Exploring the Influence of Offspring *Peg3* Expression on Maternal-Infant Communication and Maternal Mood in Mice and Humans

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Submitted for consideration for the degree of Doctor of Philosophy

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December 2021

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11β-HSD2	11β-hydroxysteroid dehydrogenase 2 (placental enzyme)
11-HSD	Hydroxysteroid 11-Beta Dehydrogenase 2 (gene)
ALSPAC	Avon Longitudinal Study of Parents and Children
ANOVA	Analysis of Variance
ANCOVA	Analysis of Covariance
ASCL2	Achaete-scute Complex Homolog
ATP	Adenosine triphosphate
BORIS	Behavioural Observation Research Interactive Software
BSID-III	Bayley Scales of Infant Development Third Edition
CDKN1C	Cyclin Dependent Kinase Inhibitor 1C
cDNA	Complementary DNA
CICS	Cardiff Infant Contentiousness Scale
CNS	Central Nervous System
DEAF1	Deformed Epidermal Autoregulatory Factor 1 Homolog
DEG	Differentially Expressed Gene
DMR	Differentially Methylated Region
DNA	Deoxyribonucleic Acid
DOHAD	Developmental Origins of Health and Disease
DSI	Direct Social Interaction
DSM	The Diagnosistic and Statistical Manual of Mental Disorders
dUTP	Deoxyuridine Triphosphate
E	Embryonic Day
EDA	Experimental Design Assistant
ELCS	Elective Caesarean
EPDS	Edinburgh Postnatal Depression Scale

EZM	Elevated Zero Maze
GiW	Grown In Wales
GO	Gene Ontology
GRB10	Growth Factor Receptor-bound Protein 10
HPA	Hypothalamic-pituitary-adrenal
hPL	Human Placental Lactogen
IBQ-R-SF	Infant Behavioural Questionnaire Revised-Short Form
IGF2	Insulin-like Growth Factor 2
IUGR	In utero Growth Restriction
IVF	*In-vitro* Fertilisation
Lab-TAB	Laboratory Temperament Assessment Battery
LDB	Light Dark Box
MRC	Medical Research Council
MEG3	Maternally Expressed Gene 3
MGI	Mouse Genome Informatics
MHINT	Mental Health Intergenerational Transmission
NADH	Nicotinamide Adenine Dinucleotide
NLGN3	Neuroligin-3
NICE	The National Institute for Health and Care Excellence
P	Postnatal Day
PBQ	Postnatal Bonding Questionnaire
PCA	Principal Component Analysis
PCR	Polymerase Chain Reaction
PE	Pair End
PEG1	Paternally Expressed Gene1
PEG3	Paternally Expressed Gene 3
Peg3KO	Paternally Expressed Gene 3 Knockout
PHLDA2	Pleckstrin Homology-like Domain Family a Member 2
Prls	Prolactin-like hormones expressed in the placenta
PVN	Paraventricular Nucleus

RNA	Ribonucleic Acid
RNA-Seq	Ribonucleic Acid Sequencing
SEM	Standard Error of the Mean
SD	Standard Deviation
SRS	Silver Russel Syndrome
STAI	State Trait Anxiety Index
tRNA	Transfer Ribonucleic Acid
Ube3	Ubiquitin Ligase Gene
UHW	University Hospital of Wales
UK	United Kingdom
USVs	Ultrasonic Vocalisations
WT	Wild Type

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Declaration

Acknowledgements of assistance received

Professor Ros John: general advice, guidance, and discussion with regard to project design, statistical testing, mouse husbandry and all things genetic.

Professor Anthony Isles: general advice, guidance and discussion regarding behavioural genetics, neuroscience, behavioural testing, and statistics.

Dr David Harrison: for help with mouse husbandry, behavioural methodology and laboratory techniques, general guidance, and mentoring.

Dr Hugo Creeth and **Susan Hunter:** for assistance with animal husbandry, behavioural methodology and laboratory techniques, including training in genotyping and embryo dissection.

Dr Rebecca Pearson for guidance using the MHINT coding scheme, Observer XT, and guidance regarding statistical analysis for human data.

Alice Chibnall for training in RNA extraction

Natalie Wellard for training in RNA-Seq data analysis

Data produced jointly

With thanks to:

Matthew Higgs (Wellcome Trust PhD student) for assisting in the generation of data for maternal anxiety and maternal care tasks, and for second scoring the pup retrieval task (Chapter 3) and scent marking (Chapter 4)

Katie Sedgewick (Wellcome Trust PhD student) for second scoring photos from the marble burying task (Chapter 4).

Dr David Harrison for dissecting both the hypothalamus and the olfactory bulb from P6 neonatal mice to be used for RNA extraction (Chapter 4).

Dr Hayley Dingsdale for validating 20% of the video coding of mother-infant dyads that were coded in the first instance by the author (Chapter 5).

Data/materials provided by someone else

With thanks to:

Dr Anna Janssen for establishing the Grown in Wales Cohort

Dr Lorna Sumption, Samantha Garay and Katrina Savory for administering the 12-month infant assessments .

GeneWiz Ltd for performing the library preparation and generation of raw RNA sequencing data.

Published work associated with this thesis:

Harrison, D.J, Creeth, H.D.J, **Tyson, H.R,** Boque-Sastre, R, Ilses, A.R, Palme, R, Touma, C and John, R.M. (2020). **Unified Behavioural Scoring for Preclinical Models.** Frontiers in Neuroscience 14.

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Funding

This PhD was funded by the Wellcome trust PhD studentship scheme

The Grown in Wales Cohort was originally funded by the MRC and has since been funded by the Waterloo Foundation.

Acknowledgements

More time in this PhD has now been spent in a global pandemic than out of it, which has come with its own challenges, but has also made me more thankful for all the support I've had along the way.

Firstly, thank you to my supervisors Ros and Anthony. Ros, thank you for taking me on as a rotation student all those years ago, and thank you for all your support and encouragement over the last 4 years. But in particular, thank you for always trying to dissuade my imposter syndrome. Anthony, thank you for taking a chance on me at interview and thank you for entertaining my endless stats questions and last-minute drafts!

Thank you also to the whole of the Preg Lab both past and present. Sam and Lorna, for that one short year pre-pandemic, you always made coming into work more enjoyable and thank you both for being there for me after the hours spent in the Mouse House. Alice, thank you for making the post-lockdown office a happier place to be. Raquel, thank you for always being a friendly face, and sticking up for me when I needed it. Finally, Harri, thank you for putting up with me over the last 4 years. You've been an incredible mentor and a better friend.

