the SIP was utilised to compare social behaviour in control and MIA adult male offspring from the 2nd cohort of mice in Chapter 3.

Chapter 2 : Materials and Methods

2.1 Husbandry and legislation

Animal studies and breeding were performed in accordance with the Animals (Scientific Procedures) Act 1986 (amended 2012). The mice were housed in an environmentally regulated holding room temperature (21°C ± 1.5) and humidity (48% ± 12%) under a 12-hour light/dark cycle with lights turning on at 06:00 h. Mice were housed in 16 x 48 x 14 cm conventional cages with free access to water and food. All cages contained the same environmental enrichment of chew-stick, nestlet bedding, a cardboard tube, and a plastic tube that was used for handling the mice. Cages were cleaned weekly unless it was experimentally necessary to delay cleaning.

Adult mice were housed in same sex pairs, except for males used as sires which were housed individually 72 hours prior to first exposure to females and remained individually housed thereafter. Pregnant dams were individually housed at embryonic day (E) 17.5 rather than at E0.5 to avoid the potential stressor effects of social separation (Soliani et al. 2017). When individually housed, dams were placed next to their original cage mate to allow them to experience familiar odours and vocalisations, thus reducing any impact of separation. Mice were left undisturbed for 1 week following arrival into the holding room, then all animals went through a 3-day programme of increased handling, outlined in Table 1. The aim of this programme was to reduce any confounds introduced by handling animals for the first time during an experiment (Gouveia and Hurst 2019), whilst providing consistency, with each animal getting approximately the same amount of handling time. Prior to first handling animals were habituated to experimental room and experimenter for 30 minutes. Adult mice were identified with an ear notch at the end of the 3-day handling programme.

Pups were left undisturbed by the experimenter until P9. From P9 to P13 pups were identified with a non-toxic marker used to mark one paw, refreshed at P11 when animals were weighed. At P13 pups were ear notched for identification purposes.

2.2 Experimental animals

The mice used in testing arose from 3 sources.

1. WT C57BL/6J mice (JAX:000664) obtained from Charles River were used as dams and sires, as well as stimulus animals for the experiments of Chapters 3 and 4. They were also used as the experimental animals in Chapter 5.
2. *Cyfip1*$^{tm2a(EUCOMM)Wtsi}$ animals (MGI:5002986, subsequently referred to as *Cyfip1*$^{+/-}$) were kindly provided by Professor Lawrence Wilkinson from the School of Psychology, Cardiff University. These animals were bred inhouse with WT C57BL/6J to produce heterozygous males used for breeding as part of the work in Chapter 4.
3. The litters generated by breeding groups in Chapter 3 were all WTs and were utilised in behavioural testing reported in Chapter 3 and 5. The breeding group in Chapter 4 produced mixed genotype (WT and *Cyfip1*$^{+/-}$) litters that were used in the behavioural tests of Chapter 4. At the end of testing, P29, animals were killed, and tail tissue collected for genotyping, which was carried out externally by TransnetYX (London, UK).

Table 2.1 Standardised approach to handling mice prior to first testing

	DAY		
	1	**2**	**3**
1. Place hands at opposite end from nest to allow exploration by mice	60 seconds	30 seconds	30 seconds
2. Remove mouse from cage with handling tube.	yes	yes	yes
3. Hold mouse in tube, before releasing on to hand in clean cage.	30 seconds	20 seconds	10 seconds
4. Return mouse to home cage, using	Tube	Tube	Hands
5. Repeat steps 1-3.	yes	yes	no
6. Transfer mouse from home cage in tube to new cage, restrain via scruffing to ear notch.	no	no	yes

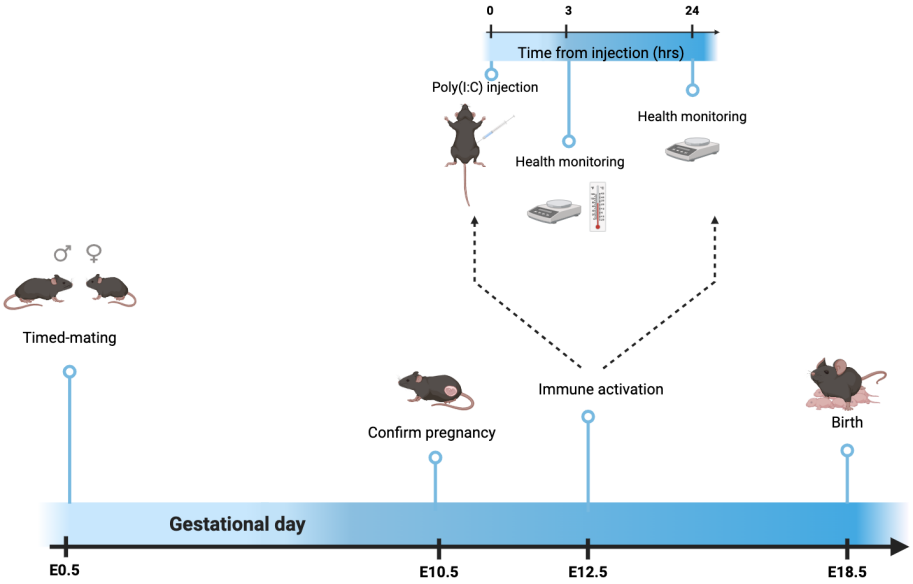


Figure 2.1 Experimental timeline for MIA. Illustration of timing of MIA and validation of immune activation in dams. Created in BioRender.com.

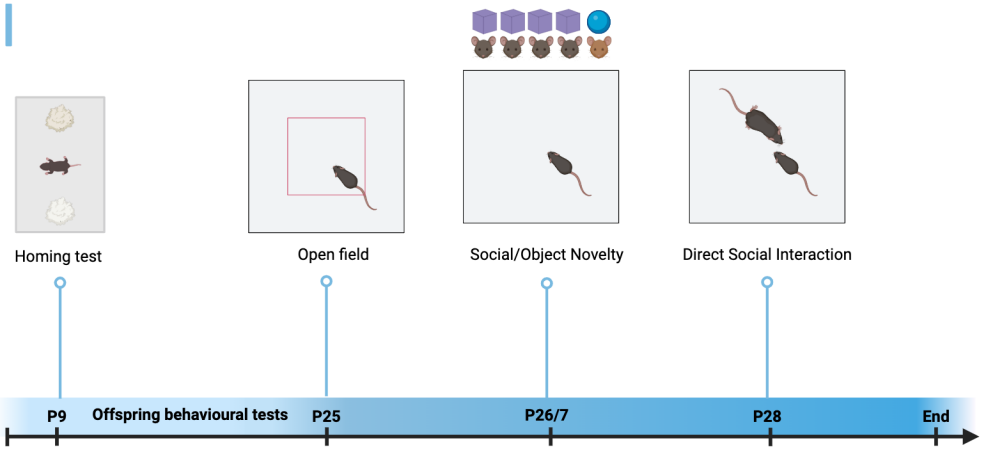


Figure 2.2 Experimental timeline for offspring behavioural tests. Illustration of timing of behavioural tests for MIA offspring. Not all offspring experienced full schedule of testing. Created in BioRender.com

2.3 Maternal immune activation

2.3.1 Breeding

Female WT C57BL/6J mice, 10-12 weeks old were set-up in timed mating with male WT C57BL/6J mice 10-16 weeks old for the work in Chapter 3. Or with *cyfip1*$^{+/-}$ males, 10-14 weeks old, for the work in Chapter 4. Prior to pairing the weight of the females was recorded. Pairs of females (cagemates) were added to a singly housed male's cage at the end of the light phase, 18:00 h. At the end of the dark phase females were examined for the presence of a vaginal plug and returned to their home cage. If a plug was present this was defined as E0.5. Females would be weighed again at E10.5 to confirm pregnancy. Pregnant mice show approximately a 20% increase in weight from the time of pairing at E10.5 (Chow et al. 2016).

2.3.2 Immune activation

Dams were randomly assigned to receive poly(I:C) or saline injections at E12.5. Immune activation was induced by low molecular weight (LMW) poly (I:C) (Invivogen lot # PIW-41-05). Poly(I:C) was prepared by adding endotoxin-free physiological water to the vial of LMW poly(I:C) and the solution was pipetted up and down until solubilization was complete, as per the manufacturer's instructions. This was then diluted with endotoxin free 0.9% saline to three different concentrations so when injected at 5µl per gram of animal body weight delivered the doses, 5, 10 or 20 mg/kg. Aliquots of each concentration were stored at -20°C and the same batch of poly(I:C) was used for all experiments. At E12.5 pregnant mice were weighed to allow calculation of injection volume and abdominal surface temperature was recorded with an infra-red thermometer (Fisher-Scientific). Injections took place between 11:30 and 13:30, dams were removed from their cage via a plastic tube and placed on the top of an empty cage. The animals were then restrained via scruffing and injected at approximately a 20° angle relative to the animal, into the abdomen, left of the midline using a 29G needle. A new needle was used for each injection. Following injections dams were returned to their home cages.

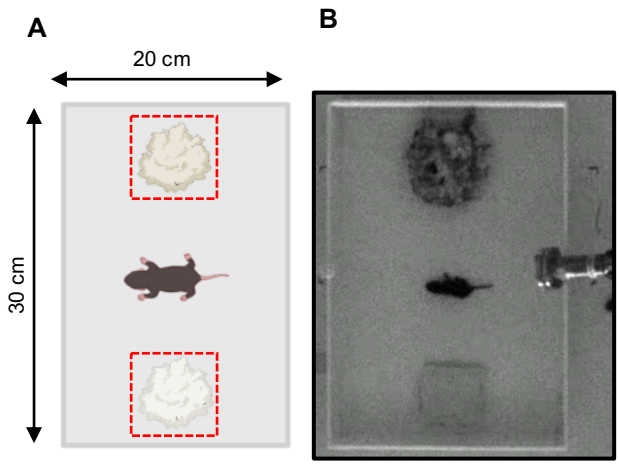


Figure 2.3 Homing behaviour test. **A**. Schematic showing test arena dimensions. Dashed red lines demark zone of interests for tracking software. **B**. Photograph showing test setup.

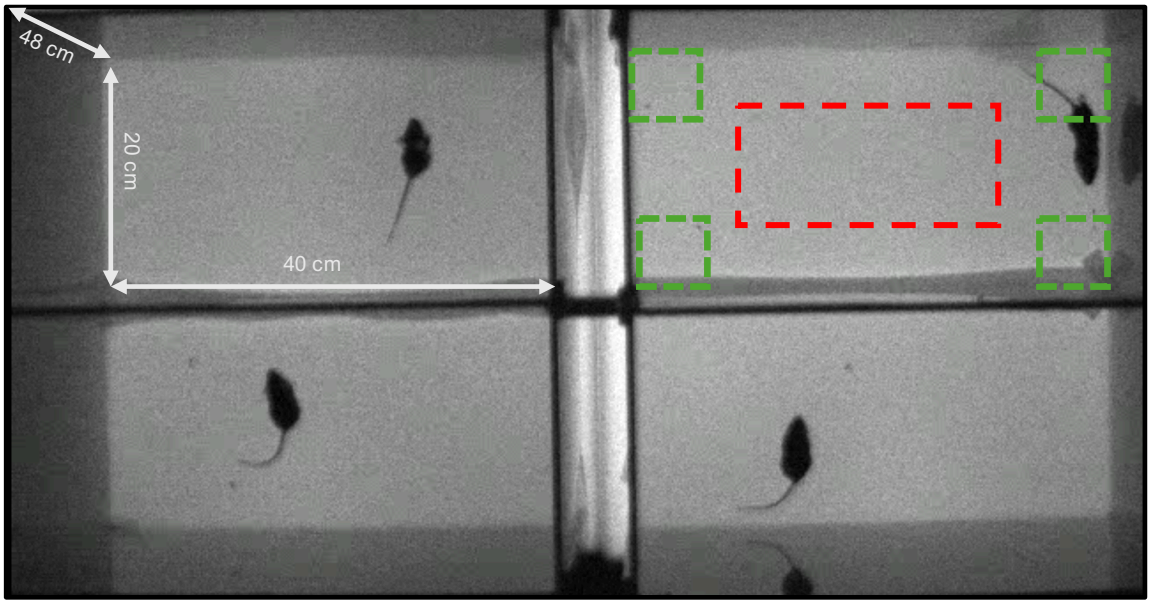


Figure 2.4 Open field Arena. Top left quadrant features dimensions of each arena. Top right quadrant shows zones of interest. Red dashed line marks the centre zone, and green dashed lines mark out the corner regions.

2.3.3 Dose response

As stated, 3 doses of poly(I:C) were used 5, 10 and 20 mg/kg. These were selected to identify the lowest dose that could activate the immune system of dams without producing a phenotype in offspring. This dose was used in the gene environment model of Chapter 4. 20 mg/kg was chosen as the upper limit as it is the most widely used dosage in in the literature when using MIA to model autism relevant phenotypes. The number of adverse outcomes for dams exposed to this dosage was too high, with deaths and abortions exceeding the number of litters produced (Table. 2.2) thus only offspring data from dams exposed to 5 and 10 mg/kg are presented.

Table 2.2 Outcomes from poly(I:C) administration to dams at E12.5.

Dosage (mg/kg)	No. of injections	Litters	Litters eaten	Pregnancy aborted	Animal died or culled
0	9	8	1	0	0
5	11	7	3	1	0
10	11	8	2	1	0
20	9	2	1	3	3
Stimulus	NA	5	2	0	0

NB. Stimulus litters were born to non-injected dams for use as stimuli in social tests and breeding occurred at the same time thus are included for comparison.

2.3.4 Validation of immune activation

Poly(I:C) is a synthetic analog of double-stranded RNA (dsRNA) toll-like receptor 3 (TLR3) agonist and is used as a viral mimetic (Alexopoulou et al. 2001). Administration of poly(I:C) produces increases in pro-inflammatory cytokines such as interleukin (IL)-6 and tumour necrosis factor (TNF)-α, as well as changes in weight, temperature and behavioural changes e.g. lethargy (Gandhi et al. 2007; Mueller et al. 2019; Estes et al. 2020). To validate immune activation, weight, surface temperature and sickness behaviours were recorded post-injection. Weight loss is a

marker of illness so pregnant dams were weight 3hrs and 24hrs post-injection. Surface temperature whilst lower than core body temperature, does track core temperature changes (Kawakami et al. 2018; Mei et al. 2018) thus using an infrared thermometer (Fisher-Scientific) each dam had abdominal surface temperature recorded 3hrs post-injection. Sickness behaviours were scored as per Gandhi et al. (2007) 3hrs and 24hrs post-injection. This involved inspecting the animals for reduced levels of activity, curled posture, lethargy, piloerection, drooping eyelids and general unresponsiveness. The presence of these symptoms contributed to a 4-point scale of scoring. 0 = no symptoms and 3 = three or more symptoms. Weight, surface temperature and sickness behaviour measures allowed for dutiful observation of any adverse outcomes for the animals. Measurements were taken in the holding room to minimise disruption to the dams. The full experimental timeline for dams, from breeding to birth is summarised in Figure. 2.1.

2.4 Behavioural assays

All behavioural tests were undertaken during the light phase and animals were habituated to the testing room for a minimum of 30 minutes prior to testing. Animals were transported to the testing room in the homecage placed on a trolley. Experiments were designed to include males and females in testing.

2.4.1 Offspring testing

Offspring born to MIA and control dams underwent behavioural testing from P9-P28 (Figure 2.2).

2.4.1.1 P9 homing test

All testing took place between 11:00 and 14:00. Testing took place at P9 as homing behaviour has typically developed in mice by P6 (Honeycutt and Alberts 2005), whilst isolation induced USV calling rates have declined by P9 in C57BL/6 mice (Yin et al. 2016; Caruso et al. 2022). Furthermore, the pup's eyes are yet to open at this stage, limiting the influence of visual input on behaviour. This timepoint is also beneficial given the hyporesponsive response to stress of rodents in their first two weeks of development (Schmidt et al. 2005), meaning a potential reduced impact of testing compared to later timepoints in development.

Adapted from (Muroyama et al. 2016), the homing test assesses a pup's social recognition via olfaction whilst indirectly providing a measure of maternal attachment (Moles et al. 2004). The stimulus used in this adapted version of the homing test was nesting material from the home cage, rather than woodchip bedding used by Muroyama and colleagues. The arena used was an acrylic container (30 cm x 20 cm x 10 cm) with a silicone mat placed at the bottom to allow the pups to get traction as they navigated the arena. A 4cm petri dish was placed either end of the arena, at the midpoint between sidewalls (Figure. 2.3). One dish would contain a clean nestlet and the other would contain nesting material from the home cage. The home cage was not cleaned from E17.5 to P10 to allow accumulation of socially salient odours.

Each pup was placed in the centre of the arena with the head orientated towards one of the sidewalls. The location of the home nesting material remained the same for the whole litter, but the starting orientation of each pup alternated with each trial. The placement of the nesting material would alternate from litter to litter. Once placed in the arena the pup had 3 minutes to freely explore the chamber. Trials were recorded with a camera mounted above the arena and movement of the pup tracked with EthoVision (Noldus). Zones of interest were demarcated virtually using EthoVision, they consisted of the home nest, and the clean nest. These zones encompassed the petri dishes (Figure. 2.3). The variables measured by Ethovsion were distance travelled, velocity, latency to find the home nest, latency to find the clean nest and time spent in either zone. During the test USVs were recorded as outlined in Section 2.4.1.1.1. Each pup was tested once, and following completion of the test the pup was removed to a heated cage until the whole litter had been tested. The heated cage and homing test were both in separate rooms from the home cage. Between tests the silicone mat was cleaned with 70% ethanol and dried.

2.4.1.1.1 Ultrasonic vocalisation (USV)

During the homing test an ultrasonic microphone (UltraSoundGate CM16, Avisoft Bioacoustics) was positioned centrally to the side of the test arena (Figure 2.3). The microphone was connected to an UltraSoundGate 416H preamplifier and for the 3-minute duration of the homing test, vocalisations were recorded. Frequency (count) and mean duration (ms) were the two parameters used for analysis.

2.4.1.1.2 Pup retrieval

Once an entire litter had completed the homing test, each animal was returned to the home cage. With the dam in the nest each pup was placed at the opposite end from the nest and the time taken (seconds) for the dam to retrieve the pup was recorded. This was not meant to replicate commonly used methods of assessing pup retrieval (Weber and Olsson 2008) but rather offer a rapid, ethnological snapshot of maternal care following a period of separation (up to 30 minutes).

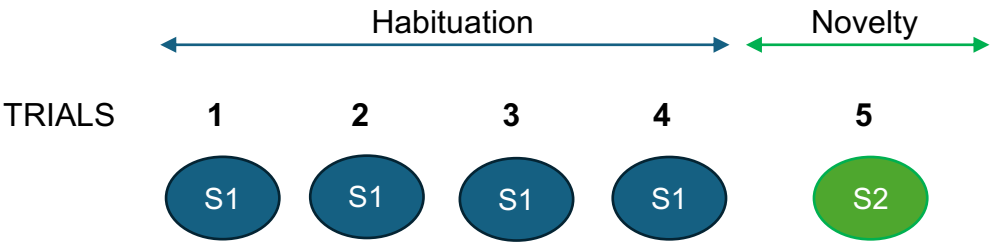
2.4.1.2 P25 Open field

Mice had their spontaneous activity recorded on two consecutive days (P24 and 25) at approximately the same time. The mice freely explored a 40 x 20 x 48 cm arena (Figure. 2.4) for 20 minutes in the dark. Infrared illumination allowed tracking of the mice with an overhead camera, and EthoVision XT (Noldus) recorded and quantified the locomotion of the mice. EthoVision was also used to define the centre of the arena (an area ¼ the overall area of the arena floor) and the corners (an area 5 x 5 cm in each corner, Figure. 2.4).

2.4.1.3 P26/27 Social and Object Novelty

Individuals with autism are often reported as having poor social recognition (Weigelt et al. 2012). To examine social recognition in juvenile mice this work adapted the protocol utilised by Hörnberg et al. (2020), (Figure 2.5). At P26 & P27 male and female mice had their social and object recognition tested with a counterbalanced design so half the mice had social recognition tested first with the other half having object recognition tested which was reversed on the 2nd day. Tests took place in the same arena as the open field test in the absence of any nesting material. Despite an existing familiarity with the arena test mice were habituated to the arena for 20 minutes on the days of testing. The 1st trial began with the introduction of a novel sex and age matched C57BL/6J stimulus mouse or a novel object (model tapir, Amazon, UK). For 2 minutes the mice were left to freely interact which was recorded by an overhead camera and EthoVision XT (Noldus). A 5-minute inter-trial interval followed before being repeated for 4 consecutive trials, facilitating habituation to stimulus mouse or object. For the 5th trial a novel stimulus mouse (littermate to 1st stimulus mouse) or a novel object (model echidna, Amazon, UK)

A



B

Figure 2.5 Social/object novelty recognition test. **A**. The 5-trial habitation - novelty recognition task used for social and object stimuli. S1 – first stimulus 1 S2 – second stimulus. **B**. The toy tapir and echidna used as object stimuli

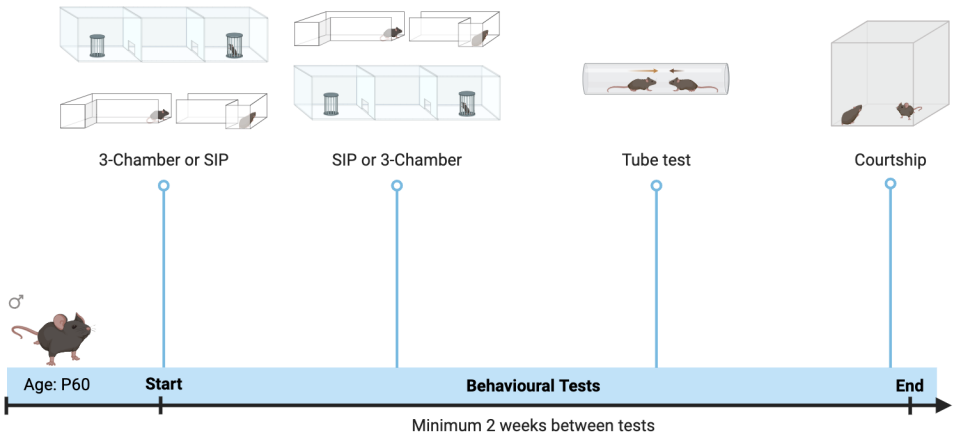


Figure 2.6 Experimental timeline for male social behaviour testing. Illustration of timing of social behavioural tests for adult male mice.

was used. Object stimuli were approximately 4 cm in length, weighed approximately 10 g and were placed in the centre of the arena at the start of the trial. Interaction time (s) with the social stimulus was only scored when the nose of the experimental animal was directed toward the stimulus mouse and within 2 cm. Object interaction was scored when the animal's nose was orientated toward the object and within 2 cm. A recognition index was calculated by subtracting the interaction time of 4th trial from the 5th.

2.4.1.4 P28 Direct Social Interaction

This test was designed to measure social interaction of juvenile mice when introduced to a familiar setting in the presence of an unfamiliar mouse. At P28, mice were tested in the same arena they completed the open field test, also in darkness. Stimulus mice for this test were adult female mice (P90+), habituated to the testing arena for 20 minutes the day before first testing. During testing, stimulus mice were placed in the arena 5 minutes before test mice were added for 3 minutes, and behaviour was recorded by an overhead IR camera using EthoVision XT (Noldus). Behaviour was scored manually using event logging software BORIS(Friard and Gamba 2016). Interaction was defined as the mice being within 2 cm of each other, excluding stimulus directed interactions and tail-to-tail interactions. Stimulus directed interactions were defined as interactions initiated by the stimulus animal to which the test animal didn't respond and include, the stimulus animal approaching the test animal and the test animal moving away but the stimulus animal follows. Another example of stimulus directed behaviour is the stimulus animal approaching and sniffing the test animal whilst it is grooming but the test animal does not respond i.e. test animal carries on grooming. Interaction time (s) and stimulus directed interaction time (s) were analysed separately.

2.4.2 Social behaviour testing of adult male mice

Social behaviour was tested in adult male (P90+) C57BL/6J mice as outlined in Figure 2.6.

2.4.2.1 Social Interaction Platform (SIP) test

All tests of sociability in mice have limitations (Jabarin et al. 2022) where direct interaction between unfamiliar adult male mice typically results in fighting (Kondrakiewicz et al. 2019). To avoid potential harm caused by fighting and allow assessment of more direct social interaction between male mice the social interaction platform was created. Consisting of two L-shaped chambers, separated by a 7 cm gap (Figure 2.7a), elevated around 80 cm form the floor. Mice could come together and interact via whisking in a region designated the interaction zone (Figure 2.6b) but also opt out and move to the rescue zone (Figure 2.7b). Mice were tested in 5 trials over consecutive days. In the first 2 trials mice were habituated to the chamber in the absence of another mouse. In the 3rd trial, mice were tested with their cagemate as the stimulus mouse. In the 4th and 5th trials mice were tested against unfamiliar mice both from the same cage, resulting in all mice from two cages being tested against one another. Trials 1 and 2 were 5 minutes long and trials 3,4 and 5 were 10 minutes long. All trials were recorded with an overhead camera and EthoVision XT (Noldus). The tracking software calculated and recorded the distance travelled (cm), time spent in the interaction zone (s) and time spent in the rescue zone (s), as well as the number of transitions between the zones. In trials 3, 4 and 5 the distance (cm) between mice was also recorded, using centre of mass as the reference point.

2.4.2.2 Three-chamber test

To aid in assessing the validity of the SIP test as a measure of sociability in adult mice the three-chamber test was also carried out to allow comparison of the outcomes of both tests. The three-chamber test is widely used to measure sociability in mice (Moy et al. 2004). The arena (40 x 60 x 22 cm, Figure 2.7~~a~~) was constructed with opaque Perspex barring the front panel which was transparent. The arena was divided into 3 equally sized chambers by 2 removable walls each 20 cm wide. Each wall featured a central door 5 cm x 7.5 cm that was removed during trials to allow the mice to move freely between chambers. The barred containers that held stimulus mice during testing were 15cm tall with a 9cm diameter. Bars were equally spaced with gaps wide enough to allow transmission of salient cues of all modalities

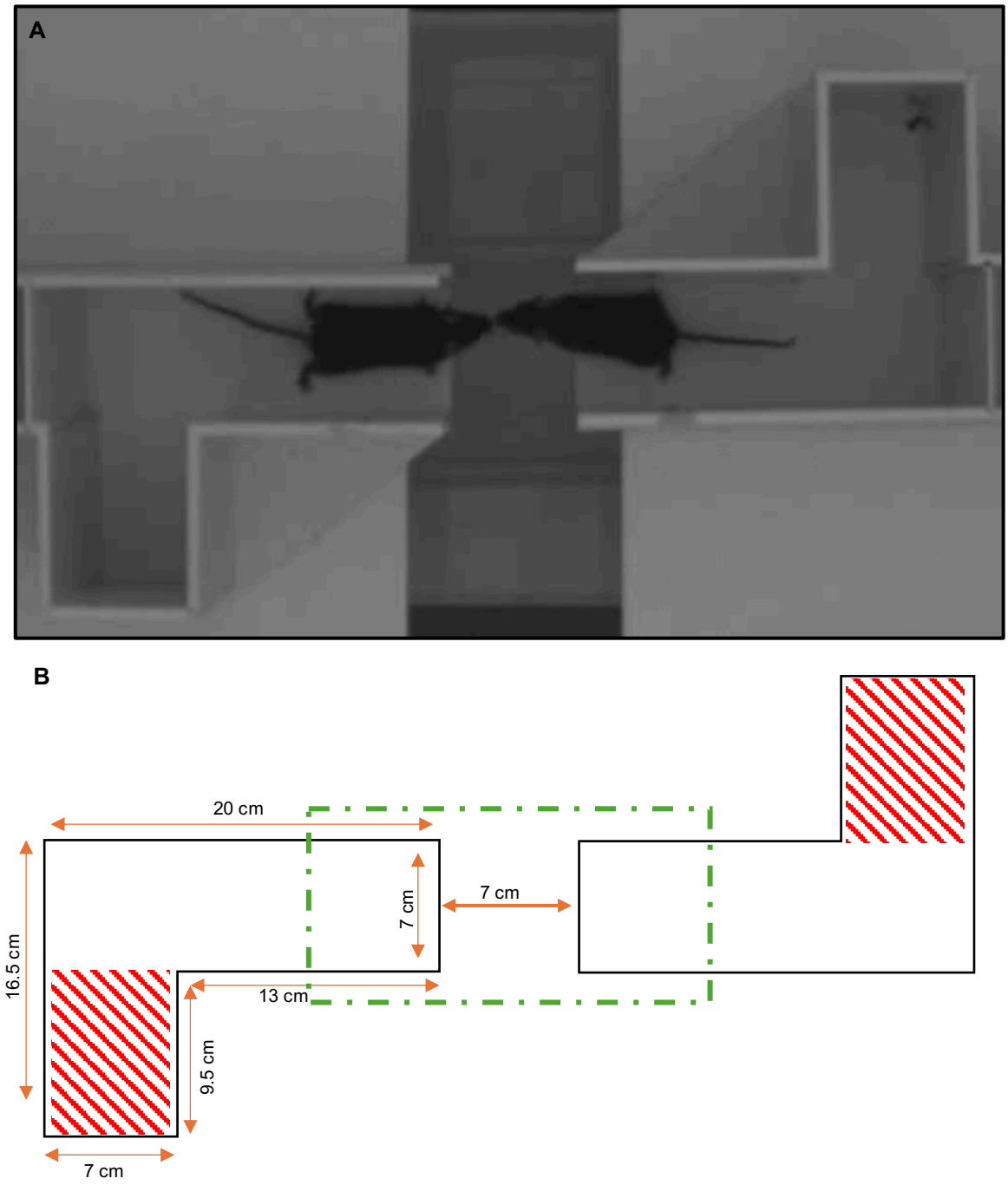


Figure 2.7 Social interaction platform (SIP) test. **A.** Photograph illustrating mice interacting across the gap between 2 chambers. **B.** Schematic of the chamber with dimensions and zones of interest. Green dashed line represents the 'social' zone and the red diagonal lines demarcate the 'rescue' zones.

including tactility but close enough to prevent direct interaction. Stimulus animals were habituated to the holding containers over 3 sessions prior to testing. By the first test, all stimulus animals would enter a container upon presentation without any physical intervention. The clear top of the containers allowed constant observation of stimulus animals, throughout testing to monitor for signs of distress.
Experimental animals underwent 3 trials each 10 minutes long. A 10-minute habituation period preceded the 1st trial, with the mouse confined to the central chamber. In the 1st trial the doors were removed, and the mouse could freely explore all three chambers, in the absence of both holding containers. At the end of the 1st trial when the mouse returned to the central chamber the doors were closed and a holding container with a stimulus mouse was added to either the left or the right chamber and the empty holding containing added to the opposite side. The doors of the central chamber were removed, and the 2nd trial commenced. This was repeated for the 3rd trial, but the empty holding container featured a novel stimulus mouse. All trials were filmed by an overhead camera and EthoVision XT (Noldus) which calculated time spent in each zone (s), distance travelled (cm) and the number of transitions between zones. Interaction time (s), when the mouse was directing attention toward the containers that held stimulus mice was scored by the experimenter using BORIS software (Friard and Gamba 2016).

2.4.2.3 Social Interaction – Courtship

Direct social interaction by adult male mice (P90+) was tested via exposure to an adult female mouse (P90+). The only parameter of interest was interaction time (s). Oestrus was not assessed in females because social interest of males in females has been shown to be unaffected by the oestrus stage (Hanson and Hurley 2012). Males were added to the arena (the open field arena described in 2.4.1.2) for 5 minutes to allow for habituation. Then a female was presented, and the mice were allowed to interact freely for 3 minutes. Male cagemates were tested against the same female. Multiple females were used as stimulus and the ID of the stimulus was used as a random effect in analysis. Trials were recorded using an overhead camera and EthoVision XT (Noldus). Social interaction time (s) was scored using BORIS (Friard and Gamba 2016), when the mice were within 2cm of one another, discounting tail-to-tail

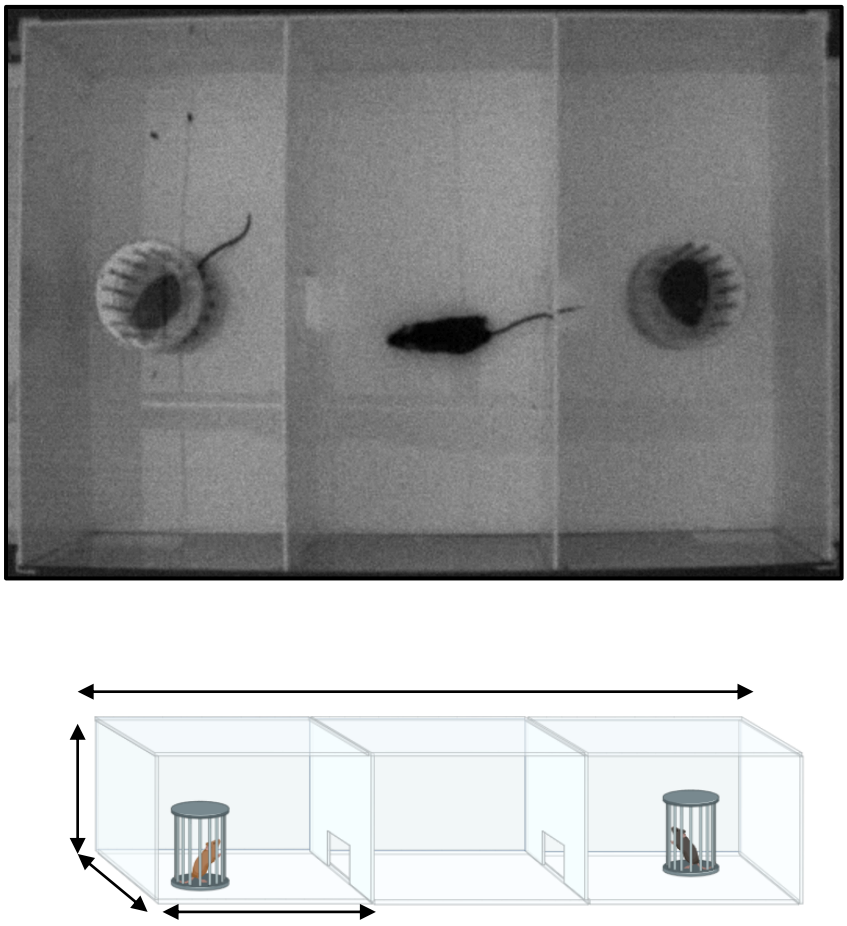


Figure 2.8 Three-Chamber sociability test. **A.** Photograph illustrating the 3-chamber test. **B.** Schematic of the arena with dimensions. Created in BioRender.com

interactions. The total time (s) spent in interaction was used in analysis. Total stimulus directed interaction time (s) was also scored and analysed.

2.4.2.4 The tube test

Mice are hierarchical animals and to assess social dominance between familiar and unfamiliar mice the tube test was utilised(Lindzey et al. 1966; Wang et al. 2011). The apparatus was constructed with a clear polycarbonate tube (30 cm long with an internal diameter of 3.5 cm) that connected to a holding chamber (15 cm x 10 cm) with a hinged lid at each end (Figure 2.9a). These were holding chambers for test mice with the entrance to the tube blocked by an acrylic barrier that could be removed readily. The tube also featured an acrylic gate at the halfway point that was removed manually during the test. Prior to testing mice were acclimatised to the tube itself and trained to the point they traversed the tube without stopping or backing out. This began with placing a 30cm tube (identical to the one used in the test) in an empty arena with each mouse, allowing free exploration. After 2 minutes, the mouse was prompted by the experimenter's hand to enter the tube was handled in the tube and released back into the homecage, this was repeated several times. The following day each mouse was exposed to the full test apparatus. To start, the mouse would be held in the holding chamber for 30 seconds before the door to the entrance of the tube was removed. Once the mouse entered the tube the centre gate was removed and the mouse allowed to freely traverse the tube back and forth between holding chambers, for 3 minutes. Exposure to the test setup was repeated once more a week later. For the test, mice would be collected from the homecage using the holding chamber and connected to the tube. Once both chambers were in position, the barriers to the tube were removed and the mice entered the tube, meeting at the gate in the middle. This gate was then removed, and one mouse would push their opponent out of the tube. The mouse that got pushed out was ranked as the submissive mouse in that trial. Adult male mice (P90+) were housed in pairs and tested against their cagemates in a best of 3 fashion to establish a dominant mouse, repeated over 3 days. Each pair of mice was matched to a pair of unfamiliar mice and dominance was assessed for each of the 3 matches (Figure 2.9b). Other than the winner of each match no other measures we recorded.

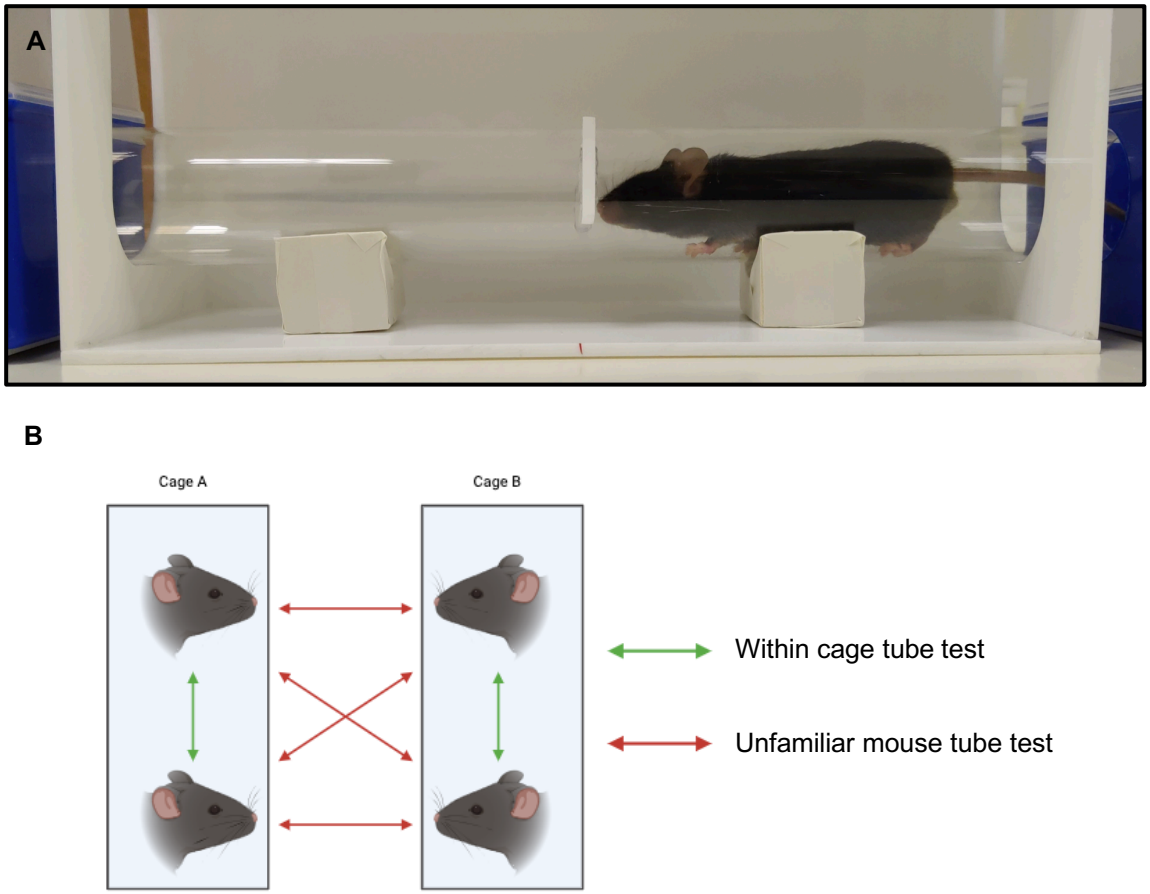


Figure 2.9 Tube test. **A.** Photograph illustrating the tube test setup. The blue containers at the edge of the frame are the holding chambers. **B.** Schematic outlining how matches were organised within cage and between unfamiliar mice to establish hierarchy.

2.5 Statistical methods

Data analysis and plots were completed using R software, version 4.3.2 (R core team 2012). To identify outliers, plots of raw data were examined and Rosner's test was utilised for confirmation. Not all confirmed outliers, as identified by a significant result in the Rosner's test were removed, Table 2.3 summarises the outliers identified and whether these were excluded from analysis. No outliers were observed in the 2nd MIA offspring cohort of Chapter 3 nor in any of data from Chapter 5.

2.5.1 Data analysis

Experiments were designed considering individual animals as the n, with all animals in a litter being tested. To account for dependency between observations, data was analysed using mixed models, with litter included as a random effect. Mixed models were analysed using the R package **lme4** (Bates et al. 2015) with p-values provided in type III ANOVA tables via the R package **lmerTest** (Kuznetsova et al. 2017). Fitted models were assessed using the **DHARMa** package (Florian Hartig 2020) which produces scaled residuals by simulating new response data from the fitted model for every observation. From here empirical density functions are calculated for the simulated observations and residuals are defined as the empirical density function at the value of the observed data. Values range from 0-1 where 0 means all simulated values are larger than observed. DHARMa outputs Q-Q and residual plots and it is the visual inspection of these plots that was used to determine if a model was acceptable. For models that returned a p-value smaller than 0.05, post-hoc tests were utilised via the **emmeans** R package (Lenth 2024) to highlight specific group differences and Tukey's Honestly Significant Difference (HSD) was used to correct for multiple comparisons. For repeated measure designs, a similar approach was taken but individual animal used as the random effect. Raw data was assessed for normality via inspection of Q-Q plot and the Shapiro-Wilk test, similarly homogeneity of variance was assessed with the Levene's test. If either of these assumptions were violated a non-parametric test was used. For mixed models, these assumptions were tested based on the DHARMa simulations and where non-parametric mixed models were deemed most appropriate, analysis was carried out using **nparLD** (Noguchi et al. 2012). In some instances, experiments were also analysed

considering the litter to be the n i.e. litter averages. Where this was done it is indicated in the text. Statistically significant results are presented in the text along with the model used. For each model the intraclass correlation (ICC) is reported as an illustration of the clustering with the random effect (Golub and Sobin 2020). Where more than one random effect was included in a model, the ICC for each effect is presented. Also reported is the marginal R^2 and conditional R^2 to allow for comparison of the variance of fixed effects and the total model.

2.5.2 Data visualisation

Raw data for all experiments are presented in the form of estimation plots, which comprise of two distinct but aligned plots. A swarm plot, that orders each data point to show the underlying distribution as well as means and error bars. The second plot displays an effect size as a bootstrapped 95% confidence interval. Estimation plots are produced with the R package **Dabestr** (Ho et al. 2019). To visualise potential interactions, plots were generated using the **emmeans** (Lenth 2024) and **ggeffects** (Lüdecke 2018) R packages.

Table 2.3 Outliers identified by Rosner's test and decisions to include or exclude.

Cohort	Test	Variable	Outliers identified	Exclusions	Reason for including/excluding
MIA Cohort 1	P9 Homing	no. of calls	3	0	Included as it is possible to make the number of USV recorded
		mean duration	1	0	Included as it is possible to have the mean duration recorded
	P25 Activity	distance	5	5	Excluded due to error in experimental set-up
		centre	10	10	Excluded due to error in tracking - not possible to have times
	P28 Direct Social Interaction	stimulus directed interaction time	1	0	Included as it was a genuine measurement
G x E	P9 Homing	weight	2	0	Included as low weight did not compromise performance in testing
		distance	4	4	Excluded due to tracking issues once animal was in the nest and not visible
		mean duration	1	0	Included as it is possible to have the mean duration recorded
		retrieval time	6	2	4 included as genuine time. 2 Excluded - another researcher entered room during test
	P25 Activity	distance	1	0	Included as it is a genuine, possible distance covered.

Chapter 3 : Optimisation of a poly(I:C)-induced maternal immune activation protocol

3.1 Introduction

Epidemiological investigations have associated maternal infection and systemic inflammation with risk of ASD (Abdallah et al. 2012; Brown et al. 2014; Jiang et al. 2016; Jones et al. 2017). The causal mechanisms behind this association remain elusive but pre-clinical models offer support for this association. For example, MIA via pre-natal administration of the viral mimetic poly(I:C) produces ASD relevant phenotypes in the offspring of exposed dams (Meyer et al. 2009; Malkova et al. 2012; Choi et al. 2016; Zhao et al. 2021; Tartaglione et al. 2022). However, despite extensive use of MIA models, a range of factors impinge on the reproducibility of findings thus optimizing any MIA protocol requires a range of considerations (Kentner et al. 2019). This is especially true when investigating the potential interactive effects of MIA with other insults such as gene x environment interaction mouse models. Some of the key considerations are described below, outlining the choices made in adapting an MIA protocol for use in this work.

3.1.1 Immune activation

A common approach to MIA is to use a mid-gestational injection of polyinosinic: polycytidylic acid (poly(I:C)), a synthetic double-stranded RNA analog that mimics viral infection, primarily via TLR-3 activation (Zhou et al. 2013). Poly(I:C) was initially, demonstrated to cause increased levels of interleukin-6 (IL-6) in maternal serum, mirroring spikes caused by influenza, and highlighted as causal in the manifestation of disease-relevant phenotypes in MIA offspring (Meyer et al. 2006; Smith et al. 2007). However, Kentner et al. (2019) drew attention to widely varied reporting in IL-6 levels in response to poly(I:C) even when dosage, administration route and source of poly(I:C) were the same. Inconsistencies in immunogenicity of poly(I:C) have been attributed to several factors such as molecular weight and endotoxin contamination. In cellular models, poly(I:C) with higher molecular weights was demonstrated to illicit a greater immune response (Mian et al. 2013; Zhou et al.

2013). Mueller et al. (2019) highlighted the effects of poly(I:C) variability on immune response in pregnant mice. Different batches from the same vendor were found to contain wide ranging sizes of poly(I:C) fragments which in turn produced variable maternal responses from IL-6 levels to thermal response, and spontaneous abortion rates with higher weights associated with a greater immune response. This was reinforced when LMW poly(I:C) was compared to HMW poly(I:C) which demonstrated a greater immune response compared to LMW poly(I:C). Endotoxin contamination was not found to contribute to the variability in poly(I:C) response unlike Kowash et al. (2019) who reported an interaction between molecular weight of poly(I:C) and the level of endotoxin contamination in producing an immune response. This was primarily attributed to the variability of poly(I:C) from Sigma. Invivogen's LMW poly(I:C) was found to be less variable in the distribution of poly(I:C) molecular weight, have lower endotoxin contamination whilst not impacting litter size (Kowash et al. 2019). Careaga et al. (2018) also found greater immune activation in response to HMW poly(I:C) compared to LMW poly(I:C), supporting previous findings highlighting the relationship between molecular weight of poly(I:C) and immune response. In consideration of the variability of molecular weight ranges of poly(I:C) and the influence this has on immune response the work in this chapter utilised Invivogen's low molecular weight (LMW) poly(I:C).