Thank you also to all the Mothers who participated in the Grown in Wales study and thank you to the Wellcome Trust for your generous funding, both of which enabled the studies in this thesis to take place.

Finally, I want to thank the people behind the scenes, who mean more to me than they know.

Parental Unit One, thank you for always believing in me when I didn't believe in myself. You can now finally stop asking, "How's the writing going?" Thank you both for everything, I wouldn't be here without either of your support over the years. Mike, thank you especially for listening to me angst over career choices for the last 10 years. Momma Bear, you say I only remember the negative, but I really do remember the positives too. Thank you for being there for me since day one. I wouldn't be where I am today without you behind me. I would say more, but I don't have the words, so I'll just say *thank you* and hope you know how much I appreciate your support. Also, the contents page wouldn't be what it is without you…

Parental Unit Two, thanks for being proud of me, even when you had no idea what I was doing. And thank you to Will, for the comedic relief, and for being there if I needed you.

Lastly Matt, just, thank you. Thank you for sticking with me through the Winter of Behavioural Testing, and through all the tears, panic, and stress muffins. But also, more importantly, thank you for making the good times even better. I'm not sure how I would have done this without you. You make my heart happy.

Summary

Maternal mental health conditions have been shown to be among the most disruptive influences on the mother-infant bond and their reciprocal social relationship, often resulting in long-lasting, often adverse infant outcomes. However, the mechanisms underlying this are poorly understood.

Reduced placental expression of the imprinted gene *PEG3* has been linked to prenatal depression in human pregnancy. In mice, intrinsic loss of function of *Peg3* in dams has previously been linked to poor maternal care. Reduced expression of *Peg3* in the murine feto-placental unit has also been shown to indirectly impair maternal care and increase anxious-like behaviour in wild-type dams. It has been hypothesised that this indirect effect on maternal behaviour may result from defects in the development of the placental endocrine lineages. Together, these studies suggested that reduced placental *PEG3* expression may play a causal role in driving maternal mood symptomology and adverse infant outcomes in human pregnancy.

Using a *Peg3* loss-of-function mouse model, this thesis replicated previous work linking reduced offspring *Peg3* expression to impaired maternal care and increased anxiety-like behaviours in wild-type dams with 100% mutant litters. For the first time, this thesis extended previous work, characterising the behaviour of wild-type dams with litters composed of both mutant and wild type pups. Behavioural characterisation of *Peg3* mutant offspring revealed a sexually dimorphic effect of *Peg3* disruption, highlighting a male-specific deficit in neonatal social behaviour in mutant mice. Later life social impairments were also observed in both mutant and wild-type littermates. Inclusion of wildtype controls allowed for these behavioural changes to be attributed to the adverse maternal environment, likely induced by placental endocrine sufficiency, as opposed to intrinsic loss of function of the *Peg3* gene.

PEG3 expression and social communication were also explored in mother-infant dyads from the Grown in Wales human cohort. There were no associations with *PEG3* and mother-infant social behaviour. However, sexually dimorphic outcomes in response to prenatal depression were observed. Specifically, prenatal maternal mood symptomology was associated with negative aspects of vocalisation in mothers with female, but not male infants.

In summary, this thesis provides support for the relationship between placental endocrine dysfunction, maternal behaviour, and offspring outcomes in a *Peg3* loss of function model. It also contributes to a growing body of literature suggesting that males are differently affected by disruption of *Peg3* expression. Finally, though no association with *PEG3* was reported in the human cohort, this thesis highlights the need to study sexually dimorphic outcomes in relation to perinatal maternal mood symptomology, as a mother's behaviour may be influenced by the sex of their infant.

1

Chapter 1: Introduction

"Parental affections" are the root of sociality" – Darwin 1871

1.1 The Mother-Infant Relationship

Most animal species are inherently social, with aspects of social behaviour such as social recognition playing a role even in animals lacking a central nervous system (Gibbs et al., 2008). Whilst many fundamentally important social behaviours are shared across the animal kingdom, such as establishing territories and some form of courtship (Seebacher and Krause, 2017), it is among mammals that social behaviours are the most crucial and complex (Silk, 2007). Of all the social interactions observed in mammals, the earliest and most important is the bond between mother and infant. This process is dynamic and bi-directional, involving both the provision of care from the mother and complimentary behaviours from the infant to elicit such care; highlighting the reciprocal nature of this bond (Sullivan et al., 2011). Quality maternal care is essential in sculpting the infant's development, and disruptions in this process can have a lasting impact on the infant's neurobiological, socioemotional, and cognitive development, extending into later life (Champagne and Curley, 2005, Sullivan et al., 2011, Slomian et al., 2019).

Maternal care may be broadly defined as "the pattern of a mother's behaviour that appears to enhance her offspring's survival and reproductive success" (Saltzman and Maestripieri, 2011). At its most rudimental, maternal care in mammals consists of the provision of the infant's primary drives: nutrients, warmth, and shelter. However, a successful mother-infant bond is dependent on more than the satisfaction of basic drives alone. Notably, studies of children in Romanian orphanages have shown that though infants raised in these environments had their primary needs met, staff shortages, shift patterns and a ratio of caregivers to children of up to 1:12 (Zeanah et al., 2005), deprived these children of a stable, intimate caregiver, resulting in high mortality, higher levels of regression and withdrawal, and impaired 'normal' functioning even after adoption (Rutter, 1985). Echoing this, findings from Harlow's work on maternal deprivation in infant monkeys were remarkably similar; whereby monkeys raised in isolation demonstrated higher mortality, increased anxiety like behaviours, impaired

social behaviour with peers, later life reproduction deficits in female infants and the significant preference of tactile comfort over food (Harlow et al., 1965). In contrast, infants who have grown up with the experience of strong maternal bonding are more likely to demonstrate healthy social and emotional development, be more resistant to stress, and in turn, have successful relationships with their own children (Chess et al., 1991, Tops et al., 2014). Together, these experiments highlight the importance of the development of a successful mother-infant relationship, beyond the satisfaction of basic needs, and the consequences disruptions in this relationship can cause.

A strong maternal bond is not defined solely by the physical proximity of the mother however, but also by a number of verbal and non-verbal behaviours, including the mother's responsiveness, sensitivity, and emotional state (Johnson, 2013). Sensitivity refers to a mother's affect, acceptance, and response to her infant's emotions (Baker and McGrath, 2011), whilst responsiveness refers to a mother's ability to be warm, affectionate and provide comfort to her infant (Johnson, 2013). Both maternal sensitivity and responsiveness have been associated with positive mother-infant relationships, alongside the development of infant vocal reactivity and attention skills (Baker and McGrath, 2011). However, the relationship between mother and infant requires participation of both individuals, and social behaviours of the infant have also been shown to influence the quality of maternal care. Mothers with infants who are perceived to cry excessively or have irritable temperaments have been shown to score lower on maternal self-report questionnaires assessing the quality of their bond with their infant (Kommers et al., 2017). Equally, infant's reciprocal facial expressions have also been shown to affect mother's perceptions of their infant bonding, with increased infant smiling shown to be associated with better mother-infant bonding (Nolvi et al., 2016).

Though the social behaviours above all take place postnatally, there is growing evidence that this relationship begins *in utero*, with a number of studies demonstrating that both newborns (Guellai and Streri, 2011, Bard and Nagy, 2017) and fetuses at 33 weeks gestation demonstrate some social ability (Reissland et al., 2011, Nagy et al., 2021). Notably, newborns show a preferential response to their mother's voice as early as 3 hours after birth (DeCasper and Fifer, 1980, Fifer and Moon, 1994) while fetuses at 33-41 weeks have been shown, via ultrasound technology, to respond differently to their mother's voice compared to a strangers (Kisilevsky et al., 2009). In addition, infants in the third trimester will attend to three-point light displays configured like a face, despite having no prior experiences of faces (Reid et al., 2017). As social communication between mother and infant is the earliest objective measure of the quality of the mother-infant bond, it is an important outcome to consider when exploring the effects of disruption in the mother-infant relationship. Maternal speech for example, has been independently associated with infant outcomes such as attachment style, cognitive development, and psychopathology (Lewis and Feiring, 1989, Allely et al., 2013). Excessive infant distress vocalisation on the other hand has been associated with impaired mother-infant bonding (Nolvi et al., 2016).

To summarise, whilst it is well established that poor quality maternal care can have a significant impact on the development of the offspring, the mother-infant dyad is reciprocal in nature. Although maternal care is provided by the mother, a successful social interaction must also involve the infant actively soliciting and receiving her care in order for the mother and infant to have the best possible outcomes in later life. Despite this reciprocal

dynamic, there has been comparatively little focus on the role of the offspring in influencing this social relationship (Potter et al., 2019). Further, models of maternal care and parenting have highlighted the multi-deterministic nature of this relationship, emphasising the importance of both the pre- and postnatal environment (Figure 1.1, Belsky, 1984, Taraban and Shaw, 2018). Although a discussion of all of these factors is beyond the scope of this thesis, perinatal mental health has been cited as one of the most disruptive factors to the maternal-infant relationship (Belsky, 1984, Taraban and Shaw, 2018) and one of the strongest, most well-replicated risk factors for a range of infant outcomes such as increased rates of psychopathology, and less stable future social interactions (Goodman and Gotlib, 1999).

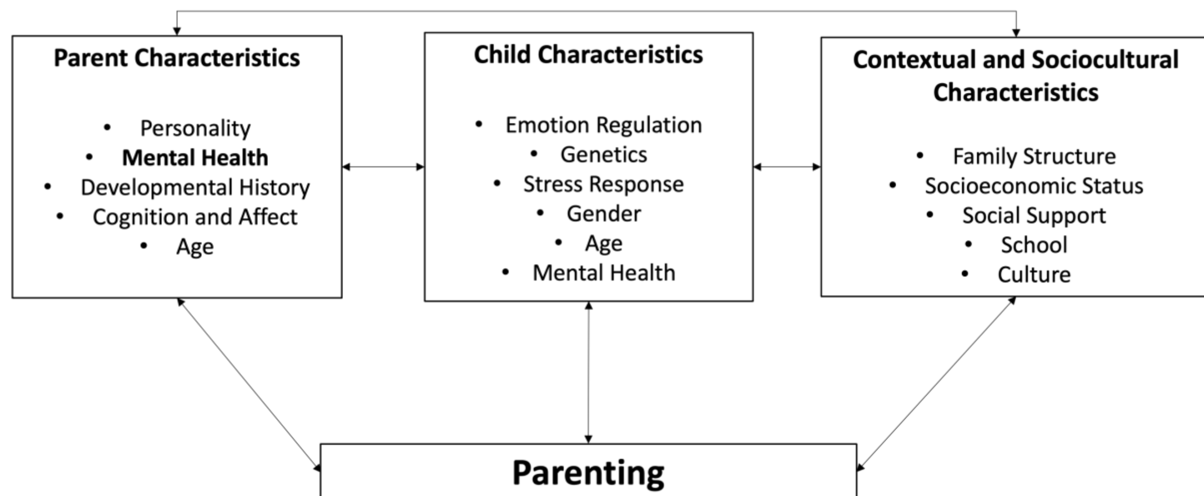


Figure 1.1. **Taraban and Shaw's (2018) Process of Parenting Model**. The updated process of parenting model highlights the parent, child and social factors that alter the quality of parenting, and as such the mother-infant bond. *Adapted from Taraban and Shaw (2018).*

1.2 Perinatal Mental Health

Perinatal mental illness refers to psychiatric conditions that manifest during pregnancy, and up to one year following childbirth (Stuart-Parrigon and Stuart, 2014). In 2016, the most recent study of its kind estimated that the total lifetime cost of perinatal depression and anxiety, per women with the condition, was £75,728 and £34,811 for depression and anxiety respectively. Aggregated, the total cost for each one-year cohort of births in the UK was estimated to be £6.6 billion per year, with around 75% of this cost attributed to adverse impact on the child (Bauer et al., 2016). Perinatal mental disorders are the most common complication of pregnancy, with around 1 in 5 women experiencing a mental health problem perinatally (NICE, 2017). Though a number of conditions can fall under the umbrella of perinatal mental health, depression and anxiety are among the most prevalent (O'Hara and Wisner, 2014).

The Diagnosistic and Statistical Manual of Mental Disorders (DSM) states that a clinical diagnosis of perinatal depression requires an onset of depressive symptomology during pregnancy or within the first four weeks after birth (American Psychiatric Association, 2013). However, it is widely acknowledged that symptoms can occur up to one year after childbirth (Stuart-Parrigon and Stuart, 2014), with the peak time of onset occurring 2-3 months

post-delivery (Wisner et al., 2010, O'Hara and Wisner, 2014). Encompassing both minor and major depressive episodes, perinatal depression is usually defined in one of two ways; either via an assessment of self-report symptoms using clinically approved measures such as the Edinburgh Postnatal Depression Scale (Cox et al., 1987), or via a clinical interview. Though reports of the prevalence of perinatal depression vary depending on the period of time that symptom manifestation is thought to begin, it is largely accepted that prevalence rates during pregnancy are between 10-25% (Field, 2017b), though in the UK, this rate is rising, with rates being 51% more common in young mothers than in mothers 25 years ago (Pearson et al., 2018). Postnatally, the prevalence of maternal depression symptoms are thought to be between 20-40% (Field, 2010).