The timing of poly(I:C) administration influences outcome, both in terms of maternal immune response and offspring phenotypes (Meyer et al. 2006) as well as the rate of spontaneous abortion following MIA (Mueller et al 2019). In the context of modelling ASD, the timepoint of MIA challenge that is most common is E12.5. This reflects the association between ASD and maternal viral infection being strongest during mid-pregnancy. Another consideration is the route of administration, i.v injection is a greater technical challenge, particularly in mice and the use of anaesthesia and/ or restraint may introduce confounds, that are more impactful when looking to establish sub-threshold doses. I.P injection is less technically challenging, takes less time but will have lower bioavailability than I.V administration. Dosage of poly(I:C) will also impact maternal and offspring outcomes and when modelling ASD relevant phenotypes, the most widely used dosage when administration is via IP injection, is 20mg/kg. To allow comparison with other work and avoid potential additional stressors involved with i.v. injections, this work used an injection timepoint

of E12.5, delivered via i.p. injection and the dosage of 20mg/kg was selected as the highest dose in a dose response curve. A dose response curve facilitated identification of a sub-threshold dose, in terms of offspring outcomes, with a view to use in gene x environment models (Kentner et al. 2019).

3.1.2 Immune activation validation

Administration of immunogens such as poly(I:C) stimulate the production of a number of proinflammatory cytokines (Gandhi et al. 2007), with the primary biomarker of immune activation being Il-6 (Smith et al. 2007). Validation of immune activation via cytokine measurements it typical achieved via two approaches. One is microsampling blood from all animals within an experiment, though this introduces the potential confound of handling stress. The second approach is to have a separate cohort of satellite animals which are used for biological sampling thus avoiding additional handling of the main experimental cohort. However, beyond the increased costs in terms of animals and time this approach may miss out on individual differences. Estes et al. (2020) demonstrated the baseline immunoreactivity (BIR) of mice to poly(I:C) was variable and could be stratified into three groups, low- medium- and high- responders. The level of response predicted behavioural outcomes in offspring with medium responders being most likely to produce offspring with a repetitive behavioural phenotype, when treated with an intermediate dose. This work points to establishing the BIR of female mice prior to breeding and may be a better alternative to use of satellite cohorts for immune activation validation as without establishing a BIR, unequal distribution of low- medium- and high- responders between cohorts makes inferences from satellite groups challenging.

Instead of analysing cytokines as a measure of immune response it is possible to validate immune activation via measurement of the behavioural, weight and thermal changes induced by cytokines (Gandhi et al. 2007). These responses have been demonstrated to be dose-specific for poly(I:C) (Estes et al. 2020), though temperature response may not be sensitive to molecular weight of poly(I:C) given LMW and HMW were shown to produce similar maternal temperature changes (Mueller et al. 2019). Surface temperature tracks changes in core temperature (Kawakami et al. 2018; Mei et al. 2018), and Mueller et al. (2019) demonstrated a

strong correlation between rectal and surface temperatures in pregnant mice, highlighting surface temperate as a potential non-invasive marker of immune activation. The work described here took a non-invasive approach to immune activation validation and measured abdominal surface temperature prior to injection and 3-hours post-injection, a time that has been shown to be the peak hypothermic response following i.v. injection (Mueller et al. 2019). Weight change was also recorded 3hrs and 24hrs post-injection and sickness behaviours scored as per Section 2.3.4.

3.1.3 Animals and environment

Mice are a commonly used organism in MIA models but also contribute to the variability in outcomes reported in these models. The same MIA protocol can yield differences in offspring phenotype for different strains of mice. Schwartzer et al. (2013) using BTBR mice, an inbred strain used as an ASD model, demonstrated more pronounced social deficits when offspring were born to dams exposed to poly(I:C). Comparison of C57BL6 mice and the NIH Swiss outbred strain found similar effects of poly(I:C) on social behaviour but NIH Swiss animals demonstrated anxiety and depression-like phenotypes (Morais et al. 2018). Most MIA mouse models use C57BL6 mice but even within this single strain, sources of variability exist. Kim et al. (2017) highlighted an absence of ASD-related phenotypes in MIA offspring of C57BL6 mice from Jackson Laboratories (Jax), in contrast to MIA offspring of C57BL6 mice from Taconic Biosciences (Tac). The source of this variation was attributed to maternal gut microbiome, specifically the presence of segmented filamentous bacteria (SFB) in Tac mice which facilitates increases in plasma interleukin-17a (Il-17a) in response to poly(I:C) challenge. Co-housing Jax mice with Tac mice or treating Jax dams with faecal slurry from SFB mono-colonised mice leads to IL-17a increase in response to poly(I:C) as well as behavioural and cortical abnormities in offspring mice. Estes et al. (2020) reproduced the finding Jax mice lack SFB but built on the work of Kim et al. by comparing Tac mice with Charles River (CR) C57BL6 mice. CR mice demonstrated SFB levels comparable to Tac mice but didn't display increases in IL-17a following immune challenge. Furthermore, Tac mice did not display the weight loss and fetal loss seen in CR mice. The lack of

SFB in Jax mice was cited by Kim et al as the reason for the absence of MIA induced phenotypes, however, there have been reports of successful MIA models using Jax mice(Malkova et al. 2012; Zhao et al. 2021). SFB levels were not reported in these papers but the difference in findings may point to the interaction of MIA with environmental factors. With the above findings in mind the work reported here used C57BL6/J mice as the model organism. Mice with SFB might be more likely to produce MIA induced phenotypes, but the presence of SFB can be viewed as a separate, additional hit to MIA which may interact with genetic variants thus C57BL6/J are more desirable to combine MIA and genetic models.

MIA models can be influenced by a range of factors, such as caging system, which has been shown to interact with timing and intensity of MIA challenge (Mueller et al. 2018). Mice housed in individually ventilated cages (IVC) experienced more adverse outcomes in response to poly(I:C) exposure at gestational day (GD) 9 compared to mice housed in open cages (OC), with an absence of behavioural abnormalities in the offspring of dams exposed to a lower dose (1 mg/kg i.v.), housed in IVCs. These effects were not seen when poly(I:C) exposure occurred at GD12. This work highlights the influence of caging system as a potential confound and source of variability in MIA models. It also reinforces the need to consider unintentional “hits” when designing experiments utilizing MIA protocols. This is of relevance when the aim is to combine MIA with a genetic model. Logge et al. (2014) were unable to demonstrate aberrant behavioural phenotypes in Neuregulin 1 (Nrg1) mutant mice raised in IVCs in contrast to Nrg1 mutants raised in filter-top cages (FTC). With a view to combining MIA with genetic models this worked used OC housed mice as this facilitated comparison with previous work of genetic models where they were raised in OCs.

Another unintentional hit that needs consideration is maternal care behaviour, which MIA has been shown to impact on. Ronovsky et al. (2017) showed C57BL6 dams exposed to poly(I:C) at E12.5 displayed decreased levels of licking and grooming behaviour toward pups, spending more time nest-building. Berger et al. (2018) reproduced this finding using the same MIA protocol in a different mouse strain, C3H/HeNCrl (C3H). Altered maternal care, like MIA is an early life adverse event and may interact with MIA to exacerbate offspring outcomes or produce

phenotypes not purely attributable to MIA. Disentangling the contribution of MIA and maternal care behaviour in a MIA model is challenging but one method that is used to counter this source of variability is the use of cross-fostering programmes. However, this approach can introduce additional confounds and highlights the challenges in attempting to control for unintended hits (Kentner et al. 2019). In the context of this work, cross-fostering was not used, nor direct testing of maternal care behaviour because both could influence the outcome of the P9 homing test which is a measure of maternal attachment. Social separation is another stressor and can impact physiology and behaviour. Yet, many protocols single house females following timed mating, this could be another source of variation and act as a further hit. Timing of single housing pregnant rats was shown to influence behaviour (Soliani et al. 2017). Rats single housed in the 3 different weeks of gestation produced offspring with varied measures of anxiety-related behaviour with offspring of the dams separated in the 2nd week of gestation being most affected. To mitigate for any impact of social separation in this work, dams were singly house at E16.5 or 17.5 and placed next to their former cagemate to allow sharing of olfactory and auditory cues.

3.1.4 Outcome measures

Variability in outcome measures is also seen in the MIA literature but one of the most consistent findings in MIA mouse models that utilise poly(I:C) is the impairment of social behaviour in offspring (Kentner et al. 2019). MIA can be a valuable tool in modelling endophenotypes of social behaviour and studying behaviour early in development will help contextualise findings in relation to ASD in humans. Ideally any model will capture physiological changes that accompany endophenotypes but as a first step this work focussed on offspring behaviour, assessing social phenotypes early in development, with a homing test at P9, a social novelty test at P27 and a direct social interaction test at P28.

All offspring were tested thus the reported sample size for behavioural outcomes is the number of offspring tested, though the litter is included as a random effect, as outlined in Section 2.5.1. The rationale for using individual offspring as experimental units comes from findings that litters can contain MIA susceptible and MIA resilient animals, at varying proportions (Mueller et al. 2021; Herrero et al.

2023). This means sampling 1 or 2 animals per litter may skew findings by chance with either susceptible or resilient animals being overrepresented. Whereas litter averages could dilute any effects. Furthermore, social behaviour, as MIA can be influenced by several factors, discussed in Section 1.5.2 thus, it is logical to test all offspring.

3.2 Aims and Objectives

Based on the reporting guidelines of (Kentner et al. 2019), the work in this Chapter aimed to optimize a well-established MIA protocol to identify a dose that stimulated a dam's immune system without producing social behaviour deficits in the offspring prior to weaning. To achieve this aim, the following study will

1. Identify a sub-threshold dose of poly(I:C) via a dose-response experiment
2. Validate immune activation in pregnant dams using non-invasive methods
3. Characterise the early social behaviour of male and female mice.
4. Determine if multiple tests in early life impacts social behaviour of control and MIA offspring.

3.3 Methods

The work in this chapter utilised two separate cohorts of WT C57BL6/J mice. Dams underwent the MIA protocol outlined in Section 2.3 and their offspring were tested in assays described in Section 2.4. Delivery of mice from Charles River was staggered to facilitate testing of all offspring at the desired developmental timepoints. Breeding for the 1st cohort of MIA dams took place between November and January, with mice housed in a regulated holding room, temperature (21.5°C ± 1.5°C) and humidity (47% ± 10%). Pregnant dams were administered LMW poly(I:C) via i.p. at E12.5 with 1 of 3 doses (5,10 or 20 mg/kg) or saline (Fig 2.1). Breeding for the 2nd cohort of MIA dams took place during April and May, with mice housed in the same regulated holding room as the 1st cohort, temperature (21.25°C ± 0.75°C) and humidity (47% ± 9%). For both cohorts the number of sires used, and offspring produced, including the male: female ratio are summarised in Table 3.1. Not all

offspring of the 1st cohort took part in all the tests illustrated in Figure 2.2, with the numbers of animals used in each test reported in Table 3.2. For the 2nd cohort, offspring were only tested in the open field at P25, and Direct social interaction at P28, with all offspring tested.

Table 3.1 Numbers of offspring produced in two independent MIA cohorts.

	Group	No. of sires	No. of litters	Total at P0	Total at P7	Total males: females
MIA Cohort 1	Controls	5	8	54	47	20:27
	5 mg/kg	4	7	43	43	17:26
	10 mg/kg	6	8	52	50	33:17
	20 mg /kg	2	2	14	12	6:6
MIA Cohort 2	Controls	4	6	43	40	18:22
	10 mg/kg	6	6	38	34	18:16

Table 3.2 **Numbers of offspring using in behavioural testing.**

	Group	P9 homing	P25 Activity	P26-27 Social novelty	P28 Direct social interaction
MIA Cohort 1	Controls	37	45	26	45
	5 mg/kg	43	43	35	43
	10 mg/kg	50	50	30	50
MIA Cohort 2	Controls	NA	40	NA	40
	10 mg/kg	NA	32	NA	32

3.4 Results

3.4.1 Poly(I:C) triggers immune response in pregnant dams

Following administration of poly(I:C) the weight of control dams increased over 24 hours but the weight of dams exposed to poly(I:C) did not (Fig.3.1a), supported by a ***dose*** x ***time*** interaction (LMM($F_{[4,54]} = 6.8$, $P < 0.001$)) when analysed with the model ***weight*** ~ ***dose*** x ***time*** + ***(1 | dam)***. There were no main effects of ***dose*** (LMM ($F_{[2,27]} = 0.2$, $P = 0.8$)) or ***time*** (LMM ($F_{[2,54]} = 3.1$, $P = 0.05$)). Control dams showed an increase in weight, 24 hours post injection ($t_{[54]} = -3.4$, $p < 0.01$). Dams in the 10 mg/kg group lost weight 3 hours post injection ($t_{[54]} = 3.1$, $p < 0.01$) and remained lighter 24 hours post injection ($t_{[54]} = 3.9$, $p < 0.001$). Dams belonging to the 5 mg/kg group saw no weight change over the 24hrs following injection ($t_{[54]} = -0.6$, $p = 0.83$). No weight differences between the groups were observed at 3 hours or 24 hours following injection. This model returns an ICC = 0.86, a marginal $R^2 = 0.06$ and a conditional $R^2 = 0.87$. MIA dams in the 10 mg/kg group displayed a reduction in surface temperature 3 hours after injection ($t_{[27]} = 2.8$, $p < 0.01$) whereas control dams ($t_{[27]} = -1.2$, $p = 0.23$) and 5 mg/kg dams did not ($t_{[27]} = -0.12$, $p = 0.9$), (Fig.3.1b). The different trajectories of surface temperature between groups resulted in 10 mg/kg mice being colder than controls, 3 hours post-injection ($t_{[27]} = 3.7$, $p < 0.01$). There were no main effects of ***dose*** (LMM ($F_{[2,27]} = 3.1$, $P = 0.06$)) or ***time*** (LMM ($F_{[1,27]} = 0.5$, $P = 0.47$)) when analysed with the model ***temperature*** ~ ***dose*** x ***time*** + ***(1 | dam)***. This model returns an ICC = 0.15, a marginal $R^2 = 0.2$ and a conditional $R^2 = 0.32$. All MIA dams, 5 mg/kg and 10 mg/kg displayed some sickness behaviour, e.g. lethargy 3 hours after injection and no control dams showed sickness behaviour. No dams from either group displayed sickness behaviour 24hrs post injection (Fig.3.1c). The differential in weight gains and sickness behaviour between

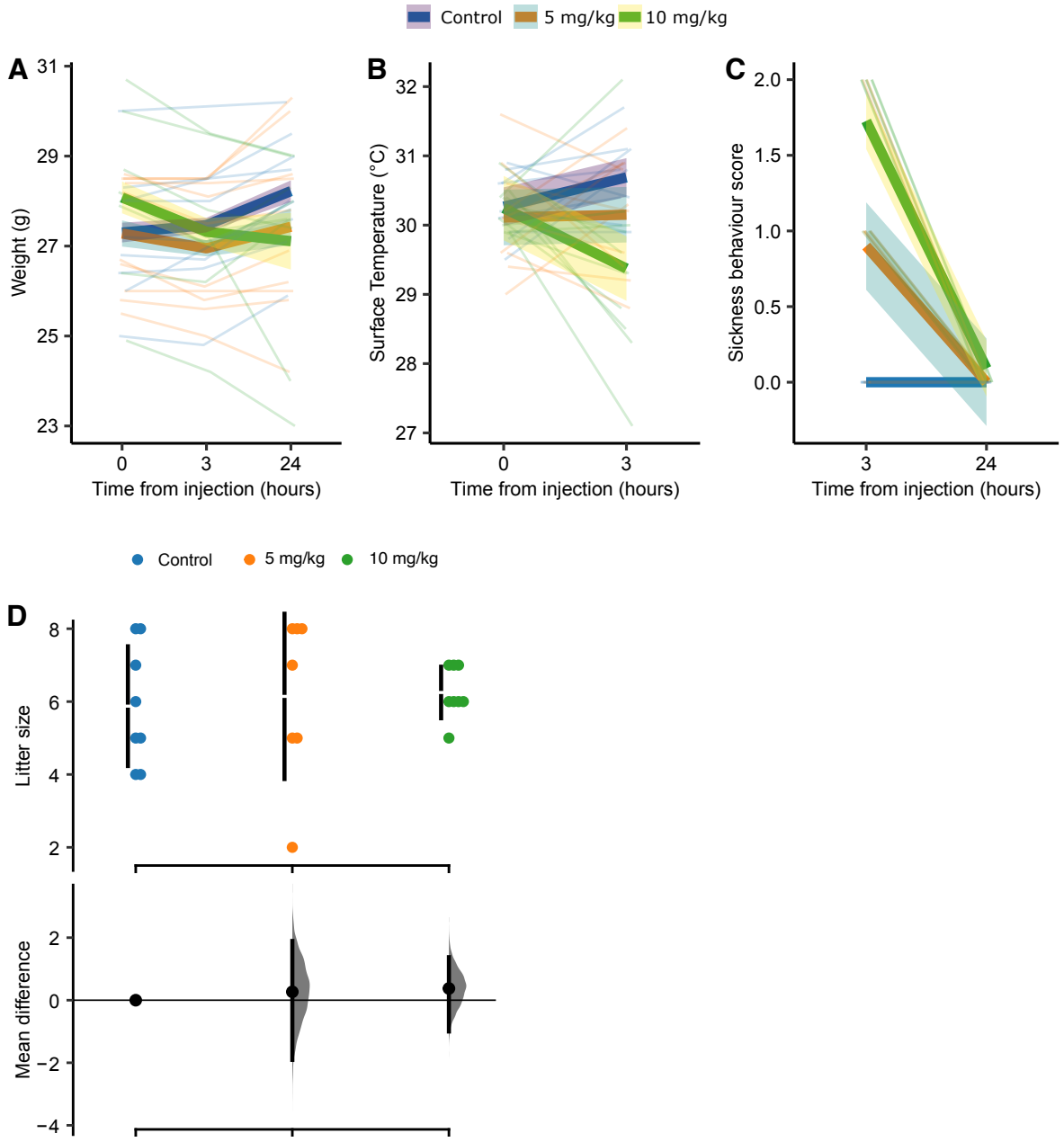


Figure 3.1 The response of dams to maternal immune activation (MIA) at 2 doses of poly(I:C). **A**. Weight change following MIA. **B**. Surface temperature change following MIA. **C**. Sickness behaviour score following MIA. **D**. Estimation plot showing litter sizes and the mean difference between control and MIA groups. A - C Ribbons represent 95% confidence intervals. A-C Controls *n* = 9, 5 mg/kg *n* =10 and 10 mg/kg *n* = 11. D controls *n*= 8, 5 mg/kg n = 7 and 10 mg/kg *n* = 8.

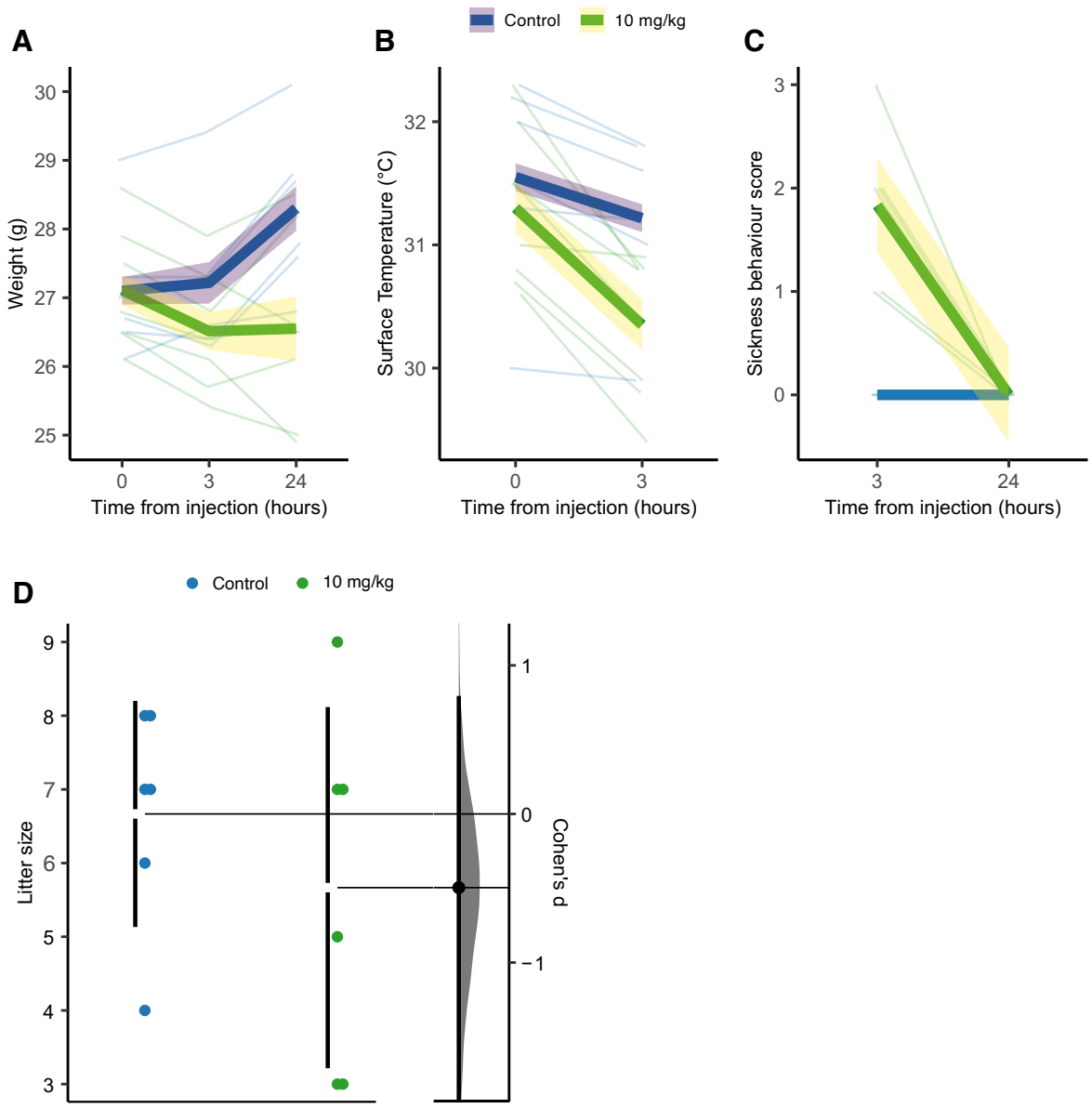


Figure 3.2 The response of dams to maternal immune activation (MIA) with 10 mg/kg of poly(I:C). **A**. Weight change following MIA. **B**. Surface temperature change following MIA. **C**. Sickness behaviour score following MIA. **D**. Estimation plot showing litter sizes and Cohen's d effect sizes for control and MIA groups. Ribbons in A, B, C represent 95% confidence intervals. A, B, C Controls n = 6 and 10 mg/kg n = 9. D controls n= 6 10 mg/kg n = 6.

groups did not lead to significant differences in litter sizes (Fig.3.1d), (Kruskal-Wallis χ^2= 0.5, df = 2, *P* = 0.79)).

In a second independent cohort using only 1 dose of poly(I:C), 10 mg/kg, MIA dams showed an absence of the weight gain seen in controls 24 hours post injection (Fig.3.2a). This was supported by a main effect ***time*** (LMM ($F_{[2,24]}$ = 6.9, *P*<0.01)) and a ***dose*** x ***time*** interaction (LMM ($F_{[2,24]}$ = 17.1, *P* < 0.001)) when analysed with the model ***weight ~ dose*** x ***time*** + ***(1 | dam)***. This model returns an ICC = 0.86, a marginal R^2 = 0.23 and a conditional R^2 = 0.89. Control dams showed an increase in weight, 24 hours post injection ($t_{[24]}$ = -5.2, *p* < 0.001). 10 mg/kg dams lost weight 3 hours post injection ($t_{[24]}$ = 3, *p* < 0.05) and remained lighter 24 hours after poly(I:C) administration ($t_{[24]}$ = 2.8, *p* < 0.05). Control dams were heavier than MIA dams 24 hours post injection ($t_{[14.6]}$ = 3.1, *p* < 0.01). At 3 hours post injection MIA dams showed a reduction in surface temperature (Fig.3.2b). This was supported by a main effect ***time*** (LMM ($F_{[1,12]}$ = 47.9, *P*<0.001)) and a ***dose*** x ***time*** interaction (LMM ($F_{[1,12]}$ = 11.1, *P* < 0.01)) when analysed with the model ***temperature ~ dose*** x ***time*** + ***(1 | dam)***. This model returns an ICC = 0.88, a marginal R^2 = 0.32 and a conditional R^2 = 0.92. MIA dams were colder 3 hours post injection ($t_{[12]}$ = 7.8, *p* < 0.001) and colder than controls at the 3-hour timepoint ($t_{[13.6]}$ = 2.3, *p* < 0.05). As with the first cohort, MIA dams displayed sickness behaviours 3 hours after injection, these were not displayed by control mice (Fig.3.2c). The differential in weight gains and sickness behaviour between groups did not lead to significant differences in litter sizes (Fig.3.2d), (Kruskal-Wallis χ^2= 0.67, df = 1, *P* = 0.4)). Taken together, both cohorts demonstrate poly(I:C) prevented expected weight gain, caused sickness behaviours, and can cause a hypothermic response at higher a higher dose, without affecting litter size.

3.4.2 Offspring born to dams exposed to 10mg/kg poly(I:C) are less likely to display homing behaviour at P9.

The proportion of offspring where homing behaviour is absent at P9 is higher in offspring born to dams exposed to 10mg/kg poly(I:C) (26/50) compared to control

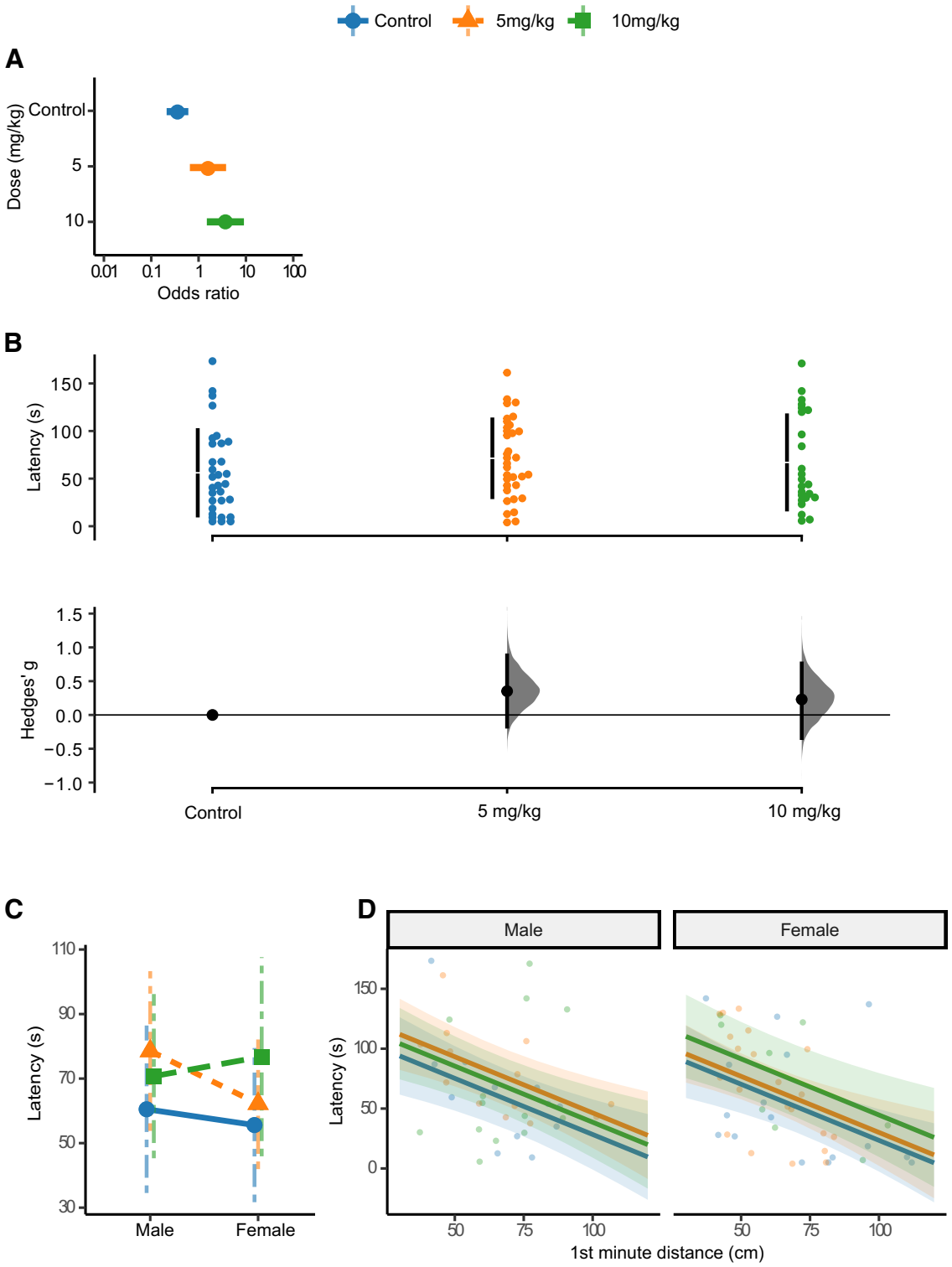


Figure 3.3 P9 homing test. **A**. Odds of mice having absent homing behaviour, with lines representing 95% CIs. **B.** Latency time to find home nest for individual offspring. The top panels show the raw data with a black bar showing the mean (break in the bar) and 95% confidence intervals for each group. In the bottom panels, black circles show the estimated effect size, Hedge's *g*, between each group and the reference group (controls). **C**. Mean latency times for individual animals with estimated marginal means plotted with 95% CIs plotted as dashed lines. **D**. Latency to find the home nest material predicted by 1st minute activity. Ribbons represent 95% CIs and individual points represent raw data. controls n = 37, 5 mg/kg n = 43, 10 mg/kg n = 50.

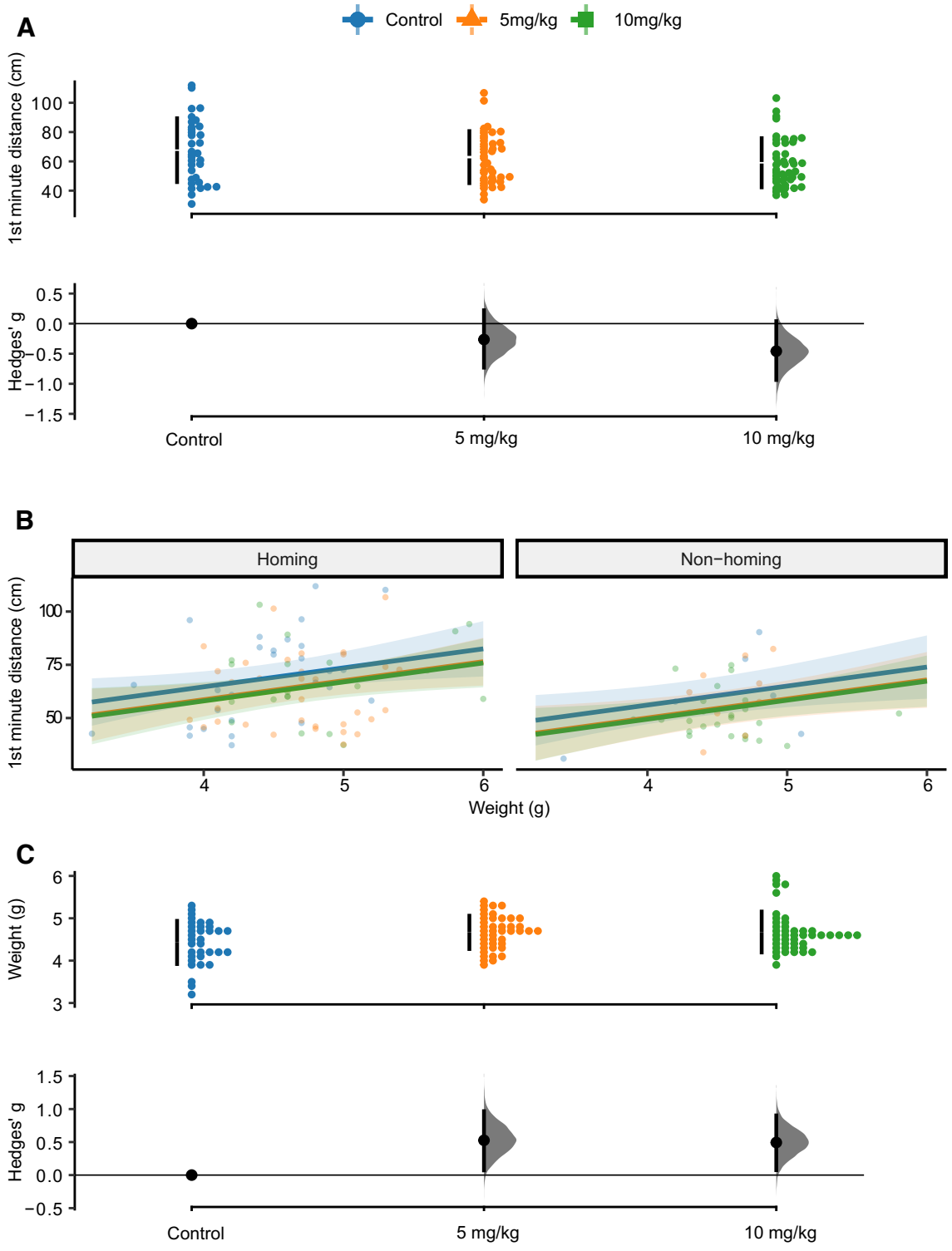


Figure 3.4 Additional variables recorded during homing test. **A**. Distance travelled in 1st minute of homing test. **B**. 1st minute predicted by homing test for homing and non-homing animals. Ribbons represent 95% CIs and individual points represent raw data. **C.** Weight of offspring at P9**.** For A and C, the top panels show the raw data with a black bar showing the mean (break in the bar) and 95% confidence intervals for each group. In the bottom panels, black circles show the estimated effect size, Hedge's *g*, between each group and the reference group (controls). controls *n* = 37, 5 mg/kg *n* = 43, 10 mg/kg *n* = 50.

offspring (6/37), reflected by a higher odds ratio (GLMM (link=logit (OR =3.69, 95% CI [1.5,9.08], P<0.01) of homing being absent (Fig.3.3a). The proportion of offspring born to dams treated with 5mg/kg poly(I:C) with absent homing behaviour is comparable to controls (9/43). The model, ***homing behaviour ~ dose + (1 | litter)***, returned an ICC = 0.1 a marginal R^2 = 0.16 and a conditional R^2 = 0.24. For homing offspring, MIA groups appear to take longer to find the home nesting materials than controls (Fig.3.3b) with no apparent differences between male and females (Fig.3.3c). Analysis showed no main effects of ***dose*** (LMM ($F_{[2,14.1]}$ = 0.76, P = 0.49)) or ***sex*** (LMM ($F_{[1,73.9]}$ = 0.26, P = 0.61)). Offspring that are less active in the first minute take longer to find the home nest, across all groups (Fig.3.3d), supported by a main effect of ***1st minute distance travelled*** (LM ($F_{[1,75]}$ = 12, P<0.001)). The model used ***latency ~ dose*** x ***sex*** + ***weight*** + ***1st minute distance travelled*** returned an ICC = 0.01, a marginal R^2= 0.18 and a conditional R^2 = 0.19.

Activity in the first minute of testing appears similar across groups (Fig.3.4a), analysis showed no effect of ***dose*** (LMM ($F_{[2,18.7]}$ = 1.2, P=0.32)) but highlighted main effects of ***homing*** (LMM ($F_{[1,107.3]}$ =5.2, P<0.05)) and ***weight*** (LMM ($F_{[1,32.6]}$ = 5.5, P<0.05)), with homing offspring and heavier offspring demonstrating greater initial activity (Fig.3.4b). The model ***1st minute distance travelled ~ homing*** + ***weight*** + ***dose*** x ***sex (1 | litter)***, returned an ICC = 0.04 a marginal R^2 = 0.15 and a conditional R^2 = 0.18. Offspring born to poly(I:C) treated dams appear to be heavier at P9 than control offspring (Fig.3.4c). However, the model ***Weight ~ homing*** + ***dose*** x ***sex (1 | litter)***, highlighted no main effects of ***dose*** (LMM ($F_{[2,18.3]}$ = 0.86, P=0.44)) or ***sex*** (LMM ($F_{[1,98.3]}$ = 0.19, P=0.66)) and returned an ICC = 0.74 a marginal R^2 = 0.07 and a conditional R^2 = 0.76.

At P9 homing behaviour is less likely to be present in offspring born to dams treated with 10mg/kg. For animals with present homing behaviour, latency to find, the nest and initial levels of activity were similar across groups.

3.4.3 Frequency of calling and mean duration of calls increases with latency to find the home nest

The number of calls emitted during the P9 homing test does not appear to vary across groups (Fig.3.5a). Homing and non-homing animals appear to show a dose x homing interaction (Fig.3.5b) but analysis did not support this ***dose x homing*** (LMM

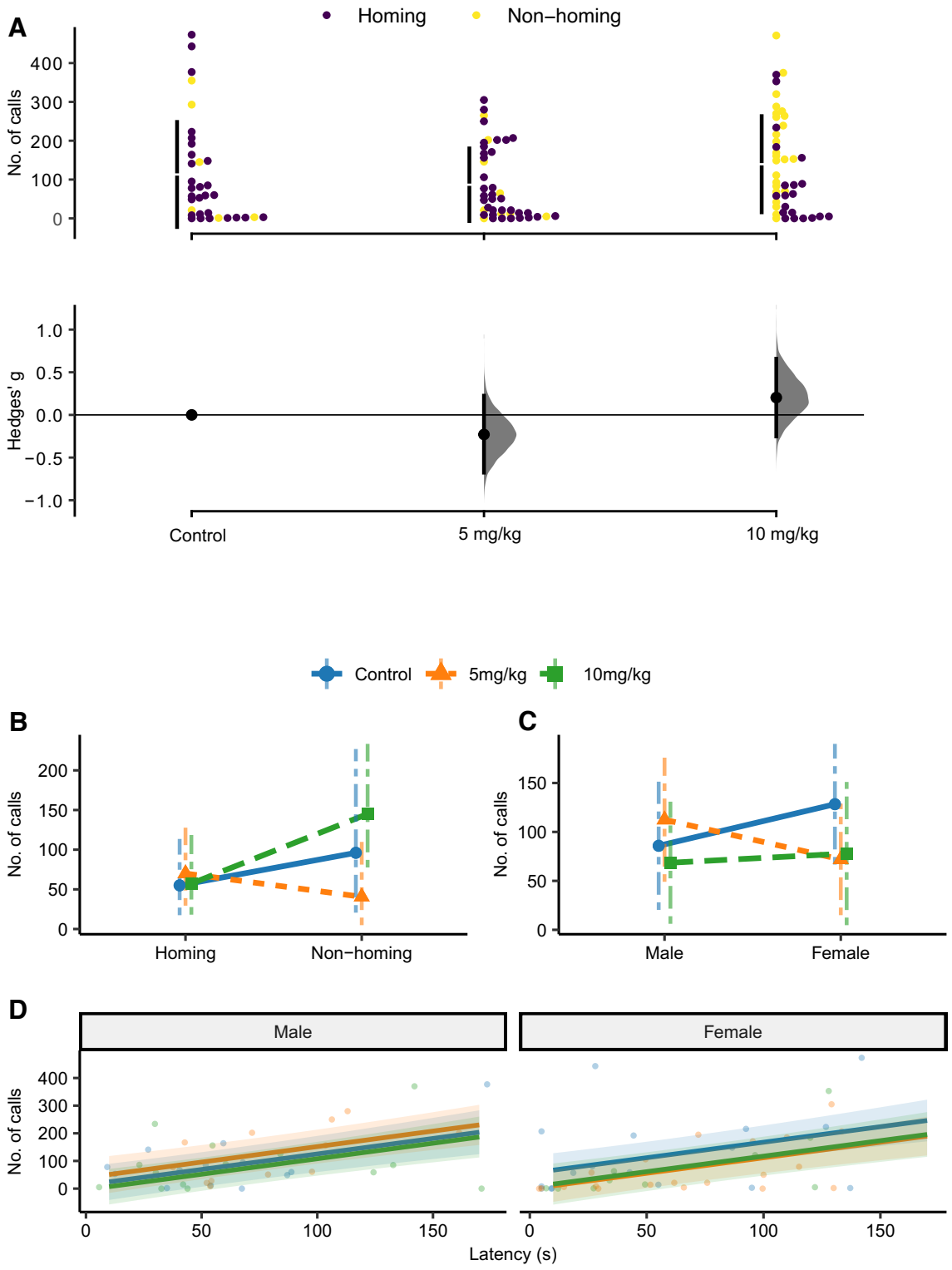


Figure 3.5 Vocalisation during the P9 homing test. **A**. Calling frequency of whole cohort. The top panel show the raw data with a black bar showing the mean (break in the bar) and 95% confidence intervals for each group. In the bottom panels, black circles show the estimated effect size, Hedge's *g*, between each group and the reference group (controls). **B.** Interaction plot for calling frequency of homing and non-homing animals. **C.** Interaction plot for calling frequency of male and female animals**.** B and C feature estimated marginal means plotted with 95% CIs plotted as dashed lines. **D.** Calling frequency precited by latency time to find the nesting material. Ribbons represent 95% CIs and individual points represent raw data. For A controls *n* = 37, 5 mg/kg *n* = 43, 10 mg/kg *n* = 50. For B, homing controls *n* = 31 non-homing controls *n* = 6, homing 5 mg/kg *n* = 34, non-homing 5 mg/kg *n* = 9, Homing 10 mg/kg *n* = 26 and non-homing 10 mg/kg *n* = 24. For C, Male controls *n* = 16, Female controls *n* = 21, Male 5 mg/kg *n* = 17, Female 5 mg/kg *n* = 26, Male 10 mg/kg *n* = 32 and Female 10 mg/kg *n* = 18. For D, Male controls *n* = 13, Female controls *n* = 18, Male 5 mg/kg *n* = 13, Female 5 mg/kg *n* = 21, Male 10 mg/kg *n* = 14 and Female 10 mg/kg *n* = 10.

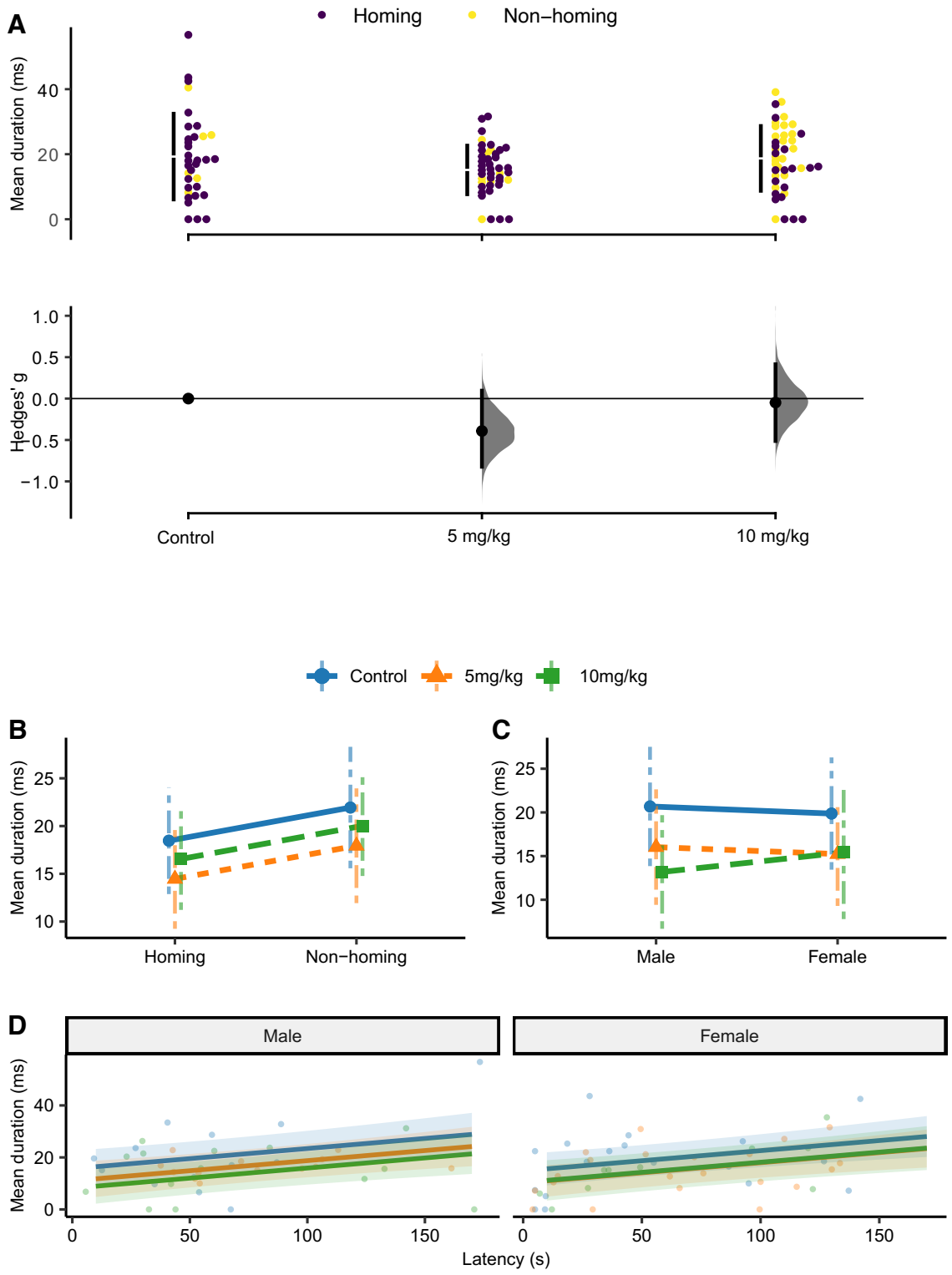


Figure 3.6 Mean duration of USVs during the P9 homing test. **A**. Mean call duration of whole cohort. The top panels show the raw data with a black bar showing the mean (break in the bar) and 95% confidence intervals for each group. In the bottom panels, black circles show the estimated effect size, Hedge's *g*, between each group and the reference group (controls). **B.** Interaction plot for mean call duration of homing and non-homing animals. **C.** Interaction plot for mean call duration of male and female animals**.** B and C feature estimated marginal means plotted with 95% CIs plotted as dashed lines. **D.** Mean call duration precited by latency time to find the nesting material. Ribbons represent 95% CIs and individual points represent raw data. For A controls *n* = 37, 5 mg/kg *n* = 43, 10 mg/kg *n* = 50. For B, homing controls *n* = 31 non-homing controls *n* = 6, homing 5 mg/kg *n* = 34, non-homing 5 mg/kg *n* = 9, Homing 10 mg/kg *n* = 26 and non-homing 10 mg/kg *n* = 24. For C, Male controls *n* = 16, Female controls *n* = 21, Male 5 mg/kg *n* = 17, Female 5 mg/kg *n* = 26, Male 10 mg/kg *n* = 32 and Female 10 mg/kg *n* = 18. For D, Male controls *n* = 13, Female controls *n* = 18, Male 5 mg/kg *n* = 13, Female 5 mg/kg *n* = 21, Male 10 mg/kg *n* = 14 and Female 10 mg/kg *n* = 10.