Despite anxiety being more prevalent in the general population than depression (McManus et al., 2016), the majority of research into perinatal mental health has predominately focused on depressive episodes. However, in recent years there has been a rapid growth in research exploring perinatal anxiety. Similar to perinatal depression, perinatal anxiety is defined as anxiety experienced during pregnancy or in the first 12 months following childbirth, with prevalence rates thought to be between 21-25% during pregnancy, and between 13-40% postnatally (Field, 2017a, 2018). Though anxiety disorders have many different subcategories including obsessive compulsive disorders, and pregnancy related anxiety (Huizink et al., 2014), the most commonly diagnosed is generalised anxiety (Misri et al., 2015), which is assessed by psychometric questionnaire and is typically broken down into state and trait anxiety (Spielberger, 1970).

Although diagnostically defined as distinct disorders, depression, and anxiety commonly present comorbidly in the perinatal period, with evidence suggesting that 30-58% of mothers presenting with major depressive disorder also present with anxiety (Field et al., 2010). Consequently, this can make disentangling the individual effects of each affective disorder difficult, and as a consequence, the two are often studied together. In line with this, there have been suggestions that the high degree of correlation in risk factors between depression and anxiety indicate a shared genetic pathway (Kendler et al., 2007). Conversely, there is also growing evidence that depression and anxiety, despite their high comorbidity, have distinct trajectories and that comorbid depression and anxiety result in a more severe symptom score trajectory than depression or anxiety presenting in isolation (Penninx et al., 2011). Though there has been little research into anxiety as a distinct disorder from depression, the growing field of perinatal anxiety research suggests that the disorders should, when possible, be studied separately (Bayrampour et al., 2016, Ahmed et al., 2018).

Despite conflicting research surrounding the nature of study and the onset of perinatal affective disorders, it is largely agreed that perinatal anxiety and depression have long lasting consequences on outcomes for the mother, infant, and their reciprocal bond (Stein et al., 2014). Though the types of outcomes vary, many social behaviours that are integral to successful mother-infant bonding are affected. Mothers with perinatal mental health conditions have an increased risk for a number of impairments which compromise their ability to initiate interactions and appropriately respond to cues from their infant including; problems breast-feeding (Taj and Sikander, 2003, Stuebe et al., 2012), poor attachment (Carter et al., 2001, Stevenson-Hinde et al., 2013), reduced maternal responsiveness; (Campbell et al., 2007, Field, 2010, Lovejoy et al., 2000, Murray et al., 1996), reduced maternal warmth and touch (Field, 1995, Righetti-Veltema et al., 2002), impaired maternal sensitivity, (Hwa-Froelich et

al., 2008), vocal communication (Hwa-Froelich et al., 2008, Porritt et al., 2014), and increased maternal intrusiveness (Weinberg and Tronick, 1998). In turn, infants of mothers with perinatal affective disorders show impairment on a number of outcomes, beginning at the fetal stage and continuing into later life. Although research is limited at the fetal stage, there is some evidence to suggest that affective mood disorders during pregnancy are associated with fetal hyperactivity and irregular fetal heartbeat (Gentile, 2017). At birth, one of the biggest risk factors of maternal mood disorders is preterm birth and low birth weight (Khashan et al., 2008, Henrichs et al., 2010, Liu et al., 2012), which confer their own risks for infant mortality, cardiovascular diseases, and later life psychiatric problems (Wojcik et al., 2013). Independent from low birth weight, maternal mental health has been linked to an infant's social-emotional development, with children of mothers with postnatal depression exhibiting less positive affect as early as 6 months old (Campbell et al., 1995). As the infant gets older, maternal mood disorders have been associated with deficits in cognition, motor development, language, poor temperament, and both internalising and externalising problems (Muzik and Borovska, 2010, Field, 2011). These effects are persistent, with increased risk of psychopathologies extending into adolescence and beyond (Pawlby et al., 2009, Plant et al., 2015), with one study observing that perinatal depression in mothers predicted antisocial behaviour in their adult offspring 30 years later (Mäki et al., 2003).

When looking at the mother-infant dyad together, a number of impairments are also observed, with interactions between mothers with perinatal mental health conditions and their infants characterised by the sharing of negative behaviour states more commonly than positive states and showing increased levels of disengagement and hostility (Campbell et al., 1995, Burke, 2003, Feldman et al., 2009).

Taken together, it is clear that there is considerable evidence that maternal mood is critically important for a successful mother-infant relationship, and in turn, offspring wellbeing. However, the mechanisms behind this are still largely unknown (Yim et al., 2015). Causes and consequences of maternal mood disorders are complex and are difficult to study in humans, ethically, and due to a reliance of self-report information in many studies. Further, though animal models have been used to study maternal mood disorders, these models cannot adequately capture the spectrum of symptoms pertaining to mood disorders, and tend to focus on one specific symptomology, an issue when multiple environmental and genetic components play a role in the aetiology of mood disorders (Stein et al., 2014). Finally, exposure to perinatal mental health conditions occurs both in the pre- and postnatal environment. Historically studies looking at these mechanisms have focused on postnatal depression, however, there is a growing interest in depression and anxiety across the whole of the perinatal period (Shonkoff et al., 2012).

1.3 Prenatal Programming

Development of the infant, and the maternal-infant bond begins before birth and it is well established that the early environment of an infant can shape their development, conferring both protective, and risk factors for later life outcomes (Barker, 2007, Seckl and Holmes, 2007, Gluckman et al., 2008). This concept, known as the 'Developmental Programming hypothesis' (Langley-Evans, 2006, Langley-Evans, 2015), the 'Fetal Programming hypothesis' (Seckl and Holmes, 2007) or most prominently, the Developmental Origins of Health and Disease (DOHaD) hypothesis (Barker, 1995, Barker, 1998, Barker, 2007), specifically refers to how exposure to non-

genetic ‘stressors’ during critical periods of development, including the *in utero* period, can ‘program’ changes in the fetus, resulting in alterations to the health and development of the child with persisting effects into adulthood (Barker, 1995, Barker, 1998). The hallmark outcome of all prenatal programming hypotheses is low birth weight, which confers a number of later life risks for the offspring and is a common outcome for infants exposed to a number of prenatal stressors, including but not limited to poor maternal nutrition (Barker, 1995, Barker, 1998) and prenatal anxiety and depression (Henrichs et al., 2010; Khashan et al., 2008; Liu et al., 2012).