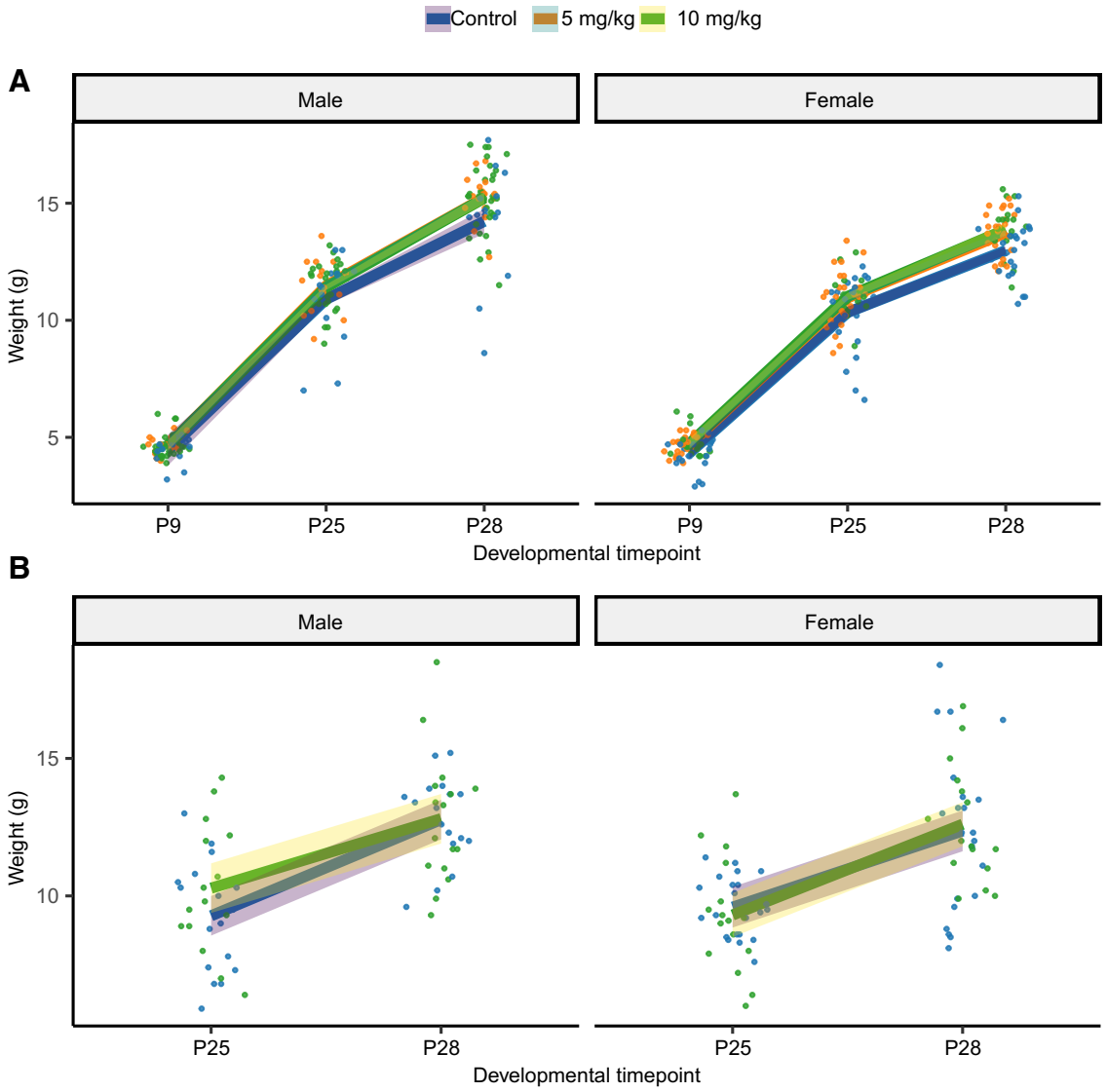


Figure 3.7. Longitudinal weight measurements for control and MIA offspring. **A**. Weight measures of control, 5 mg/kg and 10 mg/kg mice at 3 time points. **B.** Weight measures for a 2nd cohort of mice, controls, and 10 mg/kg at two timepoints. Bold lines and ribbons are means and CIs respectively with dots representing raw data. A. Male controls *n* = 17, Female controls *n* = 28, Male 5 mg/kg *n* = 17, Female 5 mg/kg *n* = 26, Male 10 mg/kg *n* = 32 and Female 10 mg/kg *n* = 18. B. Male controls *n* = 17, Female controls *n* = 23 and Male 10 mg/kg *n* = 16 and Female 10 mg/kg *n* = 16.

($F_{[2,107.6]}$ = 2.6, P =0.08)) and no main effects of ***dose*** (LMM ($F_{[2,21.8]}$ = 0.8, P=0.46)), ***homing*** (LMM ($F_{[1,108.6]}$ = 1.5, P=.22)) or ***sex*** (LMM ($F_{[1,100.5]}$ = 1, P=0.32)). The model, **sqrt** (***No. of calls)*** ~ ***dose*** x ***homing*** *x* ***sex*** + ***weight*** + ***(1 | litter)***, returned an ICC = 0.21 a marginal R^2 = 0.17 and a conditional R^2 = 0.34. Homing animals displayed a pattern of behaviour suggestive of a dose x sex interaction (Fig.3.5c). Analysis did not demonstrate such an interaction ***dose*** *x* ***sex*** (LMM ($F_{[2,75.2]}$ = 1.8, P =0.16)) nor main effects of ***dose*** (LMM ($F_{[2,16.3]}$ = 0.4, P=0.67)) or ***sex*** (LMM ($F_{[1,70.9]}$ = 0.04, P=0.85)). It did reveal a main effect of ***latency*** (LMM ($F_{[176]}$ = 25.9, P<0.001)), all groups called more as latency to find the home nesting material increased (Fig.3.5d). The model, ***No. of calls*** ~ ***latency*** + ***dose*** x ***sex*** + ***weight*** +***(1 | litter)***, returned an ICC = 0.27 a marginal R^2 = 0.25 and a conditional R^2 = 0.46. Mean duration of calls appears similar across all groups (Fig.3.6a) and appears unaffected in animals with absent homing behaviour (Fig.3.6b). Support for this comes from no effect of ***dose*** (LMM ($F_{[2,19]}$ = 0.6, P=0.55)), ***homing*** (LMM ($F_{[1,114.4]}$ = 2.7, P=0.1)) or ***sex*** (LMM ($F_{[1,104.3]}$ = 0.5, P=0.48)). The model, **Mean duration** ~ ***dose*** x ***homing*** *x* ***sex*** + ***weight*** + ***(1 | litter)***, returned an ICC = 0.24 a marginal R^2 = 0.05 and a conditional R^2 = 0.28. Considering mice that display homing behaviour, MIA animals appear to have lower mean duration of calling than controls (Fig.3.6c). This is not supported by analysis as there is no main effect of ***dose*** (LMM ($F_{[2,16.3]}$ = 1.2, P=0.3)) or ***sex*** (LMM ($F_{[1,77.6]}$ = 0.01, P=0.9)). A main effect of ***latency*** (LMM ($F_{[1,76]}$ = 11.4, P<0.01)) was returned with mean duration increasing with latency to find the nest, (Fig.3.6d). The model, ***mean duration*** ~ ***latency*** + ***dose*** x ***sex*** + ***weight*** + ***(1 | litter)***, returned an ICC = 0.27 a marginal R^2 = 0.16 and a conditional R^2 = 0.39.

3.4.5 Males are heavier than females at P28

MIA animals appear heavier than controls at P25 and P28 timepoints and males appear heavier than females across all groups, at the P28 timepoint measured, with all groups increasing in weight over time (Fig.3.7a). There is no main effect of ***dose*** (LMM ($F_{[2,19.3]}$ = 2.3, P=0.13)) but there are main effects of ***sex*** (LMM ($F_{[1,109.3]}$ = 25.6, P<0.001)) and ***timepoint*** (LMM ($F_{[1,124]}$ = 2177.8, P<0.001)). There is also an interaction of ***sex*** *x* ***timepoint*** (LMM ($F_{[1,124]}$ = 40.8, P<0.001)). Post hoc comparisons reveal control male controls ($t_{[142]}$ = 3.5, p<0.001), 5mg/kg males ($t_{[140]}$ = 4.1, p<0.001) and 10 mg/kg males ($t_{[142]}$ = 4.6 p<0.001) are heavier than their female counterparts at P28 but not at P25. ***Weight*** ~ ***timepoint*** x ***dose*** x ***sex*** + ***(1 |***

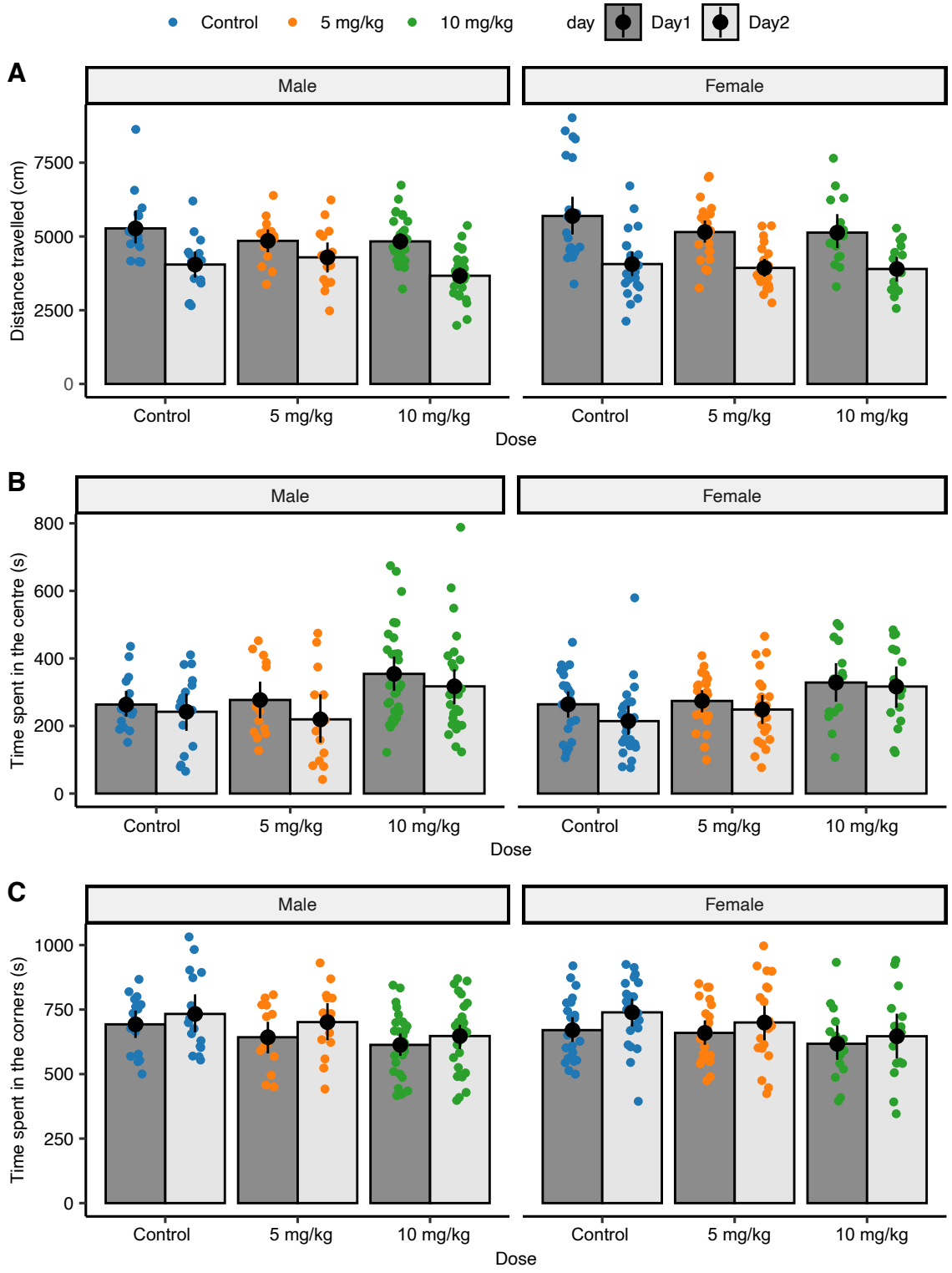


Figure 3.8 **.** Activity in open field P24 & 25 1st MIA cohort. **A**. Distance travelled day 1 v day 2. **B**. Time spent in the centre of open field day 1 to day 2. **C** Time spent in the corners of the open field day 1 to day 2. Coloured dots represent individual data points with the black dot and line representing the group mean with bootstrapped 95% CI. Male controls *n* = 16, Female controls *n* = 23, Male 5 mg/kg *n* = 14, Female 5 mg/kg *n* = 22, Male 10 mg/kg *n* = 30 and Female 10 mg/kg *n* = 14.

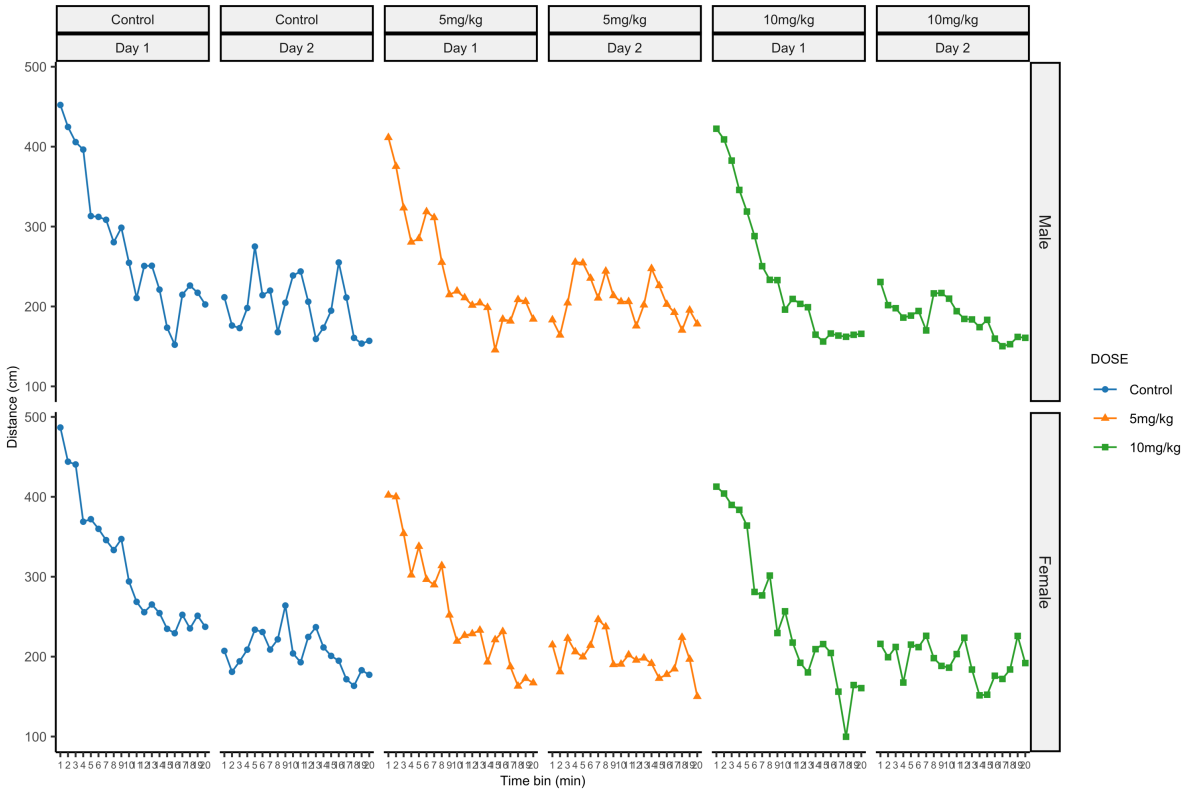


Figure 3.9 Activity in open field P24 & 25 1st MIA cohort in 1-minute time bins. Male controls *n* = 16, Female controls *n* = 23, Male 5 mg/kg *n* = 14, Female 5 mg/kg *n* = 22, Male 10 mg/kg *n* = 30 and Female 10 mg/kg *n* = 14.

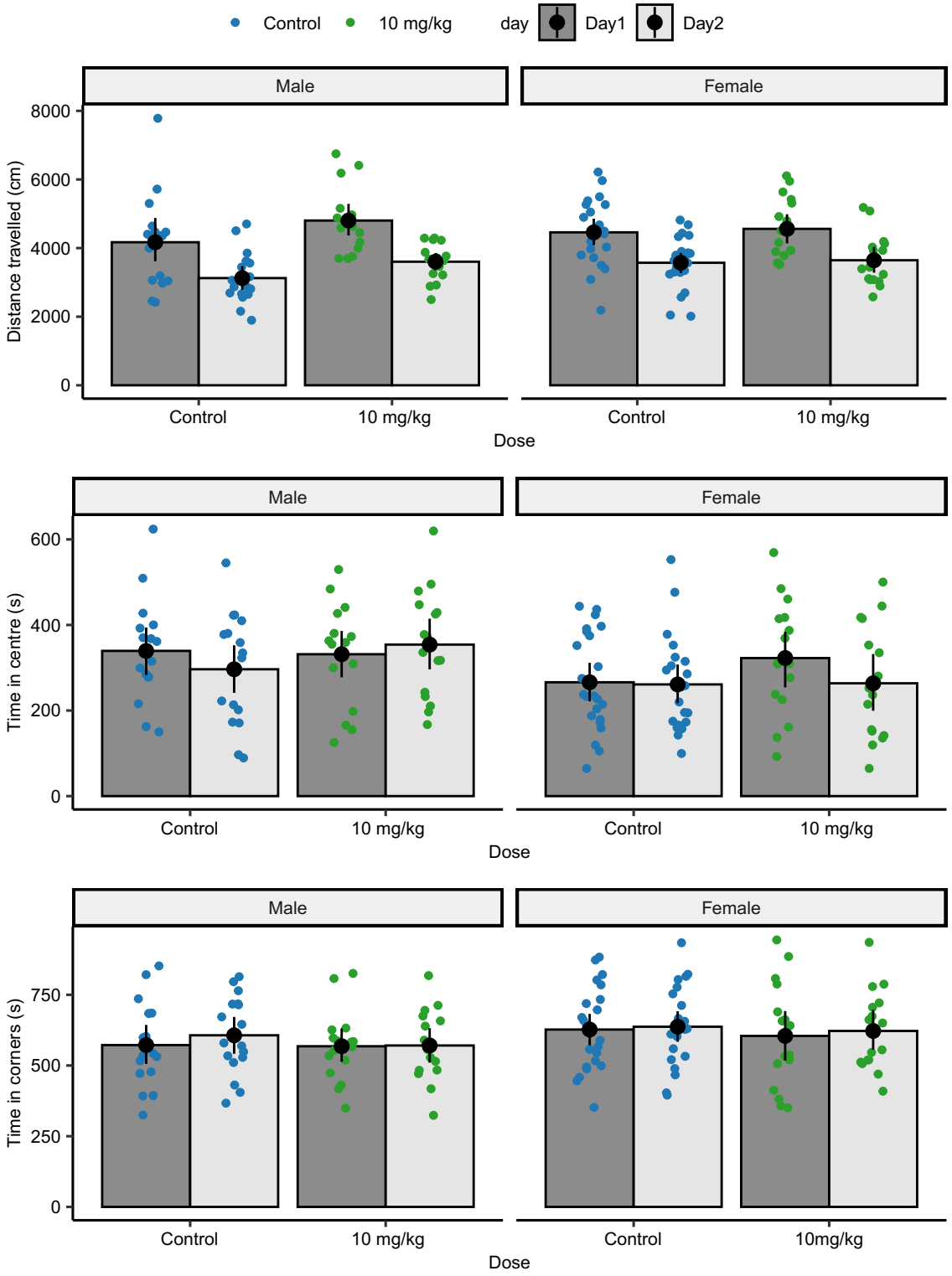


Figure 3.10. Activity in open field P24 & 25 2nd MIA cohort. **A**. Distance travelled day 1 v day 2. **B**. Time spent in the centre of open field day 1 to day 2. **C** Time spent in the corners of the open field day 1 to day 2. Coloured dots represent individual data points with the black dot and line representing the group mean with bootstrapped 95% CI. Male controls *n* = 17, Female controls *n* = 23, Male 10 mg/kg *n* = 16 and Female 10 mg/kg *n* = 16.

ID) + ***(1 | Litter)***. returns an ICC_{ID} = 0.37, ICC_{litter} = 0.48, marginal R^2 = 0.62 and conditional R^2 = 0.94. In a 2nd cohort of experimental animals using just controls and the 10mg/kg dose of poly(I:C) both groups appear to get heavier from P25 to P28 with no obvious differences between groups (Fig.3.7b). This is supported by a main effect of ***timepoint*** (LMM ($F_{[1,124.1]}$ = 91.2, P<0.001)) and no main effect of ***dose*** (LMM ($F_{[1,9.9]}$ = 0.28, P=0.6)) or ***sex*** (LMM ($F_{[1,129.4]}$ = 0.8, P=0.37)). ***Weight*** ~ ***timepoint*** x ***dose*** x ***sex*** + ***(1 | Litter)***. returns an, ICC = 0.29, marginal R^2 = 0.33 and conditional R^2 = 0.58.

All groups increase in weight from P25 to P28 for control and MIA animals in both cohorts. Males are heavier than females at P28 in the first cohort, but this sex effect is not seen in the second cohort.

3.4.6 Activity

All groups display reduced activity in the open field from day 1 to day 2 (Fig.3.8a) supported by a main effect of ***day*** (LMM ($F_{[1,114]}$ = 100.4, P < 0.001)), with no effect of ***dose*** (LMM ($F_{[2,19]}$ = 1.6, P =0.22)) or ***sex*** (LMM ($F_{[1,109.5]}$ = 0.7, P =0.4)). Post-hoc comparisons revealed the reduction in activity from day 1 to 2 in male 5 mg/kg as not statistically significant ($t_{[114]}$ = 1.69, p=0.09). There are no differences in distance travelled between groups on day 1 or 2. ***Distance travelled*** ~ ***day*** x ***dose*** x ***sex*** + ***(1 | ID)*** + ***(1 | Litter)***. returns an ICC_{ID} = 0.18 ICC_{litter} = 0.1, marginal R^2 = 0.29 and conditional R^2 = 0.5. All groups spend a reduced amount of time in the centre of the open field from day 1 to day 2 (Fig.3.8b) supported by a main effect of ***day*** (LMM ($F_{[1,114]}$ = 7.7, P < 0.01)). Mice in the 10 mg/kg group appear to spend more time in the centre, supported by a main effect of ***dose*** (LMM ($F_{[2,19]}$ = 5.1, P < 0.05)), with no effect of ***sex*** (LMM ($F_{[1,10]}$ = 0.3, P = 0.58)). Post hoc tests show 10 mg/kg males spend more time in the centre than control males on day 1 ($t_{[65.8]}$ = -2.5, p< 0.05) and 5 mg/kg males on day 2 ($t_{[62.8]}$ = -2.5, p< 0.05). ***Time spent in centre*** ~ ***day*** x ***dose*** x ***sex*** + ***(1 | ID)*** + ***(1 | Litter)***. returns an ICC_{ID} = 0.31, ICC_{litter} = 0.1, marginal R^2 = 0.13 and conditional R^2 = 0.49. Control and MIA offspring show similar patterns of behaviour considering time spent in the corners of the open field day 1 to 2, spending more time in corners on day 2. (Fig.3.8c), with a main effect of ***day*** (LMM ($F_{[1,114]}$ = 10, P < 0.01)). Male and female 10 mg/kg mice appear to spend less time

in corners on both days than controls, but this is not supported by a main effect of ***dose*** (LMM ($F_{[2,19]}$ = 3.3, P = 0.06)). ***Time spent in corners ~ day*** x ***dose*** x ***sex*** + ***(1 | ID)*** + ***(1 | Litter)***. returns an ICC_{ID} = 0.31, ICC_{litter} = 0.07, marginal R^2 = 0.09 and conditional R^2 = 0.44.

In a 2nd cohort of experimental animals using just controls and the 10mg/kg dose of poly(I:C) both groups habituate to the open field from day 1 to day 2 (Fig.3.10a) supported by a main effect of ***day*** (LMM ($F_{[1,68]}$ = 98.7, P < 0.001)), with no group differences in activity on either day 1 or 2. ***Distance travelled ~ day*** x ***dose*** x ***sex*** + ***(1 | ID)*** + ***(1 | Litter)***. returns an ICC_{ID} = 0.31 ICC_{litter} = 0.24, marginal R^2 = 0.25 and conditional R^2 = 0.66. Female animals from both groups appear to spend less time in the centre than males, on both days (Fig.3.10b), supported by a main effect of ***sex*** (LMM ($F_{[1,61.2]}$ = 10.1, P < 0.01)). There was no effect of ***dose*** (LMM ($F_{[1,9.9]}$ = 0.1, P = 0.74)) of ***day*** (LMM ($F_{[1,68]}$ = 1.6, P = 0.21)). Post hoc comparisons highlighted differences between males and females on day 1($t_{[124]}$ = 2., p<0.05) or day 2 ($t_{[123]}$ = 2.8, p<0.01). ***Time spent in centre ~ day*** x ***dose*** x ***sex*** + ***(1 | ID)*** + ***(1 | Litter)***. returns an ICC_{ID} = 0.09, ICC_{litter} = 0.27, marginal R^2 = 0.08 and conditional R^2 = 0.41. Control and MIA offspring show similar patterns of behaviour considering time spent in the corners of the open field day 1 to 2 (Fig.3.10c), with a no main effect of ***day*** (LMM ($F_{[1,68]}$ = 0.6, P = 0.43)), ***dose*** (LMM ($F_{[1,9.4]}$ = 0.2, P =0.66)) or ***sex*** (LMM ($F_{[1,64.3]}$ = 2.7, P =0.1)). ***Time spent in corners ~ day*** x ***dose*** x ***sex*** + ***(1 | ID)*** + ***(1 | Litter)***. returns an ICC_{ID} = 0.25, ICC_{litter} = 0.04, marginal R^2 = 0.03 and conditional R^2 = 0.32.

Taken together, MIA doesn't appear to influence activity levels or habituation in the open field test, a finding replicated in a 2nd independent cohort. MIA does appear to influence exploratory behaviour in 10 mg/kg males, but this was not replicated in the 2nd cohort where females of both groups showed lower levels of exploration compared to males.

3.4.7 Offspring born to dams exposed to 10mg/kg poly(I:C) show reduced social novelty preference.

Offspring in all groups showed habituation to repeated exposure of the same mouse in the first four trials of the social novelty test at P26 and 27, followed by increased

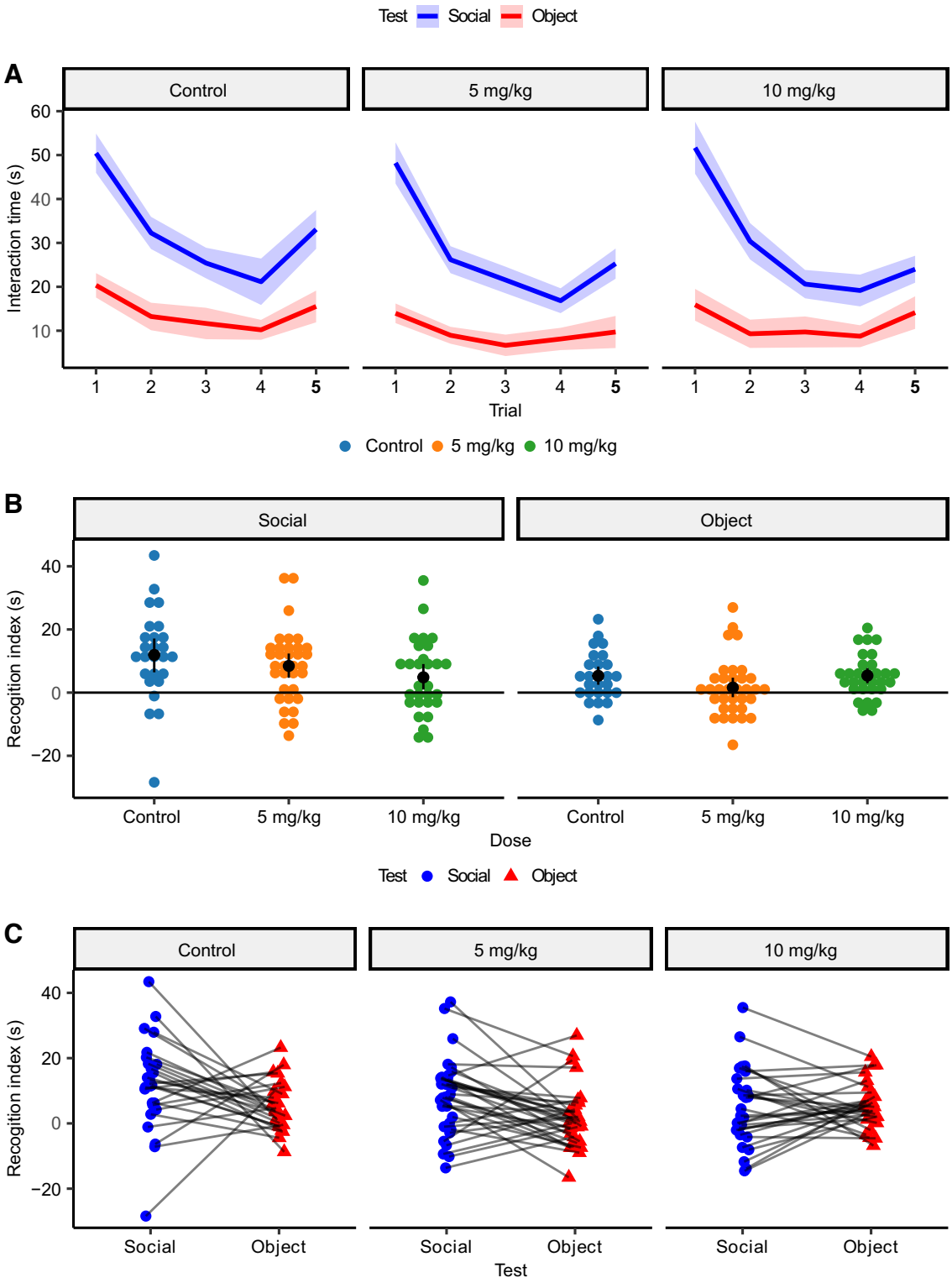


Figure 3.11. Social and Object novelty recognition task. **A.** Mean interaction time with social and object stimuli in 5-trial habituation/recognition task at P26 & 27, ribbons represent 95% CIs and the emboldened number 5 on the x-axis represents the presentation of a novel stimulus. **B.** Recognition indices for novel social and object stimuli at P26 & 27, black dots represent means with black line represent bootstrapped 95% CIs. **C.** Recognition indices novel social versus object stimuli at P26 & 27 for each animal. For A, B and C, Controls *n* = 26, 5mg/kg *n* = 35 and 10mg/kg *n* = 30.

interest to a novel mouse in trial 5 (Fig.3.11a), supported by a main effect of ***trial*** (LMM ($F_{[4,336]} = 129.8$, $P<0.001$)). No main effects of ***dose*** (LMM ($F_{[2,12.25]} = 1.1$, $P=0.38$)) or ***sex*** (LMM ($F_{[1,79.4]} = 0.17$, $P=0.68$)) were returned. Considering females and males together, post-hoc comparisons showed reduced interaction times in controls ($t_{[51.9]} = 10.5$, $p <0.001$), and the 5mg/kg group ($t_{[39.8]} = 13$, $p <0.001$) from trial 1 to 4 and controls ($t_{[51.9]} = -4.3$, $p <0.001$), and the 5mg/kg group ($t_{[39.8]} = -3.6$, $p <0.01$) demonstrated increased interest from trial 4 to 5. The 10mg/kg group also show habituation from trial 1 - 4 ($t_{[44.8]} = 10.9$, $p <0.001$) but do not show an increase from trial 4 – 5 ($t_{[44.8]} = -2$, $p =0.28$). The model, ***social interaction time*** ~ ***dose*** *x* ***sex*** x ***trial*** + ***(1 | ID)*** + ***(1 | litter)***, returned an $ICC_{[ID]} = 0.22$, an $ICC_{[litter]} = 0.11$ a marginal $R^2 = 0.48$ and a conditional $R^2 = 0.65$. In the object novelty test all groups showed habituation to repeated exposure of the same object in the first four trials (Fig.3.12a), supported by a main effect of ***trial*** (LMM ($F_{[3.44,306.1]} = 25.7$, $P<0.001$) Greenhouse-Geisser corrected). Post-hoc comparisons showed reduced object interest from trials 1 – 4 in all groups, controls ($t_{[44.4]} = 4.8$, $p <0.001$), the 5mg/kg group ($t_{[34]} = 4.6$, $p <0.001$) and 10mg/kg group ($t_{[38]} = 4.1$, $p <0.01$). None of the groups showed an increase in interest from trial 4 - 5 controls ($t_{[44.4]} = -2.8$, $p =0.05$), the 5mg/kg group ($t_{[34]} = -0.97$, $p =0.87$) and 10mg/kg group ($t_{[38]} = -2.7$, $p =0.08$). The model **sqrt (*object interaction time)*** ~ ***dose*** x **sex** x ***trial*** + ***(1 | ID)*** + ***(1 | litter)***, returned an $ICC_{[ID]} = 0.43$, an $ICC_{[litter]} = 0.08$ a marginal $R^2 = 0.17$ and a conditional $R^2 = 0.6$.

The recognition index reduced as dose of poly(I:C) increased for the social test but not the object test (Fig.3.11b) supported by a main effect of ***test*** (LMM ($F_{[1,87]} = 8.1$, $P<0.01$)). There was no main effect of ***dose*** (LMM ($F_{[2,87]} = 2.1$, $P=0.13$) but post hoc comparisons revealed a higher social recognition index in control offspring compared to 10mg/kg offspring ($t_{[173]} = 2.6$, $p <0.05$). Comparison of the recognition indices between social and object tests (Fig.3.11c) highlighted higher social recognition indices compared to object recognition indices in controls ($t_{[173]} = 2.4$, $p <0.05$) and the 5mg/kg group ($t_{[173]} = 2.8$, $p <0.01$) but not the10mg/kg group ($t_{[173]} = -0.2$, $p =0.84$). The model ***recognition index*** ~ ***dose*** x ***test*** + ***(1 | ID),*** returned an $ICC_{[ID]} = 0.05$, a marginal $R^2 = 0.09$ and a conditional $R^2 = 0.14$. Offspring born to dams exposed to 10mg/kg of poly(I:C) show a reduced preference for social novelty, with a lower social recognition index compared to controls and demonstrate a similar level of preference for novel social or object stimuli.

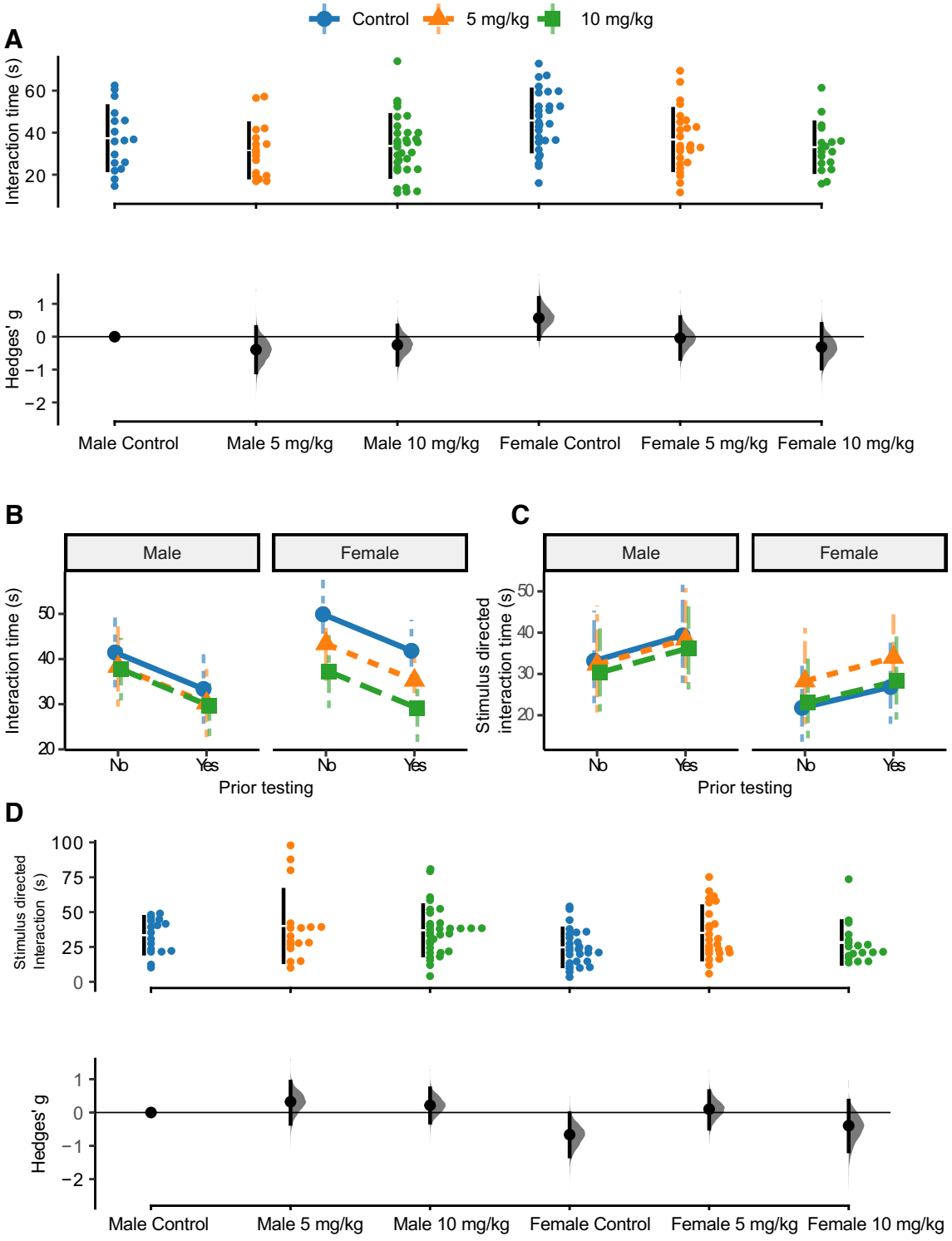


Figure 3.12 . Social interaction at P28 for 1st MIA cohort. **A**. Interaction times for individual offspring, the top panel show the raw data with a black bar showing the mean (break in the bar) and 95% confidence intervals for each group. In the bottom panel, black circles show the estimated effect size, Hedge's *g*, between each group and the reference group (male WT controls). **B**. Interaction plot for social interaction times for offspring tested at P26 & 27 versus naïve mice. **C**. Interaction plot for stimulus directed interaction times for offspring tested at P26 & 27 versus naïve mice. For B and C estimated marginal means plotted with 95% CIs plotted. **D.** Stimulus directed interaction times for individual offspring, the top panel show the raw data with a black bar showing the mean (break in the bar) and 95% confidence intervals for each group. In the bottom panel, black circles show the estimated effect size, Hedge's *g*, between each group and the reference group (male WT controls). Male controls *n* = 17, Female controls *n* = 28, Male 5 mg/kg *n* = 17, Female 5 mg/kg *n* = 26, Male 10 mg/kg *n* = 32 and Female 10 mg/kg *n* = 18.

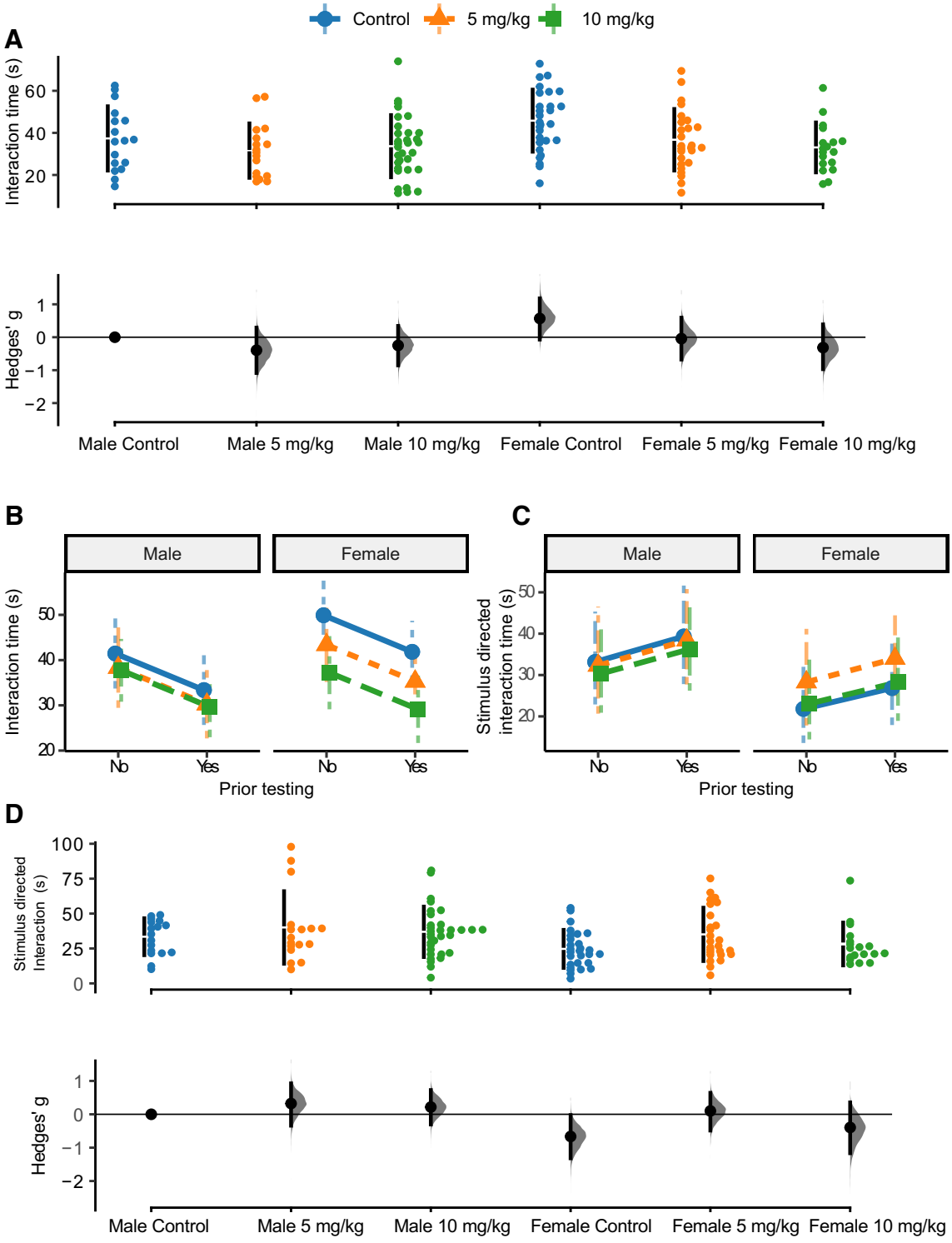


Figure 3.13. Social interaction at P28 for 2nd MIA cohort. **A**. Interaction times for individual offspring. **B.** Stimulus directed interaction times for individual offspring. For A and B the top panels show the raw data with a black bar showing the mean (break in the bar) and 95% confidence intervals for each group. In the bottom panels, black circles show the estimated effect size, Hedge's *g*, between each group and the reference group (male WT controls). **C.** Stimulus directed interaction time predicted by weight of the experimental animal. Ribbons represent 95% CIs and individual points represent raw data. Male controls *n* = 17, Female controls *n* = 23 and Male 10 mg/kg *n* = 16 and Female 10 mg/kg *n* = 16.

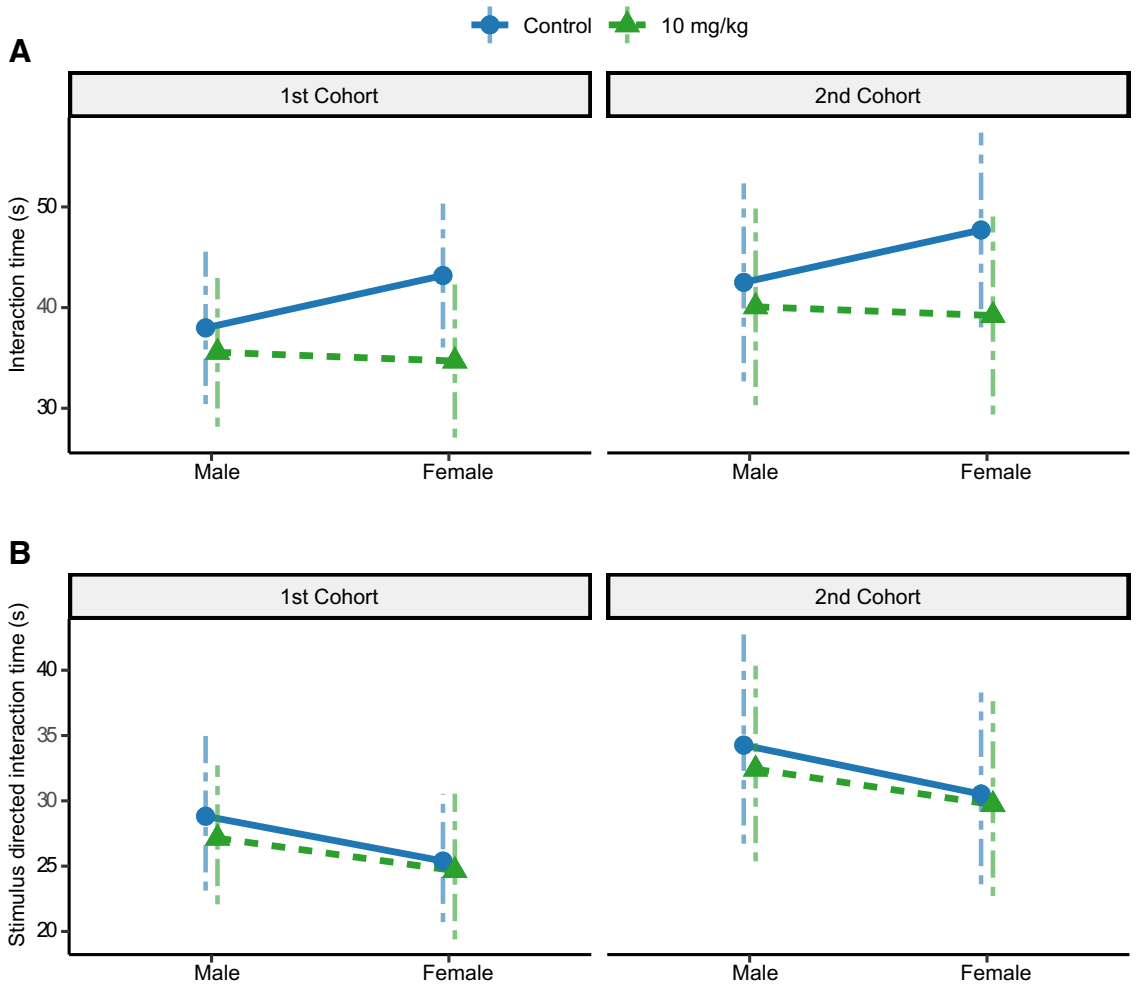


Figure 3.14. Comparison of P28 interaction times between 1st and 2nd MIA cohorts. **A.** Mean interaction times of male and female mice. **B.** Mean stimulus directed interaction times towards male and female mice. A and B feature estimated marginal means plotted with 95% CIs plotted as dashed lines. 1st cohort Male controls *n* = 17, Female controls *n* = 28, Male 5 mg/kg *n* = 17, Female 5 mg/kg *n* = 26, Male 10 mg/kg *n* = 32 and Female 10 mg/kg *n* = 18. 2nd cohort Male controls *n* = 17, Female controls *n* = 23 and Male 10 mg/kg *n* = 16 and Female 10 mg/kg *n* = 16.

3.4.8 Prior social testing reduces time spent in social interaction at P28

Raw data for direct social interaction at P28 shows similar levels for males but points to differences across female groups (Fig.3.12a). Analysis does not support this with no main effect of ***dose*** (LMM ($F_{[2,18.2]} = 2.9$, $P=0.08$) or ***sex*** (LMM ($F_{[1,127]} = 2.4$, $P=0.12$). Post hoc testing demonstrated a difference between female controls and females from the 10mg/kg group ($t_{[27.3]} = 2.8$, $p < 0.05$) which was not present between male groups ($t_{[22.4]} = 0.8$, $p = 0.69$). Animals who underwent the social recognition test at P26 and 27 showed lower interaction times than untested animals (Fig.3.12b) supported by a main effect of ***prior testing*** (LMM ($F_{[1,15.5]} = 6.8$, $P<0.05$). The model ***interaction time*** ~ ***weight*** + ***stimulus directed interaction time*** + ***prior testing*** + ***dose*** x ***sex*** + ***(1 | Stimulus ID)*** + ***(1 | Litter)*** returned an $ICC_{[Stim\ ID]} = 0.01$, an $ICC_{[litter]} = 0.07$ a marginal $R^2 = 0.19$ and a conditional $R^2 = 0.25$. The raw data for stimulus directed interaction time points to higher levels in male groups (Fig.3.12d), supported by a main effect of ***sex*** (LMM ($F_{[1,122.9]} = 7$, $P<0.01$) with no main effect of ***dose*** (LMM ($F_{[2,18.9]} = 0.3$, $P=0.72$) or ***prior testing*** (LMM ($F_{[1,22.2]} = 1.2$, $P=0.27$). The sex difference seems biggest between controls (Fig.3.12c), supported by post hoc testing which highlighted a difference between male and female controls ($t_{[31.3]} = 2.5$, $p < 0.05$) but not in the MIA groups. The model **sqrt** (***stimulus directed interaction time)*** ~ ***weight*** + ***interaction time*** + ***prior testing*** + ***dose*** x ***sex*** + ***(1 | Stimulus ID)*** + ***(1 | Litter)*** returned an $ICC_{[Stim\ ID]} = 0.18$, an $ICC_{[litter]} = 0.18$ a marginal $R^2 = 0.16$ and a conditional $R^2 = 0.46$.

In a 2nd cohort of experimental animals using just controls and the 10mg/kg dose of poly(I:C), raw data for direct social interaction at P28 similar interaction times across groups (Fig.3.13a). This view is reinforced with no main effect of ***dose*** (LMM ($F_{[1,9.2]} = 0.16$, $P=0.69$) or ***sex*** (LMM ($F_{[1,56.6]} = 0.5$, $P=0.48$). The model ***interaction time*** ~ ***weight*** + ***stimulus directed interaction time*** + ***dose*** x ***sex*** + ***(1 | Stimulus ID)*** + ***(1 | Litter)*** returned an $ICC_{[Stim\ ID]} = 0.03$, an $ICC_{[litter]} = 0.42$ a marginal $R^2 = 0.09$ and a conditional $R^2 = 0.51$. The raw data for stimulus directed interaction time shows no difference across groups (Fig.3.13b), supported by no main effect of ***dose*** (LM ($F_{[1,62]} = 0.66$, $P=0.42$) or ***sex*** (LM ($F_{[1,62]} = 2.7$, $P=0.1$). Stimulus directed interaction appears to be greater for heavier females (Fig.3.13c) supported by a main effect of ***weight*** (LM ($F_{[1,62]} = 5$, $P<0.05$) but not by an interaction of ***weight*** *x* ***sex*** (LM ($F_{[1,62]}$

= 3.2, *P*<0.08) The model ***interaction time ~ weight + stimulus directed interaction time + dose x sex*** returned an adjusted R^2 = 0.11.
Comparing control and 10mg/kg groups from both cohorts suggests higher interaction times in the 2nd cohort (Fig.3.14a) but this is not supported by analysis when controlling for the prior testing some of the 1st cohort experienced. There is no main effect of ***cohort*** (LMM ($F_{[1,24.8]}$ = 0.8, *P*=0.39) as well as no main effects of ***dose*** (LMM ($F_{[1,23.6]}$ = 2.1, *P*=0.16) or ***sex*** (LMM ($F_{[1,149.6]}$ = 0.9, *P*=0.34). The model ***interaction time ~ cohort + weight + stimulus directed interaction time + prior testing + dose x sex + (1 | Stimulus ID) + (1 | Litter)*** returned an $ICC_{[Stim\ ID]}$ = 0.06, an $ICC_{[litter]}$ = 0.3 a marginal R^2 = 0.22 and a conditional R^2 = 0.5. Animals from the second cohort appear to experience more stimulus directed interaction than the first cohort, with females having less stimulus directed interaction than males in all groups (Fig.3.14b). Analysis did not provide evidence of a difference between cohorts with no main effect of ***cohort*** (LMM ($F_{[1,15.45]}$ = 2.23, *P*=0.16). The model ***sqrt (stimulus directed interaction time) ~ cohort + weight + interaction time + prior testing + dose x sex + (1 | Stimulus ID) + (1 | Litter)*** returned an $ICC_{[Stim\ ID]}$ = 0.06, an $ICC_{[litter]}$ = 0.02 a marginal R^2 = 0.16 and a conditional R^2 = 0.22.

Overall, neither 5 or 10 mg/kg of poly(I:C) impacted the social interaction of offspring at P28 nor did it influence the level of interest stimulus mice displayed towards offspring. The temporal proximity of the social habituation test did appear to impact the performance of all groups at P28, with the greates effect seen in the 10 mg/kg group.

3.5 Discussion

This study achieved its primary aim to identify a sub-threshold dose of poly(I:C) that would stimulate the immune system of pregnant dams without producing social impairments in the offspring. The validation of immune activation via non-invasive methods was also successful with surface temperature, weight change and sickness behaviour demonstrating dose dependent responses. For offspring born to dams exposed to the higher dose, 10 mg/kg showed potential social impairments early in development in 2 out 3 tests. At P9, the pups of the 10 mg/kg dams were less likely

to successfully locate the home nest than controls and the sub-threshold group, 5 mg/kg. The higher dose group also displayed a reduced preference for social novelty in a social recognition test at P26 and P27. In a P28 direct social interaction test there was no overall effect of poly(I:C) dose but female controls appeared to spend more time in interaction than 10 mg/kg females. However, all groups showed reduced interaction times at P28 if they were tested at P26 and P27. A second independent cohort was assayed using only the higher dose and immune activation was demonstrated just like the first cohort. Offspring were only tested at P25 for activity and P28 Direct social interaction test. Social interaction times at P28 showed no difference between cohorts with no observed effect of poly(I:C) dose. The work here displayed the variability associated with MIA models as males were heavier than females at P25 and P28 in the first cohort, an effect not seen in the second cohort. In the open field at P25 in the first cohort 10 mg/kg males showed more exploratory behaviour on day 1 compared to control males and 5 mg/kg males as measured by time in the centre with no observed sex effect. However, in the second cohort there was a sex effect when considering time in the centre of the open field with females from both groups showing less exploratory behaviour than males, on both days. In the first cohort stimulus directed interaction at P28 showed a sex effect with stimulus animals apparently more interested in males than females. This was not apparent in the second cohort but there was an interaction with sex and weight with stimulus animals seemingly more interested in heavier females.

3.5.1 Maternal immune activation

Administration of poly(I:C) has consistently demonstrated the production of hypothermic response, causation of weight loss/ inhibition weight gain and induction of sickness behaviours (Mueller et al. 2019; Estes et al. 2020; Tillmann et al. 2024). The work here replicated these finding with 10 mg/kg poly(I:C) administration at E12.5 in two independent cohorts validating the immune activation within the dams. Furthermore, a lower dose of 5 mg/kg prevented weight gain in the 24-hours post injection, induced some sickness behaviour, primarily lethargy but did not produce a hypothermic response. This reinforces the robust immune activation potential of poly(I:C). Neither 5 nor 10 mg/kg influenced litter size. This agrees with previous

work that demonstrated LMW poly(I:C) from Invivogen did not impact litter sizes in rats (Kowash et al. 2019). A key strength of this work was the use of non-invasive methods to validate immune activation, even surface temperature was measured with an inexpensive pocket IR thermometer, which has applications in general animal welfare not just MIA models. With that said, there are some clear refinements to be made. C57BL/6J mice were selected because they lack segmented filamentous bacteria which can modulate outcomes in MIA models (Kim et al. 2017), and this was viewed here as a potential undesirable phenotype for combination with a genetic model. However, the mice used here were not assessed for possessing SFB. Whilst it has been demonstrated that LMW poly(I:C) from Invivogen is more consistent with regards to poly(I:C) fragment size there was no quality check inspecting fragment size in the lot used for these experiments. This is also true for assessment of endotoxin contamination. Baseline immunoreactivity of the dams was not assessed either, this has been shown to modulate phenotypic outcomes in offspring (Estes et al. 2020). These steps would have increased confidence in the findings presented here but the omission of them does not invalidate the observations regarding immune activation.

3.5.2 P9 homing behaviour

Assessment of homing behaviour has been assessed previously in an MIA model using C57BL/6 mice and no deficits were observed (Morais et al. 2018). The work here observed a high proportion of (26/50) 10 mg/kg offspring did not find the home nest in time indicating lower levels of maternal attachment possibly caused by olfaction deficits (Moles et al. 2004; Muroyama et al. 2016). The main source of disagreement with other work likely comes down to technical differences. The assay here is designed to more challenging; the home nest is nesting material and is a small target within a relatively large arena which is like Muroyama et al. (2016b). However most other paradigms cover large proportions of the arena in home cage bedding and making locating the home nesting material easier (Morais et al. 2018; Luchetti et al. 2021; Morais et al. 2021). Even in Muroyama et al. (2016), the virtually demarked home nest zone was a third of the arena whereas the home nest zone here was just the home nest material which covered a much smaller proportion of the arena than other tests (Fig 2.3). Furthermore, the test here is shorter, only 3

minutes with no habituation period, this was a conscious decision to test maternal attachment as a reflex whilst minimising the potential stress of separation and colder temperatures being outside the nest. There was no effect of MIA on initial levels of activity during the homing test, but 10 mg/kg animals did appear to have lower levels of activity (Fig 3.4a). The absence of homing could be driven by a hypoactive phenotype, or the animals may appear hypoactive due to lower levels of social motivation. It is an important consideration as initial activity is strongly correlated with locating the home nest in homing animals. The lower numbers of homing animals in the 10 mg/kg group may be due to a developmental delay but from an activity and physical perspective i.e. weight this did not appear to be the case.

If development was delayed it would be expected to be reflected in higher numbers of calls in non-homing animals. This appeared to be true for controls and 10 mg/kg animals, but analysis did not support this. The unbalanced group numbers and variability in calling may have masked a true effect in differences between homing and non-homing animals. In homing animals longer latency to locate the home nest predicts higher numbers of emitted calls, which may reflect adaptive communication. The animals can detect the social cue and may call at increasing rates as their affective state changes. This makes sense as the presence of home nesting material can reduce USV emission whilst distress induces increased calling (Moles et al. 2004), though in a rat poly(I:C) model of MIA the calming effect of home nesting material was not present in male pups (Potasiewicz et al. 2020) This work appears to be the first effort in recording USVs during the homing test in an MIA model. Studies typically assess isolation-induced USVs in pups and whilst this work examines calling in a different context. The absence of an MIA effect on call number agrees with Morais et al. (2018) but disagrees with other reports. For example, Malkova et al. (2012) observed reduced calling at P8 and P10 in MIA males, which agrees with a report of reduced UVS at P6 in poly(I:C) exposed male rats (Potasiewicz et al. 2020). Direct comparisons between these findings are difficult given the different contexts. However, observations of reduced isolation-induced calls and an absence of reduced calling in presence of homing nest material in males of the same MIA model support the notion that social communication is not perturbed in this work (Potasiewicz et al. 2020). It also points to the need to establish baseline calling behaviour through isolation-induced calling. A refinement to this protocol could be to partition call traces

in before and after finding the nest as it would be expected that animals would call less once the nest is located given previous findings. This is partially supported by the data here given animals with longer latencies emit more calls. Similarly, calling duration increased with time to find the nest, pointing to an adaptation to social context. Mean duration as with number of calls emitted does not appear to be influenced by maternal immune activation.

3.5.3 Open field activity

MIA did not appear to influence activity in the open field at P25, a finding reproduced in two independent cohorts. This finding is supported by recent work using the same vendor for poly(I:C) and found no effect of MIA on activity levels in 8-week-old males and females, at 10 mg/kg and 20 mg/kg (Tillmann et al. 2024). It is also supported by Mueller et al. (2021) who saw no effect of MIA on open field activity in adult mice (12 weeks-old) whether the litter or the individual was considered as the n. Rat MIA models investigating behaviour in juveniles (P35-39) found also no effect of MIA on activity levels in the open field for males and females (Su et al. 2022; Lan et al. 2023). Poly(I:C) has been reported to reduce activity (Malkova et al. 2012) and increase activity (Vigli et al. 2020). These differences may arise from technical differences, the open field here was carried out in the dark but isn't in Malkova et al. (2012). In the protocol here the test was only 20 minutes, whereas Vigli et al. (2020) used a 1-hour long protocol but it is worth noting that in the first 20 minutes there were no differences between control and MIA offspring, supporting the observation reported here. For both cohorts, controls and 10mg/kg groups, male and females showed habituation from day 1 to day 2. However, in cohort 1 males from the 5 mg/kg group did not display a reduction in activity that indicates habituation which is typically interpreted as a potential deficit in non-associative learning (Leussis and Bolivar 2006). However, whilst the males in the 5 mg/kg group may not show a statistically significant reduction from day 1 to day 2, activity levels split into 1-minute time bins suggest intact intra- and intersession habituation to the open field (Fig 3.9). This finding needs further interrogation to see if it can be reproduced but the focus of the open field protocol here was identifying differences in activity that may confound results observed in social tests that followed, as they we carried out in the same

arena. There are no reports of intersession habituation to the open field in MIA models to allow comparison of findings. Increased exploratory behaviour in MIA offspring has not been previously reported but here males from the 10 mg/kg group spent more time in the centre of the open field on day 1 than their control counterparts. Males and females from the 10 mg/kg group appeared to spend less time in the corners of the open field which suggests increased exploratory behaviour (Gillette et al. 2014). Reduced exploration in female MIA rat offspring has been reported, with no change in males (Su et al. 2022; Lan et al. 2023). There was no increase in exploration in MIA animals in a second independent cohort where a sex difference exists with males showing more exploratory behaviour than females as measured by time spent in the centre. These differences highlight the sensitivity of MIA models to unintentional sources of variation (Weber-Stadlbauer and Meyer 2019). One such source may have been the time of year the animals were tested, winter v spring having interactive effects with the MIA group. It may also have arisen due to the skewed sex ratios in the 10 mg/kg litters of the first cohort where the ratio of males to females was more than 2:1.

3.5.4 P26 and P27 Social and object novelty recognition

Social novelty recognition appeared reduced in offspring of 10 mg/kg poly(I:C) exposed dam at P26/P27 whilst habituation appeared unaffected. There were no observed sex effects. This agrees with the observation that P35 MIA mice, males and females show intact sociability but no preference for social novelty (O'Leary et al. 2014). The work here assessed the recognition in a habituation-based test not the three-chamber paradigm. Schaafsma et al. (2017), used a similar paradigm at P45 to investigate the interactive effects in a *Cntnap2* mouse model exposed to LPS-induced MIA and found impaired habituation and recognition in male mutant MIA offspring but not effects of MIA on WTs. The protocol used here was adapted from Hörnberg et al. (2020), testing mice at a similar age, though the mice hear were tested prior to weaning not post-weaning. The performance of offspring in the 10mg/kg poly(I:C) group here appeared to phenocopy the *Nlgn3* KO mice displaying intact social habituation but perturbed social novelty recognition. This is suggestive of a convergent pathological mechanism between *Nlgn3* KO and MIA via poly(I:C). Impaired social recognition is a feature of ASD making the observed phenotype here

particularly relevant. However, this is the first time this has been demonstrated in a poly(I:C) model in mice this young thus it is important to reproduce this finding in an independent cohort. There is an argument that the S1 and S2 mice may have been too similar given they were siblings, but two of the three groups could discriminate the novelty of S2 suggesting a genuine deficit in the 10 mg/kg group. This may be more of a valid argument for the object portion of the test which was designed to be equally as challenging. The two stimuli used we a toy tapir and echidna that were made from the same material and were similar size and weight thus there were less features to use in discrimination. All groups showed habituation to objects, but none showed a preference for novelty suggesting the discrimination may have been too challenging. Recognition indices were calculated by subtracting interaction times (trial 5 - trial 4). For controls and the 5 mg/kg group social recognition indices were greater than object recognition indices but not in the 10 mg/kg, providing support for impaired social novelty recognition in this group.

3.5.5 P28 Direct social interaction

Having identified potential deficits in the first two social tests, the P28 direction interaction test did not reveal an overall effect of MIA on the time spent in interaction with an adult female mouse. A sex dependent effect was observed with female controls showing higher interaction times than females in the 10 mg/kg group. This was not replicated in a second cohort though general patterns were conserved between cohorts, namely female controls appear to spend more time in social interaction than MIA counterparts. The consensus between cohorts was that MIA does not produce an overt social phenotype in this test. The difference between control and MIA females in the first cohort may have been a genuine effect but unplanned sources of variation may have contributed to this observation. One was identified via analysis which revealed the mice that underwent the social novelty recognition test spent less time in interaction in at P28. This was evident in all groups, but the impact may have been greater in magnitude for some groups i.e. 10 mg/kg females driving the difference reported. Another contributing source of variation could be the sex ratio of the 10 mg/kg group in the first cohort that was skewed towards males. This would have meant a potential altered social environment for the females compared to other groups which may have influenced

social behaviour. No sex dependent effects were reported in the social novelty recognition test so the variation was likely specific to the P28 test and may have arisen from the stimulus animals. The animals used as stimulus in social tests are crucial to social interactions but are typically overlooked in analysis (Jabarin et al. 2022). To address this, the behaviour of the stimulus was record here. MIA did not appear to affect stimulus behaviour. Stimulus directed interaction displayed a sex effect in the first cohort with stimulus animals showing higher levels of interest in males, an effect not seen in the second cohort. There appeared to be an interactive effect of sex and weight in the second cohort with stimulus animals showing increased levels of interest in females. Whilst this differs to the first cohort it suggests larger animals, which may be more developed are more salient to the stimulus mice. Males in the first cohort were heavier than females at the time of testing showing agreement with the interest in heavier females in the second cohort. This observation is another example of the sensitivity of MIA protocols to unintentional environmental influences. It was highlighted that the general laboratory environment can impact the outcomes of MIA protocols. The exact same protocol used in two different labs produced a phenotype in the offspring in one lab but not the other (Tillmann et al. 2024). Therefore, pursuing the same MIA protocol in the same lab 5-6 months apart is likely sensitive to small variations like those observed here, especially when examining something as complex and inherently variable as social behaviour. Reports of social behaviour impairments in MIA offspring are commonplace but there are no reports of direct interaction tests prior to weaning. In the context of social behaviour assays a key strength of this test is the consideration of stimulus behaviour but a major limitation is the reduction approach to the variables measured.

Importantly, males and females in the 5 mg/kg group did not display any differences in interaction times or stimulus interaction times meaning there was no aberrant social phenotypes in any of the three pre-weaning tests thus 5 mg/kg was confirmed as the sub-threshold dose to use in the gene x environment model with *Cyfip1*$^{+/-}$.

3.6 Conclusions

The work here successfully identified a dose of poly(I:C) that stimulates the immune system of pregnant dams without impacting the social behaviour of young mice as assayed in three tests, P9 homing, P26 social novelty and P28 Direct social interaction. Using a higher dose, 10 mg/kg this work demonstrated a potential deficit in social interest at P9 with pups more likely to display absent homing behaviour suggesting lower levels of maternal attachment. This appears to be the first such identification of a social interest deficit observed in mice this young when using an MIA protocol. Mice born to dams exposed to the higher dose of poly(I:C) displayed reduced interest in social novelty at P26 and P27 but did not display a reduction in social interest at P28 highlighting the need to probe social behaviour with different tests. A second independent cohort only experienced one social test, the P28 social interaction and reproduced the finding that 10 mg/kg of poly(I:C) did not impact social interest as measured by this test. This observation validates the use of multiple social tests in early life, though the order of tests is worth consideration as mice that underwent social novelty test at P26 and P27 showed lower levels of interaction at P28. Therefore, it may be better to introduce more time between tests or place the most demanding task last. Together the findings from examining social behaviour prior to weaning highlight the ability of MIA in modelling social endophenotypes. Studying behaviour early in development may be a more appropriate approach in the context of social impairment arising from neurodevelopmental disorders compared to phenotyping only adult animals.

Chapter 4 : The combined effect of MIA and *Cyfip1* haploinsufficiency on the early life social behaviour of mice.

4.1 Introduction

ASD are a group of highly heterogeneous neurodevelopmental disorders that manifest in the early years of life (Lord et al. 2020c). Characterized by impairments in social communication, restricted interests and repetitive behaviour and processing sensory information (Lai et al. 2014). Attempts to unpick the aetiology of ASD have revealed a strong genetic contribution for ASD risk but nearly all genetic risk factors for ASD can be found in individuals without a diagnosis as demonstrated by carriers of 16p11.2 deletions, most of whom do not cross diagnostic thresholds (Hanson et al. 2015). This suggests the need for additional risk factors to yield phenotypes that meet diagnostic criteria. It is now understood that ASD manifests following the interaction of a combination of genetic and environmental factors that influence brain development (Chaste and Leboyer 2012).

The core domains by which ASD is diagnosed are very broad, facilitating the phenotypic heterogeneity observed in ASD. Furthermore, the categorical nature of neuropsychiatric diagnoses does not account for intermediate phenotypes nor the overlap of symptoms with other conditions. Social behaviour can be profoundly impacted in numerous conditions such as schizophrenia and major depression, not just in ASD. Furthermore, social behaviour in healthy populations is highly variable (Robinson et al. 2011) and unaffected family members of individuals with ASD often display sub-threshold traits (Murphy et al. 2000). Genetic influences on ASD risk also influence variation of social behaviour in the general population (Robinson et al. 2016b). This points to symptoms of ASD existing at the extreme tails on a continuum of normality implying the same biological mechanisms underpin normal and pathological brain functions. The RDoC framework also supports this view, promoting the modelling of endophenotypes i.e. symptoms when using animals rather than attempting to recapitulate a nosological entity (Anderzhanova et al. 2017). Developing multiple-hit preclinical models of specific endophenotypes may offer further insight into the complex aetiology and presentation of ASD adding to

existing knowledge gained from single gene models aimed at fully replicating ASD symptomology. The hope is such an approach will increase the translatability of basic research to clinical populations. In Chapter 3, the viral mimetic poly(I:C) was used to induce MIA and a dosage of 5 mg/kg at E12.5 stimulated the immune system of dams without producing social behaviour deficits in offspring at P9 or P28. The work here builds on Chapter 3 examining a gene x environment model by administering 5 mg/kg to dams mated with *Cyfip1*$^{+/-}$ males before assessing social behaviour early development i.e. pre-weaning.

CYFIP1 is one of four genes (*NIPA1, NIPA2, TUBGCP5,* the other 3) located within the 15q11.2 locus and considered to be the prime candidate for causing phenotypes associated with 15q11.2 BP1-BP2 CNV that is linked with a range of neurodevelopmental and psychiatric conditions, including ASD(Clifton et al. 2020). In the context of ASD*, CYFIP*1 confers risk though 15q11.2 CNVs, common variants and SNVs (van der Zwaag et al. 2010; Burnside et al. 2011; Waltes et al. 2014; Wang et al. 2015; Mariano et al. 2024). Additionally reports of altered (high and low) levels of *CYFIP1* mRNA in patients with ASD highlight the influence of gene dosage (Nowicki et al. 2007; van der Zwaag et al. 2010). *Cyfip1* animal models consistently report biological phenotypes that overlap with other preclinical models of ASD, but behavioural phenotypes are mild, with social deficits rarely reported (Clifton et al. 2020). *Cyfip1* cKO adult (P90-120) male mice displayed reduced social approach and recognition behaviour whereas conditional *Cyfip1* overexpression mice only displayed altered social recognition behaviour (Kim et al. 2022). In a haploinsufficient *Cyfip1* mouse model that may more accurately model reduced CYFIP1 levels in humans, Bachmann et al. (2019)demonstrated reduced interest of *Cyfip1* adult (P60+) male mice in social odours compared to their WT littermates. In the same model, Sledziowska et al. (2020)reported a similar deficit in *Cyfip1*$^{+/-}$ adult (P60-70) female mice without replicating the finding in males. The lack of a robust social phenotype in genetic single-hit *Cyfip1* models may be representative of human carriers of the mutation, many of whom don't display behavioural phenotypes (Cafferkey et al. 2014) though carriers who present as typically developing, may possess subclinical phenotypes (Stefansson et al. 2014). The low penetrance of *CYFIP1* variants coupled with an associated risk for a range of neurodevelopment and psychiatric conditions suggests phenotypes associated with altered *CYFIP1*

dosage may require secondary hits to produce clinical phenotypes. The additional hits may be either genetic or environmental, such as infection during pregnancy which, like *CYFIP1* is recognised as a risk factor for a range of neuropsychiatric conditions including ASD, schizophrenia and bipolar disorder (Brown and Meyer 2018).

MIA is used to model infection during pregnancy using various agents that stimulate the immune system including poly(I:C) and the bacterial endotoxin LPS (Brown and Meyer 2018). MIA via poly(I:C) administration has been used in combination with several genetic models to study the interactive effects on phenotypes relevant to neuropsychiatric disorders, such as social behaviour impairment. Evidence for MIA interacting with genetic factors known to impact behavioural phenotypes comes from studies of gene x environment models, targeting genetic loci associated with a range of neurodevelopmental conditions. Haddad et al. (2023) reported a synergistic interaction between MIA via poly(I:C) at E9.5 and a *Cntnap2*$^{-/-}$ genotype to decrease pre-pulse inhibition (PPI) in male juvenile (P47-48) *Cntnap2*$^{-/-}$ rats, but not juvenile females. Furthermore, this effect was not evident when the males were fully developed (P90+). There were no reported interactive effects regarding social behaviour. Schaafsma et al. (2017) demonstrated MIA and *Cntnap2* interact to produce male offspring that display lower levels of vocalisation at P3, compared to MIA or mutation alone. A similar interaction was seen as MIA *Cntnap2* males also lacked social recognition at P45. The interactive effects observed did not impact the female mice tested. In *DISC1* models, social interest measured by the 3-chamber paradigm was diminished in mutants exposed to MIA, unlike WTs exposed to MIA (Abazyan et al. 2010). A finding reproduced in the *DISC1* model used by Lipina et al. (2013). In a *Shank*3Δ11−/− model, MIA appeared to have an additive effect increasing the social deficits observed in adult male mice (P150+) compared to single hit models. The double-hit reduced sociability and social novelty preference in the three-chamber and reduced social approach in a resident intruder paradigm. MIA-only males did not display social deficits and *Shank*3Δ11−/− only males showed intact sociability but not social novelty preference (Atanasova et al. 2023). These works highlight the ability of MIA to interact in an additive fashion with ASD risk genes to influence social behaviour in animal models.

Variants of *CYFIP1* have low penetrance, meaning most carriers will typically not have a diagnosis for the neuropsychiatric disorders *CYFIP1* is associated with. People with deletions of the 15q11.2 interval present with pathological phenotypes around 10% of the time (Kirov et al. 2014). The low penetrance of this CNV is reinforced by estimates suggesting it is inherited from unaffected parents more often than affected parents (Cox and Butler 2015). In a study of 2 probands, each carrying 2 missense point mutations in *CYFIP1* and presenting with complex phenotypes including developmental delay and ASD it was revealed both SNVs were inherited, one from each parent. The parents were unaffected as was a sibling with only one SNV, reflecting the low penetrance of possessing a single *CYFIP1* variant (Mariano et al. 2024). Low penetrance is potentially beneficial in developing gene x environment models as it may avoid ceiling effects associated with using a penetrant risk factor. The 15q13.3 microdeletion is like *CYFIP1* variants in being low in penetrance and varied in clinical presentation (Ben-Shachar et al. 2009; Shinawi et al. 2009). A hemizygous 15q13.3 microdeletion mouse model displayed an interaction with peripubertal stress to impair sensorimotor gating. It also demonstrated interactive effects in locomotor reaction to amphetamine administration. Hemizygotes exposed to peripubertal stress showed an increase locomotor response to amphetamine compared to hemizygotes who did not. This effect was reversed in WTs with mice exposed to peripubertal stress showing decrease amphetamine sensitivity compared to WTs that did not experience peripubertal stress displaying (Giovanoli et al. 2019). No social phenotype arose through this interaction, but the work highlights the potential in using less penetrant genetic insults to determine gene x environment interactions. Giovanoli et al. (2019), also highlighted the importance of timing when it comes to environmental insults as the interactive effects were observed when stress exposure occurred P30-40 but were absent when exposure took place P50-60.

Infection during pregnancy is associated with risk for a number of neuropsychiatric disorders that are also associated with *CYFIP1* variants, such as ASD, schizophrenia and major depression (Reisinger et al. 2015; Brown and Meyer 2018). This phenotypic overlap suggests both factors may converge on common biological pathways. MIA is well-established as a model for infection during

pregnancy and protocols that utilise poly(I:C) to stimulate the dam immune system consistently demonstrate altered social behaviour in offspring (Kentner et al. 2019). This makes MIA an attractive candidate as a second hit to combine with *Cyfip1* haploinsufficiency in examining potential interactive effects on early life social behaviour. Modelling a specific endophenotype is hoped to improve the translatability of animal models but there still needs to correspondence between symptom-based models and clinical symptoms. Therefore, as ASD manifests in early life if is important to study social behaviour early in development. This will aid the contextualisation of any findings as typical social behaviour will be different in young versus fully developed mice and similarly in a child compared to an adult. In the context of gene x environment interactions, it is important to study more than one timepoint in development as interactive effects may be temporally sensitive. The same phenotype may arise in both the two-hit and one-hit models, but an interaction may cause an impairment to manifest earlier or delay its presentation.

ASD is diagnosed more in males than females, a contributing factor to this sex bias is a dysmorphism in the presentation of ASD with social dysfunction potentially arising later in females (Lai and Szatmari 2020). This is reflective of sex differences in typical social behaviour, and if the desire is to study ASD through endophenotypes that exist on a continuum of normality then it is important to study both sexes. Furthermore, gene x environment interaction models provide an opportunity to integrate the biological basis of the sex bias observed in ASD. For example, one explanation for the sex bias is the female protective effect which posits females require greater disruption to biological pathways to present with ASD symptoms (Ferri et al. 2018). A multiple hit model will test this because at a very basic level a single-hit model might produce males with a social behaviour phenotype but not for females whereas, a double-hit would offer greater disruption so females exposed to the double-hit may present with a phenotype that looks like males in the single-hit model. Furthermore, the presence of interactive effects in only one sex provides a chance to probe the basis of this difference which can speed up the elucidation of mechanisms underlying any observed interaction.

A consideration when examining social behaviour is the influence of the social environment. The haploinsufficient *Cyfip1* mouse model produces litters of mixed

genotypes, WTs and *Cyfip1*$^{+/-}$. The presence of two genotypes can itself modulate the social phenotype observed in both the WT and mutant, as outlined in Section 1.5.2.2. This is understandable in the context of social behaviour, if gene dosage of *Cyfip1* is important for social behaviour in an individual, it will be important for the group, especially a developing group where social behavioural responses are being learned. This is an additional reason to study both males and females as the modulatory effect of mixed genotypes can be sex dependent (Kalbassi et al. 2017). In this chapter, we describe the early social behavioural phenotypes of *Cyfip1*$^{+/-}$ offspring born to dams exposed to poly(I:C). To provide insight into potential sex differences male and female mice were studied. The impact of mixed genotype litters was considered by analysing the effect of the proportion of *Cyfip1*$^{+/-}$ mice in a litter had on WT social behaviour.

4.2 Aims and objectives

Building on the work of Chapter 3, the work here aimed to determine if MIA at a lower dose of 5 mg/kg and haploinsufficiency of *Cyfip1* interact to affect the social behaviour of male and female mice early in development i.e. pre-weaning. To achieve this aim, the following study will.

1. Characterise the early social behaviour of MIA exposed offspring to determine if
    a. *Cyfip1*$^{+/-}$ and MIA interact to produce different social behaviour phenotypes compared to *Cyfip1*$^{+/-}$ or MIA alone.
    b. *Cyfip1* is important in the development of social behaviour by comparing *Cyfip1*$^{+/-}$ mice with WT littermates.
    c. There is a sex difference in the behaviour of *Cyfip1*$^{+/-}$ mice born to MIA and control dams.
2. Determine if the presence of *Cyfip1*$^{+/-}$ mice in a litter modulates the behaviour of WT littermates.

4.3 Methods

In this work female WT C57BL/6J mice underwent timed-mating with *Cyfip1*$^{+/-}$ males to generate an experimental cohort for behavioural testing. Breeding took place during June and July, with mice housed in the same regulated holding room as the experimental cohorts used in Chapter 3, temperature (21.5°C ± 0.75°C) and humidity (47% ± 10%). Pregnant dams underwent the MIA protocol described in Section 2.3. The numbers of offspring generated including genotype split and sex ratios are found in Table 4.1. Offspring underwent 3 behavioural tests. At P9, prior to eyes opening, mice underwent the homing test where calling behaviour was also recorded. This protocol was updated from Chapter 3 and pup retrieval times were assessed following the test. Activity levels were assessed in the open field at P24 & 25 before mice were finally tested in a direct social interaction assay, carried out in the dark at P28. *Cyfip1*$^{+/-}$ offspring born to dams exposed to poly(I:C) were compared to their WT littermates as well as control WT and *Cyfip1*$^{+/-}$ offspring in all 3 tests. Social tests were selected and adapted to place a greater emphasis on olfaction for acquiring social cues, outlined in Section 2.4.1. The numbers of mice used in each test are reported in Table 4.2.

Table 4.1 Numbers of offspring produced via timed mating.

Group	Sires	Litters	Offspring at P0	Offspring at P7	Males: Females	Males (WT: *Cyfip1*$^{+/-}$)	Females (WT: *Cyfip1*$^{+/-}$)
Controls	11	16	117	114	48:66	16:32	36:30
5 mg/kg	9	15	115	115	56:59	30:26	29:30

Table 4.2 Numbers of offspring used in testing.

Group			P9 Homing	Proportion of non-homing mice	P25 Activity	P28 Social interaction
Genotype	**MIA**	**Sex**				
WT	Control	Male	16	0.3	16	16
		Female	36	0.25	34	34
	5 mg/kg	Male	30	0.3	30	30
		Female	29	0.17	29	29
Cyfip1$^{+/-}$	Control	Male	32	0.19	29	29
		Female	30	0.3	30	30
	5 mg/kg	Male	26	0.27	26	26
		Female	30	0.23	30	30

4.4 Results

Table 4.3 Summary of results for Wildtype mice

	WT Control		WT MIA	
Assay	**Males**	**Females**	**Males**	**Females**
P9 Homing				
Latency to find home nest	=	=	=	=
USVs (no. of. Calls)	=	=	=	=
USVs (mean duration)	=	=	=	=
Retrieval time	=	=	=	=
P25 Open Field				
Distance	=	=	=	=
Habituation	Present	Present	Present	Present
P28 Social Interaction				
Interaction time	=	=	=	=
Stimulus directed interaction	=	=	↓	↓
Effect of higher proportion of Cyfip1$^{+/-}$ mice on interaction time	↓	↓	↓	↓

Table 4.4 Summary of results for *Cyfip1*$^{+/-}$ mice

Assay	*Cyfip1*$^{+/-}$ Control Males	*Cyfip1*$^{+/-}$ Control Females	*Cyfip1*$^{+/-}$ MIA Males	*Cyfip1*$^{+/-}$ MIA Females
P9 Homing				
Latency to find home nest	=	=	↑	=
USVs (no. of. Calls)	=	=	=	=
USVs (mean duration)	=	=	=	=
Retrieval time	=	=	=	=
P25 Open Field				
Distance	=	=	=	=
Habituation	Present	Present	Present	Present
P28 Social Interaction				
Interaction time	↓	↓	↓	↓
Stimulus directed interaction	=	=	=	=
Effect of higher proportion of Cyfip1$^{+/-}$ *mice on interaction time*	↓	↓	↓	↓

4.4.1 Low dose of poly(I:C) triggers immune response in pregnant dams

In the 24 hours following injection the weight of control dams increased while the weight of dams exposed to poly(I:C) decreased (Figure.4.1a), main effects of ***dose*** (LMM($F_{[1,33]}$ = 4.3, P < 0.05)), ***time*** (LMM($F_{[1,66]}$ =8.6, P<0.001)) and a ***dose*** x ***time*** interaction (LMM($F_{[1,66]}$ = 20.2, P < 0.001)) when analysed with the model ***weight*** ~ ***dose*** x ***time*** + ***(1 | dam)***. Control dams showed an increase in weight, 24 hours post injection ($t_{[1,66]}$ = -6.588, p < 0.001), driving a difference in weight between groups at 24 hours ($t_{[1,41.4]}$ = 4, p < 0.001). This model returns an ICC = 0.83, a marginal R^2 = 0.17 and a conditional R^2 = 0.86. MIA dams showed a reduction in surface temperature 3 hours after injection whereas control dams did not (Figure.4.1b), main effects of ***dose*** (LMM($F_{[1,33]}$ = 4.2, P < 0.05)), ***time*** (LMM($F_{[1,33]}$ = 54.8, P < 0.001))

and a ***dose*** x ***time*** interaction (LMM($F_{[1,33]}$ = 32.6, P < 0.001)), when analysed with the model ***temperature ~ dose*** x ***time*** + ***(1 | dam)***. MIA animals were colder than controls ($t_{[1,45.2]}$ = 4.1, p < 0.001) 3 hours post injection. This model returns an ICC = 0.68, a marginal R^2 = 0.33 and a conditional R^2 = 0.79. All MIA dams displayed some sickness behaviour e.g., lethargy 3 hours after injection and no control dams showed sickness behaviour. No dams from either group displayed sickness behaviour 24hrs post injection (Figure. 4.1c). The differential in behavioural and physiological immune responses between groups did not lead to significant differences in litter sizes (Figure. 4.1d), (Mann-Whitney-Wilcoxon (W = 125, P = 0.85)). MIA with a low dose of poly(I:C) causes a hypothermic response, presentation of sickness behaviours and prevents the weight gain seen in unchallenged dams, all without affecting litter size between groups.

4.4.2 MIA offspring are lighter than control offspring after P13

Males appear heavier than females across all groups, at every timepoint measured (Figure. 4.2), main effect of ***sex*** (npLMM ($t_{[1]}$ = 7.8, P < 0.01)). At P25 and P28 MIA offspring appear lighter, main effect of ***dose*** (npLMM ($t_{[1]}$ = 7.5, P < 0.01)) and a ***dose*** x ***timepoint*** interaction (npLMM ($t_{[2.2]}$ = 8.6, P < 0.001)). There was no effect of ***genotype*** (npLMM ($t_{[1]}$ < 0.001, P < 0.99)). Males are heavier than females from early in development with control offspring becoming heavier than MIA offspring after P13.

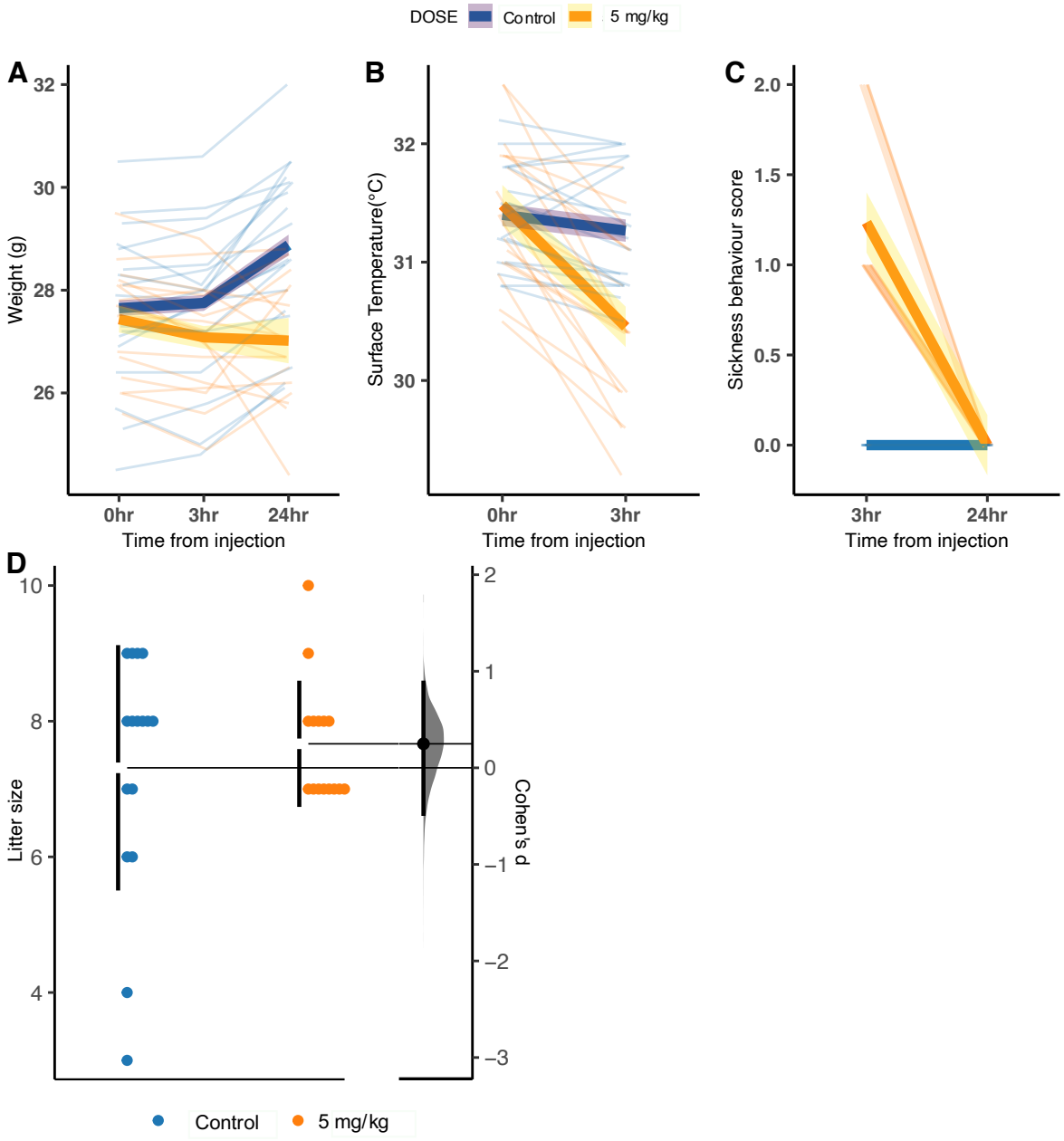


Figure 4.1 The response of dams to maternal immune activation (MIA) via 5 mg/kg of LMW poly(I:C). **A**. Weight change following MIA. **B**. Surface temperature change following MIA. **C**. Sickness behaviour score following MIA. **D**. Estimation plot showing litter sizes and Cohen's d effect sizes for control and MIA groups. Ribbons in A, B, C represent 95% confidence intervals. A, B, C 0mg/kg n= 18, 5mg/kg n=17 and D n= 16, 5mg/kg n=15.

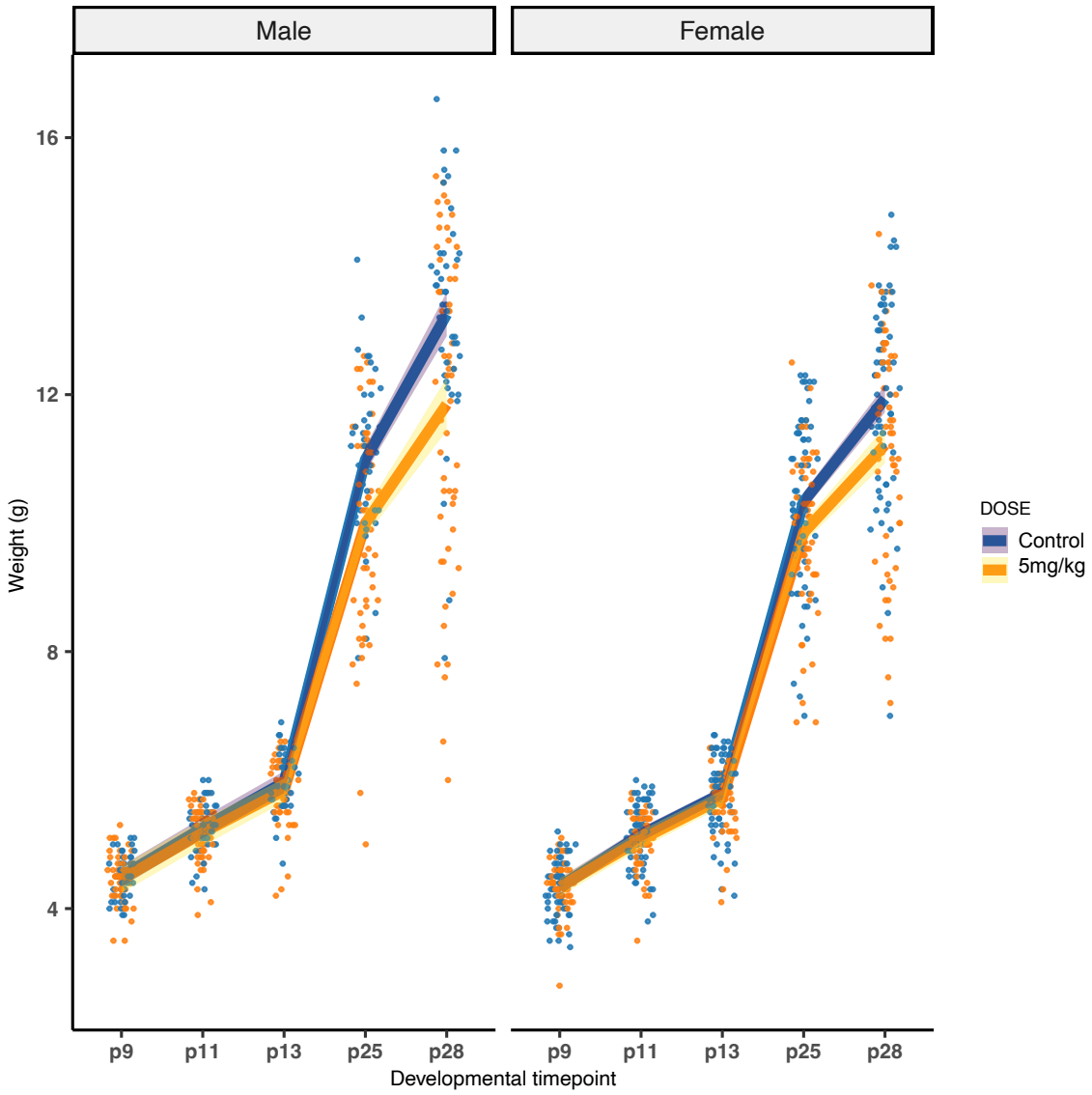


Figure 4.2. Longitudinal weight measurements for control and MIA offspring. Bold lines and ribbons are means and CIs respectively with dots representing raw data *n* = 45 control males, 64 female controls, 56 male MIA and 59 females MIA offspring.