Originally, the term ‘stressor’ referred to poor maternal nutrition, with evidence for prenatal programming based on epidemiological studies in numerous cohorts associating low birth weight in infants, (a proxy for a poor intrauterine environment), with heart disease, type II diabetes and hypertension in adulthood (Barker, 1995, Barker, 1998). However, as research has progressed, the term ‘stressor’ has taken on a broader meaning, encompassing a number of factors including both perinatal anxiety and depression (Glover et al., 2018).

Though a number of studies have documented an association between prenatal maternal mood and infant outcomes, many of these associations have been linked to additional factors such as socioecomonic status, smoking, alcohol consumption whilst pregnant or the quality of infant attachment (Heron et al., 2004, Bergman et al., 2010). Importantly however, a number of community studies in different populations have found that even when controlling for these factors, an association between prenatal mood and later life infant outcomes remained, suggesting a direct casual pathway (DiPietro et al., 2006, Koutra et al., 2013, O'Donnell et al., 2014, Glover et al., 2018). It remains difficult to explore the effects of *prenatal* versus *postnatal* mental health, as both prenatal depression and anxiety are among the strongest risk factors for the development of the disorders postnatally (Vesga-López et al., 2008). However, a cohort in the United Kingdom have shown in a 14-year longitudinal study, that both maternal anxiety and depression during pregnancy predicted internalising and externalising problems in infants throughout ages 4-13 years, independently of sociodemographic status or any maternal mood symptoms reported after pregnancy (O'Donnell et al., 2014). Together, this highlights the importance of the *in utero* environment in altering infant outcomes. However, whilst there is substantial evidence for the DOHaD hypothesis, the mechanisms underlying the emergence of prenatal mood disorders are still unclear.

More recently, an alternative prenatal programming hypothesis, the ‘Placental Programming’ hypothesis (Creeth and John, 2020) has addressed this, suggesting that in some pregnancies, placental endocrine insufficiency may result in maternal mood disorders, and infant outcomes which confer later life risk such as low birth weight (Creeth and John, 2020).

Located at the boundary between the maternal and fetal environment, the placenta is a super-endocrine organ often cited as the physical interface, and initial site of mother-infant interaction (Maltepe & Fisher, 2015). Derived from extraembryonic tissues, the placenta is primarily fetal in origin, and is responsible for creating an environment suitable for fetal growth, and supporting both the growth, and development of the fetus (John and Hemberger, 2012). Throughout pregnancy, the placenta plays a role in a number of maternal-fetal interactions with its key functions including the transport of nutrients to the fetus, protecting the fetus, and the production of hormones responsible for priming the mother, both physiologically and psychologically for pregnancy and

childbirth (Ferner and Mess, 2011, John and Hemberger, 2012). Consequently, optimal placental functioning is critical to fetal and later life development (John and Hemberger, 2012).

Assessment of placental function, and the consequences of a suboptimal placenta represent a challenge in human research, due to the need to avoid harm to both the mother and fetus. As such, animal models are necessary to compensate for this. Though there is no one ideal species to model human pregnancy and placental development, rodents are often used, as though the gross architecture of the human and mouse placenta differ, there are core similarities between the cell types, structure, and mechanisms underlying development (Figure 1.2, Hemberger et al., 2020).

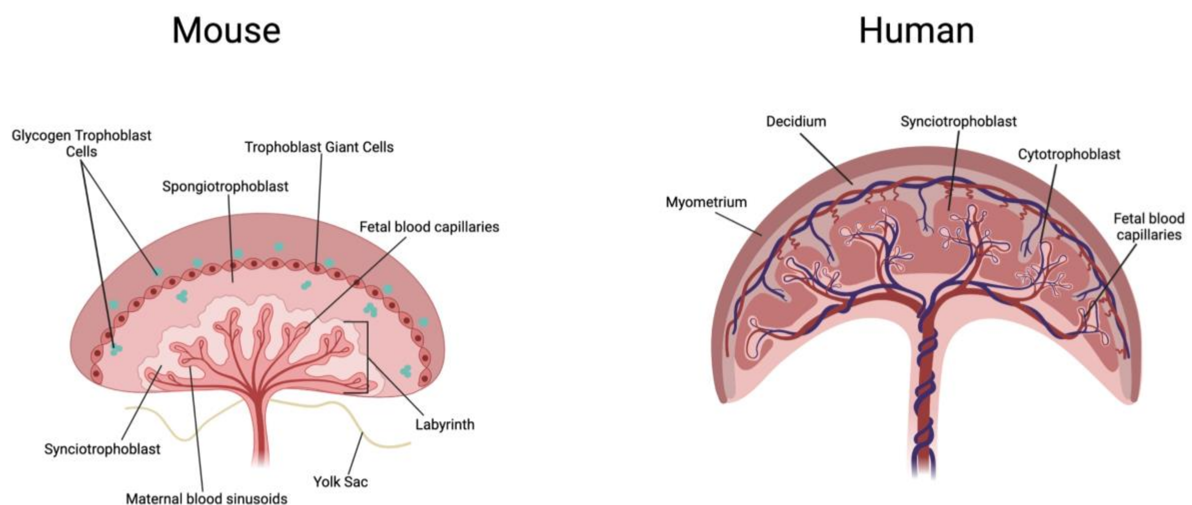


Figure 1.2 The Placental structure of both the Mouse and Human Placenta.
Adapted from Hemberger et al. (2020)

At the core of the placental programming hypothesis is placental endocrine insufficiency (Creeth and John, 2020). As a super-endocrine organ, communication between the mother and fetus via the placenta occurs primarily through the secretion of hormones into the blood (Costa, 2016). The major endocrine lineages in human and mice are the syncitiotrophoblast and spongiotrophoblast (including trophoblast giant cells) respectively, where the majority of placental hormones in each species are produced. These hormones, such as progesterone, and human placental lactogen (*hPL*) in humans, and members of the prolactin like family (*Prls*) in mice, are thought to target the maternal brain, and be involved in priming the mother, both physiologically and psychologically for pregnancy and childbirth (Ferner and Mess, 2011, John and Hemberger, 2012). The key premise of the placental programming hypothesis is that during pregnancy, *in utero* exposure of the fetus and placenta to stressors, drives changes in the endocrine function of the placenta, influencing hormone production, and subsequently leading to low birth weight in the infant (Creeth and John, 2020). In addition, placental endocrine insufficiency can result in a failure to prime the maternal brain for motherhood, leading to inappropriate maternal care (see section 1.4) which may in turn manifest as symptoms of maternal mood disorders, through which continued exposure may lead to potentially adverse outcomes in children (See Figure 1.3).

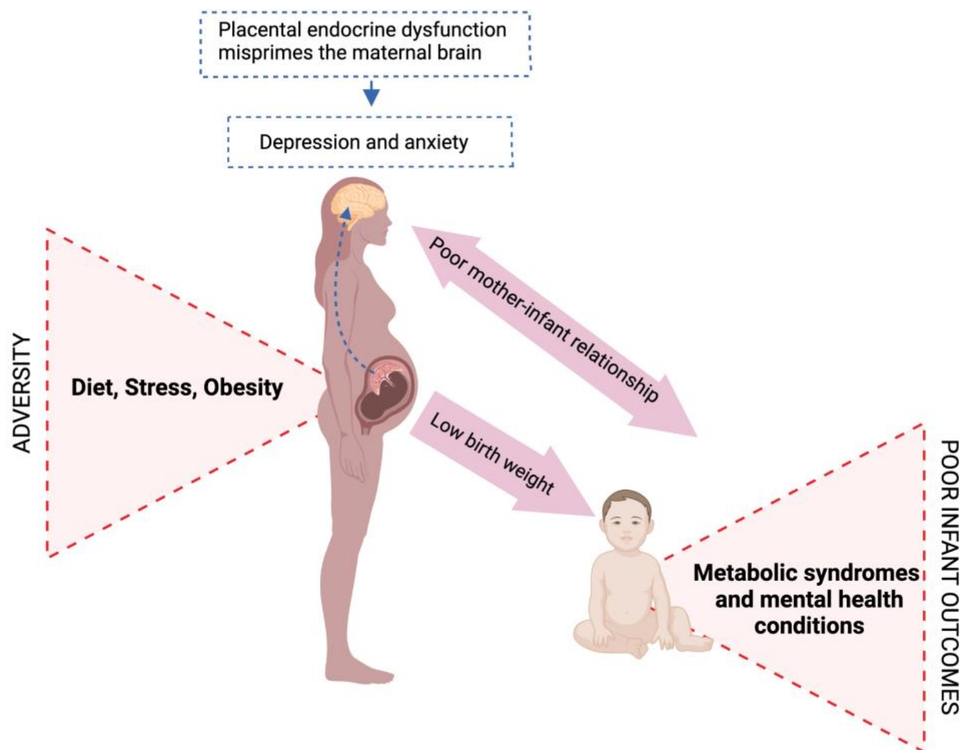


Figure 1.3 The Placental Programming Hypothesis. The fetus and placenta are exposed to adversities *in utero*. These adversities cause changes in the placental endocrine lineages of the placenta, altering hormone production, consequently impacting nutrient supply resulting in low birth weight. Changes in placental endocrine function may also result in the maternal brain failing to adapt for motherhood, manifesting as symptoms of maternal mood disorders. Low birth weight and continued exposure to perinatal maternal health conditions may further contribute to poor outcomes in children. *Adapted from Creeth et al., (2020).*

Though the two hypotheses suggest different causes of adverse outcomes in infants in the context of maternal mood disorders; via a direct effect on the fetus (fetal programming) and an indirect effect via the placenta (placental programming), the key occurrence of low birth weight or fetal growth restriction is common to both models. Further, as the placenta is fetally derived, consequently sharing the same genetics and environment as the fetus (John and Hemberger, 2012), the two models are not mutually exclusive, and may even be seen as complementary as the placental programming hypothesis does not exclude the possibility of direct changes to the fetus via placental endocrine insufficiency or other environmental adversity (Creeth and John, 2020). Regardless of hypothesis, stresses in the maternal environment must be transmitted across the placenta to affect the fetus. Consequently, research into placental function and outcomes of pregnancy have suggested a key role of the placenta, either directly, or indirectly, in mediating the relationship between prenatal stress and adverse infant outcomes (Lewis et al., 2015, Janssen et al., 2016, Creeth and John, 2020).

Although a relatively new area of research, in both humans and mice, the majority of research into the mediating role of the placenta between prenatal stresses, including maternal mental health, and adverse infant outcomes has focused on two main areas, the first being the hypothalamic-pituitary-adrenal (HPA) axis, and the gene *Hydroxysteroid 11-Beta Dehydrogenase 2 (11-HSD*), which codes for the placental enzyme, 11β-hydroxysteroid dehydrogenase 2 (11β-HSD2*).* In humans, anxiety and depression during pregnancy have been associated with

reduced expression of *11-HSD* (O'Donnell et al., 2014, Seth et al., 2015, Togher et al., 2017). As 11β-HSD2 regulates fetal exposure to cortisol via inactivation to cortisone, reduction in its expression may increase fetal exposure to cortisol, which in conjunction with exposure to maternal depression, has been shown to influence infant temperament (Davis et al., 2007), and increase infant risk for neurosensory deficits and affective disorders later in life (Moisiadis and Matthews, 2014). A caveat to this, however, is that it is not clear how much maternal cortisol crosses the placenta under either normal or stressed conditions (Stirrat et al., 2018).

The second area is placental endocrine insufficiency. As highlighted previously, *hPL* is a pregnancy hormone important for both the fetus and mother, playing a role both in supporting fetal growth, and in the induction of maternal care behaviours (Freemark et al., 1987, Bridges and Freemark, 1995, Shingo et al., 2003, Vergara-Castañeda et al., 2016). *HPL* is secreted by the syncitiotrophoblast in the placenta during pregnancy, and has been linked to the suppression of maternal anxiety during this time (Torner et al., 2001). Importantly, expression of *hPL* has also been shown to be reduced in both the placentas of low birth weight infants, and in the placentas of mothers who meet clinical, and sub-clinical self-report thresholds for postnatal depression (Dutton et al., 2012, Janssen et al., 2016, Sumption et al., 2020). In humans, further evidence that placental endocrine function and hormone production can influence mothers' mental health comes from research exploring placental corticotrophin hormone, which via the pituitary in the brain, acts as a stimulant to release cortisol (Glynn and Sandman, 2014). Importantly, increased levels of placental corticotrophin hormones have also been associated with postnatal depression (Glynn and Sandman, 2014). Though together, this suggests an indirect link between placental hormone production, maternal mood symptomology and infant outcomes in humans, these studies are often confounded by a number of factors including timing of birth, postnatal maternal care and the severity and type of measure of prenatal stress, including maternal mood symptomology Sumption, 2020). Further, much of the evidence listed above is *indirect* evidence, as manipulation of the placenta in humans, and the risk it poses to both mother and infant is unethical. As a consequence, more direct evidence linking placental endocrine insufficiency to maternal mood and infant outcomes is difficult to come by.