4.4.3 MIA affects homing behaviour of *Cyfip1*$^{+/-}$ offspring in a sex dependent fashion but not wild type littermates

Offspring at P9 were more likely to display homing behaviour than not (GLMM (link=logit (OR = 3, 95% CI [2.24,4.09], $P < 0.001$). For the whole cohort the total proportion of non-homing offspring was 25% (57/229), Table 4.2 contains the proportions of non-homing mice for each group. The absence of homing behaviour was not affected by ***genotype*** (GLMM (link=logit (OR = 1.03, 95% CI [0.67,1.59], $P = 0.88$), ***dose*** (GLMM (link=logit (OR = 1.05, 95% CI [0.69,1.61], $P = 0.82$) or ***sex*** (GLMM (link=logit (OR = 1.08, 95% CI [0.71,1.66], $P = 0.71$). For homing offspring, the time taken to find the home nest did not differ across WT groups but did for *Cyfip1*$^{+/-}$ groups (Figure.4.3a). Main effects were observed, ***dose*** (LM ($F_{[1,156]} = 4$, $P < 0.05$)) and ***1st minute distance travelled*** (LM ($F_{[1,156]} = 11.2$, $P < 0.01$)), (Figure. 4.3b) as well as a three-way interaction ***genotype*** *x* ***dose*** *x* ***sex*** (LM ($F_{[1,156]} = 5.4$, $P < 0.05$)), (Figure. 4.3c) when analysed with the model ***Latency to find home nest ~ 1st minute distance travelled + genotype*** x ***dose*** x ***sex,*** which has an adjusted $R^2 = 0.11$. No effects were observed for ***genotype*** (LM ($F_{[1,156]} = 0.001$, $P = 0.97$)) or ***sex*** (LM ($F_{[1,156]} = 2.9$, $P = 0.09$)) Post-hoc comparisons show *Cyfip1*$^{+/-}$male MIA offspring took longer to find the nesting material than *Cyfip1*$^{+/-}$male control offspring, ($t_{[156]} = 3.3$, $p < 0.01$) and *Cyfip1*$^{+/-}$female MIA offspring, ($t_{[156]} = 2.7$, $p < 0.05$).

Considering the *n* to be the litter, thus using the average times per litter (Figure.4.4a) there is no longer a main effect of ***dose*** (LM ($F_{[1,81]} = 0.31$, $P = 0.58$)), however the main effect of ***1st minute distance travelled*** (LM ($F_{[1,81]} = 5.6$, $P < 0.05$)), (Figure. 4.4b) and the three -way interaction ***genotype*** x ***dose*** x ***sex*** remains (LM ($F_{[1,81]} = 4.1$, $P < 0.05$)), when analysed with the model ***Latency to find home nest ~ 1st minute distance travelled + genotype*** x ***dose*** x ***sex***. Patterns of behaviour remain the same when comparing *n* = individual animal v litter averages (Figure. 4.4c).

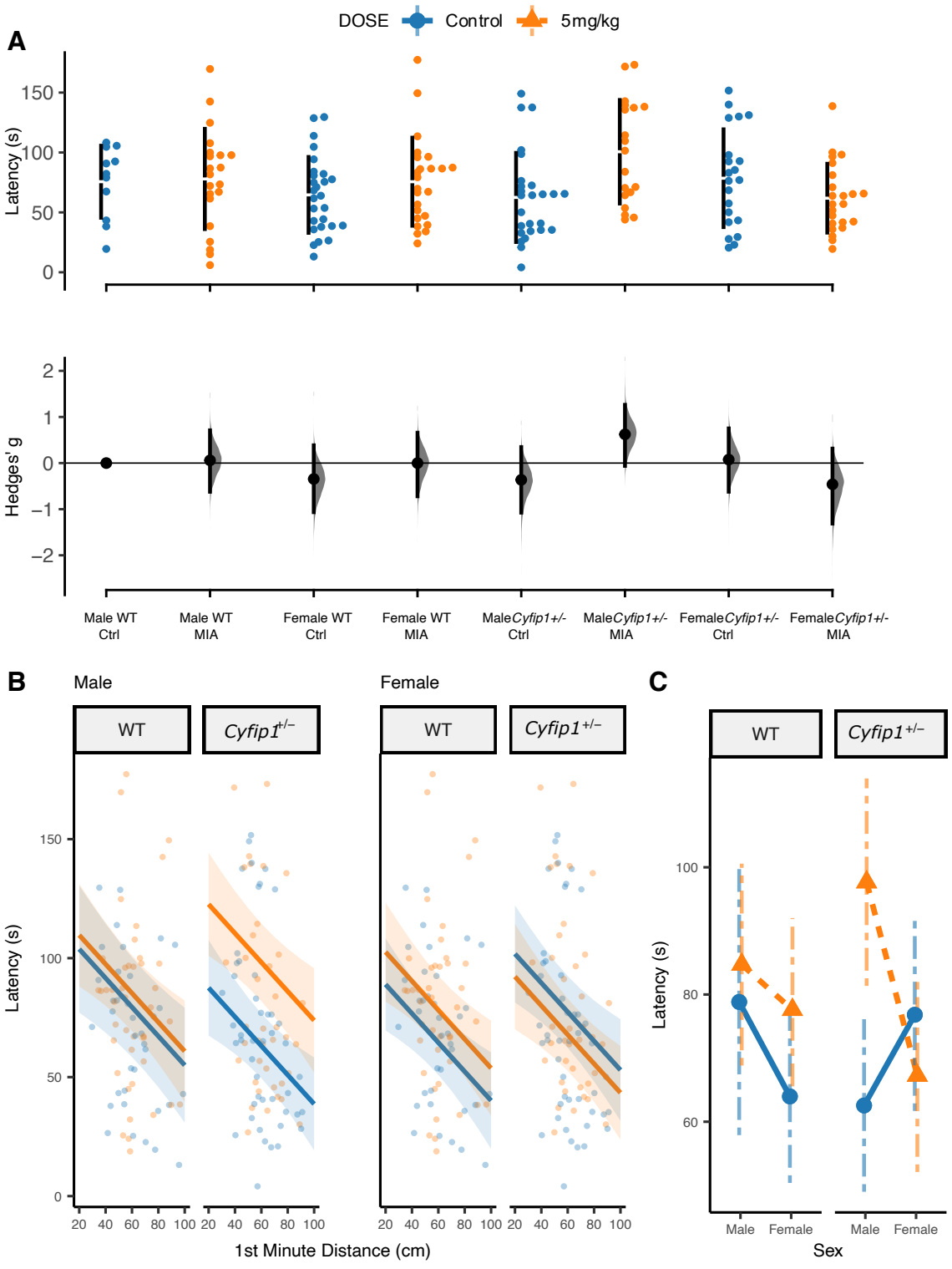


Figure 4.3. Latency times to find home nesting material at P9 for WT and *Cyfip1*$^{+/-}$ offspring of control and MIA dams. **A**. Latency times for individual offspring. The top panels show the raw data with a black bar showing the mean (break in the bar) and 95% confidence intervals for each group. In the bottom panels, black circles show the estimated effect size, Hedge's *g*, between each group and the reference group (male WT controls). **B**. Latency to find the home nest material predicted by 1st minute activity. Ribbons represent 95% CIs and individual points represent raw data. **C**. Interaction plot for individual animals with estimated marginal means plotted with 95% CIs plotted as dashed lines. *n* = 11 Male WT controls, 21 Male WT MIA, 27 Female WT controls, 24 Female WT MIA, 26 Male *Cyfip1*$^{+/-}$ controls, 19 Male *Cyfip1*$^{+/-}$ MIA, 21 Female *Cyfip1*$^{+/-}$ controls and 23 Female *Cyfip1*$^{+/-}$ MIA animals.

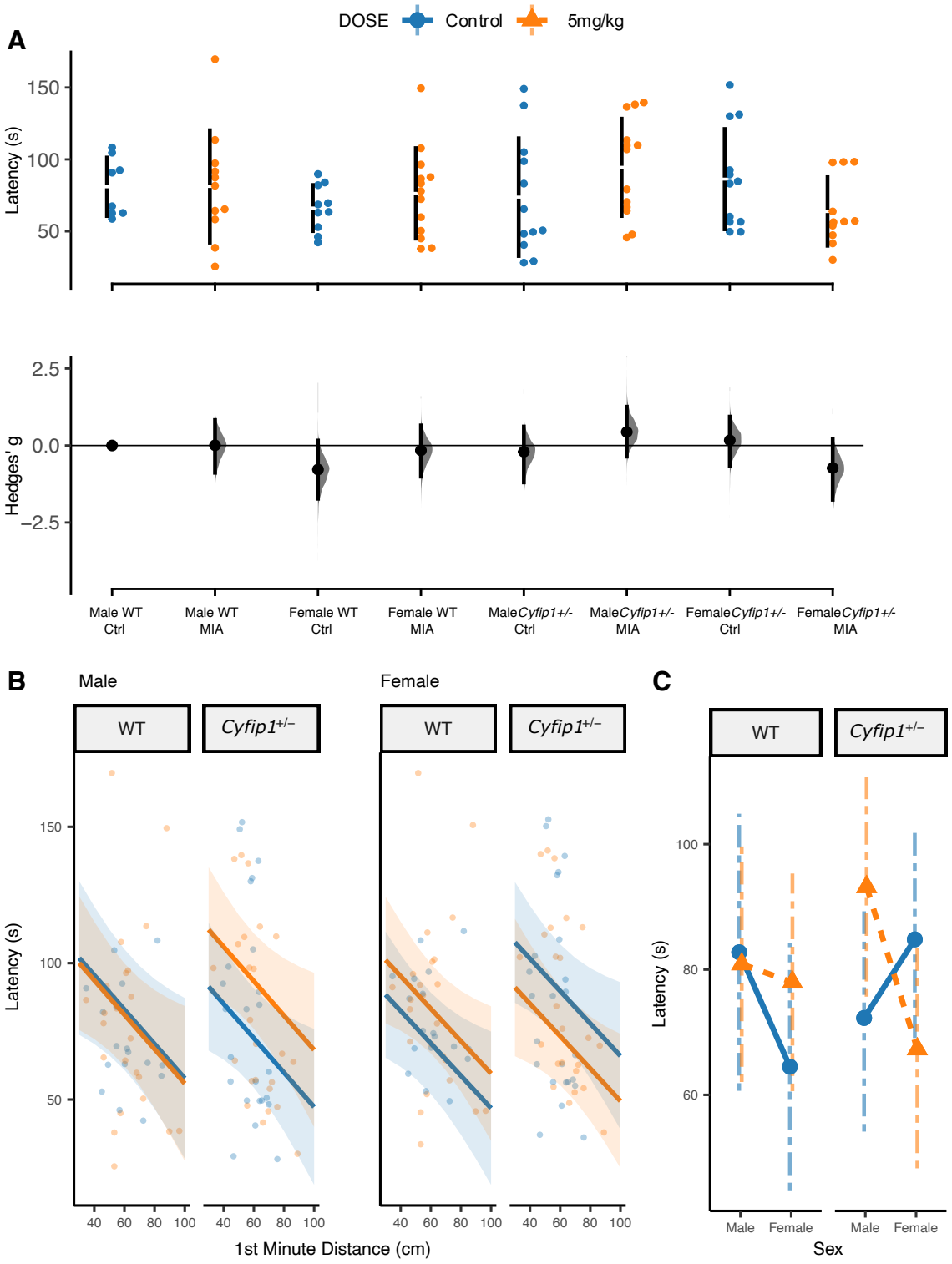


Figure 4.4. Litter average latency times to find home nesting material at P9 for WT and *Cyfip1*$^{+/-}$ offspring of control and MIA dams. **A**. Litter average latency times for individual offspring. The top panels show the raw data with a black bar showing the mean (break in the bar) and 95% confidence intervals for each group. In the bottom panels, black circles show the estimated effect size, Hedge's *g*, between each group and the reference group (male WT controls). **B**. Litter average latency to find the home nest material predicted by 1st minute activity. Ribbons represent 95% CIs and individual points represent raw data. **C**. Interaction plot for litter average latency times with estimated marginal means plotted with 95% CIs plotted as dashed lines. *n* = 8 Male WT controls, 11 Male WT MIA, 10 Female WT controls, 13 Female WT MIA, 12 Male *Cyfip1*$^{+/-}$ controls, 13 Male *Cyfip1*$^{+/-}$ MIA, 12 Female *Cyfip1*$^{+/-}$ controls and 11 Female *Cyfip1*$^{+/-}$ MIA animals.

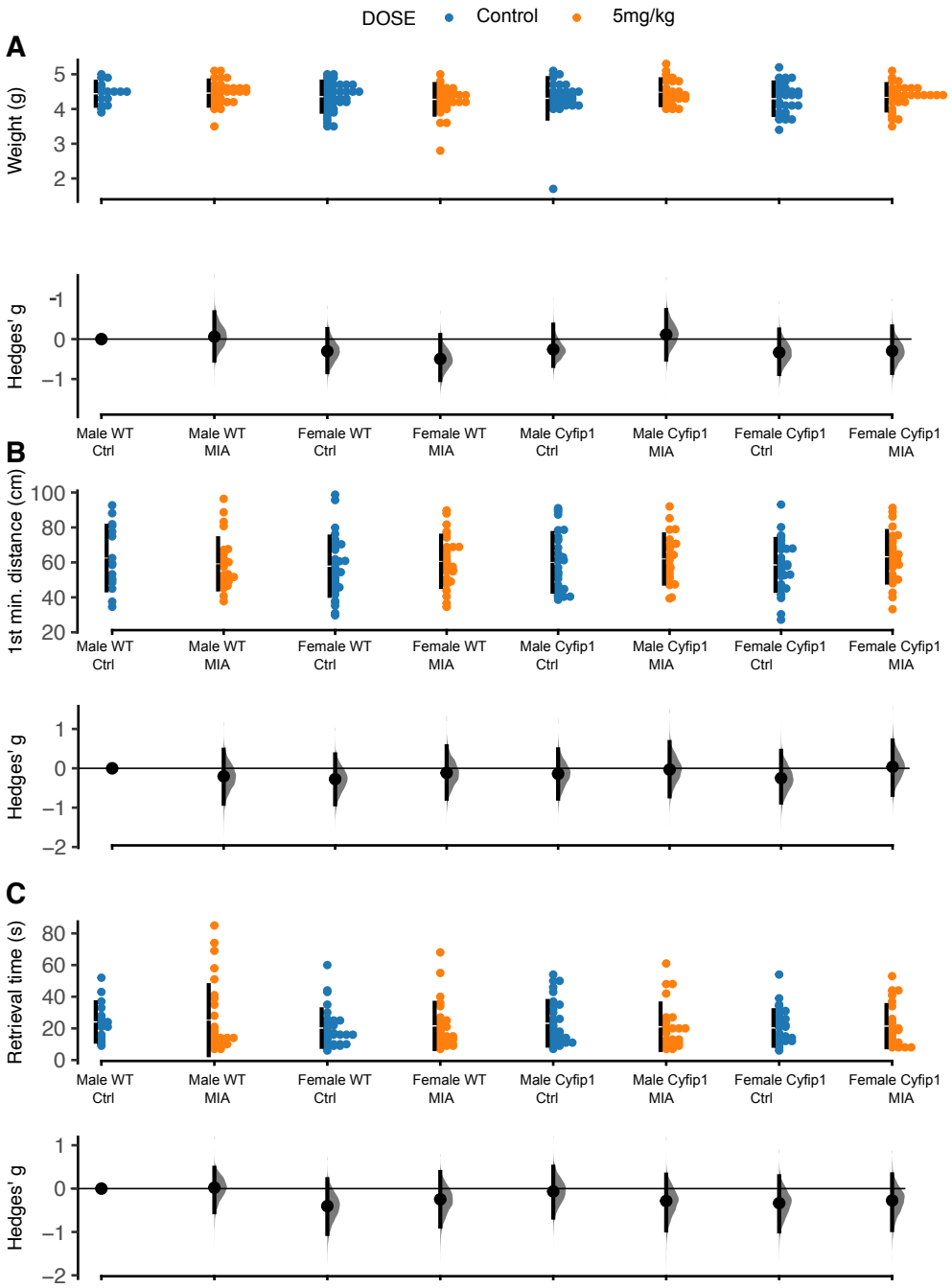


Figure 4.5 **.** Additional variables recorded during homing test. **A**. Weight of offspring at P9. **B**. Distance travelled in 1st minute of homing test. **C.** Pup retrieval time at P9 following return to homecage. The top panels show the raw data with a black bar showing the mean (break in the bar) and 95% confidence intervals for each group. In the bottom panels, black circles show the estimated effect size, Hedge's *g*, between each group and the reference group (male WT controls).For A *n* = 16 Male WT controls, 30 Male WT MIA, 36 Female WT controls, 29 Female WT MIA, 32 Male *Cyfip1*$^{+/-}$ controls, 26 Male *Cyfip1*$^{+/-}$ MIA, 30 Female *Cyfip1*$^{+/-}$ controls and 30 Female *Cyfip1*$^{+/-}$ MIA animals. For B *n* = 16 Male WT controls, 30 Male WT MIA, 33 Female WT controls, 28 Female WT MIA, 32 Male *Cyfip1*$^{+/-}$ controls, 25 Male *Cyfip1*$^{+/-}$ MIA, 30 Female *Cyfip1*$^{+/-}$ controls and 30 Female *Cyfip1*$^{+/-}$ MIA animals. For C *n* = 16 Male WT controls, 30 Male WT MIA, 36 Female WT controls, 29 Female WT MIA, 32 Male *Cyfip1*$^{+/-}$ controls, 26 Male *Cyfip1*$^{+/-}$ MIA, 30 Female *Cyfip1*$^{+/-}$ controls and 27 Female *Cyfip1*$^{+/-}$ MIA animals.

Females appear lighter than males at P9 (Figure. 4.5a) supported by a main effect of ***sex*** (LMM ($F_{[1,202.7]} = 4.9$, $P < 0.05$)) when analysed using the model ***Weight ~ homing + genotype x dose x sex.*** A model with an ICC = 0.41, a marginal R^2 = 0.03 and a conditional R^2 = 0.42. Initial activity i.e. the distance travelled in the 1st minute, for the whole cohort, homing and non-homing offspring appears similar across all groups (Figure. 4.5b) supported by the observation of no significant main effects or interactions, even when controlling for weight. There was no difference in the time taken by the dam to retrieve pups from any of the groups (Figure. 4.5c). Overall, the proportion of offspring with present homing behaviour is equal across groups, with all groups also showing a similar level of initial activity. MIA affects the homing behaviour of *Cyfip1*$^{+/-}$ offspring in a sex dependent fashion, impacting males but not females with no differences across wild-type groups.

4.4.4 Vocalisation behaviour differs between non-homing and homing offspring

The number of ultrasonic vocalisations emitted by pups was highly variable across each group (Figure. 4.6a). Pups that displayed homing behaviour called fewer times than pups with absent homing behaviour, main effect of ***homing*** (GLMM (link=negative binomial ($P < 0.01$)) no other effects were observed. Mean duration of USVs showed similar patterns to the number of calls but no significant main effects or interactions were observed when considering the whole cohort (Figure. 4.6b). In non-homing animals, no significant main effects were observed, but an interaction between ***genotype, dose,*** and ***sex*** was observed (GLMM (link=negative binomial ($P < 0.05$). Male WT control offspring call more often than female WT controls with the reverse seen in *Cyfip1*$^{+/-}$ controls. MIA offspring showed the opposite pattern (Figure. 4.6c). Non-homing animals showed no main effects or interactions when considering mean call duration (Figure. 4.6d).

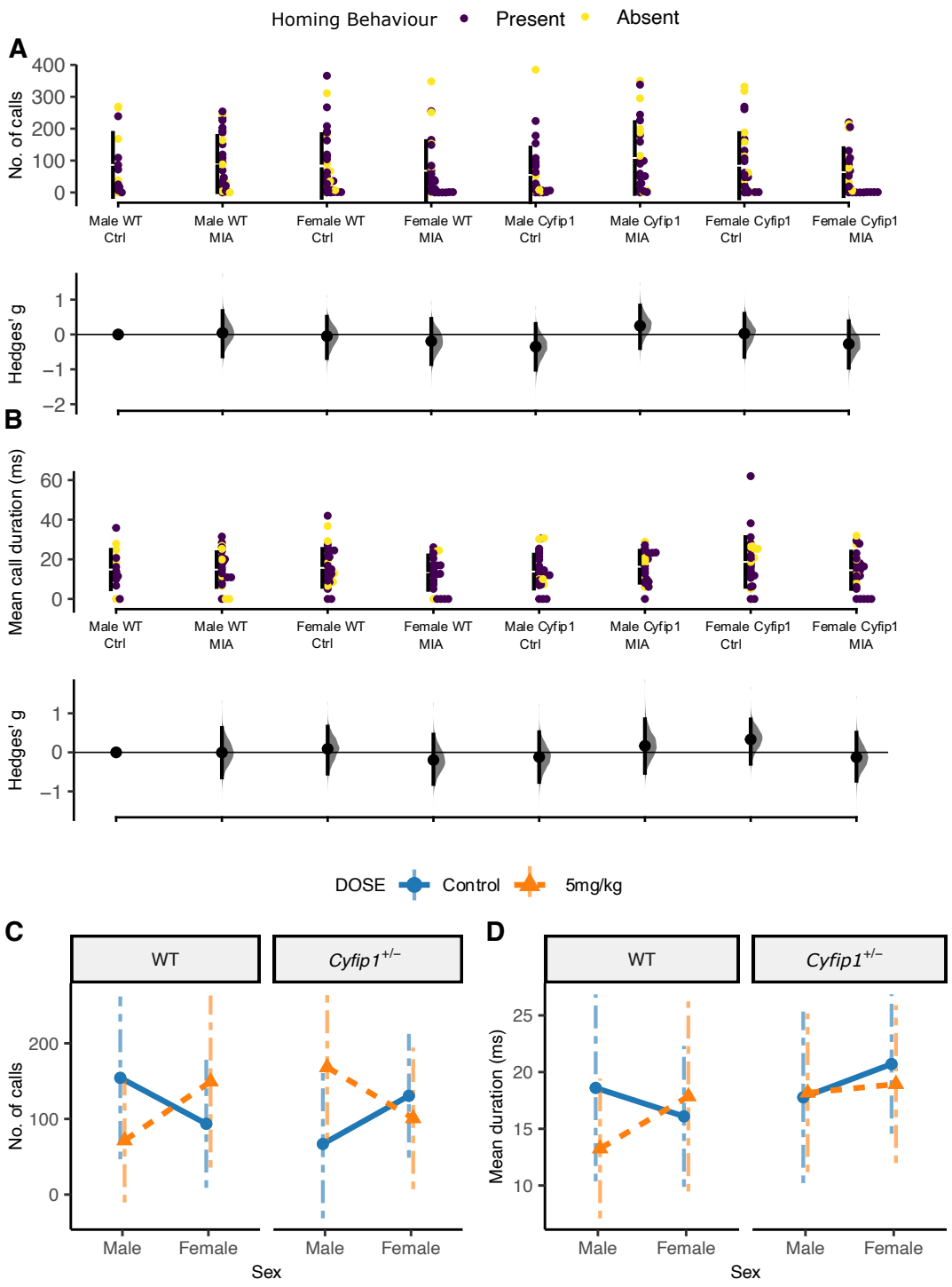


Figure 4.6. Vocalisation behaviour during the homing test. **A**. Calling frequency of whole cohort. **B**. Mean call duration of whole cohort. The top panels show the raw data with a black bar showing the mean (break in the bar) and 95% confidence intervals for each group. In the bottom panels, black circles show the estimated effect size, Hedge's *g*, between each group and the reference group (male WT controls). **C.** Interaction plot for calling frequency of animals with absent homing behaviour **D.** Interaction plot for mean call duration of animals with absent homing behaviour**.** C and D feature estimated marginal means plotted with 95% CIs plotted as dashed lines. For A and B *n* = 16 Male WT controls, 30 Male WT MIA, 36 Female WT controls, 29 Female WT MIA, 32 Male *Cyfip1*$^{+/-}$ controls, 26 Male *Cyfip1*$^{+/-}$ MIA, 30 Female *Cyfip1*$^{+/-}$ controls and 27 Female *Cyfip1*$^{+/-}$ MIA animals. For C and *n* = 5 Male WT controls, 9 Male WT MIA, 9 Female WT controls, 5 Female WT MIA, 6 Male *Cyfip1*$^{+/-}$ controls, 7 Male *Cyfip1*$^{+/-}$ MIA, 9 Female *Cyfip1*$^{+/-}$ controls and 7 Female *Cyfip1*$^{+/-}$ MIA animals.

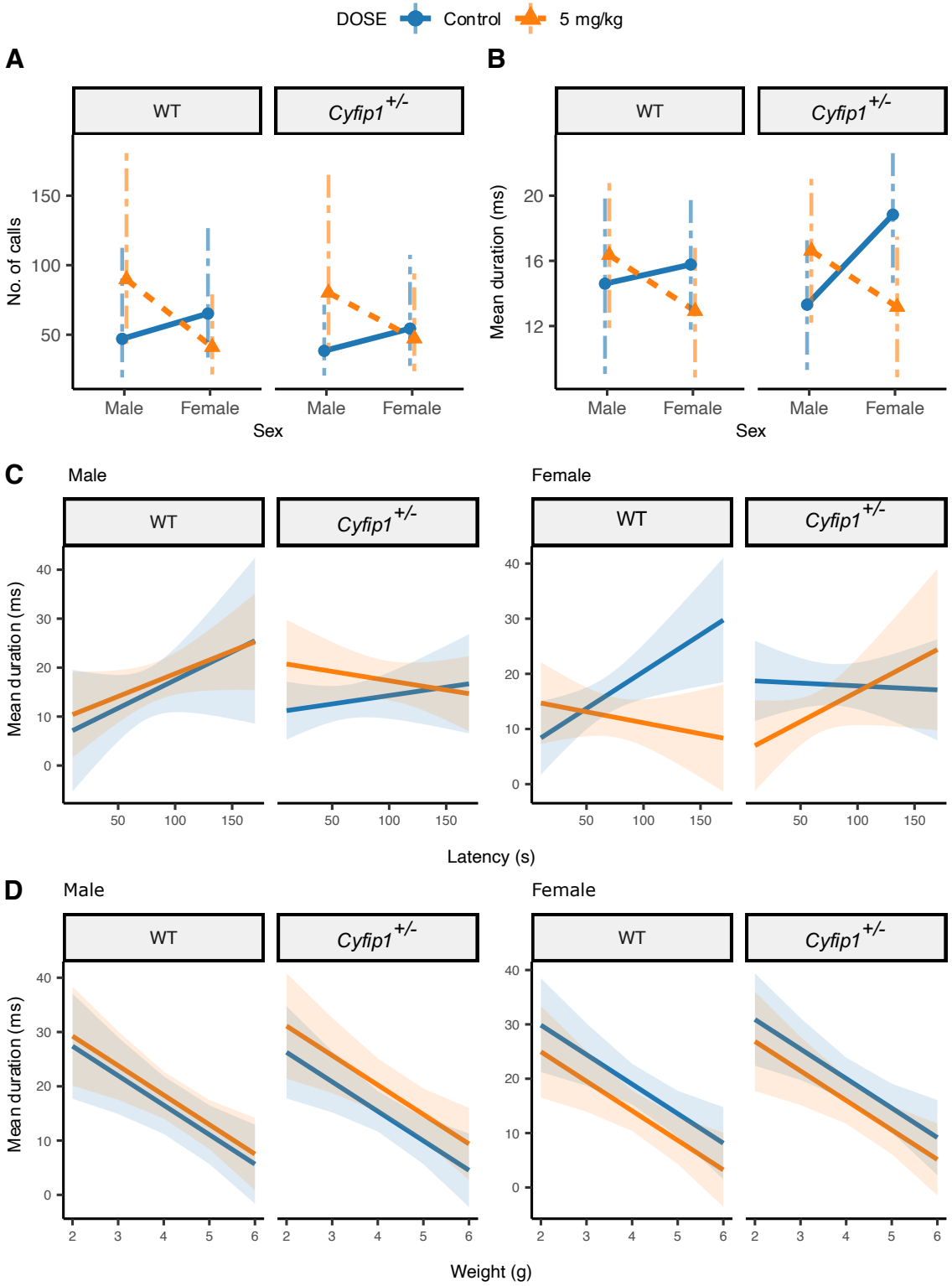


Figure 4.7. Vocalisation behaviour of animals with present homing behaviour at P9. **A**. Interaction plot for calling frequency of animals **B**. Interaction plot for mean call duration of animals A and B feature estimated marginal means plotted with 95% CIs plotted as dashed lines. **C.** Mean call duration predicted by latency to find home nesting material. **D.** Mean call duration predicted by weight. In C and D ribbons represent 95% CIs and individual points represent raw data. *n* = 11 Male WT controls, 21 Male WT MIA, 27 Female WT controls, 24 Female WT MIA, 26 Male *Cyfip1*$^{+/-}$ controls, 19 Male *Cyfip1*$^{+/-}$ MIA, 21 Female *Cyfip1*$^{+/-}$ controls and 23 Female *Cyfip1*$^{+/-}$ MIA animals.

In homing animals, the number of calls emitted showed a similar pattern between WT and *cyfip1*$^{+/-}$ groups (Figure. 4.7a), supported by a ***dose x sex*** interaction (GLMM (link=negative binomial (P<0.05). No other effects were observed using the model ***Number of calls ~ genotype x dose x sex*** which returns an ICC = 0.15, a marginal R^2 = 0.06 and a conditional R^2 = 0.2. Mean call duration showed a similar pattern compared to number of calls emitted (Figure. 4.7b). When modelling mean call duration to consider homing latency (Figure. 4.7c) and weight (Figure. 4.7d) there is a main effect of ***weight*** (LMM ($F_{[1,122.8]}$ = 9.7, P < 0.01)) and ***latency*** (LMM ($F_{[1,43.4]}$ = 5.8, P < 0.05)). Mean duration also demonstrates a ***genotype x dose x sex*** interaction when controlling for weight and latency (LMM ($F_{[1,145.1]}$ = 4.1, P < 0.05)). ***Mean duration ~ weight + latency x genotype x dose x sex*** returns an ICC = 0.07, a marginal R^2 =0.18 and a conditional R^2 = 0.23.

At P9 animals with absent homing behaviour call more but show no differences in mean duration of calls emitted. MIA appears to alter vocalisation of animals with homing behaviour in a sex dependant fashion for both WT and *Cyfip1*$^{+/-}$ groups. Heavier animals have lower mean call duration across all groups. Genotype, dose, and sex interact to produce different call duration as latency to find the home nest increases. WT male control animals who take longer to find the home nest emit longer calls, whereas male *Cyfip1*$^{+/-}$ MIA animals have shorter calls for longer latency times. *Cyfip1*$^{+/-}$ female MIA animals increase their call duration with longer latency times.

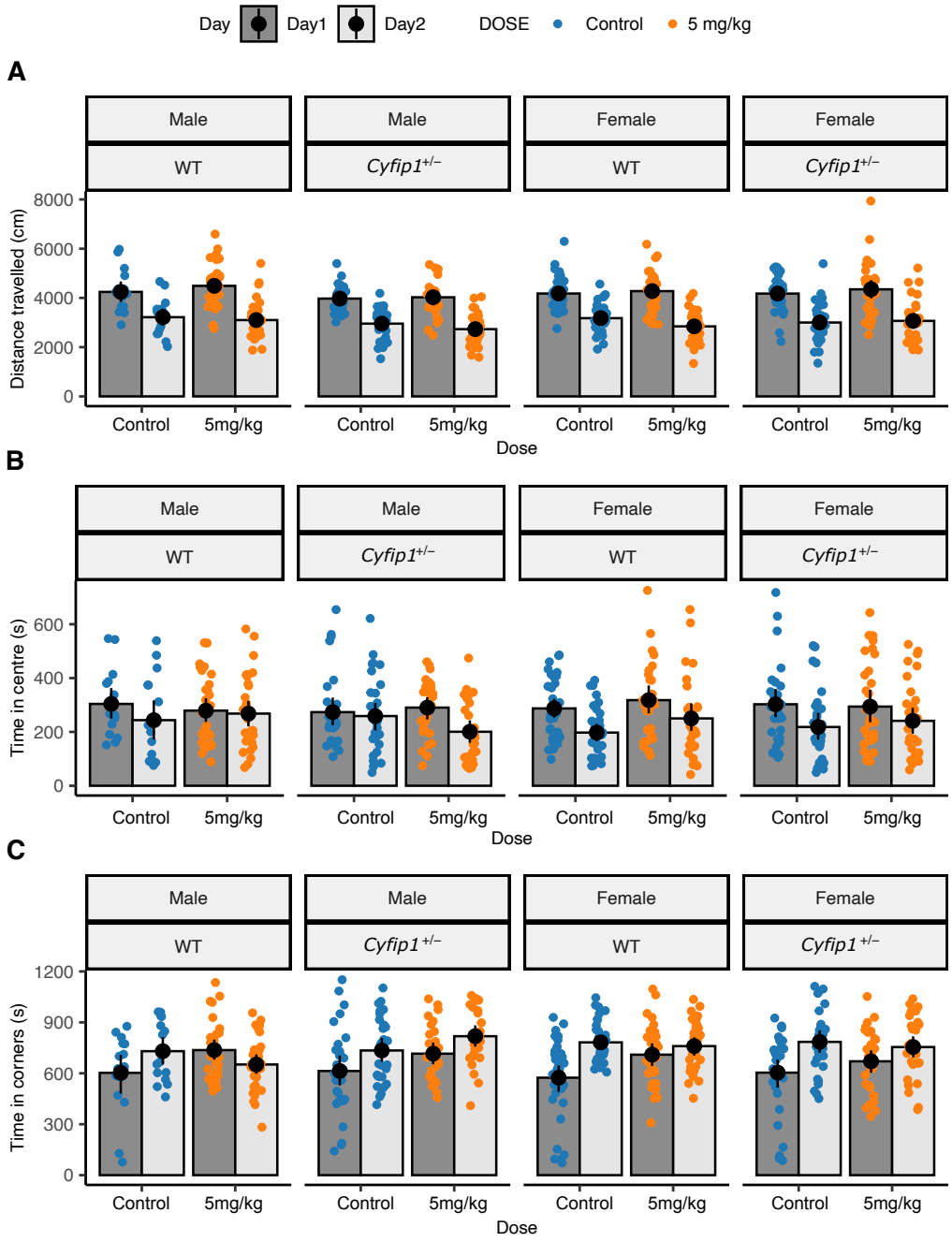


Figure 4.8. Activity in open field P24 & 25 **A**. Distance travelled day 1 v day 2. **B**. Time spent in the centre of open field day 1 to day 2. **C** Time spent in the corners of the open field day 1 to day 2. Coloured dots represent individual data points with the black dot and line representing the group mean with bootstrapped 95% CI. *n* = 16 Male WT controls, 30 Male WT MIA, 34 Female WT controls, 29 Female WT MIA, 29 Male *Cyfip1*$^{+/-}$ controls, 26 Male *Cyfip1*$^{+/-}$ MIA, 30 Female *Cyfip1*$^{+/-}$ controls and 30 Female *Cyfip1*$^{+/-}$ MIA animals.

4.4.5 MIA offspring show less exploratory behaviour than controls at P24 on day 1 of open field

At P24 & 25, all groups habituate to the open field from day 1 to day 2 (Figure. 4.8a) supported by a main effect of ***day*** (LMM ($F_{[1,216]}$ = 501.7, P < 0.001)), with no group differences in activity on either day 1 or 2. ***Distance travelled*** ~ ***day*** x ***genotype*** x ***dose*** x ***sex*** + ***(1 | ID)*** + ***(1 | Litter)***. returns an ICC_{ID} = 0.33 ICC_{litter} = 0.17, marginal R^2 = 0.39 and conditional R^2 = 0.69. All groups spend a reduced amount of time in the centre of the open field from day 1 to day 2 (Figure. 4.8b) supported by a main effect of ***day*** (LMM ($F_{[1,216]}$ = 51.5, P < 0.001)). **Sqrt (*Time spent in centre)*** ~ ***day*** x ***genotype*** x ***dose*** x ***sex*** + ***(1 | ID)*** + ***(1 | Litter)***. returns an ICC_{ID} = 0.45, ICC_{litter} = 0.05, marginal R^2 = 0.08 and conditional R^2 = 0.54. Control and MIA offspring show different patterns of behaviour considering time spent in the corners (5 cm x 5 cm virtually demarked zone in each corner of the arena) of the open field day 1 to 2 (Figure. 4.8c), with a main effect of ***day*** (LMM ($F_{[1,216]}$ = 36.2, P < 0.001)). An interaction is also present between ***day*** and ***dose*** (LMM ($F_{[1,216]}$ = 13.6, P < 0.001)) with post-hoc comparisons revealing control offspring spent less time in the corners on day 1 than day 2 ($t_{[144]}$ = -6.6, p<0.001) whereas their MIA counterparts show no change from day 1 to day 2. On day 1 controls spend less time in corners than MIA offspring ($t_{[144]}$ = -3.1, p<0.05) with no difference between the groups on day 2. ***Time spent in corners*** ~ ***day*** x ***genotype*** x ***dose*** x ***sex*** + ***(1 | ID)*** + ***(1 | Litter)***. returns an ICC_{ID} = 0.07, ICC_{litter} = 0.15, marginal R^2 = 0.13 and conditional R^2 = 0.32. Taken together, habituation and activity levels in the open field are the same across groups but control offspring have higher levels of exploration on day 1 than their MIA counterparts as less time is spent in corners of the arena.

4.4.6 *Cyfip1*$^{+/-}$ offspring show reduced levels of social interaction than WT littermates

At P28, *Cyfip1*$^{+/-}$ offspring spend less time in social interaction with an adult female than WT offspring (Figure. 4.9a), supported by a main effect of ***genotype*** (LMM ($F_{[1,201.7]}$ = 16.3, P < 0.001)). There are no apparent interactions (Figure. 4.9b) and none were identified when analysed with the model ***Interaction time*** ~ ***weight*** + ***stimulus directed interaction time*** + ***grooming time*** + ***homing*** + ***genotype*** x ***dose*** x ***sex*** + ***(1 | Litter)***. This model returns an ICC = 0.19, marginal R^2 = 0.14 and

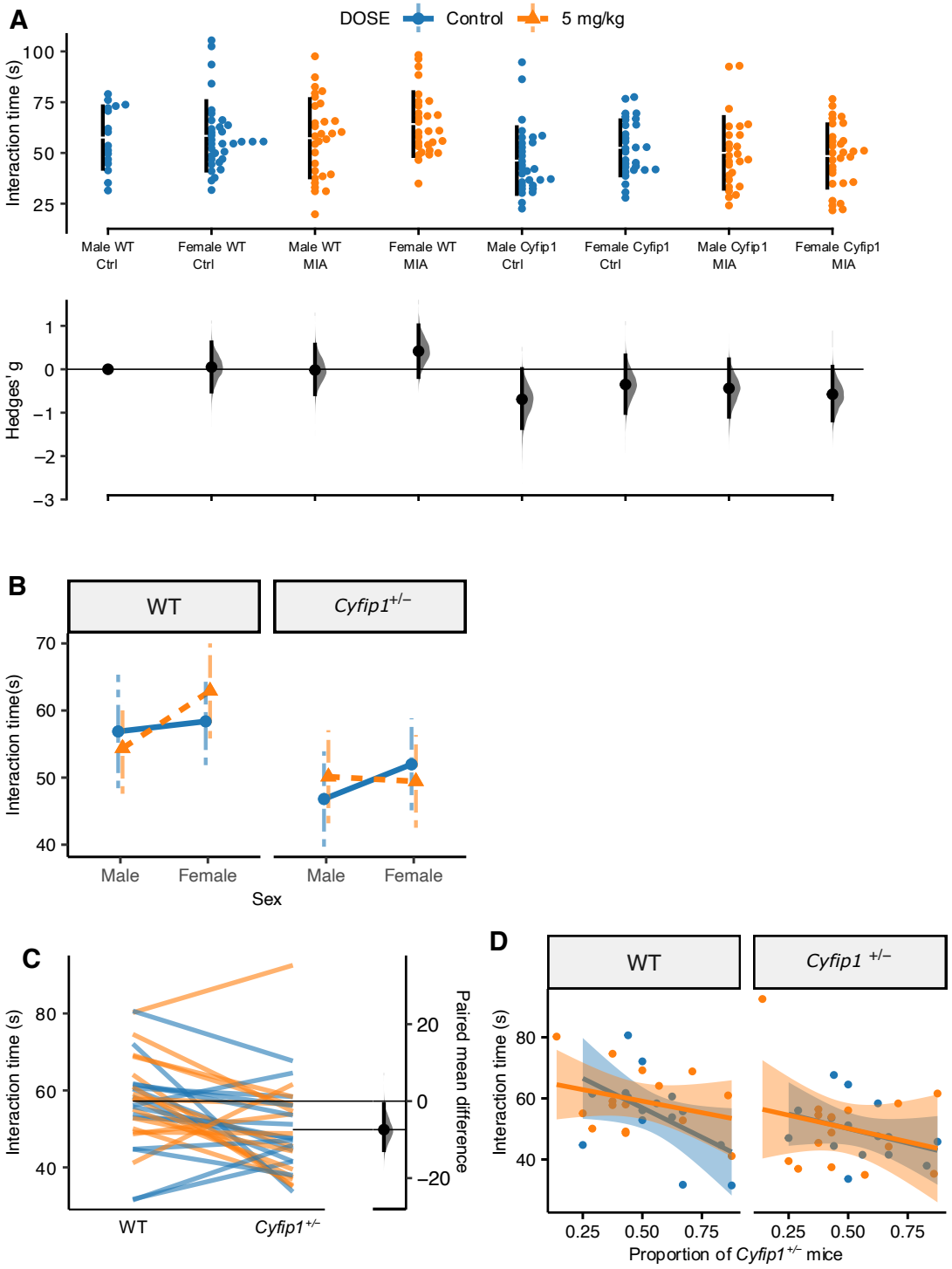


Figure 4.9. Social interaction time at P28 for WT and *Cyfip1*$^{+/-}$ offspring of control and MIA dams. **A**. Interaction times for individual offspring, the top panel shows the raw data with a black bar showing the mean (break in the bar) and 95% confidence intervals for each group. In the bottom panel, black circles show the estimated effect size, Hedge's *g*, between each group and the reference group (male WT controls). **B**. Interaction plot for offspring estimated marginal means plotted with 95% CIs plotted as dashed lines. **C.** Paired estimation plot comparing litter averages for WTs and *Cyfip1*$^{+/-}$. The right-hand panel is the effect size of the difference between groups. **D.** Interaction time varies with the proportion of *Cyfip1*$^{+/-}$ mice in a litter. For A and B, *n* = 16 Male WT controls, 30 Male WT MIA, 34 Female WT controls, 29 Female WT MIA, 29 Male *Cyfip1*$^{+/-}$ controls, 26 Male *Cyfip1*$^{+/-}$ MIA, 30 Female *Cyfip1*$^{+/-}$ controls and 30 Female *Cyfip1*$^{+/-}$ MIA animals. For C and D, *n* = 30 litters (15 control, 15 MIA).

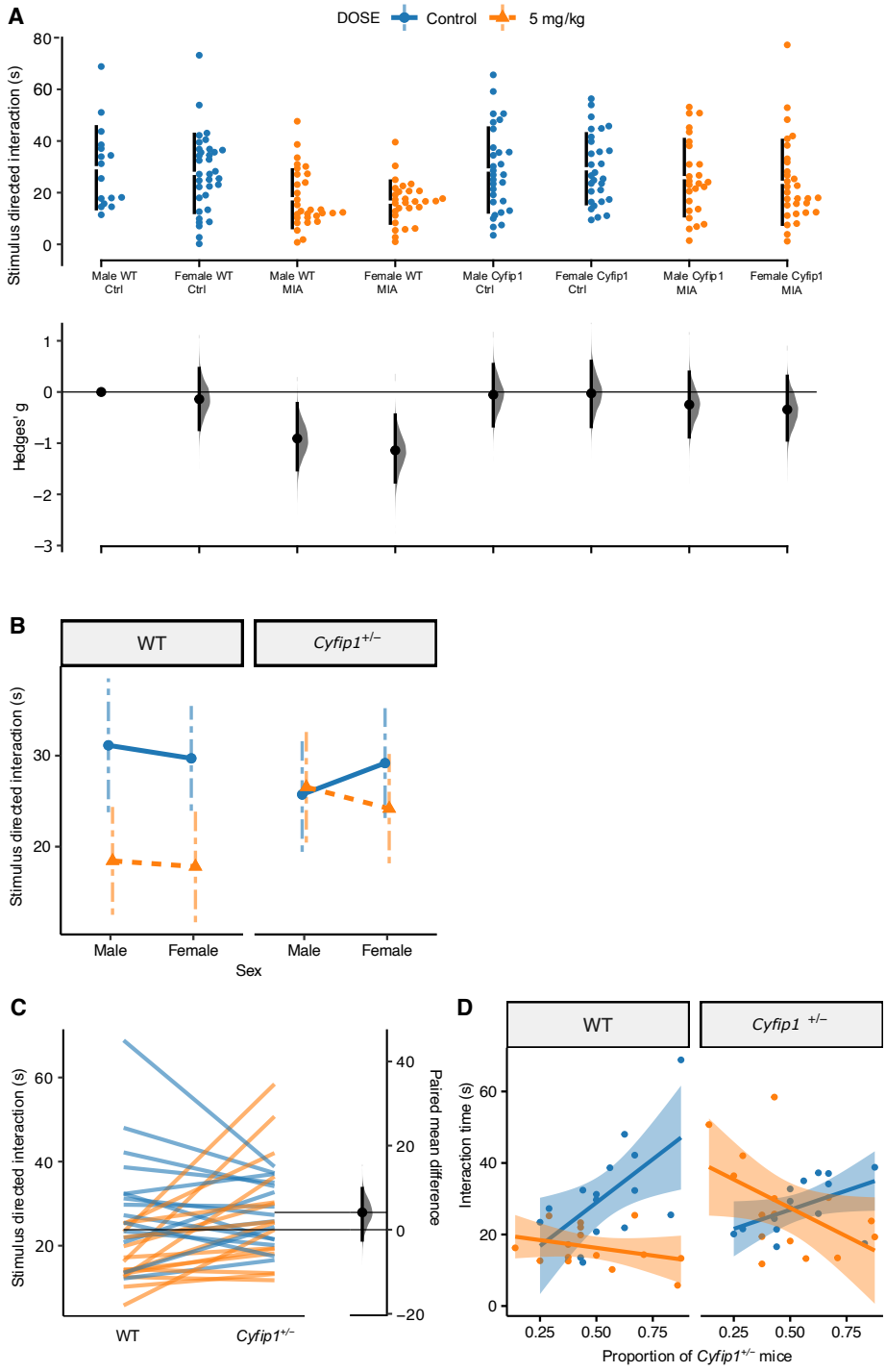


Figure 4.10**.** Stimulus directed interaction time at P28 for WT and *Cyfip1*$^{+/-}$ offspring of control and MIA dams. **A**. Stimulus directed interaction times for individual offspring, the top panel show the raw data with a black bar showing the mean (break in the bar) and 95% confidence intervals for each group. In the bottom panel, black circles show the estimated effect size, Hedge's *g*, between each group and the reference group (male WT controls). **B**. Interaction plot for offspring with estimated marginal means plotted with 95% CIs plotted as dashed lines. **C.** Paired estimation plot comparing litter averages for WTs and *Cyfip1*$^{+/-}$. The right-hand panel is the effect size of the difference between groups. **D.** Interaction time varies with the proportion of *Cyfip1*$^{+/-}$ mice in a litter. For A and B, *n* = 16 Male WT controls, 30 Male WT MIA, 34 Female WT controls, 29 Female WT MIA, 29 Male *Cyfip1*$^{+/-}$ controls, 26 Male *Cyfip1*$^{+/-}$ MIA, 30 Female *Cyfip1*$^{+/-}$ controls and 30 Female *Cyfip1*$^{+/-}$ MIA animals. For C and D, *n* = 30 litters (15 control, 15 MIA).

conditional R^2 = 0.31. Post-hoc comparisons show female WT offspring interact more than female *Cyfip1*$^{+/-}$ offspring, ($t_{[94.4]}$ =3.3, p<0.01).