Crucially, shared genetic factors are also something that need to be considered, as, as highlighted by Rice et al., (2010) many prenatal factors that impact offspring development *in utero* are influenced by inheritable maternal characteristics (Rutter et al., 2001). Notably, a number of infant outcomes are to some degree inheritable, such as depression (Shadrina et al., 2018), an outcome which is complicated further by the relationship between prenatal and postnatal depression in mothers (Faisal-Cury and Menezes, 2012). To combat this, cross-fostering studies are often used in animal research as a means of disentangling the effects of shared genetics versus a shared environment, however, the availability and advances in *in vitro* fertilisation (IVD) also means that this is now more feasible in human studies. A notable example of this is from Rice et al., (2010), who using a sample of children born from IVF, were able to explore whether a range of offspring outcomes were observed in related, or unrelated mother-infant dyads. Importantly, many of the associations between prenatal stress and offspring outcomes, such as birthweight and anxiety, were seen in both related and unrelated groups suggesting a role of the shared environment, rather than solely shared genetics. While this approach provides the first steps toward a way in which the effect of shared genetics on offspring outcomes can be accounted before in human studies, the use of children born from IVF raises the question of whether the results of this study are generalisable to the

general population. Further, given the specificity of such a population, much of the research disentangling the effects of shared genetics, the research supporting the placental programming hypothesis, and the placenta as a mediator between maternal mood and infant outcomes, has been performed in animal models, as discussed in Section 1.4.

Alongside the growing body of literature supporting the role of the placenta as a biological mechanism mediating the relationship between maternal perinatal mental health and infant outcomes, is a need to understand the mechanisms that underlie this relationship. One popular suggestion is that epigenetic processes underlie prenatal programming (Seckl et al., 2000, Seckl and Holmes, 2007, Glover et al., 2018). Specifically, adversities in pregnancy leads to changes in the epigenome, and consequently, the changes in the expression potential of genes important to placental development. Generally, epigenetics refers to alterations in gene function / expression that do not entail a change in the underlying DNA sequence (Peschansky and Wahlestedt, 2014). A subset of mammalian genes, the imprinted genes, are epigenetically regulated, and have been established as regulators of both fetal growth and placental development (Li et al., 1999, Tucci et al., 2019). Consequently, imprinted genes have been hypothesised to play a role in prenatal programming, with aberrant expression of imprinted genes in the placenta potentially thought to underlie the relationship between maternal prenatal mood disorders and low birth weight in infants.

1.4 Imprinted Genes

Genomic imprinting is a parent-of-origin dependent epigenetic modification which leads to monoallelic gene expression as a consequence of epigenetic events in the germline (Surani, 1998). Though the majority of autosomal gene expression is derived from both inherited parental alleles, imprinted genes are either paternally expressed (silencing of the maternal allele) or maternally expressed (silencing of the paternal allele) depending on which parent the allele is inherited from. Consequently, though the alleles are in a diploid state in the cell, functionally they are haploid (Surani, 1998).

To date, 260 and 228 imprinted genes have been identified in mice and humans respectively, with 63 conserved between the two species (Tucci et al., 2019). Though imprinted genes make up less than 1% of the mammalian genome, they play a disproportionately important role in mammalian development, and are well established to regulate fetal growth and placental development (Lefebvre et al., 1998, Li et al., 1999, Tunster et al., 2013).

Although there are many theories surrounding the evolution of imprinted genes, the most widely recognised is parental conflict theory which predicts that genomic imprinting evolved as a response to conflict between the maternal and paternal genomes. The starkest, and most obvious example of this is, is the *in utero* allocation of resources to the fetus, where the fetus' maternal genes are 100% shared with the mother, whilst the paternal genes are not (Moore, 1991). Differential interests can also arise when there is asymmetries of relatedness in social groups. For example, in a nest where the remaining females are all related (sisters, half-sisters, maternal aunts etc), but the male(s) are not necessarily related to the females at all. Here the conflict may not just be about resources but about maternal care more broadly (see Isles et al., (2006) for a review). Using the example of *in utero* resource allocation, conflict theory suggests that there is inequality of parental investment in offspring, with

pregnancy incurring little cost to the father, but a greater investment of the mother, whose resources, via natural selection, are redirected to support the offspring. As such, to afford the maximum chance of all offspring surviving, the paternal genome maximises extraction of maternal resources for the offspring, whilst the maternal genome balances resource allocation to protect her reproductive fitness for future pregnancies (Moore, 1991, Haig, 2000). In support of this, paternally expressed genes tend to be fetal growth stimulators, whilst maternally expressed genes are growth suppressors, with many of these genes being co-regulated within an imprinted gene network involved in the control of the conceptus (Gabory et al., 2009, Gabory et al., 2010, Fowden et al., 2011).

One means through which imprinted genes regulate fetal development is by the regulation of placental growth and nutrient transfer. *Insulin-like Growth Factor 2* (*Igf2)* was the first imprinted gene discovered in mammals and has since been established as a positive fetal growth regulator (DeChiara et al., 1991). In mice, disruption of the P0 transcript of *Igf2* results in a smaller placenta, impaired nutrient supply to the fetus, and subsequent fetal growth restriction (Constancia et al., 2002). In humans, lower expression of *IGF2* has been linked to *in utero* growth restriction (IUGR), and the imprinting disorder Silver-Russel Syndrome (SRS) (Azzi et al., 2015). Notably, the expression of imprinted genes are not only altered in infants with imprinting disorders, but also in the placenta of infants who are small for gestational age, but are otherwise healthy (Diplas et al., 2009).

Critically, in addition to their role in the regulation of fetal growth, imprinted genes are regulated by epigenetic marks that respond to environmental adversities. In humans for example, fetal *IGF2* levels are associated with prenatal stressors such as maternal nutrition (Wallace et al., 2015) or gestational diabetes (Bowers et al., 2013b). While it is challenging to establish causality in human studies on imprinted genes, work with experimental models formally demonstrate that adversity in pregnancy can cause epigenetic changes in offspring. For example, offspring of dams fed on calorie controlled, low protein diets, show permanent disruption of the maternally expressed gene *Cyclin Dependent Kinase Inhibitor 1C* (*Cdkn1c*), despite themselves being fed on a normal diet from birth (Van de Pette et al., 2017). Taken together, this evidence suggests that imprinted genes represent highly promising candidates as a mechanism for linking perinatal adversity to later life outcomes in infants.