Litter averages we calculated from the entire cohort of offspring and the interaction times of WT offspring were higher than their *Cyfip1*$^{+/-}$ litter mates (Figure. 4.9c) (Paired t-test ($t_{[29]}$ = 3.1, P <0.01, mean difference =7.4s, 95%CI [2.5,12.3]). Litter averages were also analysed with the model ***Interaction time ~ genotype*** + ***proportion of Cyfip1*$^{+/-}$ *animals in the litter*** + ***(1|Litter)***. Interaction time decreases with an increasing proportion of *Cyfip1*$^{+/-}$ animals in the litter (Figure. 4.9d) supported by a main effect of ***proportion of Cyfip1*$^{+/-}$ *animals in the litter*** (LMM ($F_{[1,28]}$ = 6, P < 0.05)) and the model also returns a main effect of ***genotype*** (LMM ($F_{[1,29]}$ = 9.4, P < 0.01)). This model returns an ICC = 0.33, a marginal R^2 = 0.2 and a conditional R^2 = 0.46. *Cyfip1*$^{+/-}$ animals interact less than WT animals when considering individuals or litter averages, with the biggest difference occurring between female WTs and *Cyfip1*$^{+/-}$ animals from MIA groups. The presence of *Cyfip1*$^{+/-}$ influences the social interest of WT littermates with WT animals from litters with a higher proportion of *Cyfip1*$^{+/-}$ animals showing lower interaction times.

4.4.7 Stimulus animals show reduced interest in WT MIA offspring

Stimulus animals in the P28 social interaction test spend less time in non-reciprocal interaction with WT MIA offspring than *Cyfip1*$^{+/-}$ MIA offspring and control groups (Figure. 4.10a). This supported by a main effect of **dose** (LMM ($F_{[1,29]}$ = 6.4, P < 0.05)) and a **genotype x dose** interaction (LMM ($F_{[1,200.8]}$ = 8.5, P < 0.01)), (Figure. 4.10b). Post-hoc comparisons show stimulus animals spend less time in non-reciprocal interaction with WT MIA offspring than *Cyfip1*$^{+/-}$ MIA offspring ($t_{[79.8]}$ = -2.7, p<0.05), WT controls ($t_{[69.1]}$ = -3.6, p<0.01), and *Cyfip1*$^{+/-}$ controls ($t_{[72]}$ = -2.8, p<0.05). The model ***Stimulus directed interaction time ~ weight + interaction time + grooming time + genotype*** x ***dose*** x ***sex*** + ***(1|Litter) +(1|Stimulus ID)*** returns an ICC_{litter} = 0.19, $ICC_{stimulus}$ = 0.07, marginal R^2 = 0.14 and conditional R^2 = 0.36

Considering litter averages of the entire cohort of offspring for stimulus directed interaction times *Cyfip1*$^{+/-}$ animals appear higher than WT littermates (Figure. 4.10c). Litter averages were also analysed with the model ***Stimulus directed interaction time ~ genotype*** x ***dose*** x ***proportion of Cyfip1*$^{+/-}$ *animals in the litter*** + ***(1|Litter)***.

Stimulus interest in the offspring demonstrates a relationship with proportion of *Cyfip1*$^{+/-}$ animals in the litter affected by both dose and genotype (Figure. 4.10d), supported by a main effects of ***dose*** (LMM ($F_{[1,26]} = 5.7$, $P < 0.05$)) and ***genotype*** (LMM ($F_{[1,26]} = 9.1$, $P < 0.01$)), as well as interactions between ***dose*** and ***proportion of Cyfip1*$^{+/-}$ *animals*** (LMM ($F_{[1,26]} = 11.6$, $P < 0.01$)) and ***genotype*** and ***proportion of Cyfip1*$^{+/-}$ *animals*** (LMM ($F_{[1,26]} = 6$, $P < 0.05$)). This model returns an ICC = 0.42, a marginal $R^2 = 0.41$ and a conditional $R^2 = 0.66$.

MIA appears to interact with *Cyfip1*$^{+/-}$ in an indirect fashion as stimulus animals have less non-reciprocal interaction with WT MIA animals than other groups. Non-reciprocal interaction by the stimulus animals is influenced by the proportion of *Cyfip1*$^{+/-}$ animals in a litter with differing patterns for control and MIA animals.

4.5 Discussion

The work presented here provides the first evidence suggesting *Cyfip1* haploinsufficiency and MIA can interact to modulate the social behavioural phenotypes of mice early in development. At P9, homing behaviour of *Cyfip1*$^{+/-}$ males was impacted by MIA but not *Cyfip1*$^{+/-}$ females. An effect observed when either the individual or litter was considered as the *n*. Genotype, MIA and sex also interacted to influence USV duration during the homing test for pups that displayed homing behaviour. For pups, with absent homing behaviour a three-way interaction was observed in the number of vocalisations emitted during the test. The findings here also highlight a potential role of *Cyfip1* in the pre-weaning social behaviour of mice. Compared to WT littermates, *Cyfip1*$^{+/-}$ mice showed reduced social interaction at P28 in control and MIA groups. Social environment appeared to influence social behaviour at P28 with WTs from litters with a higher proportion of *Cyfip1*$^{+/-}$ mice displaying lower interaction times. Furthermore, *Cyfip1*$^{+/-}$, MIA and social environment displayed evidence of interacting as WT MIA offspring appeared less interesting to stimulus mice at P28 compared to control offspring and *Cyfip1*$^{+/-}$ MIA offspring.

4.5.1 Maternal immune activation

A 5 mg/kg dose of poly(I:C) was enough to illicit an immune response in dams. MIA dams failed to gain weight 24 hrs post-injection and displayed sickness behaviours 3 hrs after administration, which replicates the findings of Chapter 3. However, the dams exposed to MIA in this study displayed a drop in temperature 3 hrs post-injection which wasn't seen in Chapter 3. This could be explained by the individual variability in response to poly(I:C) reported previously (Estes et al. 2020) . However, comparison of the data shows dams were colder prior to injection in Chapter 3 than Chapter 4 and temperatures post-injection are comparable. This points to a potential floor effect of hypothermic response to 5 mg/kg and starting temperature is important. At least in the context of surface temperature. MIA did not impact on litter sizes or the weight of pups in the first two weeks after birth. This was observed in Chapter 3 but unlike Chapter 3, both WT and *Cyfip1*$^{+/-}$ mice born to dams exposed to poly(I:C) were lighter than control groups at P25 and P28. Mice born to poly(I:C) exposed dams have been reported to be lighter in early development (Arsenault et al. 2014). Given this was not observed for purely WT litters in Chapter 3, the weight differences here may be representative of an interactive effect between *Cyfip1*$^{+/-}$, MIA and social environment.

4.5.2 P9 Homing behaviour.

Examination of homing behaviour in *Cyfip1*$^{+/-}$ mice is previously unreported and this work revealed a social deficit early in development in *Cyfip1*$^{+/-}$ male mice are born to dams exposed to MIA. Control cyfip1$^{+/-}$ mice and MIA *Cyfip1*$^{+/-}$ female mice showed comparable homing times, and the 5 mg/kg dose of poly (I:C) did not impact homing behaviour of WTs replicating the finding in Chapter 3. Mice with higher initial activity found the homing nest quicker but this was consistent across all groups with no differences in distance travelled in first minute (Fig 4.4b). Males were heavier than females, but this was not impacted by MIA or *Cyfip1* haploinsufficiency. Furthermore, pup retrieval times upon return to home cage were similar between groups suggesting latency to find the home nest was not influenced by altered levels

of maternal care, weight, or activity differences (Fig 4.5). The homing test is reliant on intact olfaction (Honeycutt and Alberts 2005; Fiori et al. 2017) and lower interest in social odours has been previously reported for adult *Cyfip1*$^{+/-}$ male mice (Bachmann et al. 2019). Therefore, taking longer to find the home nest points to a deficit in social interest in *Cyfip1* haploinsufficient males born to MIA dams, particularly in response to olfactory cues.

Comparison of calling behaviour between mice with present and absent homing behaviour is previously unreported. At P9 mice with absent homing behaviour emit more USVs than homing mice, without displaying a difference in mean call duration. This increased calling rate coupled with a failure to locate home nest material may highlight delayed development in non-homing mice. Previous reports have highlighted a reduction in isolation-induced calls by P9 (Scattoni et al. 2008; Caruso et al. 2022) and homing behaviour is typically established by P9 (Honeycutt and Alberts 2005). Increased calling may also point to a lower level of maternal attachment in mouse pups as previous work demonstrated reduced calling in P8 mice in the presence of nesting material from the homecage in comparison to isolation-induced calls (Moles et al. 2004). Direct comparison with this finding is difficult as isolation-induced calls were not measured here and the pups had to physically locate the homing nest in this work. Within non-homing mice there are no individual effects of genotype, MIA, or sex but a 3-way interaction is present with opposing calling patterns between WT and *Cyfip1*$^{+/-}$ mice. In WT mice, control males call more than MIA males with control *Cyfip1*$^{+/-}$ males calling fewer times than MIA *Cyfip1*$^{+/-}$ males. An opposing trend in this pattern was seen in WT females with WT males. There appeared to be no difference in calling rates between control and MIA *Cyfip1*$^{+/-}$ females. This finding suggests the absence of homing behaviour may represent different phenotypes dependent of genotype, MIA and sex. Control WT males may display delayed development whereas MIA *Cyfip1*$^{+/-}$ males may be demonstrating decreased maternal attached. This assumes homing behaviour is truly absent in these mice, the assignment of a cut-off point i.e. the end of the 3-minute test does not mean the mice would not find the home nest, particularly for MIA *Cyfip1*$^{+/-}$ males. The variability in the data coupled with smaller group sizes in the non-homing groups (n=5-9) could also point to a lack of power to detect differences.

For homing mice increased calling was seen in WT and *Cyfip1*$^{+/-}$ males but not females born to MIA dams. When considering calling frequency in conjunction with the time taken to find the home nest i.e. latency, a difference emerges between MIA WT and *Cyfip1*$^{+/-}$ males. Both groups call more than male control counterparts but as established MIA *Cyfip1*$^{+/-}$ males take longer to find the nest thus in the context of locating the homing nest similar calling levels may not indicate the same phenotype. The variability and distribution of the number of calls across groups prevented fitting models with more variables that can influence calling, e.g. weight. Considered in isolation mean call duration was similar across groups. However, it varied with weight, and heavier mice showed lower call duration in all groups. Call duration varied by the latency to home in a genotype, MIA and sex-dependent manner. It was positively correlated with latency to find the home nest in WT control and MIA males and WT control and *Cyfip1*$^{+/-}$ MIA females. This may reflect an adaptive element to social communication with mice adapting the type of calls emitted as they take longer to locate the home nest. Duration of USVs in young mice (P8) has been previously shown to be modulated by social context, in response to clean versus soiled bedding (Wöhr 2015). *Cyfip1*$^{+/-}$ control males and females, MIA *Cyfip1*$^{+/-}$ males and MIA WT females display potentially aberrant adaptation in social communication as longer latency does not predict increased call duration. This suggestive evidence points to a complex interaction of genotype, MIA and sex in adaptive social communication. Spectrographic analysis of call traces may offer greater insight potential communication differences, i.e. syllables sequences. Studying a mouse model of 16p11.2 deletion it was observed WTs emitted USVs with a structured syllable pattern whereas 16p11.2 deletion males made fewer calls, and the syllable structure appeared random, whilst 16p11.2 deletion females had a structured pattern of syllables (Agarwalla et al. 2020). It may also be pertinent to partition calls traces into calls prior to finding the nest versus calls once nest is found, like the presence of homing versus non-homing behaviour. Such an approach may help tease out interactive effects as some groups may reduce calls once in the nest and others may call more which would be more suggestive of altered adaptive communication. Whilst reports have demonstrated the modulation of USVs by social context (Moles et al. 2004; Wöhr 2015), it is important to acknowledge the difference in complexity of the tasks. The assay used here is more challenging for mice as home nest material and

clean material are present in the same arena and mice need to discriminate to locate the home nest. Therefore, the increase in call duration might reflect changes in the affective state of mice as they take longer to find the home nest. Distress has been demonstrated as modulator of USVs (Moles et al. 2004). Three out of the four cyfip1 groups don't show greater call duration for longer latency times which could signify an inflexibility in adapting to social context which may be underpinned by a deficit in processing socially pertinent olfactory cues. This is a possibility as behavioural inflexibility has been observed in a rat *Cyfip1*$^{+/-}$ model, though it should be noted this was observed in adults (Silva et al. 2019a).

Overall, recording of USVs in the context of the P9 homing test has highlighted potential sex-dependent interactive effects of *Cyfip1*$^{+/-}$ and MIA on adaptive social communication. In mice with absent homing behaviour the number of calls emitted during testing displayed an interaction between genotype, MIA and sex and all groups call more than their homing counterparts. This difference in calling behaviour suggests the absence of homing behaviour may represent different phenotypes across groups. The number of calls and call duration show a potential interaction between MIA and sex when analysed in isolation. When considered in the context of the homing test mean duration displays a possible interaction between genotype, MIA and sex that suggests altered social communication in specific contexts. The partitioning of homing and non-homing mice was valuable to uncovering differences in calling behaviour. Furthermore, efforts to analyse calling behaviour in the context of the homing test illuminated potential interactive effects that would have been otherwise masked, reinforcing the design choice to record USVs during the test. Improvements could be made to this test, particularly the partitioning of USV traces. Splitting call traces into before and after finding the nest (nearly all animals stayed in the nesting material once located) would assist interpretation, in a similar way as splitting homing and non-homing mice.

4.5.3 Activity in the open field at P25

Activity levels were comparable across all groups with no apparent influence of genotype, MIA or sex. In the context of *Cyfip1*$^{+/-}$ mice this replicates previous findings (Bachmann et al. 2019; Sledziowska et al. 2020b), which demonstrated WT-level activity in adult (P60+) *Cyfip1*$^{+/-}$ mice. Other gene x environment models have observed no interactive effects on locomotor activity in the open field (Giovanoli et al. 2019; Petroni et al. 2022) whilst others have. Schaafsma et al. (2017) examined the interactive effects of *Cntnap2* and LPS-induced MIA and found male mutants born to MIA dams had higher activity levels at P40 than controls. Haddad et al. (2023) used a *Cntnap2* rat model exposed to poly(I:C)-induced MIA and found no interactive effects on activity in adolescence but did in adulthood. Specifically, it was observed that female MIA *Cntnap2*$^{+/-}$ rats were less active than their control counterparts whereas female MIA *Cntnap2*$^{-/-}$ rats were more active than controls. Direct comparison of activity across studies is limited given the differences in genetic models, environmental insults, age of testing and the open field protocol used. In this study all groups demonstrated habituation to the open field, including *Cyfip1*$^{+/-}$ males which contrasts the finding of (Sledziowska et al. 2020b). This difference in findings is most likely explained by the larger sample size used here as protocols used were the same. Though given the difference in the age of mice tested P25 v P60+, it is possible that *Cyfip1*$^{+/-}$males might lose the ability to habituate post-weaning. The absence of an effect of 5 mg/kg of poly(I:C) on activity levels or habituation of WTs reinforces the work of Chapter 3 and suggests social environment does not influence behaviour of WTs in the open field at this stage of development. All groups show comparable levels of exploratory behaviour within the open field, with a lower time spent in the centre on day 1 compared to day 2. MIA does appear to influence exploratory behaviour on day 1 with MIA animals spending more time in corners than controls. However, on day 2 control groups and MIA groups spend similar amounts of time in the corners of the open field. This could reflect higher levels of anxiety on day 1 in MIA mice, but controls spend a similar amount of time in the centre so this likely represents place preference rather than an affective state. MIA mice may prefer the corners and controls prefer to be

alongside a single edge. This finding would need to be reproduced to facilitate proper inferences. In summation genotype, MIA nor sex influence activity at P25 with no evidence of interactive effects between these factors.

4.5.4 P28 Social behaviour

Cyfip1$^{+/-}$ male and female mice show lower levels of social interaction compared to WT mice at P28, when considering the individual animals and litter averages. Lower social interest in *Cyfip1*$^{+/-}$ animals in direct social interaction has not been previously reported. Bachmann et al. (2019) demonstrated lower interest of adult male *Cyfip1*$^{+/-}$ mice in social odours and Sledziowska et al. (2020)highlighted the same effect in adult female *Cyfip1*$^{+/-}$ mice, without reproducing the finding in males. Given these findings relate to olfactory cues the assay utilised here was designed to place more of a reliance on olfaction. Being carried out in the dark reduced the influence of vision in guiding social behaviour. Sensory processing issues are a feature of ASD with people experiencing hypo- and hypersensitivities across all modalities and there is evidence for sensory phenotypes underpinning behavioural phenotypes in a number of genetic mouse models (Orefice et al. 2016; Robertson and Baron-Cohen 2017). Domínguez-Iturza et al. (2019), report a possible sensory related phenotype in a *Cyfip1*$^{+/-}$ mice mouse model with *Cyfip1*$^{+/-}$ mice showing reduced preference for novel textures.

A consideration is the impact of prior testing, i.e. the P9 homing test, which may have been a stressor for the *Cyfip1*$^{+/-}$ animals. The P9 homing test was chosen because it took place in the animals' stress hyporesponsive period, where stressful stimuli don't typically produce the same level of stress response as in older animals. However, it is possible that this period is not the same in *Cyfip1*$^{+/-}$ animals and testing at P9 had the impact of an additional 'hit' in this model. This could be examined in any attempt to reproduce these findings by having a cohort that does not undergo the homing test at P9. Considering MIA *Cyfip1*$^{+/-}$ males displayed an olfactory dependent social deficit at P9, it is likely that the homing test was not an additional stressor and the effect at P28, where all *Cyfip1*$^{+/-}$ groups showed lower levels than WTs is evidence *Cyfip1*$^{+/-}$ is important in social behaviour. Kim et al. (2022) has previously highlighted the importance of *Cyfip1* gene dosage for social behaviour in cKO and

overexpression models and the work here reinforces that. Furthermore, gene dosage of *Cyfip1* may be important for the litter as suggested by the observation that WTs from litters with higher proportions of *Cyfip1*$^{+/-}$ mice displayed reduced interaction times compared to WTs from litters with lower proportions of *Cyfip1*$^{+/-}$ mice. This observation adds weight to the growing body of work highlighting the importance of social environment in phenotypic outcomes(Yang et al. 2011b; Yang et al. 2015b; Kalbassi et al. 2017; Sledziowska et al. 2020b). A more robust assessment would need to take place to validate this observation, namely the testing of a pure WT cohort alongside a *Cyfip1*$^{+/-}$ cohort using the same stimulus animals.
MIA did not appear to influence social interaction times at P28 suggesting there were no interactive effects at this stage of development, but this could be test specific. Tests of social behaviour with an age-matched stimulus might unveil interactive effects or even tests with littermates would offer insights as these would examine different aspects of social behaviour. Interestingly the absence of homing behaviour at P9 did not predict reduced social interaction at P28 which may be due to differences in what each test is measuring in terms of social behaviour. Or it could reflect the absence of homing behaviour as a developmental delay that no longer exists at P28. Further work would be required to pick this apart.

Quantification of stimulus directed interaction towards experimental animals is typically overlooked and here it is demonstrated stimulus mice show lower levels of interest in MIA WT mice at P28. This finding may be a result of MIA causing WT offspring to produce lower levels of mouse urinary proteins (MUPs) which are crucial for social behaviour. The MUP, Darcin has been shown to be downregulated by acute immune activation (Lopes and König 2016) thus MIA could programme offspring to have lower baseline levels of MUPs leading to lower interest from stimulus mice. However, this effect was not observed in the same test in solely WT litters Chapter 3 which points to an indirect interaction between MIA and *Cyfip1*$^{+/-}$ impacting on WT littermates. It is also possible MIA *Cyfip1*$^{+/-}$ mice are affected but it is masked by the lower levels of social interest they display compared to WT littermates leading to more stimulus directed interaction. Stimulus mice display more interest in control mice, particularly WTs that show lower levels of interest, but this is likely an absence of social reciprocity rather than a compensation mechanism but adds weight to the notion of control animals having intact MUP levels. Social

environment can impact MUP production (Janotova and Stopka 2011) and *Cyfip1*$^{+/-}$ and MIA may interact to yield WT offspring that produce lower levels of MUPs. This could be tested in future work through analysis of MUP concentration and by using urine from MIA animals as a social stimulus for other animals. This work reinforces the need to consider the role of the stimulus animal in social tests.

4.6 Conclusions

For the first time, this work has demonstrated an interaction between MIA and *Cyfip1*$^{+/-}$ in impacting social behaviour early in development. This can be observed directly at P9 with MIA *Cyfip1*$^{+/-}$ males demonstrating less maternal attachment by taking longer to find the homing nest. However, by P28 all *Cyfip1*$^{+/-}$ groups show a social deficit with lower interaction times than WT groups. Given the importance of olfaction in both tests it is reasonable to suggest that MIA *Cyfip1*$^{+/-}$ males present with a social deficit earlier than control *Cyfip1*$^{+/-}$ males, females, or MIA *Cyfip1*$^{+/-}$ females. There is also evidence of an indirect interaction at P28 as stimulus animals show lower levels of interest in MIA WT animals than other groups. This suggests MIA and *Cyfip1*$^{+/-}$ can interact to result in MIA WT animals that produce less appetitive signals, such as MUPs, to other animals. As well as a gene x environment interaction, for the first time this work shows *Cyfip1*$^{+/-}$ resulting in a social deficit in direct interaction with other animals early in development. Additional evidence for a role of *Cyfip1*$^{+/-}$ in social behaviour comes from the impact on the social behaviour of WTs in mixed genotype litters. Higher numbers of *Cyfip1*$^{+/-}$ mice in a litter leads to lower interaction times of WTs which suggests a gene dosage effect on a group's social behaviour, in developing animals.

Chapter 5 : The Social Interaction Platform as a test of social behaviour in adult mice.

5.1 Introduction

Social behaviour is complex and requires an individual to actively process numerous cues of various sensory modalities to produce dynamic responses to the behaviour of others (Jabarin et al. 2022). The expression of social behaviour is diverse and can be classified as aggressive, cooperative, mutualistic, altruistic and parental (Ko 2017; Raam and Hong 2021). Studying social behaviour is inherently challenging due to the complexity of its underpinning characteristics, meaning progress in understanding the molecular and neuronal processes that mediate the observed behaviour is impeded (Cacioppo and Decety 2011). Inability to correctly identify and respond to social cues, as well as struggling to initiate and maintain social relationships is core to the symptomology of ASD (Lai et al. 2014). In fact, aberrant social behaviour is a devastating feature of many psychiatric (e.g. depression), neurodevelopmental, and neurodegenerative (e.g. dementia) conditions, highlighting the importance of resolving the underpinning neurobiological mechanisms (Thom et al. 2020; Voldsbekk et al. 2023).

Current tools for interrogating the substrates of social behaviour are largely unsuitable for use in humans (Jabarin et al. 2022). Therefore, elucidation of mechanisms that produce pathology in diseases impacting social behaviour requires and relies upon the development of robust animal models (Silverman et al. 2010; Silverman et al. 2022). Mice and rats are widely used to model neurodevelopmental disorders, as they are easy to maintain, display a complex repertoire of social behaviour and can undergo genetic manipulation to possess mutations that mimic those found in humans. Despite widespread use and impressive progress made in rodent models of neurodevelopment disorders direct comparison with humans remains inappropriate. One explanation for this is how well human conditions map on to rodents. Can you have an autistic mouse? This can be partially addressed by moving away from disease models and producing endophenotype models as proposed by the RDoC diagnostic framework, discussed in Section.1.5.1. By focussing on the domain, Systems for Social Processes or an individual construct

within said domain, as opposed to a specific disease, animal models are more likely to yield translatable results (Anderzhanova et al. 2017). A second explanation for the slow progress in producing translatable outcomes in the context of social behaviour is our limited understanding of rodent social behaviour (Shemesh and Chen 2023).

Mice and rats are social creatures, but the social behaviour of mice differs from rats, showing more aggression, having a simpler repertoire of social behaviours as well as showing lower levels of reward in social interactions (Kondrakiewicz et al. 2019). For both animals, social behaviour is typically assessed using simple paradigms which are criticized for lacking ecological validity (Kondrakiewicz et al. 2019; Shemesh and Chen 2023). The ideal social behaviour test would have extensive behavioural readouts, from animals living in a natural environment with continuous tracking of behaviour. Such an approach would undoubtedly further our understanding of rodent social behaviour and likely increase the translatability of results. Indeed elaborate, elegant set-ups such as the Visible Burrow System (VBS), do exist but are extremely intensive regarding operation and data collection (Shemesh and Chen 2023). Simple, reductionist tests are typically used because of the high level of experimental control whilst being less demanding in terms of labour and computation. Currently the most widely use examination of social behaviour in rodent models is the three-chamber test (Jabarin et al. 2022)

5.1.1 Three-Chamber Test

The three-chamber test assesses an animal's sociability by measuring its preference for a social environment compared to a non-social environment. It is also used to evaluate an animal's preference for a novel versus a familiar conspecific, which is termed social novelty preference (Rein et al. 2020). Section 2.4.2.2 provides an overview of the protocol used in the three-chamber test. Within the three-chamber test stimulus animals are contained within wired cups meaning only the subject animal is free to move thus is in complete control of seeking out and investigating the stimulus. Furthermore, the nature and composition of social interactions is limited with the stimulus physically constrained, though the subject is free to explore the stimulus through olfaction and touch such as whisking, though this will be restricted by the wire cups. Compared to tests where subject and stimulus are freely moving

the three-chamber test offers more control and easier scoring making it a desirable option. In fact, the three-chamber test has a diverse range of applications, from studying the development of social behaviour (Opendak et al. 2021), phenotyping genetic and environmental models to identify social deficits (Moy et al. 2004; Malkova et al. 2012; Rein et al. 2020) and evaluating pharmacological interventions and manipulations (Peñagarikano et al. 2015; Jabarin et al. 2021). Despite its almost ubiquitous use, there are several limitations of the three-chamber test. Two such limitations are considered below 1) Behavioural readouts 2) Stimulus behaviour.

5.1.1.1 Limitations of the three-chamber test

The key readouts in the three-chamber test are the time spent in a specific chamber or time spent in proximity to the wired cups. Ultimately, the complexity of social behaviour is reduced to one or two variables, that may not accurately reflect social interest. The subject animal can be in the chamber where a social stimulus is present but not be engaged in social behaviour but the “Time spent in” approach scores it as such. Expanding the nature and number of variables scored in the three-chamber test has been shown to increase our understanding of mouse behaviour within the test. Using multiple variables to model behaviour in the sociability phase of the test reveals two distinct phases, an “exploratory phase” and an “interaction phase” Curiosity and exploration appear to drive the earlier “exploratory phase” with an increased tendency to interaction with the stimulus signifying the “interaction phase” (Netser et al. 2017). This highlights the need to consider multiple variables and the dynamic nature of social behaviour.

Social interaction is typically not a one-way phenomenon, it involves more than 1 conspecific with the behavioural output of all parties being continually updated based on feedback from multimodal sensory inputs. The three-chamber test focusses solely on the subject animal with the physical experience of the stimuli the opposite to the subject. It is not an ethological test; the subject mouse is in essence investigating trapped conspecifics. Whilst the focus is on the subject animal, the stimulus animals will be producing a range of cues, olfactory, auditory and visual than elicit responses

from the subject. These cues will be impacted by numerous factors such as dominance status in the homecage, affective state, physical health, housing conditions and possessing genetic mutations that may impact social behaviour, in the same way they would impact the subject. The key difference being the stimulus is unable to express its social behaviour in the same way as the subject. This may impact the behaviour of the subject as anticipated feedback mechanisms are interrupted as typical behaviour from the subject mouse can't elicit the typical response/cue from the stimulus. Where mice encounter multiple mice, held in arenas that facilitate varying levels of sensory cues to be conveyed, they are more likely to investigate the stimuli held in arenas that allow the flow of the most complex level of sensory information (Contestabile et al. 2021). In the resident-intruder test, an anesthetised intruder evokes a USVs pattern from the stimulus that differs from the USV pattern emitted when encountering an awake stimulus (Hammerschmidt et al. 2012). Stimulus mice exhibit different movement patters based on familiarity with the subject mouse, which in turn affected the level of social interaction of the subject (Netser et al. 2020). Taken together, these few examples highlight the importance of considering the arousal/affective state of stimulus as well as the physical environment of the test, ensuring maximal sensory cues can be shared between subject and stimulus. In attempts for experimental control in genetic models, some work will use stimulus animals of the same genotype as the subject. This approach observed no effect of stimulus genotype on social behaviour in 16p11.2 deletion and *Cntnap2* mouse models (Brunner et al. 2015). The inbred mouse strain BTBR are commonly used as a model of ASD due to frequently observed low sociability (Yang et al. 2007b; McFarlane et al. 2008). Arakawa (2020b) observed BTBR subject mice displaying initial interest in BTBR stimulus mice in a sociability test before actively avoiding the stimulus. This avoidance was not observed when using other mouse strains such as C57BL/6J. It was revealed that BTBR mice produced olfactory cues that promote avoidance by other mice. These findings highlight the need to consider the genotype of the stimulus animal, if a mouse carries a mutation that impacts social behaviour can it be expected to induce typical social behaviour in subject mice. It also demonstrates the value of using different stimulus animals. A full behavioural readout from a stimulus animal can offer insights into the social behaviour of the subject animal.

Increased behavioural readouts and full access to cues can be achieved with direct interaction tests. However, given that male mice are aggressive, to the point stranger mice can be killed if placed into a common cage, welfare is a vital consideration (Kondrakiewicz et al. 2019). Ideally, even a simple social behaviour paradigm will provide the same physical experience for subject and stimulus, allowing the maximum exchange of sensory information whilst avoiding harm caused by aggression between unfamiliar conspecifics. Furthermore, it should be versatile, facilitating tests with different contexts and allow an array of data to be collected, yielding numerous variables. To this end, the Social Interaction Platform was designed, the protocol for which is outlined in Section 2.4.2.1.

5.1.2 Social Interaction Platform

The Social Interaction Platform consist of two elevated L-shaped arenas each with one open end with an adjustable gap between the arenas (Fig 2.7). This physical design is intended to allow adult male mice to physically interact across a gap via sniffing and whisking whilst avoiding bouts of aggression. The layout also allows each mouse to opt-out and retreat to a “rescue zone”, making interactions more ethological as in other simple social behaviour paradigms this is not an option. The layout of the SIP test will prevent certain types of interaction such as anogenital sniffing but facilitate tactile inputs such as facial investigation that are a crucial aspect of rodent social behaviour and merits further study (Arakawa 2020a). Mice that have their whiskers repeatedly trimmed in early development, display lower levels of sociability in the three-chamber test as an adult. Reduced sociability was also observed in mice that underwent whisker trimming in adulthood (Arakawa 2020a). Therefore, a social test that allows stranger mice to engage in facial investigation may add to our understanding of social behaviour. Whisking across a gap has been studied in a social context using rats and revealed whisking amplitude decreased on social approach, trimming of whiskers reduced the frequency of social interactions and highlighted sex differences. Females whisked with smaller amplitudes in encounters with males compared to females. Follow-up work demonstrated that social stimuli triggered greater firing in the barrel cortex of rats compared to whisking with an object stimulus. The pattern of whisking was also dependent on the nature of the stimulus with smaller, irregular whisking movements

for social touch compared to larger more regular movements for object touch (Bobrov et al. 2014). Taken together, this work demonstrates the value of studying social interaction across a gap. Moving forward with this concept, the next step was to decide how big of a gap. The work with rats used a 20cm gap, too large for mice, as such a gap of 7cm was chosen for the SIP. This was based on a study using mice that looked at object localisation through whisking which used an adjustable gap of up to 6cm, specifically to allow spontaneous crossing of the gap (Voigts et al. 2015). A 7cm gap was confirmed as appropriate using a small pilot study to see how individual mice behaved within the physical set-up and no mice crossed the gap.

The physical set-up addresses lack of consideration of the stimulus animal many social behaviours paradigms display. Within the SIP, subject and stimulus are essentially interchangeable as labels, they both have the same physical experience and have the same variables recorded. The behavioural readouts are automatically generated by the tracking software which uses centre point tracking. Table 5.1 includes the list of variables recorded. To account for the dyadic nature of social interaction, a number of the variables were attached to the other animal. For example, proportion of time spent in the social zone, is the amount of time spent in the social zone of mouse A / total time spent in the interaction zone of mouse B. The variables selected are a first step in developing a social behaviour test that allows comparison with existing tests such as the three-chamber.

The initial protocol using the SIP, consists of 5 trials each on consecutive days. In the first two trials, animals are tested individually, providing an opportunity for the animal to habituate to the arena and provides a baseline of exploratory behaviour in the absence of social stimulus. This is important as hyper – or hypoactivity may bias or mask results from the social phase of testing (Jarbarin et al. 2022). The first trial of the social phase (3rd trial overall) pairs cagemates together to see how familiar conspecifics behave in a new context. The final two trials are more akin to existing tests, pairing unfamiliar animals together.

5.2 Aims and Objectives

The Social Interaction Platform (SIP) looks to provide a behavioural paradigm that gives the experimental and stimulus animals the same experience facilitating the assessment of social interaction as a dyad. To this end, the work in this chapter aimed to evaluate the SIP as a test of social behaviour. To achieve this aim, the following study will.

1. Compare the performance of mice in the SIP examining the effect of social rank
    a. In the presence and absence of a conspecific
    b. Against the performance in other tests of social behaviour
2. Investigate how behaviour in the SIP varies with age.
3. Use the SIP to examine social behaviour in an ASD mouse model, specifically the male control and MIA offspring from the 2nd cohort of Chapter 3.

5.3 Methods

The SIP protocol, as described in Section 2.4.2.1 was utilised in 3 experiments, each with an independent cohort.

5.3.1 Experiment 1

Adult male mice, P90+ underwent the SIP along with the three-chamber, courtship and tube test to allow comparison of the readouts to assess if the SIP measures social behaviour. The cohort was split to allow counter balancing of testing, so half the cohort experienced the SIP first with the other half experiencing the three-chamber first. The order of testing was, SIP/three-chamber, tube test and courtship. There was a minimum of 2 weeks between tests.

5.3.2 Experiment 2

Sociability in adult male mice has been reported to decline with age (Shoji et al, 2016). A 2nd cohort of male mice was tested using the SIP at 3, 4, 5 and 6 months of age, as well as being used as stimulus mice in experiment 3.

5.3.3 Experiment 3

Adult male control and MIA offspring from the 2nd cohort of Chapter 3 were tested using the SIP at P120. In a change to the SIP protocol outlined previously, the 5th trial was a 2nd encounter with the cagemate. The protocol was adapted to examine how encountering an unfamiliar mouse affected an established relationship in the short-term.

Table 5.1 Variables measured in the SIP test

	Variable	Explanation
Habituation Phase	Distance travelled	
	Time in Social zone	
	Time in Rescue zone	
Social Phase	Total time spent in social zone	
	Time spent in social zone	Measured when both mice were present in social zone
	Proportion of time spent in social zone	Time spent in social zone by mouse A / Total time spent in social zone by mouse B
	Time spent in rescue zone	Measured when the other mouse was present in social zone
	Proportion of time spent in rescue zone	Time spent in rescue zone by mouse A / Total time spent in social zone by mouse B
	Mean distance from mouse in social zone	

5.4 Results

Experiment 1- Comparison of Social Interaction Platform with other tests of social behaviour

Table 5.2 Summary of results for Experiment 1

Assay	**Dominant**	**Submissive**
SIP		
Habituation	Present	Present
Social interest in cagemate	↓	**=**
Social interest in unfamiliar mice	**=**	**=**
3-Chamber		
Sociability	**=**	**=**
Social novelty preference	**=**	**=**
Courtship		
Interest in female	**=**	**=**

5.4.1 Habituation to SIP arenas

Activity of male mice in the habituation phase of the SIP protocol was higher on day 1 compared to day 2 and appeared similar for dominant and submissive animals (Fig. 5.1a). This is supported by a main effect of ***day*** (LMM ($F_{[1,33]}$ =4.8, P<0.05)), and no main effect of ***homecage status*** (LMM ($F_{[1,32]}$ =0.1, P=0.73)). when analysed with the model ***distance*** ~ ***weight*** + **chamber** + ***homecage status*** x ***day*** + ***(1 | ID)***. This model returns an ICC = 0.41, a marginal R^2 = 0.08 and a conditional R^2 = 0.45. Time spent in the social zone during the habituation phase appeared similar from day 1 to day 2 for dominant and submissive mice. (Fig. 5.1b). This is supported by no main effect of ***day*** (LMM ($F_{[1,33]}$ =0.1, P=0.75)), or ***homecage status*** (LMM ($F_{[1,33]}$ =3.4, P=0.07)) when analysed with the model ***time in social zone*** ~ ***weight*** + **chamber** + ***homecage status*** x ***day*** + ***(1 | ID)***. This model returns an ICC = 0.1, a marginal R^2 = 0.16 and a conditional R^2 = 0.06. Time spent in the rescue zone during the habituation phase of the SIP protocol increased on day 2 with dominant mice spending more time in the rescue zone than submissive mice on both days (Fig. 5.1c). This is supported by main effect of ***day*** (LMM ($F_{[1,33]}$ =27.5, P<0.001)) and ***homecage status*** (LMM ($F_{[1,31]}$ =5.4, P<0.05)). The model ***time in rescue zone*** ~ ***weight*** + **chamber** + ***homecage status*** x ***day*** + ***(1 | ID)*** returns an ICC = 0.14, a marginal R^2 = 0.31 and a conditional R^2 = 0.4. Post-hoc analysis did not confirm time spent in the rescue zone was higher for dominant mice on day 1 ($t_{[1,53.1]}$ = 1.8, p = 0.07) and day 2 ($t_{[1,53.1]}$ = 1.7, p = 0.09). These results highlight reduced exploration in the SIP arena on day 2 compared to day 1.

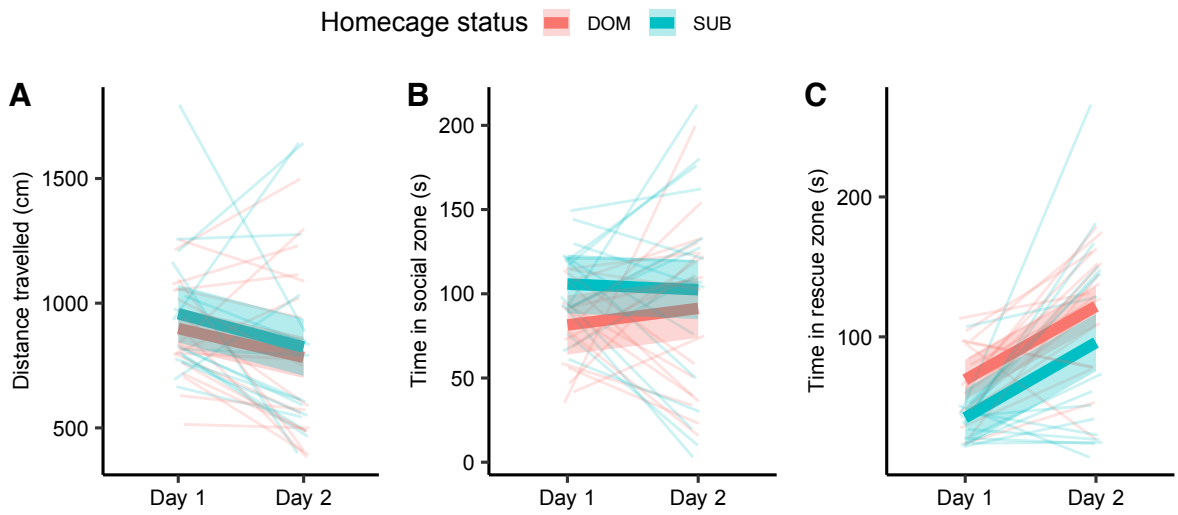


Figure 5.1. Habituation to social interaction platform chambers. **A**. Distance travelled Day 1 v. Day 2. **B.** Time spent in the social zone Day 1 v. Day 2. **C.** Time spent in rescue zone Day 1 v. Day 2. Bold lines and ribbons are means and CIs respectively with individual lines representing raw data *n* = 18 dominant males and 18 submissive males.

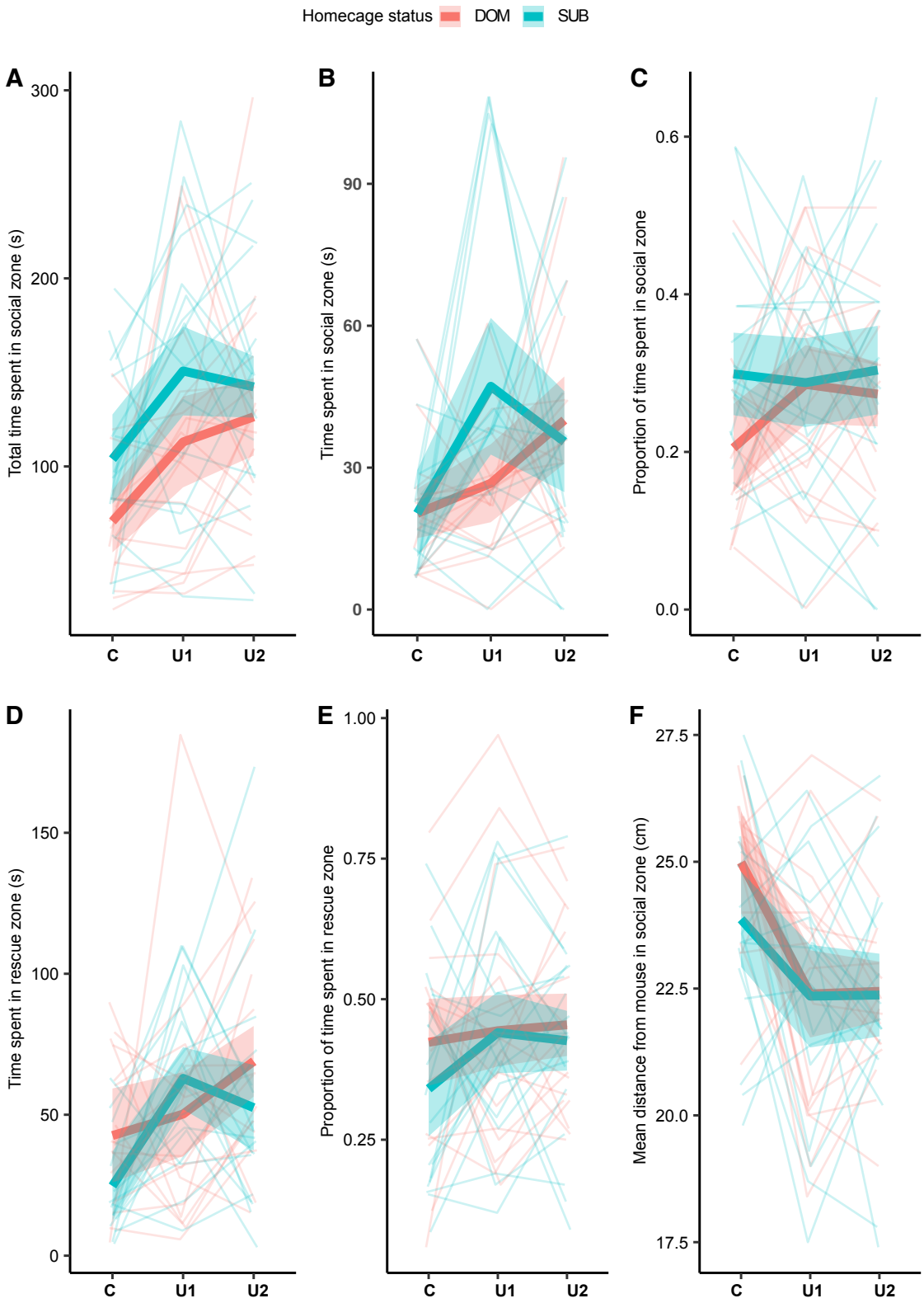


Figure 5.2. Test phases of social interaction platform. **A**. Total time spent in social zone. **B.** Time spent in the social zone at the same time as other mouse. **C.** Proportion of time spent in social zone when another mouse is in social zone. **D.** Time spent in rescue zone when the other mouse is in the social zone. **E.** Proportion of time spent in rescue zone when another mouse is in social zone. **F.** Mean distance from mouse in social zone. Bold lines and ribbons are means and CIs respectively with individual lines representing raw data *n* = 18 dominant males and 18 submissive males. (**C** = cagemate, **U1** = unfamiliar mouse 1 and **U2** = unfamiliar mouse 2).

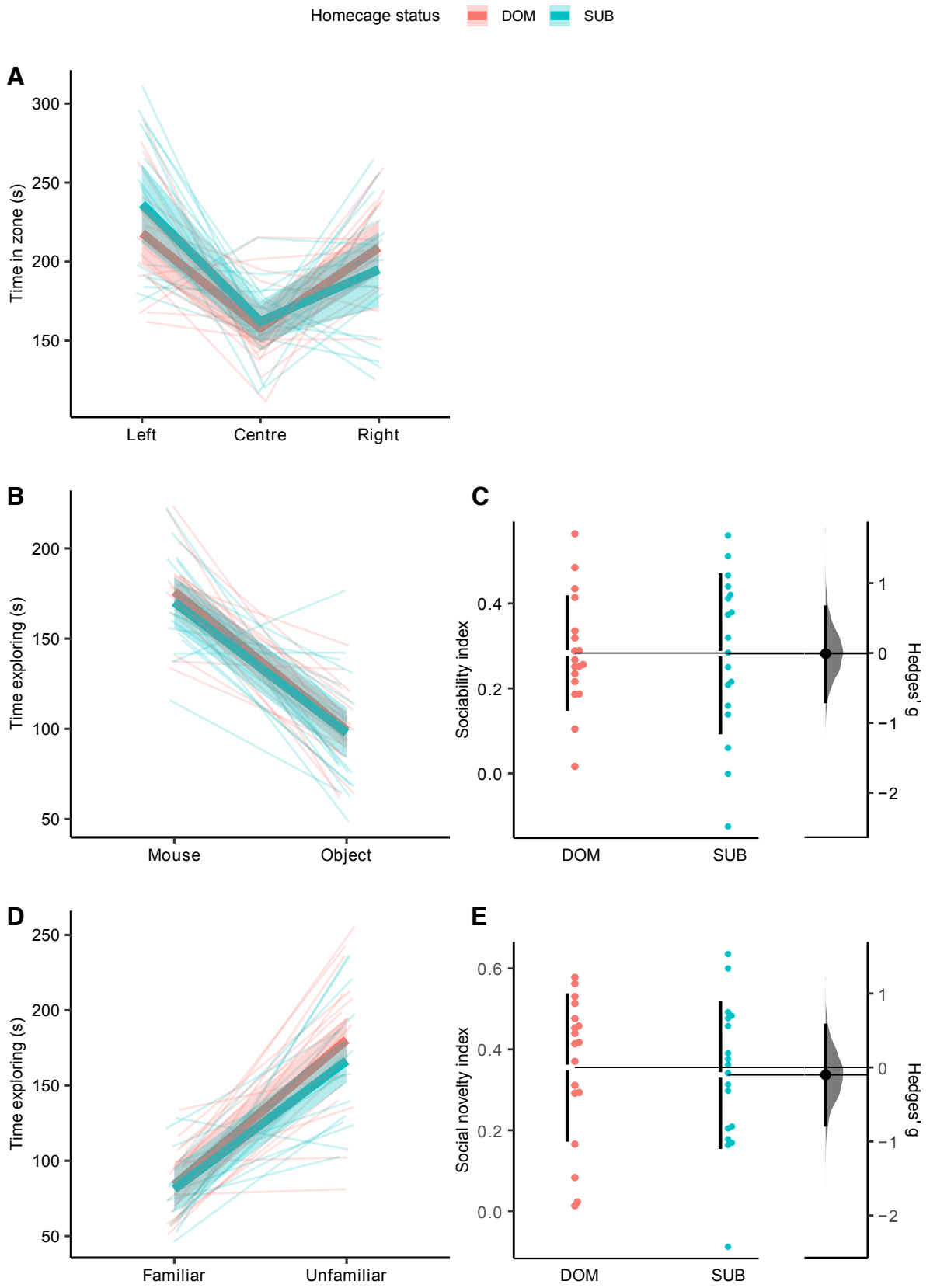


Figure 5.3. Three-Chamber test. **A**. Time spent in each chamber during habituation phase. **B.** Time spent exploring social and object stimuli during sociability test phase. **C.** Sociability index. **D.** Time spent exploring familiar and unfamiliar mice during social novelty test phase. **E.** Social novelty index. Bold lines and ribbons are means and CIs respectively with individual lines representing raw data *n* = 18 dominant males and 18 submissive males.

5.4.1.2 The social interaction platform appears to measure social interest

Total time spent in the social zone during the test phases of the SIP protocol is greatest when the mice are unfamiliar to each other and is lower in each test for dominant mice compared to submissive mice (Fig. 5.2a). This is supported by a main effect of ***test*** (LMM ($F_{[2,68]}$ =11.2, P<0.001)) and ***homecage status*** (LMM ($F_{[1,25.5]}$ =5.7, P<0.05)). The model ***Total time spent in social zone ~ weight + homecage status*** x ***day*** + ***(1 | ID) + (group)***. This model returns an ICC_{ID} = 0.31, an ICC_{group} = 0.02, a marginal R^2 = 0.2 and a conditional R^2 = 0.46. Post-hoc analysis revealed dominant and submissive mice spent longer in the social zone with 1st unfamiliar mouse (**dominant** $t_{[1,59.2]}$ = 2.7, p < 0.05), (**submissive** $t_{[1,59.2]}$ = 3, p < 0.05) and 2nd unfamiliar mouse (**dominant** $t_{[1,59.2]}$ = 3.5, p < 0.01), (**submissive** $t_{[1,59.2]}$ = 2.4, p < 0.05) than with cagemates. Post-hoc analysis highlighted submissive mice spent more time in the social zone with the 1st unfamiliar mouse than dominant mice ($t_{[1,59.2]}$ = 2.2, p < 0.05) but not the 2nd unfamiliar mouse ($t_{[1,59.2]}$ = 1.1, p < 0.05) or cagemates ($t_{[1,56]}$ = 2, p = 0.05).

The time spent in the social zone with another mouse increased when presented with an unfamiliar mouse compared to cagemates, though submissive mice demonstrated a different pattern for the two unfamiliar tests compared to dominant mice (Fig. 5.2b). This is supported by a main effect of ***test*** (LMM ($F_{[2,68]}$ =7.1, P<0.01)) with no main effect of ***homecage status*** (LMM ($F_{[1,34]}$ =1.3, P=0.26)), but an interaction of ***homecage status x test*** (LMM ($F_{[2,68]}$ =3.2, P<0.05)). ***Time spent in social zone with other mouse ~ homecage status*** *x* ***test*** + ***(1 | ID)***. This model returns an ICC = 0.06, a marginal R^2 = 0.16 and a conditional R^2 = 0.21. Post-hoc analysis revealed submissive mice spent more time in the social zone with the 1st unfamiliar mouse than a cagemate ($t_{[1,101]}$ = 3.6, p <0.01) but not the 2nd unfamiliar mouse ($t_{[1,101]}$ = 2, p =0.1). Conversely, dominant mice spent longer in the social zone with the 2nd unfamiliar mouse than cagemates ($t_{[1,101]}$ = 2.7, p <0.05) but not the 1st unfamiliar mouse ($t_{[1,101]}$ = 0.9, p =0.67). Dominant mice spent less time in the social zone with the 1st unfamiliar mouse than submissive mice ($t_{[1,101]}$ = -2.7, p <0.01) but not with the 2nd unfamiliar mouse ($t_{[1,101]}$ = 0.6, p =0.55).

The proportion of the time mice spend in the social zone with another mouse appeared similar for dominant and submissive mice when encountering unfamiliar mice, but dominant mice appeared to spend less time in the social zone with a

submissive cagemate (Fig. 5.2c). Analysis did not support these trends with no main effect of ***test*** (LMM ($F_{[2,68]}$ =1.1, P=0.34)) or ***homecage status*** (LMM ($F_{[1,25.4]}$ = 2.3, P = 0.14)). **Proportion of *time spent in social zone with other mouse ~ weight + homecage status x day + (1 | ID) + (group)*** returns an ICC_{ID} = 0.27, an ICC_{group} = 0.05, a marginal R^2 = 0.09 and a conditional R^2 = 0.38. Post-hoc analysis revealed dominant mice spend a smaller proportion of time in the social zone with a submissive cagemate ($t_{[1,53.4]}$ = -2.3, p <0.05).

Patterns for time spent in the rescue zone when the other mouse was in the social zone differed between dominant and submissive mice with both tending to spend more time in the rescue zone with unfamiliar mice (Fig. 5.2d). This is supported by a main effect of ***test*** (LMM ($F_{[2,68]}$ =7.8, P<0.001)) but not by an interaction of test *x* ***homecage status*** (LMM ($F_{[2,68]}$ =2.7, P=0.07)). ***Time spent in rescue zone with other mouse in social zone ~ weight + homecage status x day + (1 | ID)*** returns an ICC = 0.1, a marginal R^2 = 0.19 and a conditional R^2 = 0.27. Post-hoc analysis revealed submissive mice spent more time in the rescue zone with the 1st unfamiliar mouse ($t_{[1,82.6]}$ = 3.7, p <0.01) and the 2nd unfamiliar mouse ($t_{[1,82.6]}$ = 2.7, p <0.05) compared to their cagemate. Dominant mice spent more time in the rescue zone with the 2nd unfamiliar mouse compared to a cagemate ($t_{[1,84.9]}$ = 2.5, p <0.05) but not the 1st unfamiliar mouse ($t_{[1,84.9]}$ = 0.7, p =0.75). Dominant and submissive mice spend a similar proportion of time spent in the rescue zone across tests (Fig. 5.2e). This is supported by analysis with no main effect of ***test*** (LMM ($F_{[2,68]}$ =1.8, P=0.18)) or ***homecage status*** (LMM ($F_{[1,32]}$ =1.6, P = 0.22)).

Dominant and submissive mice were on average closer to unfamiliar mice in the social zone but when tested with a cagemate, dominate mice would be further away, on average from a submissive mouse in the social zone whereas submissive mice would be closer to the dominant cagemate when in the social zone (fig. 5.2f). This is supported by a main effect of ***test*** (LMM ($F_{[2,68]}$ = 12.7, P<0.001)) with no main effect of ***homecage status*** (LMM ($F_{[1,32]}$ =1, P = 0.31)). ***Mean distance from mouse in social zone ~ weight + homecage status*** x ***test*** + ***(1 | ID)***. This model returns an ICC = 0.2, a marginal R^2 = 0.18 and a conditional R^2 = 0.35. Post-hoc analysis did not support the observation that cagemates were further away from each other when a dominant cagemate was in the social zone compared to a submissive cagemate ($t_{[1,72.8]}$ = 1.7, p =0.1). Taken together the data suggest the Social Interaction Platform

test measures social interest, highlighting differences between cagemates based on hierarchical status.

5.4.1.3 Dominant and submissive mice show similar levels of sociability and interest in social novelty

In the habituation phase of the 3-chamber test dominant and submissive animals appeared to spend less time in the centre than the left or right zones, with submissive animals showing a preference for the left zone over the right (Fig. 5.3a). Analysis supported this observation with a main effect of ***zone*** (LM ($F_{[2,101]}$ = 14.6, P<0.001)) with the model ***Time in zone ~ distance travelled*** + ***zone*** *x* ***homecage status*** which returned an adjusted R^2 =0.37. Post hoc tests highlighted dominant animals spent more time in the right ($t_{[1,101]}$ = 4.2, p <0.001) and left zone ($t_{[1,101]}$ = 5, p <0.001) compared to the centre zone with no difference between left and right ($t_{[1,101]}$ = 0.8, p = 0.72). Similarly, submissive animals spent more time in the right ($t_{[1,101]}$ = 2.7, p <0.05) and left zones ($t_{[1,101]}$ = 6.2, p <0.001) compared to the centre but also spent more time in the left zone compared to the right zone ($t_{[1,101]}$ = 3.4, p <0.01).

During the sociability phase of the 3-chamber test dominant and submissive mice spent similar amounts of time exploring the stimulus mouse compared to the empty container (Fig. 5.3b). Analysis supported this observation with a main effect of ***stimulus*** (LM ($F_{[2,63]}$ = 56.7, P<0.001)) with the model ***Time exploring stimulus ~ cohort*** + ***stimulus*** + ***homecage status + weight*** which returned an adjusted R^2 = 0.65. The sociability index was similar for dominant and submissive mice (Fig. 5.3c) support by no main effect of ***homecage status*** (LMM ($F_{[1,31.3]}$ = 0.02, P = 0.89)). In the social novelty preference phase dominant and submissive mice showed a preference for exploring an unfamiliar mouse compared to a familiar mouse (Fig 5.3d). Analysis supported this observation with a main effect of ***stimulus*** (LM ($F_{[2,63]}$ = 45.6, P<0.001)) with the model ***Time exploring stimulus ~ cohort*** + ***stimulus*** + ***homecage status + weight*** which returned an adjusted R^2 = 0.66. The social novelty index was similar for dominant and submissive mice (Fig. 5.3e) supported by no main effect of ***homecage status*** (LM ($F_{[1,31]}$ = 0.04, P = 0.83)). The potential bias of submissive mice for one chamber in the habituation phase did not appear to

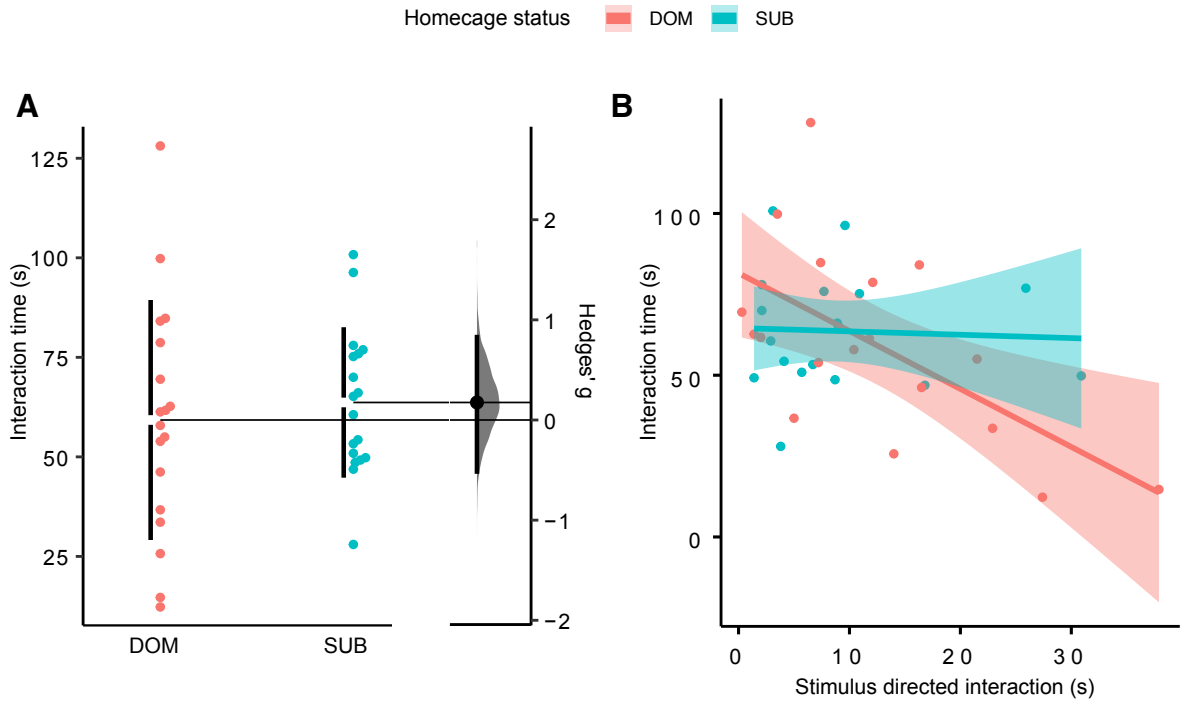


Figure 5.4. Courtship test. **A**. Estimation plot showing interaction time of males with females and Hedges' *g* effect size for dominant and submissive males. **B.** Correlation between male interaction times and stimulus directed interaction times. Bold lines and ribbons are regression lines and CIs respectively with individual points representing raw data *n* = 18 dominant males and 18 submissive males.

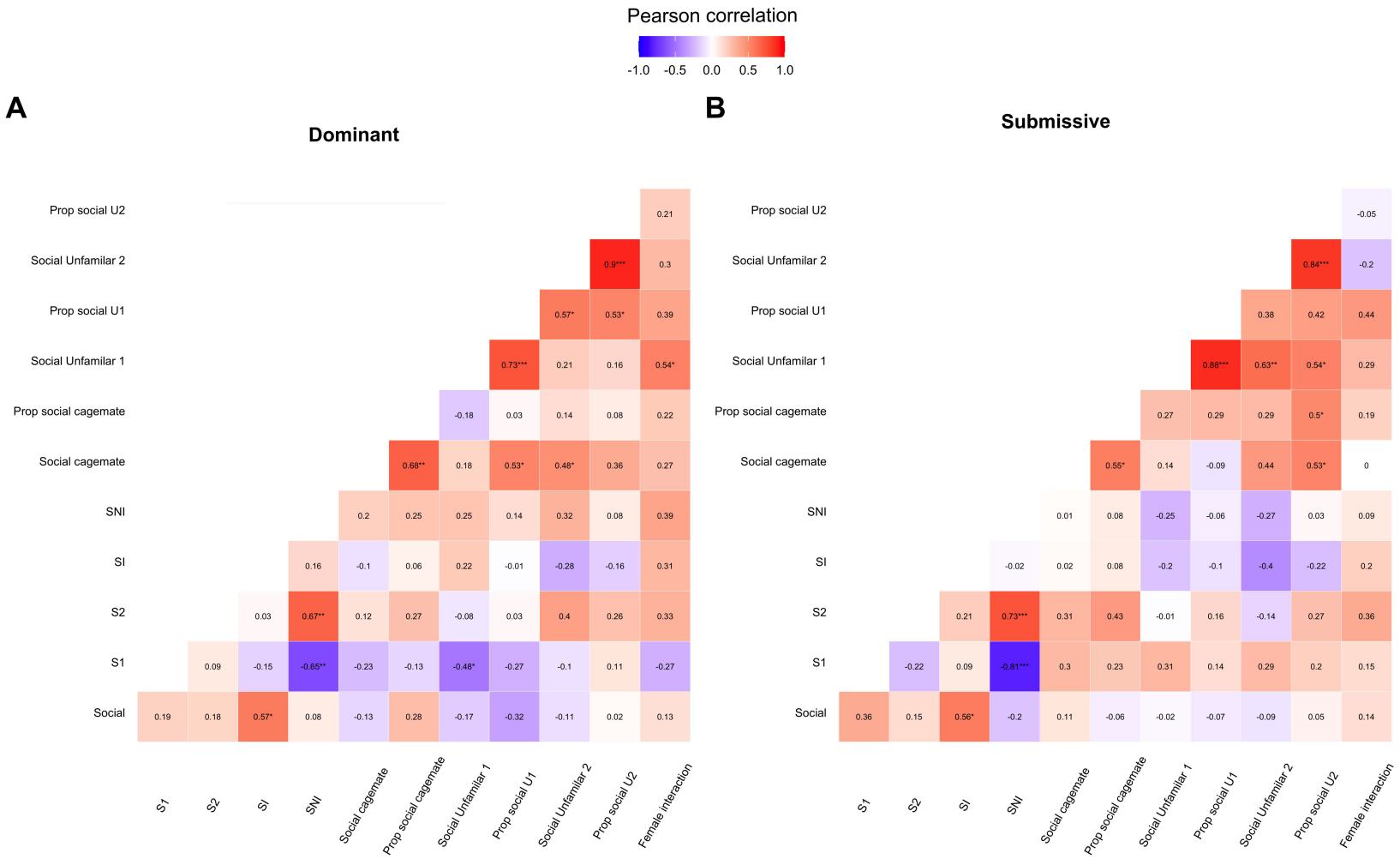


Figure 5.5. Correlation heatmaps comparing social tests. **A**. Dominant **B.** Submissive Correlation between male interaction times and stimulus directed interaction times. *n* = 18 dominant males and 18 submissive males.

impact the test phases as both dominant and submissive mice show a preference for a social stimulus over and object. Social novelty preference is also similar for dominant and submissive mice.

5.4.1.5 Female mice retain higher levels of interest in disinterested dominant males in the courtship assay

Dominant and submissive mice appeared to spend a similar amount of time interaction with a female (Fig. 5.4a), which was supported by analysis with no main effect of ***homecage status*** (LMM ($F_{[1,26]}$ = 0.3, P = 0.6)). The interaction time of dominant male mice appeared be lower at higher stimulus directed interaction times, a trend not seen for submissive male mice (Fig. 5.4b). This was supported by a main effect of ***stimulus directed interaction time*** (LM ($F_{[1,32]}$ = 11.6, P<0.01)) but not by a main effect of ***homecage status*** (LM($F_{[1,32]}$ = 2.2, P = 0.14)) or an interaction ***stimulus directed interaction time*** *x* ***homecage status*** (LM($F_{[1,32]}$ = 4.1, P = 0.05)). The model ***Interaction time ~ stimulus directed interaction time*** *x* ***homecage status*** returned an adjusted R^2 =0.2. Dominant and submissive mice show the same level of interest in female mice. However, these results highlight female mice as demonstrating higher levels of interest in dominant males that display lower levels of social interest compared to submissive males.

5.4.1.6 Correlation between social interest variables across tests show differing trends for dominant and submissive mice

Variables attributed to appetitive aspects of social behaviour demonstrate strong correlation within tests for dominant and submissive mice (Fig. 5.5). No significant correlations exist between variables of different tests but opposing trends exist for dominant and submissive mice. The social novelty index for dominant mice positively correlates with social interest variables from the SIP and courtship tests, a pattern not seen in submissive mice. Similarly for submissive mice, the time spent investigating the familiar mouse in the social novelty phase of the 3-chamber test positively correlates with variables from the SIP and courtship tests, unlike dominant mice which show a negative trend.

Considering all results from experiment 1 the SIP appears to measure social interest but may probe different aspects of social interest from other tests. Furthermore, the SIP demonstrates potential in highlighting differences between dominant and submissive cagemates.

Experiment 2 Longitudinal assessment of social behaviour using the SIP test.

Table 5.3 Summary of results for Experiment 2

Assay	
SIP	
Habituation	Present
Social interest in cagemate	Declines with age
Social interest in unfamiliar mice	Declines with age

5.4.2.1 Exploratory behaviour of SIP arenas decreases with age

Activity of male mice in the habituation phase of the SIP protocol was higher on day 1 compared to day 2 at 3 and 4 months of age but this trend reversed at 5 months (Fig. 5.6a). This is supported by main effect of ***day*** (LMM($F_{[1,53.6]}$ =7.3, P<0.01)), ***age*** (LMM($F_{[2,54.5]}$ =5.1, P<0.01)) and an interaction between ***day x age*** (LMM($F_{[2,53.6]}$ =11.8, P<0.001)) when analysed with the model ***distance ~ weight* + chamber + *age* x *day* + *(1 | ID) + (1|Cagemate ID)***. This model returns an ICC_{ID} = 0.17, $ICC_{cagemate}$ = 0.002, a marginal R^2 = 0.36 and a conditional R^2 = 0.47. Post-hoc analysis revealed activity was higher on day 1 than on day 2 at 3 months ($t_{[1,39.7]}$ = 4.8, p < 0.001) but not at 4 months ($t_{[1,41.8]}$ = 1.8, p =0.07) or 5 months ($t_{[1,38.6]}$ = -2, p =0.05). Time spent in the social zone during the habituation phase decreases from day 1 to day 2 at all ages but appears reduced on day 1 at 5 months compared to 3 and 4 months. (Fig. 5.6b). This is supported by main effect of ***day*** (LMM ($F_{[1,53.1]}$ =45.6, P<0.001)), ***age*** (LMM ($F_{[2,62.7]}$ =24.5, P<0.001)) and an interaction between ***day x age*** (LMM ($F_{[2,53.1]}$ =8.7, P<0.001)). when analysed with the model ***time in social zone ~ weight* + chamber + *age* x *day* + *(1 | ID) + (1|Cagemate ID)***. This

model returns an ICC_{ID} = 0.33, $ICC_{cagemate}$ = 0.24, a marginal R^2 = 0.51 and a conditional R^2 = 0.79. Post-hoc analysis revealed lower time spent in the social zone on day 2 compared to day 1 at 3 months ($t_{[1,33.4]}$ = 6.8, p < 0.001), 4 months ($t_{[1,23.5]}$ = 3.9, p < 0.001) but not 5 months ($t_{[1,34.2]}$ = 0.9, p =0.35). Furthermore at 5 months of age time spent in the social zone was lower than at 3 months on day 1($t_{[1,33.4]}$ = 6.4, p < 0.001) and day 2 ($t_{[1,33.4]}$ = 2.9, p < 0.05) and 4 months on day 1 ($t_{[1,23.5]}$ = 7, p < 0.001) and day 2 ($t_{[1,23.5]}$ = 4.7, p < 0.001). Time spent in the rescue zone during the habituation phase of the SIP protocol increased on day 2 at all ages (Fig. 5.6c). This is supported by main effect of ***day*** (LMM ($F_{[1,53.3]}$ =23.6, P<0.001)) and ***age*** (LMM ($F_{[2,58.5]}$ =10.2, P<0.001)). when analysed with the model ***time in rescue zone*** ~ ***weight*** **+ chamber +** ***age*** x ***day*** + ***(1 | ID) + (1|Cagemate ID)***. This model returns an ICC_{ID} = 0.02, $ICC_{cagemate}$ = 0.3, a marginal R^2 = 0.46 and a conditional R^2 = 0.63. Post-hoc analysis revealed time spent in the rescue zone was higher at 5 months on day 1 compared to 3 months ($t_{[1,38.8]}$ = -3.5, p < 0.01) and 4 months ($t_{[1,38.8]}$ = -3.4, p < 0.01). This was also observed on day 2 when comparing 5 months to 3 months ($t_{[1,38.8]}$ = -4.1, p < 0.01) and 4 months ($t_{[1,38.8]}$ = -2.7, p < 0.05). Taken together exploratory behaviour in the habituation phase of the SIP is lower on day 2 compared to day 1 and reduces with age.

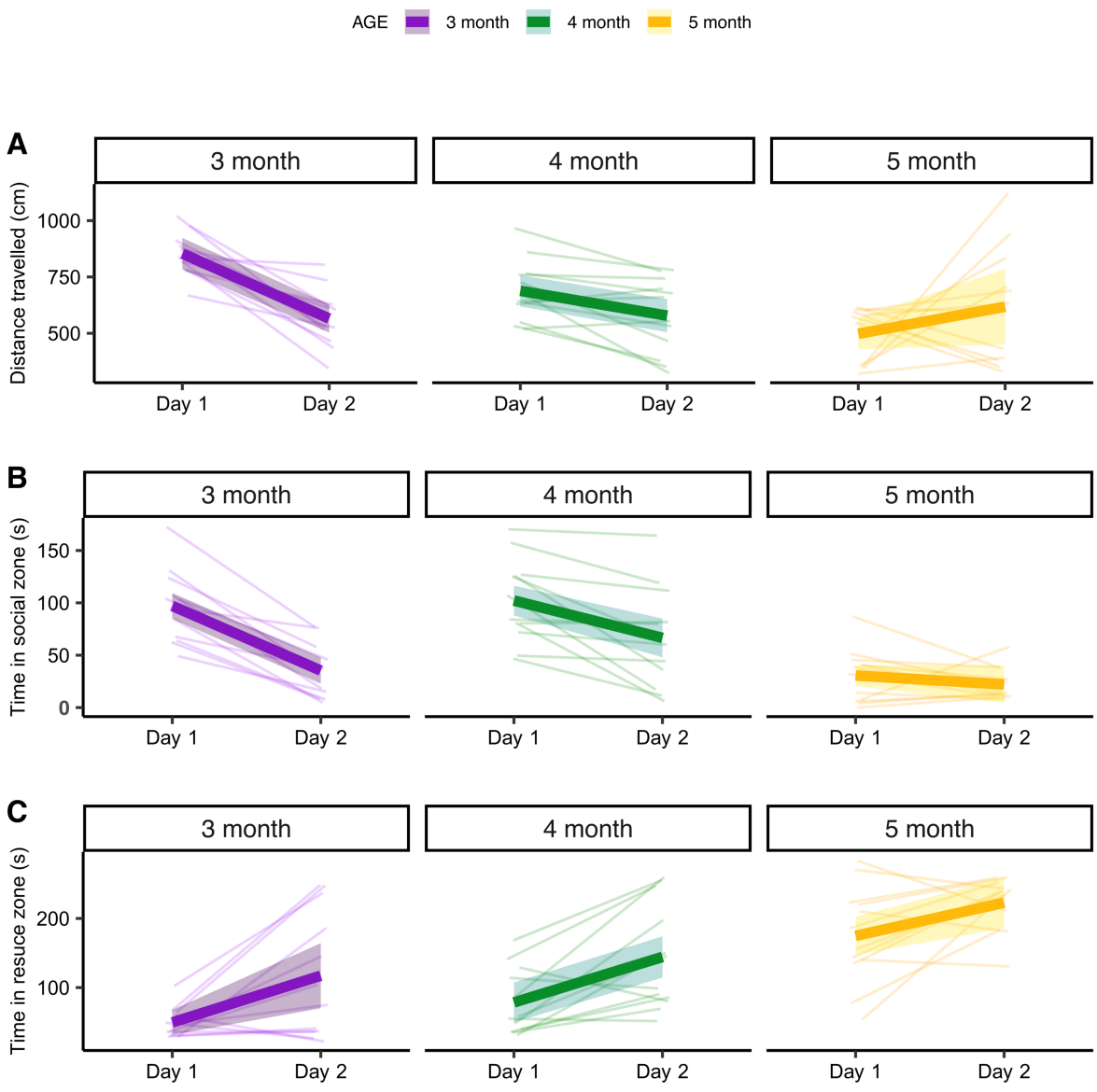


Figure 5.6. Habituation to social interaction platform chambers at 3, 4 and 5 months of age. **A**. Distance travelled Day 1 v. Day 2. **B.** Time spent in the social zone Day 1 v. Day 2. **C.** Time spent in rescue zone Day 1 v. Day 2. Bold lines and ribbons are means and CIs respectively with individual lines representing raw data *n* = 12

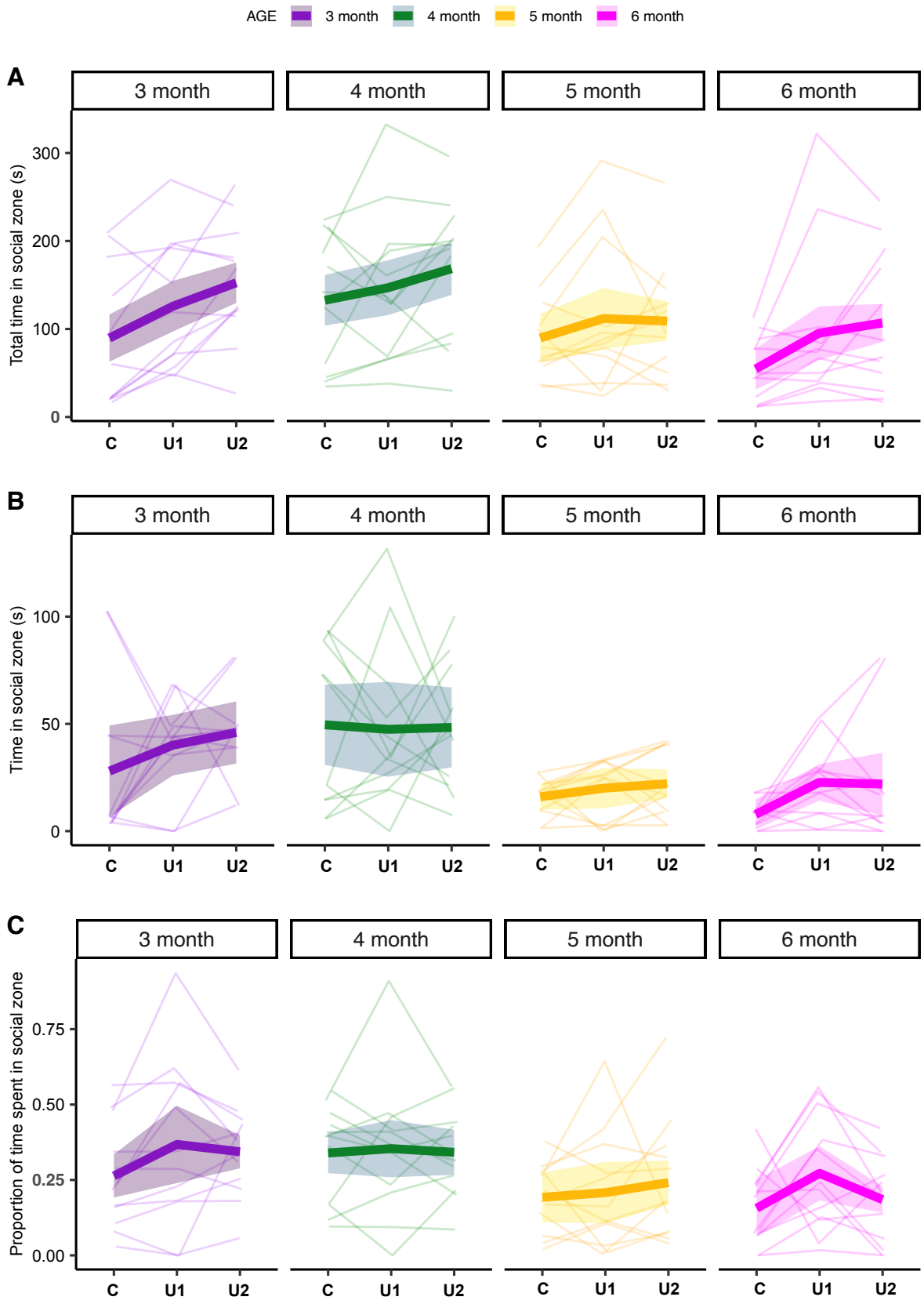


Figure 5.7. Test phases of social interaction platform at 3, 4, 5 and 6 months of age. **A**. Total time spent in social zone. **B.** Time spent in the social zone at the same time as other mouse. **C.** Proportion of time spent in social zone when another mouse is in social zone. Bold lines and ribbons are means and CIs respectively with individual lines representing raw data *n* = 12. (**C** = cagemate, **U1** = unfamiliar mouse 1 and **U2** = unfamiliar mouse 2).

5.4.2.2 Social interest decreases with age

Total time spent in the social zone during the test phase of the SIP protocol appeared higher when presented with an unfamiliar mouse compared to cagemate which persisted with age though overall time in the social zone reduced after 4 months (Fig. 5.7a). This is supported by main effect of ***test*** (LMM ($F_{[2,112.8]}$ =13, P<0.001)) and ***age*** (LMM ($F_{[3,38.4]}$ =9.7, P<0.001)) when analysed with the model ***Total time in social zone*** ~ **age** x ***day*** + ***(1 | ID) + (1|Cagemate ID) + (1|Paired cage ID)***. This model returns an ICC_{ID} = 0.3, $ICC_{cagemate}$ = 0.33, $ICC_{paired\ cage}$ = 0.04, a marginal R^2 = 0.16 and a conditional R^2 = 0.73. Post-hoc analysis revealed at 3 months of age mice spent more time in the social zone with the 2nd unfamiliar mouse than the cagemate ($t_{[1,35.5]}$ = -3.7, p < 0.01) with no difference between the 1st unfamiliar mouse and cagemate ($t_{[1,35.5]}$ = -2.1, p =0.1). No differences between tests were seen at 4 or 5 months of age but at 6 months mice spent more time in the social zone with the 2nd unfamiliar mouse than the cagemate ($t_{[1,40.7]}$ = -3.1, p < 0.01) with no difference between the 1st unfamiliar mouse and cagemate ($t_{[1,40.7]}$ = -2.4, p =0.05).

The time spent in the social zone when the other mouse was also in the social zone appeared similar between tests of cagemates and unfamiliar mice and appeared to vary with age (Fig. 5.7b). This is supported by main effect of ***age*** (LMM ($F_{[3,36.2]}$ =3.1, P<0.05)) but not ***test*** (LMM ($F_{[2,111.7]}$ =2.5, P=0.09)) when analysed with the model ***Time in social zone ~ weight + age*** x ***day*** + ***(1 | ID) + (1|Cagemate ID) + (1|Paired cage ID)***. This model returns an ICC_{ID} = 0.01, $ICC_{cagemate}$ = 0.12, $ICC_{paired\ cage}$ = 0.19, a marginal R^2 = 0.23 and a conditional R^2 = 0.48. Post-hoc analysis revealed the time spent in the social zone with a cagemate was lower at 5 months ($t_{[1,41.5]}$ = -2.8, p < 0.05) and 6 months $t_{[1,43.7]}$ = -2.7, p < 0.05) compared 4 months.

The proportion of time spent in the social zone when another mouse was also in the social zone appeared similar between cagemates and unfamiliar mice and appeared to reduce with age (Fig. 5.7c). This is supported by main effect of ***age*** (LMM($F_{[3,46.8]}$ =6.7, P<0.001)) but not ***test*** (LMM($F_{[2,112.7]}$ =2.9, P=0.06)) when analysed with the model ***Proportion of time in social zone ~ weight + age*** x ***day*** + ***(1 | ID) + (1|Cagemate ID) + (1|Paired cage ID)***. This model returns an ICC_{ID} = 0.27,

$ICC_{cagemate}$ = 0.21, $ICC_{paired\ cage}$ = 0.02, a marginal R^2 = 0.17 and a conditional R^2 = 0.59. Post-hoc analysis revealed cagemates spent a smaller proportion of time in the social zone together at 6 months compared to 3 months of age ($t_{[1,51]}$ = -2.9, p < 0.05) and 4 months ($t_{[1,40]}$ = -3.8, p < 0.01). Cagemates also spent a smaller proportion of time in the social zone together at 5 months compared to 4 months of age.

Time spent in the rescue zone appeared higher for tests with unfamiliar mice compared to cagemates but stayed consistent with age (Fig. 5.8a). This is supported by main effect of ***test*** (LMM ($F_{[2,112.7]}$ =3.5, P<0.05)) but not ***age*** (LMM ($F_{[3,40.1]}$ =1.4, P=0.27)) when analysed with the model ***Time in rescue zone ~ weight + age*** x ***day*** + ***(1 | ID) + (1|Cagemate ID) + (1|Paired cage ID)***. This model returns an ICC_{ID} = 0.02, $ICC_{cagemate}$ = 0.04, $ICC_{paired\ cage}$ = 0.09, a marginal R^2 = 0.1 and a conditional R^2 = 0.23. Post-hoc analysis revealed no specific difference between ages. The proportion of time spent in the rescue zone when the other mouse was in the social zone appeared to vary between tests of cagemates and unfamiliar mice whilst generally increasing with age (Fig. 5.8b). Analysis did not support a main effect of ***test*** (LMM($F_{[2,112.4]}$ =0.5, P=0.59)) but did support a main effect of ***age*** (LMM($F_{[3,42.5]}$ =4.8, P<0.01)) when analysed with the model ***Proportion of time in rescue zone ~ weight + age*** x ***day*** + ***(1 | ID) + (1|Cagemate ID) + (1|Paired cage ID)***. This model returns an ICC_{ID} = 0.21, $ICC_{cagemate}$ = 0.15, $ICC_{paired\ cage}$ = 0.04, a marginal R^2 = 0.24 and a conditional R^2 = 0.54. Post-hoc analysis revealed cagemates spent a smaller proportion of time in the rescue zone at 3 months compared to 5 months ($t_{[1,50.9]}$ = -2.7, p < 0.05) and 6 months ($t_{[1,62]}$ = -3.4 p < 0.01). No differences were seen across the age groups for the 1st test with an unfamiliar mouse. However, in the 2nd unfamiliar test, mice spent a greater proportion of time in the rescue zone at 6

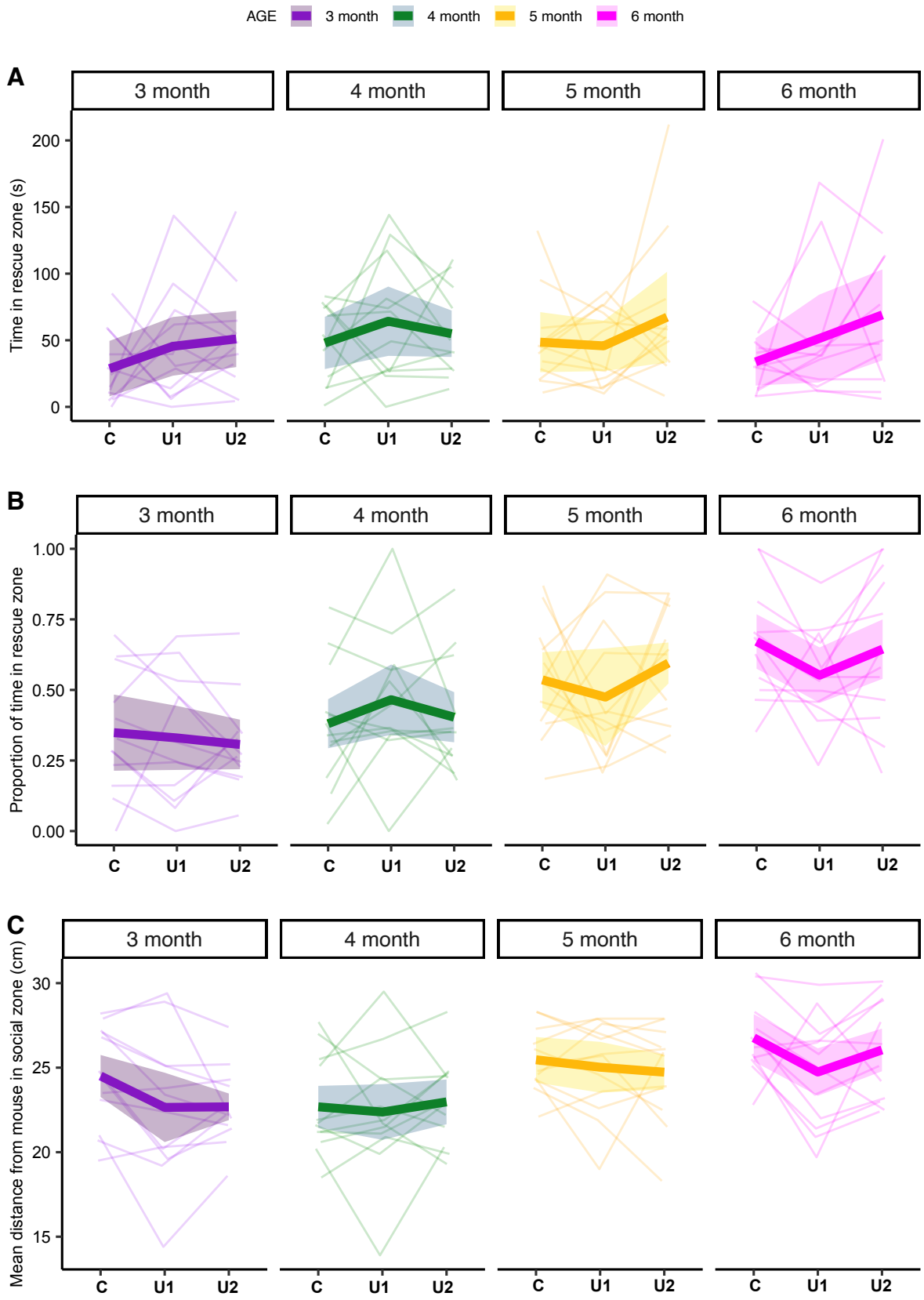


Figure 5.8. Test phases of social interaction platform at 3, 4, 5 and 6 months of age. **A**. Time spent in rescue zone when the other mouse is in the social zone. **B.** Proportion of time spent in rescue zone when another mouse is in social zone. **C.** Mean distance from mouse in social zone. Bold lines and ribbons are means and CIs respectively with individual lines representing raw data *n* = 12. (**C** = cagemate, **U1** = unfamiliar mouse 1 and **U2** = unfamiliar mouse 2).

months compared to 3 months ($t_{[1,62]} = 3.5$, $p < 0.01$) and 4 months ($t_{[1,53.2]} = 2.9$, $p < 0.05$). Similarly at 5 months, mice spent a greater proportion of time in the rescue zone compared to 3 months ($t_{[1,50.9]} = 3.8$, $p < 0.01$) and 4 months ($t_{[1,50.9]} = 2.7$, $p < 0.05$).

The mean distance between mice when one is present in the social zone of the SIP increase with age whilst varying between tests at some ages (Fig. 5.8c). This is supported by main effect of ***age*** (LMM ($F_{[3,42]}$ =6.8, P<0.001)) and ***test*** (LMM ($F_{[2,112.3]}$ =3.8, P<0.05)) when analysed with the model ***mean distance between mice ~ weight + age*** x ***day*** + ***(1 | ID) + (1|Cagemate ID) + (1|Paired cage ID)***. This model returns an ICC_{ID} = 0.4, $ICC_{cagemate}$ = 0.06, $ICC_{paired\ cage}$ = 0.04, a marginal R^2 = 0.21and a conditional R^2 = 0.6. Post-hoc analysis revealed the mean distance between cagemates when one was occupying the social zone at 6 months was larger compared to 3 months ($t_{[1,52.9]} = 2.9$, $p < 0.05$) and 4 months ($t_{[1,42.1]} = 4.3$, $p < 0.001$). At 5 months the mean distance between cagemates when one was occupying the social zone was larger than at 4 months ($t_{[1,39.5]} = 3.5$, $p < 0.01$). During the 1st unfamiliar test, the mean distance from a mouse in the social zone was larger at 6 months compared to 3 months ($t_{[1,52.9]} = 2.8$, $p < 0.05$) and 4 months ($t_{[1,42.1]} = 3$, $p < 0.05$), as well as being larger at 5 months compared to 3 months ($t_{[1,39.5]} = 3.2$, $p < 0.05$) and 4 months ($t_{[1,39.5]} = 3.3$, $p < 0.01$). During the 2nd unfamiliar test, the mean distance from a mouse in the social zone was larger at 6 months compared to 3 months ($t_{[1,52.9]} = 3.6$, $p < 0.01$) and 4 months ($t_{[1,42.1]} = 3.6$, $p < 0.01$), as well as being larger at 5 months compared to 3 months ($t_{[1,39.5]} = 2.9$, $p < 0.05$). Post-hoc analysis did not reveal any specific differences between tests at any age. All together, these data demonstrate a reduction in social interest with age, as measured by the SIP.

Experiment 3 Using the SIP to compare social behaviour in adult male MIA and control offspring

Table 5.4 Summary of results for Experiment 3

Assay	**Controls**	**MIA**
SIP		
Habituation	Present	Present
Social interest in cagemate	↓	↓
Social interest in unfamiliar mice	↑	↑
Social interest in cagemate after unfamiliar encounter	↑	**=**

5.4.3 Control and MIA offspring habituate to the SIP arenas

Control and MIA Male offspring from the 2nd experimental cohort of Chapter 3 showed similar patterns of activity in the habituation phase of the SIP protocol which both groups showing reduced activity on day 2 (Fig. 5.9a). This was supported by a main effect of ***day*** (LMM ($F_{[1,28]}$ =86.2, P<0.001)), with no main effect of ***dose*** (LMM ($F_{[1,27]}$ =0.1, P=0.72)) when analysed with the model ***distance ~ weight + dose*** x ***day*** + ***(1 | ID)***. This model returns an ICC = 0.4, a marginal R^2 = 0.46 and a conditional R^2 = 0.69. Both groups spent less time in the social zone on day 2 (Fig. 5.9b), supported by a main effect of ***day*** (LMM($F_{[1,28]}$ =116, P<0.001)), with no main effect of ***dose*** (LMM($F_{[1,27]}$ =0.16, P=0.9)) when analysed with the model ***Time in social zone ~ weight + dose*** x ***day*** + ***(1 | ID)***. This model returns an ICC = 0.72, a marginal R^2 = 0.36 and a conditional R^2 = 0.82. Both groups spent more time in the rescue zone on day 2 (Fig. 5.9c) supported by a main effect of ***day*** (LMM($F_{[1,28]}$ =33.6, P<0.001)), with no main effect of ***dose*** (LMM($F_{[1,27]}$ =0.0004, P=0.98)) when analysed with the model ***Time spent in rescue zone ~ weight + dose*** x ***day*** + ***(1 | ID)***. This model returns an ICC = 0.32, a marginal R^2 = 0.28 and a conditional R^2 = 0.51. Control and MIA offspring behave similarly, showing reduced exploration on day 2 of the habituation phase.

5.4.4 MIA males show reduced social interest in cagemates after encountering an unfamiliar mouse.

Total time spent in the social zone during the test phases of the SIP protocol is greatest when the mice are unfamiliar to each other and returns to lower levels upon the second test with a cagemate (Fig. 5.10a). This is supported by a main effect of ***test*** (LMM ($F_{[2,54]}$ =14.8, P<0.001)). MIA offspring appear to show less of an increase in the presence of an unfamiliar mouse than controls and show a reduction in time spent in the social zone upon second test with the cagemate (Fig. 5.10a). Analysis did not return a main effect of ***dose*** (LMM ($F_{[1,26]}$ =0.13, P=0.72)), nor an interaction of ***dose x test*** (LMM ($F_{[2,54]}$ =2.8, P=0.07)). ***Total time spent in social zone ~ weight + dose*** x ***test*** + ***(1 | ID) +(1|cagemate ID)***. This model returns an ICC_{ID} = 0.01, $ICC_{cagemate}$ = 0.42, a marginal R^2 = 0.21 and a conditional R^2 = 0.55. Post-hoc analysis supported the observation control offspring spent longer in the social zone in the presence of unfamiliar mice ($t_{[1,56]}$ = -3.4, p < 0.05) but not for MIA offspring ($t_{[1,56]}$ = -1.4, p =0.72). Post-hoc analysis also supported the observation MIA offspring spend less time in social zone upon the second presentation of a cagemate ($t_{[1,55.9]}$ = 3, p < 0.05) whereas there is no difference for controls ($t_{[1,55.9]}$ = 1.2, p = 0.8). The time spent in social zone when the other mouse was also in the social zone

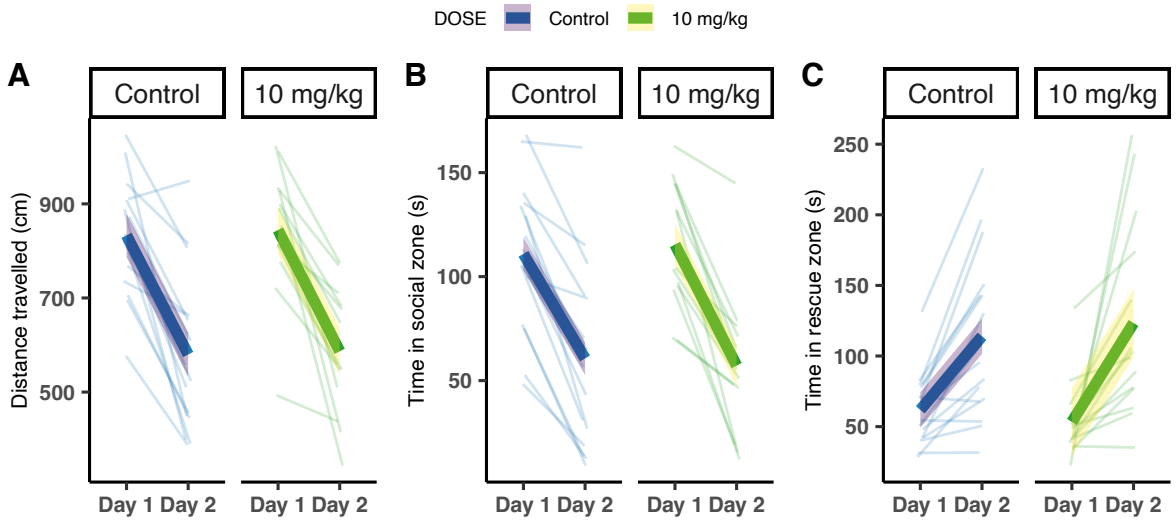


Figure 5.9. Habituation to social interaction platform chambers in control and MIA offspring. **A**. Distance travelled Day1 v. Day2. **B.** Time spent in the social zone Day1 v. Day2. **C.** Time spent in rescue zone Day1 v. Day3. Bold lines and ribbons are means and CIs respectively with individual lines representing raw data *n* = 16 control males,14 male MIA offspring.

increased from 1st cagemate presentation to encounter with an unfamiliar mouse and lowered upon 2nd cagemate presentation (Fig. 5.10b). This is supported by a main effect of ***test*** (LMM ($F_{[2,54]}$ =13.2, P<0.001)) with no main effect of ***dose*** (LMM ($F_{[1,26]}$ =0.91, P=0.35)), nor an interaction of ***dose x test*** (LMM ($F_{[2,54]}$ =2.2, P=0.11)). ***Time spent in social zone with other mouse ~ weight + dose*** x ***test*** + ***(1 | ID)***. This model returns an ICC = 0.05, a marginal R^2 = 0.26 and a conditional R^2 = 0.3. Post-hoc analysis supported the observation both control ($t_{[1,79]}$ = -3.1, p <0.01) and MIA offspring ($t_{[1,78.9]}$ = -3.3, p <0.01) spend significantly longer in the social zone in the presence of an unfamiliar mouse compared to a cagemate. Control mice ($t_{[1,79]}$ = 1.7, p =0.2) did not show a reduction in time spent in the social zone with the cagemate following exposure to an unfamiliar mouse but MIA offspring did ($t_{[1,78.9]}$ = 4.3, p <0.001). This was reinforced by the time spent in the social zone with the cagemate on 2nd presentation being higher in control than MIA offspring ($t_{[1,78.9]}$ = 2.2, p <0.05).

The time spent in the social zone by unfamiliar stimulus mice was similar between groups (Fig. 5.10c). This was supported by no main effect of ***dose*** (LMM ($F_{[1,15.7]}$ =0.1, P=0.75)) when analysed with the model ***Stimulus time in social zone ~ weight + dose (1 |*** **Stimulus** ***ID)***. This model returns an ICC = 0.73, a marginal R^2 = 0.05 and a conditional R^2 = 0.75. The proportion of the time mice spend in the social zone with another mouse present was greatest when mice were paired with an unfamiliar mouse (Fig. 5.10d), supported by a main effect of ***test*** (LMM ($F_{[2,54]}$ =7.1, P<0.01)) with no main effect of ***dose*** (LMM ($F_{[1,26]}$ =0.59, P=0.45)). **Proportion of *time spent in social zone with other mouse ~ weight + dose*** x ***test*** + ***(1 | ID)*** returns an ICC = 0.23, a marginal R^2 = 0.14 and a conditional R^2 = 0.33. Post-hoc analysis revealed control offspring spend a greater proportion of time in the social zone when the other mouse present is unfamiliar ($t_{[1,70.4]}$ = -3, p <0.05). MIA offspring show a similar pattern, but this is not supported by post-hoc analysis ($t_{[1,70.4]}$ = -2.3, p =0.06). Controls appear to spend a greater proportion of time in the social zone with cagemates on the 2nd presentation compared to MIA offspring, but this is not supported by analysis ((controls) $t_{[1,70.4]}$ = -2.2, p =0.06) versus ((MIA) $t_{[1,70.4]}$ = -0.2, p =0.97). The time spent in the rescue zone when the other mouse was also in the social zone increased from 1st cagemate presentation to encounter with an unfamiliar mouse and lowered upon 2nd cagemate presentation (Fig. 5.10e). This is supported by a main effect of ***test*** (LMM ($F_{[2,54]}$ =3.3, P<0.05)) with no main effect of

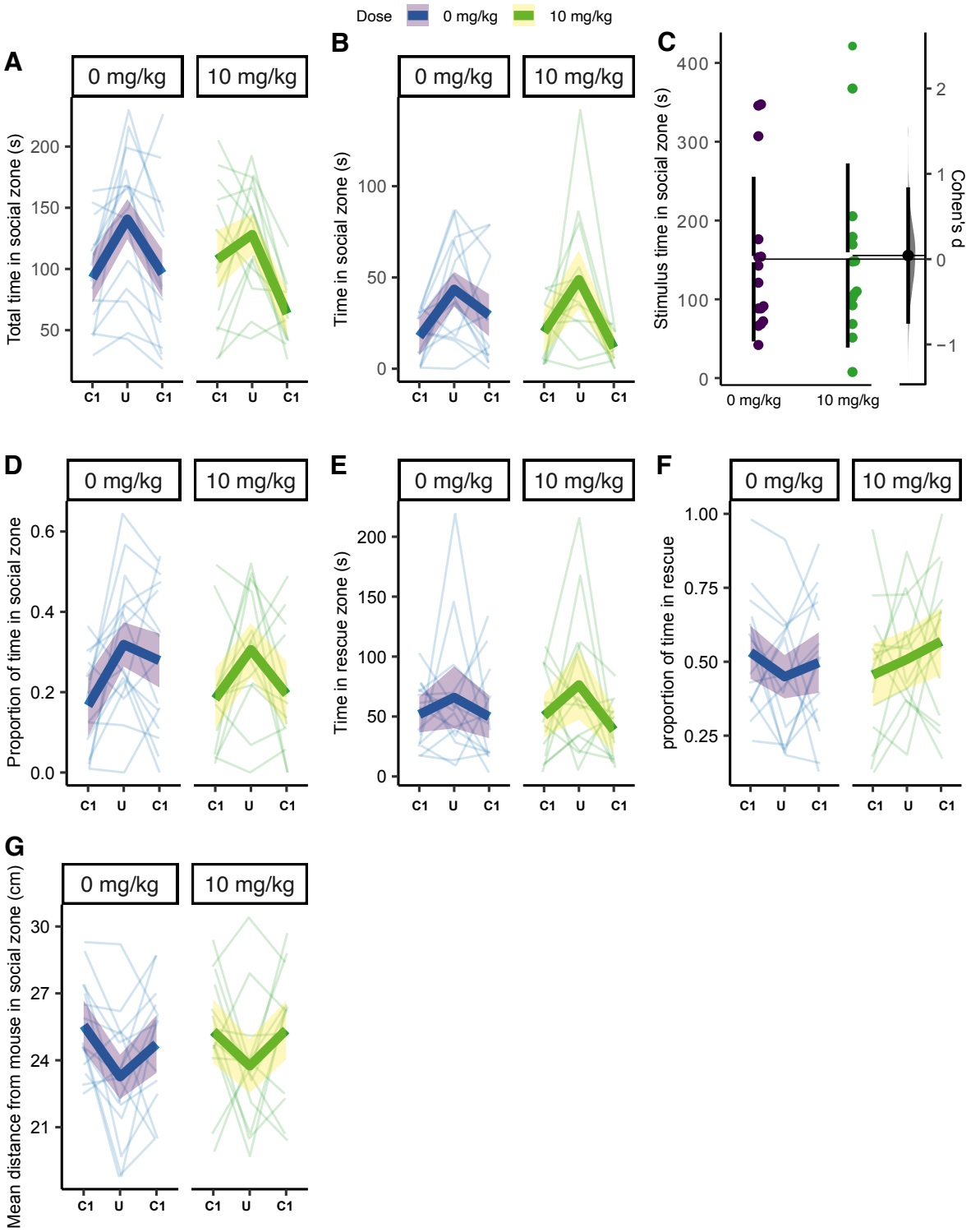


Figure 5.10. Test phases of social interaction platform. **A**. Total time spent in social zone. **B.** Time spent in the social zone at the same time as other mouse. **C.** Estimation plot showing time spent in social zone by stimulus mice and Cohen's *d* effect size. **D.** Proportion of time spent in social zone when another mouse is in social zone. **E.** Time spent in rescue zone when the other mouse is in the social zone. **F.** Proportion of time spent in rescue zone when another mouse is in social zone. **G.** Mean distance from mouse in social zone. Bold lines and ribbons are means and CIs respectively with individual lines representing raw data *n* = 16 control males,14 male MIA offspring. (**C1** = cagemate, **U** = unfamiliar mouse).

dose (LMM ($F_{[1,26]}$ =0.03, P=0.87)), ***Time spent in rescue zone with other mouse in social zone ~ weight + dose*** x ***test*** + ***(1 | ID)***. This model returns an ICC = 0.03, a marginal R^2 = 0.08 and a conditional R^2 = 0.1. Post-hoc analysis revealed MIA offspring spent a reduced amount of time in the rescue zone upon the second presentation with the cagemate compared to the unfamiliar animal ($t_{[1,79.5]}$ = 2.4, p <0.05). MIA offspring show an increasing proportion of time spent in the rescue zone across tests and control offspring appear to show reduced time in the rescue zone in the presence of an unfamiliar mouse (Fig. 5.10f). This is not supported by analysis with no main effect of ***test*** (LMM ($F_{[2,54]}$ =0.6, P=0.54)) or ***dose*** (LMM ($F_{[1,26]}$ =0.2, P=0.65)). When a mouse was in the social zone control mice and MIA offspring were on average closer to unfamiliar mice than cagemates (Fig. 5.10g). This is supported by a main effect of ***test*** (LMM ($F_{[2,54]}$ =5.1, P<0.01)) with no main effect of ***dose*** (LMM ($F_{[1,26]}$ =0.22, P=0.64)). ***Mean distance from mouse in social zone ~ weight + dose*** x ***test*** + ***(1 | ID)***. This model returns an ICC = 0.21, a marginal R^2 = 0.09 and a conditional R^2 = 0.28. Post-hoc analysis revealed controls were closer to unfamiliar mice in the social zone than cagemates at 1st presentation ($t_{[1,72]}$ = 2.6, p <0.05), but not MIA offspring ($t_{[1,71.9]}$ = 1.7, p =0.22). Considered together these data suggest exposure to an unfamiliar mouse potentiates social interest in a cagemate for control mice but not MIA mice.

5.5 Discussion

The work presented here demonstrates the potential of the SIP as a test of social behaviour. Experiment 1 revealed differences between dominant and submissive cagemates with dominant mice spending a smaller proportion of time in the social zone with submissive cagemates, no differences were present when cagemates encountered unfamiliar mice. This is supported by the performance in the three-chamber test which did not show differences based on homecage status. Dominant and submissive males spent similar amounts of time interreacting with an adult female in a direct social test. However, females showed similar levels of interest in submissive mice regardless of the level of interest displayed by submissive males but show higher levels of interest in dominant males with lower interaction times. Comparison of variable across social tests that are associated with appetitive

aspects of social behaviour did not show strong correlations but did display different patterns for dominant and submissive cagemates. For example, the social novelty index (SNI) from the three-chamber tests shows positive correlation with variables measure in the SIP. In submissive males SNI doesn't correlate with SIP variables and tends toward a negative for correlation. In experiment 2 the SIP was used in a longitudinal design and social interest as measured by the SIP appeared to decline with age as did exploratory behaviour in the habituation phase. Interestingly, between 5 and 6 months of age the mice of experiment 2 were used as stimulus animals in experiment 3. In this test they displayed increased time spent in the social zone when presented with younger mice (3 – 4 months) compared to the 5- and 6-month timepoints in experiment 2. The SIP protocol was amended in experiment 3 and mice encountered their cagemate a second time following a test with an unfamiliar mouse. This appeared to uncover differences between controls and MIA males. In the second encounter with the cagemate, controls spent the same amount of time in the social zone, but a greater proportion was as the same time as their cagemate. On the other hand, MIA males spent less time in the social zone in the second encounter with the cagemate though the proportion of time occupying the social zone with their cagemate remained the same as the first test.

5.5.1 Experiment 1

The design of the SIP provides mice with the same opportunity for interaction and avoidance of interaction. In the first set of experiments mice showed intersession habituation to the arenas, showing reduced activity and spending more time in the rescue zone on day 2. Exploratory behaviour was similar for dominant and submissive cagemates which is supported by observations mice with different social ranks display similar levels of activity in the open field (Kunkel and Wang 2018; Varholick et al. 2019). During the social phase of the SIP dominant and submissive cagemates appeared to spend more time in the social zone in the presence of unfamiliar mice. This was expected as cagemates are familiar and novelty stimulates interest so in theory unfamiliar mice are more salient. However, when other variables are inspected this become less clear. Considering the time spent in the social zone with the other mouse, this increased for dominant mice from the first trial with the cagemate to the final trial with the 2nd unfamiliar mouse. For submissive mice they

spent more time in the social zone with the 1st unfamiliar mouse. When considering the proportion of time mice spent in the social zone when the other mouse was also in the zone there were no differences between dominant and submissive cagemates in trials with unfamiliar mice. However, dominant cagemates appeared less interested in submissive cagemates potentially due to a lack of novelty. Analysis considering total wins or dominance rank including the unfamiliar mice did not reveal any different patterns. This suggests when mice are in a stable hierarchy, the submissive mice are less interesting in novel contexts. The SIP is a unique design and testing cagemates in social behaviour task is not commonplace so comparison with other works is difficult.

To try and contextualise behaviour in the SIP with regards to sociability, mice were assayed in established tests. In the three-chamber test no differences in sociability or social novelty preference were observed between dominant and submissive mice. There was no difference between groups in time spent with the social stimulus in the sociability phase. This is contrary to (Kunkel and Wang 2018) who reported dominant mice as spending more time with the social stimulus than submissive mice. The observed difference between reports may arise from differences in protocol. All cages in this work contained 2 mice, whereas Kunkel and Wang (2018), had cages of 3-5 mice and used the top and bottom ranked mice. Potentially having just 2 mice means the submissive mice in this test may be closer in hierarchical status to its cagemate than the mice test by Kunkel and Wang (2018). The affective state or social rank of the stimulus animals may also have influenced the behaviour of animals in both tests. The key takeaway from both reports is sociability is intact regardless of rank. Comparison of the SIP and three-chamber tasks did not reveal any robust correlations between variables but there were patterns suggesting differences in the social behaviour of dominant mice compared to submissive. The SNI of dominant mice showed positive trends with the key variables of the SIP, which was not seen in submissive mice. Perhaps this work missed an opportunity for a fairer comparison of the two tests namely, mice should have been tested in the SIP against the stimulus animals they encountered in the three-chamber. This would likely introduce its own confound as regardless of efforts to counterbalance designs, in one or other of the tests, mice would be familiar with each other. Both tests likely probe and facilitate different aspects of social behaviour. A useful refinement in

comparing tests like this would be to explore homecage behaviour before and after the test to see how the SIP or the three-chamber. This was done in a study probing the effect of dominance of phenotypic variation (Varholick et al. 2019). Ethological observations may offer insights into the impact of social testing on baseline social behaviour. For the SIP protocol this would be extremely pertinent following the test between cagemates. How do dominant and submissive mice interact immediately after being in a context that prevents agonistic behaviours?
Comparison of the SIP with the direct interaction test with a female did not reveal a strong correlation between interaction times of the tests. This is expected given a female is far more salient than a male conspecific and physical interaction is not limited as it is in the SIP. Dominant and submissive mice showed similar levels of interest in females during direct interaction. The SIP could be used to test males and females together this would facilitate a more direct comparison between tests. Particularly if paired with USV recordings, would calling behaviour change between the different physical environments?

5.5.2 Experiment 2

Using the SIP in a longitudinal design revealed a reduction in exploratory behaviour in the habituation phase and reduced social interest with age. These observations are supported by a previous report of reductions in locomotor activity and sociability with age (Shoji et al. 2016). Strikingly, the test animals here were used as stimulus animals in another SIP experiment between 5-and 6-months and demonstrated increased time in the social zone compared to the 5- and 6-month timepoint. The mice in the other experiment were younger, approximately 3 months. This suggests younger mice are more salient, and social interest only appears to decline with age when the stimulus is older. If valid, this merits further investigation as studies typical age-match subjects and stimulus animals for social behaviour tests. If a test examining behaviour in a 6-month-old mouse the potential lack of saliency of the stimulus could mask a genuine deficit.

The 3-month timepoint replicated the observations of experiment one, in that cagemates spent less time together than with unfamiliar mice. Social rank was not

assessed in this cohort thus its influence on performance in this test cannot be judged. The potential lower interest between cagemates in the first trial dissipates by the second trial and is more on a par with interest levels in unfamiliar mice. It is possible that prior exposure to unfamiliar mice in this test potentiated interest in cagemates. A more confident interpretation could be made if social status was known, potentiation of interest would likely occur in the dominant cagemate. This would appear as increased time in the social zone in the dominant mice. At 6-months cagemates appear less interested in each other this could potentially be habituation to the context as it was the fourth test with the same mouse. Future studies of the SIP that utilise a longitudinal design may benefit from using cages of more than 2 mice. This would also allow greater interrogation of the influence of social rank on social behaviour displayed within the SIP

5.5.3 Experiment 3

The third experiment compared the behaviour of male control and MIA offspring from the 2nd cohort of Chapter 3. Using time in the social zone as the measure of social interest suggests MIA males display lower interest in males and encountering and unfamiliar leads to lower interest in their cagemates. However, time in the social zone with another mouse and proportion of time in the social zone suggest intact levels of social interest. This observation is supported by a lack of a social phenotype for these animals in a direct social interaction test at P28. The stimulus animals showed similar levels of interest in control and MIA mice, which agrees with the same observation when tested at P28. These results point to unaffected social behaviour in MIA males. However, there does appear to be a difference between controls and MIA males when tested against the cagemate on the second occasion. Time in the social zone with the other mouse does not return to baseline i.e. the first trial with cagemate, for control males but does in MIA males. This is similar to the longitudinal observation in experiment 2 where cagemates spent more time in the social zone together at 4-months compared to the initial test at 3-months. The time between tests was greater in experiment 2 but it is possible the same effect is responsible. Once the mice have faced an unfamiliar mouse in the SIP, they associate the arenas with the possibility of encountering other mice and this

potentiates interaction between dominant and submissive cagemates. Assessing social rank in these mice may have supported this interpretation.

The return to baseline in MIA males is a subtle effect but merits further investigation. If the observation is valid, it may point to an absence of a hierarchical structure within MIA males. Interactions with unfamiliar mice may challenge the status quo of hierarchy in controls leading to increased interaction within the second trial. Absence of a defined hierarchy would inhibit this effect. The influence of MIA on social hierarchy has not been previously reported but it has been observed that individuals within litters born to MIA dams can be stratified into susceptible and resilient groups (Mueller et al. 2021; Herrero et al. 2023). This may be comparable to having mixed genotype litters in genetic models of ASD which can interfere with the development of typical social hierarchies, as demonstrated in a *Nlgn3* mouse model (Kalbassi et al. 2017). Without further work this is merely speculation, but social rank can influence social behaviour therefore any model of social processes should consider the impact of social hierarchy.

5.5.4 Limitations

Despite showing potential in assessing the social behaviour of adult male mice, the SIP, in its current form, has several limitations. The first is the small amount of time mice occupy the social zone as the same time as one another. The ability to opt-out by moving to the rescue zone was likely to produce lower levels of time spent in the social zone of interest compared to social tests where opting out is not an option. The physical design of the test may contribute to the lower levels of time in the social zone. By preventing full interaction, the mice can't engage in their full repertoire of behaviours so opt-out. The low amount of time spent in the social zone may not represent low levels of social interaction. The 'time in the zone' approach to assessing social behaviour is a reductionist feature of simple tests of social behaviour that don't accurately reflect true social behaviour in the model organism (Kondrakiewicz et al. 2019; Shemesh and Chen 2023). This can be remedied in several ways as will be discussed in the context of future directions. Whilst the 'time in zone' approach is limited, the choice to link it to the paired animal is a strength of the current SIP protocol.

5.5.5 Future directions

The next step with the SIP is refinement, starting with data acquisition. Rather than just using tracking software to record time in zones, the SIP can be augmented to acquire data with much greater depth in its current form. For example, mice will vocalise differently depending on the social action they are preforming e.g. a mouse avoiding interaction emits different calls compared to a mouse dominating another mouse (Sangiamo et al. 2020). The real strength of the work by Sangiamo et al. (2020) was developing an accurate means of attributing USVs to a mouse in a group setting. This can be applied to the SIP to see how calling varies in each zone of interest, potentially predicting approach or retreat? The physical separation would facilitate the recording of scent marking for both mice and important chemical signal between mice (Arakawa et al. 2008). It is possible to monitor movement patterns through use of piezoelectric sensors in the floor of the arena, this may be best applied to the rescue zone as a means of identifying cues that trigger approach or avoidance behaviour in the paired animal. This is valuable as the movement patterns of stimulus C57BL/6J mice influence the social investigation of the subject conspecific. Large movements promote avoidance (Netser et al. 2020). These adjustments alone would allow a deeper interpretation of what social interaction in the SIP looks like.

The ultimate goal with the SIP would be to pair it with machine learning techniques that would allow automatic tracking and classification of behaviours. Current tools are available such as DeepLabCut (DLC) that allow accurate pose estimation and tracking of user defined body parts (Mathis et al. 2018). DLC can be used in conjunction with tools such as SimBA (SIMple Behavioural Analysis) which can automatically and accurate detect predefined behaviours (Goodwin et al. 2024). This raises a barrier to such an approach, the standardisation of classifying behaviours. When inspecting a frame displaying a behaviour even skilled observers will not agree with each other all the time. It also requires definition of when a behaviour starts. A long-term goal when designing the SIP protocol was the use of machine learning techniques classify behaviours with an extreme resolution. For example, Mouse A approaches the social zone with a speed x and a stretch posture, 99/100 Mouse B responds by approaching or avoiding Mouse A. Basically, identifying social

cues in mice so when you model socially relevant endophenotypes ‘time in the social zone’ may be equal but your group of interest may not respond to social cues in the anticipated way.

5.6 Conclusions

The work here demonstrated the ability of the SIP to highlight differences in social behaviour, representing a promising first step in developing a new test of social behaviour. It points to the benefits of testing animals against cagemates, and the set-up seems to lend itself well to repeat testing of the same animal. With refinement of the SIP, it would be possible to have a group of thoroughly phenotyped animals that can be used as stimulus animals in the SIP paired with animals that aim to model social impairment. The readout from the stimulus animals could then be used to compare control and treatment groups, potentially uncovering deficits current tests focussing solely on the subject lack the resolution to reveal. However, the SIP as utilized here, is faced with many of the limitations other simple social behaviour tests encounter such as the reductionist ‘time in a zone’ approach. The protocol needs refinement to maximise the time mice spend together because the ability to opt-out of interaction means the absolute amount of time in interaction is small. If the SIP can be coupled with automated algorithms it has the potential to be an incredibly versatile paradigm from monitoring approach v avoidance behaviours to whisker tracking in social interactions. It will not replace current tests but has the potential to complement and enhance the current toolbox of investigating social behaviour.

Chapter 6 :General Discussion

The aetiology of ASD is a complex interaction of genetic and environmental factors that can manifest with a vast degree of phenotypic heterogeneity. The genetic and phenotypic overlap with other neuropsychiatric disorders and behavioural traits within the general population calls for a different approach to modelling human conditions. This thesis aimed to investigate the potential interactive effects on social behaviour when combining haploinsufficiency of *Cyfip1* with a sub-threshold dose of poly(I:C)-induced MIA. Each factor is individually associated with heterogenous phenotypic outcomes, but this work focussed on social behaviour early in develop to facilitate contextualisation of findings in relation to ASD. Following a summary of the principal findings the potential mechanisms underpinning these observations will be considered before outlining refinements that can build on the work presented here.

6.1 Summary of results

To identify a sub-threshold dose of poly(I:C) a dose-response experiment was undertaken with the early life social behaviour of the offspring the key outcome measure. A dose of 5 mg/kg was sufficient to stimulate the immune system of pregnant dams without producing an overt social phenotype in three social paradigms assayed prior to weaning. A higher dose of 10 mg/kg produced evidence of altered social behaviour in two of the three tests selected. At P9 10 mg/kg pups were less likely to locate the homing nest than controls and the lower dose group. There was no evidence of altered social communication between groups at this timepoint. All groups showed adaptive communication with longer latency times associated with increased calling and longer mean duration of calls. Mice in the 10m/kg group also displayed reduced social novelty recognition at P26 and P27 a deficit not predicted by an absence of homing at P9. MIA did not produce evidence of social impairment in a direct social interaction test with an adult female at P28. Both sexes were studied but no overt sex differences were observed in social behaviour pre-weaning.

The sub-threshold dose was then used with the same protocol as Chapter 3 in the haploinsufficient *Cyfip1* mouse model to inspect potential interactive effects in early life social behaviour. *Cyfip1*$^{+/-}$ males born to MIA dams took longer to find the home nest at P9 than *Cyfip1*$^{+/-}$ male controls and *Cyfip1*$^{+/-}$ MIA females. This effect was observed whether considering the individual animal or the litter as the n. There were no differences in homing times in WT groups replicating the finding of Chapter 3 that utilised purely WT litters. Pups with absent homing behaviour vocalised more than pups with present homing behaviour. For non-homing animals, *Cyfip1*$^{+/-}$ and MIA appeared to interact in a sex dependent manner. Male WT controls emitted more calls than female WT controls whereas male *Cyfip1*$^{+/-}$ controls called less than *Cyfip1*$^{+/-}$ females. MIA animals displayed the opposite pattern. Further interactive effects were observed at P9 in homing animals. As observed in Chapter 3, mean call duration increased with latency but only in WT control and MIA males and WT control and *Cyfip1*$^{+/-}$ MIA females. At P28, *Cyfip1*$^{+/-}$ animals displayed lower levels of social interaction than WT littermates in a direct social interaction test. Stimulus animals were less interested in WT MIA animals, an effect not seen in Chapter 3. Social environment appeared to modulate the behaviour of WT animals because the interaction times of WTs were lower when a litter had a greater proportion of *Cyfip1*$^{+/-}$ animals.

Finally, a new test of social behaviour, the Social Interaction Platform (SIP) was evaluated using WT male mice. Homecage dominance status appeared to modulate social behaviour when tests involved cagemates but not unfamiliar conspecifics. Social interest appeared to diminish with age, but older mice displayed increased time in the social zone when paired with younger mice. The SIP was also able to highlight differences between adult control and MIA offspring. Following testing with an unfamiliar mouse, MIA animals showed reduced interest in cagemates whereas controls displayed increased interaction.

In summation, *Cyfip1*$^{+/-}$ interacts with sub-threshold MIA to induce earlier presentation of social deficits in males. *Cyfip1* appears important in the development of social behaviour for the individual and the group, in the context of mixed genotype litters. At higher doses poly(I:C) can produce social phenotypes in some but not all tests of social behaviour.

6.2 The interaction between *Cyfip1* and maternal immune activation

The present study offers the first evidence of the potential interactive effects between haploinsufficiency of *Cyfip1* and maternal immune activation to impact social behaviour of male mouse pups at P9. Working on the basis this observation is valid the next priority is to probe potential shared biological mechanisms underpinning this interaction. A prime candidate for convergence between these two risk factors is synaptic dysfunction, which is implicated as a convergent pathway for many of the risk genes associated with ASD. CYFIP1 has a well-defined role at the synapse as a regulator of protein translation and cytoskeleton dynamics. Through these processes CYFIP1 influences the formation and morphology of dendritic spines (Pathania et al. 2014; Oguro-Ando et al. 2015). Animals models of poly(I:C)-induced MIA have reported dendritic phenotypes. MIA at E9.5 was shown to yield a 20% increase in spine density in the somatosensory cortex of 8-week-old mice, that also displayed impaired social interaction (Soumiya et al. 2011). Using a similar MIA protocol, Ikezu et al. (2021) reported an increase in spine density and higher numbers of filopodial spines in layer V of the medial prefrontal cortex (mPFC) from mice that displayed social deficits and social behaviour. When poly(I:C) was administered at E12.5 spine density was reduced in the somatosensory cortex of mice at P17-19 and P30 (Coiro et al. 2015). How MIA alters dendrite morphology is not clear. Ikezu et al. (2021) rescued the observed cellular and behavioural phenotypes by depleting the microglial population before local repopulation. This is a technique that has been shown to reverse damage cause by neuroinflammation (Elmore et al. 2018). Coiro et al. (2015), was able to reverse the loss of dendrites in younger mice P17-19 but not in the slightly older mice P30. This was achieved by administering lactating dams a course of anti-inflammatories in the first two weeks after birth. The rescue of MIA-induced phenotypes highlights inflammatory processes. Haploinsufficiency of *Cyfip1* and MIA may converge at dendritic spines via a combination of impaired actin polymerization and inflammatory processes mediated by microglia. There is emerging evidence of the role of CYFIP1 in microglia, so it is possible both risk factors converge on microglial function. The earlier timepoint investigated by Coiro et al. (2015) is interesting as it hints that the male specific *Cyfip1* x MIA P9 phenotype

observed here could be alleviated by administering dams and anti-inflammatory treatment. Early presenting phenotypes are appealing in the context of studying neurodevelopmental disorders thus the presynaptic role of CYFIP1 as reported by Hsiao et al. (2016) is an appealing candidate for interrogation in this GxE model. At P10 the hippocampus of *Cyfip1*$^{+/-}$ mice displayed larger presynaptic terminals and higher vesicle release probability than WT mice. This merits investigation given the temporal proximity to the homing test but also because it is a transient phenotype as it was not present at P21. Attempted rescue of the P9 homing phenotype with anti-inflammatory intervention coupled with attempts to replicate the findings of Hsiao et al. (2016) are worthy next steps in trying to disentangle the biological contributions to the interactive effects reported here.

The findings reported here are novel contributions to the knowledge base, but they come with limitations. Perhaps the main limitation is the lack of replication, thus far it is a one-off. This study only looked at pre-weaning behaviour and it would have been beneficial to see how social behaviour developed into adulthood, it is possible further interactive effects would have emerged. This could have been probed with the habituation/novelty test, given higher dose MIA animals in Chapter 3 phenocopied a *Nlgn3* mouse model (Hörnberg et al. 2020). This would be of interest as translational dysregulation was implicated in the *Nlgn3* KO impaired social novelty recognition and NLGN3 is known to interact with CYFIP1(Sledziowska et al. 2020a). The lack of biological data is a disappointment but in a purely MIA model, the initial outcome measure should be the behaviour of the offspring, thus it is reasonable to focus on behaviour in a GxE model.

An advantage of GxE models is the ability to probe the phenotypic heterogeneity and overlap seen across human disorders. *CYFIP1* and MIA convey risk for multiple conditions making them excellent candidates for GxE. The protocol could be adapted to examine the influence of gestational timing and intensity of MIA. Interactive effects may emerge at a different timepoint if poly(I:C) is administered earlier or at a higher dose. Alternatively, dosage effects can be probed with conditional KOs or overexpression models to see how interactive effects emerge, if they do. The behavioural phenotypes reported here are quite mild suggesting the model could tolerate an additional hit. Candidates for the additional hit could be environmental or

genetic. Environmental hits would need to be chosen based on timing, such as peripubertal stress may yield further interactive effects. Additional genetic hits should be selected based on known interactions with CYFIP1 such as *Fmr1* or *Nlgn3.* These options are potentially fruitful but there is a lot to elucidate regarding the *Cyfip1* x MIA interaction, but it appears to be a valuable tool for addressing a plethora of questions.

6.3 Studying pre-weaning social behaviour

Observing interactive effects in a behavioural test at P9 is validation for focussing on social behaviour in early development. The adapted protocol from Muroyama et al. (2016) presented pups with a challenging discrimination task home nest vs clean nest and across two cohorts, MIA alone and GxE, most animals successfully located the home nest. The behaviour displayed was robust, once located pups would climb into the nest and typically would not leave. This is far easier to label as homing behaviour than usual designs that cover a relatively large area with bedding material and pups can merely wander over. Such designs lack the specificity of the protocol used in this work. An additional strength of the P9 homing protocol here is the level of data acquisition, locomotor activity in terms of distance covered, weight and USVs were recorded. Furthermore, in a refinement from Chapter 3 to Chapter 4, pup retrieval times were recorded when pups were returned to the homecage. High levels of data acquisition, when the opportunity presents allows for deeper phenotyping and a potential reduction in the number of tests required, which is an important consideration at such an early developmental timepoint. The recording of USVs during the homing test has not been reported before which adds to the novelty of findings reported here. Previous work has recorded isolation-induced USVs in the presence of home bedding or clean bedding (Moles et al. 2004; Luchetti et al. 2021), but never together, and not in the context of a discrimination task. The task itself seemed well tolerated by pups and dams, with no incidents of pups being rejected and testing at P9 did not appear to influence performance in subsequent tests. Taken together, the P9 homing protocol described in this work is very attractive for probing early social behaviour and it should be utilised more often than it is in the literature.

Improvements can be made, firstly the arena could have a transparent bottom, and the camera could film from the underside. Deeplabcut could then be utilised to track the gait of the pups allowing the identification of potential motor issues thus from a single 3-minute test an investigator could probe social interest/attachment, communication and motor co-ordination (Mathis et al. 2018). The potential of the USV data can be realised by 1) analysing the syllables and the pattern of their emission 2) partitioning the traces into in- or out- of the nest. Both refinements would offer deeper understanding of the potential interactive effects observed in the work of Chapter 4.

Future experiments could expand on the findings from the homing test as they have inspired a number of questions. Could pups be tested at several timepoints? Which is often done with isolation-induced USVs, but the homing test is more disruptive and the test itself may alter the olfactory cues produced thus confounding the additional tests. A possible compromise would be to test at P9 then test again just prior to weaning e.g. P21. This would require an adjustment to the protocol due to the physical differences between a P9 and P21 mouse. My suggestion is at P21 an open field arena is used, one side is the homecage, with the dam in it and on the other is a clean cage with bedding. Then as with the P9 protocol, time to find, and time spent with either cage can be measured. USVs could still be recorded, and these will potentially come from the dam as well but with the correct set-up calls can be recorded in groups and assigned correctly to the animal that made them. (Sangiamo et al. 2020). This would represent a technical challenge, but it would be useful to assess attachment behaviour at more than one timepoint. In the context of a gene x environment model, it would help validate interactive effects observed a P9 or detect different ones.

An important question when a phenotype is observed in animal models is, can it be rescued? For deficits in the P9 homing test this has been shown to be true. Mice born via Caesarean-section (C-section) display reduced preference for the home nest at P10, as well as reduced social novelty preference in adulthood (10 weeks). Early life (P1-5) administration of oxytocin rescued homing and social novelty preference phenotypes. Attempts to rescue the absence of homing behaviour in 10

mg/kg MIA animals is an interesting prospect. In human males, oxytocin has been shown to alleviate the cytokine response to LPS administration, including TNF-α. This is noteworthy given LPS is used to model maternal immune activation, and TNF-α is elevated in maternal, placental and fetal compartments following poly(I:C) administration (Arsenault et al. 2014; Mueller et al. 2019). The rescue of social novelty preference in adulthood also stands out. At P27 10mg/kg animals displayed impaired social novelty recognition but intact habituation which phenocopies a *Nlgn3* mouse model that was revealed to have impaired oxytocinergic signalling (Hörnberg et al. 2020). An experiment examining the potential of oxytocin in rescue the absence of homing behaviour in higher dose MIA offspring merits consideration. It would be fascinating to see the impact of oxytocin on social novelty recognition at P27 as it could represent convergent pathological mechanisms between genetic and environmental models associated with ASD. The social habituation/ recognition novelty task could help probe further interactive effects of the *Cyfip1* x MIA model. Particularly if the observation stimulus animals are less interested in MIA WTs from mixed genotype litters is valid. If a conspecific is less interested the subject animal may compensate and increase interactions or withdrawal quicker, either may confound results. Therefore, it would be appropriate to score the behaviour of the stimulus animal.

The behavioural paradigms used in this work have proved useful but lack ecological validity due to the artificial nature of the set-up, time of day the testing took place and the reductionist measures of behaviour. There is a school of thought that assessment of social behaviour should shift to more complex setups that are more natural in terms of environment, provide richer readouts of behaviour and track behaviour for much longer periods (Kondrakiewicz et al. 2019; Shemesh and Chen 2023). The value in such an approach is the ability to explore the circadian rhythms of social behaviour. Arakawa et al. (2007) utilised the Visual Burrow System (VBS) to assess the social behaviour of mice day and night. It was observed the main social behaviour displayed during the day was huddling whereas active approach was the predominant social behaviour displayed at night. This finding adds weight to the idea that behavioural testing should be carried during the dark phase due to the nocturnal nature of mice. Indeed, it has been demonstrated that mice tested during the light

phase show lower levels of sociability than mice tested during the dark phase (Richetto et al. 2019). Conversely, it has also been demonstrated that social approach behaviours are similar for mice tested during light or dark phase, for both inbred mice and vasopressin receptor subtype 1b (*Avpr1b*) mutant mice (Yang et al. 2007a). Whilst questions remain regarding the examination of social behaviour during the light phase, it does not invalidate the findings of this work, but rather highlights a possible source of variability when comparing to other works. Impairments may have been masked or exacerbated by control groups potentially displaying lower levels of social behaviour in the light phase. It is also possible that MIA and/or *Cyfip1* haploinsufficiency disrupt circadian cycles with respect to social behaviour thus the simple tests used here may fail to detect an effect, which would also apply to the dark phase. Questions perhaps better addressed with 24 hr monitoring of behaviour.

Studying social behaviour with an approach like VBS is extremely valuable but also resource intensive in terms of skills and time. A compromise in approach would facilitate leaps in our understanding of the social behaviour displayed the organisms used as models. Prior to weaning I think the tests used in this work are extremely valuable in investigating how aspects of social behaviour develop. It is important to use more than one test given that each test may probe different features of social behaviour. That's said, periods of homecage monitoring would certainly augment the findings from simple tests. If the work here was reproduced it would be fascinating to monitor the homecage before and after the paradigms used here. For example, how do siblings interact before they encounter a novel conspecific in a new environment? How does the experience of testing influence behaviour on return to the homecage? No differences may arise in the P28 interaction test, but the MIA exposed offspring may behave differently with siblings following the test. Homecage monitoring could have strong applications in examining the effects of weaning to see how controls adapt versus MIA offspring. Whatever the approach, the focus needs to be on understanding social behaviour in the context of the model organism, if we do, differences that arise as consequences of risk genes and environmental challenges will emerge. Some of the findings in this work point to an improvement that can make simple behavioural tests more ethological and that is consideration of the stimulus animal.

6.4 A role for stimulus animals in revealing social impairments

Social interactions are not unidirectional, but animal models typically only consider the subject mouse. The work presented here attempted to account for the contribution of the stimulus animals to social interactions. In the P28 and courtship paradigms the stimulus directed interaction was scored, this was occasions when the stimulus would be sniffing or following the subject without the subject responding to the stimulus. This is a crude measure, but it highlighted potential interactive effects at P28 in the *Cyfip1* x MIA model with stimulus animals showing lower levels of interest in WT MIA animals. This was not observed in the purely WT MIA cohort at the same dosage of poly(I:C).

The SIP was designed to give subject and stimulus equal billing, with both parties having the same physical experience. Even with this consideration the SIP was able to highlight the importance of the stimulus animal. The longitudinal experiment in the SIP illustrated a decline in social interest with age. However, when the animals from the experiment were paired with younger animals, they spent longer in the social zone than they did at 5- months of age, then showed a decrease when tested against 6-month-old mice. This points to a decrease in interest in older mice rather than a general reduction in sociability. I believe readouts of stimulus behaviour could prove more informative than readouts of subject behaviour. For example, the SIP once refined could be used with a group of well-phenotyped stimulus animals that are used in tests with subjects. If a subject is displaying aberrant social behaviour the stimulus behaviour will reflect this, possibly even electrophysiological recordings from a stimulus may reflect atypical social behaviour in a conspecific. This is heavily reliant on investigators understanding the social behaviour of the model organism. Well-phenotyped stimulus animals could even be used within a SIP as an intervention in affected animals, promoting social development. At this stage of the SIP development this is purely speculation but the idea of using stimulus readouts to identify impairments in subject mice is feasible.

6.5 Relevance in humans

A key appeal of maternal immune activation models is the potential for identifying biomarkers for a specific disorder such as ASD. Elevated cytokines have been reported in children with ASD and some are associated with symptom severity. However, these studies do not reproduce the finding, in part due to small sample sizes (Molloy et al. 2006; Krakowiak et al. 2017; Masi et al. 2017). The key finding in this work was the apparent earlier presentation of 'symptoms' in males from the two-hit model. Follow-up studies, if they replicate this finding could analyse the inflammatory markers. It is possible specific cytokines may drive earlier presentation of symptoms in humans thus epidemiological studies could analyse cytokine profiles in children with ASD and compare this with age of onset. Such an approach encounters the main problems of chasing nosological entities. A more fruitful approach would be to look for association between cytokines and variation in social behaviour in the general population e.g. how does elevated TNF-α predict measures from the Social and Communication Checklist in a general population cohort? If a model is of an endophenotype then we should look to assess the equivalent in humans where possible Taking the P9 homing test as an example, it is believed to be a measure of attachment which can be assessed in humans. The Strange Situation procedure is one such paradigm and can be used when infants are 12-20 months old (Benoit 2004). The P9 and Strange situation procedure may not map onto each particularly well but identifying endophenotypes is only the first step, finding equivalents across species is required to drive translatable outcomes. It could be that USVs within the homing test represent the endophenotype that can be replicated in humans more easily? Crying has been suggested as a potential biomarker for ASD. Atypical crying is even suggested to a self-perpetuating risk factor because atypical crying is harder to understand so caregivers can't resolve leading to prolonged distress (Esposito et al. 2017).

6.6 Conclusions

This thesis presents the first demonstration of interactive effects between *Cyfip1*$^{+/-}$ and MIA in modulating social behaviour early in development. *Cyfip1*$^{+/-}$ displayed an interaction with MIA to impact the social behaviour of *Cyfip1*$^{+/-}$ males at P9 suggesting these factors can interact to produce presentation of social deficits at earlier ages. This work also demonstrated the importance of *Cyfip1* in early social behaviour with all *Cyfip1*$^{+/-}$ groups displaying lower levels of social interaction at P28. *Cyfip1*$^{+/-}$ also appeared to modulate the behaviour of their WT littermates with WTs showing lower interaction times when they originate in litters with higher proportions of *Cyfip1*$^{+/-}$ animals. This reinforces the role of *Cyfip1* in social behaviour and highlights the need to inspect and account for social environment when analysing social behaviour. The observation that stimulus animals appear less interested in MIA WTs when born into litters containing *Cyfip1*$^{+/-}$ animals adds further weight to the interactive effects of *Cyfip1*$^{+/-}$ and MIA, though social environment could have modulated this effect. Furthermore, it highlights the importance of considering the stimulus animal when studying social behaviour. The ability of a higher dose of MIA to induce social behavioural phenotypes across development, but crucial not in all tests demonstrates the value of studying endophenotypes and reiterates the importance of not relying on a single test at one timepoint. Overall, the findings presented here provide a model, *Cyfip1*$^{+/-}$ x MIA that can be used to further interrogate endophenotypes of diseases these risk factors are associated with. This model may also present an opportunity to examine convergent pathological pathways of genetic and environmental risk.

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