More recently, a number of imprinted genes have been shown to play a key role in the regulation of endocrine lineages in the placenta (John and Hemberger, 2012, Creeth and John, 2020), including *Pleckstrin homology-like domain family a member 2 (Phlda2)* (Tunster et al., 2016a), *Achaete-scute complex homolog 2 (Ascl2)* (Tunster et al., 2016b*)*, *Cdkn1c* (Tunster et al., 2011) and *Paternally expressed gene 3 (Peg3)* (Tunster et al., 2018). As previously highlighted, the placental programming hypothesis suggests that disruption of the endocrine lineages, and subsequent aberrant placental hormone production is thought to contribute to the mis-priming of the maternal brain, subsequently leading to altered maternal care, and adverse outcomes, including low birth weight in infants (Creeth and John, 2020). This has recently been elegantly demonstrated using a genetically manipulated 'dosage' model of *Phlda2* in mice (Creeth et al., 2018).

Phlda2 is a maternally expressed gene shown to negatively regulate the expansion of the spongiotrophoblast lineage (Tunster et al., 2016a), the most substantial endocrine lineage implicated in the manufacture of placental hormones in mice (Coan et al., 2006). Loss-of-function models of *Phlda2* have shown a 200% increase in the

contribution of the spongiotrophoblast to the mature mouse placenta, alongside increased expression of placental hormones expressed from this lineage, including the placental lactogen *prl3bl*. Conversely, two-fold expression of *Phlda2* results in a 50% loss of the spongiotrophoblast lineage, and lower expression of the same hormones (Tunster et al., 2016). To test the function of increased and decreased expression of these placental hormones, embryo transfer was used to to generate wild-type (WT) dams carrying and caring for offspring with different doses of *Phlda2*, and thus the dams were exposed to different levels of placental hormones. Dams carrying mutant offspring were found to have prenatal changes in the hypothalamus and hippocampus, areas which are both associated with maternal care behaviour, and displayed altered behaviour towards their pups postnatally (Creeth et al., 2018). Importantly, though no differences were found in the anxiety levels of dams with different levels of exposure to placental hormones, lack of pup-directed behaviour, may be seen as a 'depression-like' quality in mice, with some research showing that dams show anti-depression like behaviours in traditional depression measures, and that anti-depressants have been shown to elevate pup retrieval and other pup directed behaviours (García-Baos et al., 2022).

The offspring exposed to placental endocrine insufficiency (two-fold *Phlda2* expression), although born to genetically WT dams, also demonstrated alterations in both brain and behaviour, including increased anxiety-like behaviour and deficits in social behaviour (Harrison et al., 2021). Critically, due to the inclusion of a WT control group, these alterations were able to be attributed to the adverse maternal environment induced by placental endocrine insufficiency, rather than the intrinsic elevation of the *Phlda2* gene. Together, this provides, in rodents, evidence for the placental programming hypothesis, demonstrating that aberrant expression of imprinted genes can effect both maternal behaviour, and the later life behaviour of their offspring through placental endocrine sufficiency (Creeth et al., 2018, Creeth and John, 2020, Harrison et al., 2021). Although it should be noted, that further research is still required to demonstrate a *direct* link between placental programming and maternal mood symptomology.

Given the difficulties in exploring cause and effect of adverse outcomes in human pregnancies, there has been little research exploring the placental programming hypothesis in humans, though a number of imprinted genes including *PHLDA2, IGF2* and *CDKN1C* have been linked to fetal growth restriction in humans (Jensen et al., 2014, Azzi et al., 2015). Recently however, *PEG3* expression has been associated with both clinically diagnosed, and sub-threshold symptomology of prenatal depression, coinciding with *hPL* expression (Janssen et al., 2016, Sumption, 2020). Abberant expression of *Peg3* in mice, has been implicated in maternal care, and in the pre- and postnatal development, and behaviour of offspring. Importantly, these phenotypes have been observed both as a consequence of loss of function in the dam (Li et al., 1999, Curley et al., 2004), and independently, in the fetus (McNamara et al., 2018a). Consequently, dysregulation of *Peg3* represents a promising means of modelling preclinical prenatal mood disorders, and exploring the reciprocal social relationship between mother and infant.

1.5 Paternally Expressed Gene 3

Peg3 is one of the earliest identified imprinted genes, discovered in the second screening for novel maternal, or paternally expressed genes in the mammalian genome (Kuroiwa et al., 1996). Both *Peg3* and *PEG3* have been

shown to be relatively ubiqutiously expressed in both the brain and body (Papatheodorou et al., 2020; Karlsson et al., 2021;). In mice, *Peg3* has been found to be predominately expressed in brain, adrenal gland and pituitary gland, and more recently, has been shown to be expressed in the placenta (Curley et al., 2004). Within the brain, expression is highest in the midbrain, the basal ganglia, and a number of areas in the brain crucial for maternal behaviour and for social behavoiur more broadly, including the hypothalamus, hippocampus and olfactory bulb (Li et al., 1999; Karlsson et al., 2021).

Give the complexity of social interactions, the neural basis for social behaviour is also complex, involving a number of specific brain regions and interconnecting circuitry (see Figure 1.4 and Chen and Hong (2018) for a review). As can be seen in Figure 1.4, some of the main regions in the brain that play a role in social behaviour include the amygdala and the principal nucleus of the bed nucleus of the stria terminalis, both of which are thought to play a role in social anxiety-like behaviour, contributing to activations of the HPA axis (Davis et al., 2010, Petrulis, 2013,Wang et al., 2014). Although not depicted in the networks of social behaviour seen in Figure 1.4, the prefrontal cortex is also thought to play a role in moderating social behaviour, including social recognition and dominance hierarchy (see Bicks et al., 2015 for a review). Interestingly, many of the regions shown to be implicated in social behaviour in Figure 1.4, are, as mentioned above, also regions where *Peg3* is highly expressed, including the olfactory bulb and many regions of the hypothalamus, including the paraventricular nucleus (PVN) of the hypothalamus. Lesions in these key regions have been shown to impact on a number of social behaviours, with lesions to the olfactory bulb resulting in impaired social recognition, and sexual behaviour in mice (Cooper, 1974; Dantzer et al., 1990; Popik et al., 1991; Jacupovic et al., 2008; Sanchez-Andrade and Kendrick, 2009). Lesions to the hypothalamus however have been shown to reduce aggression in male mice and increase social investigation in female mice (Rigney et al., 2021).

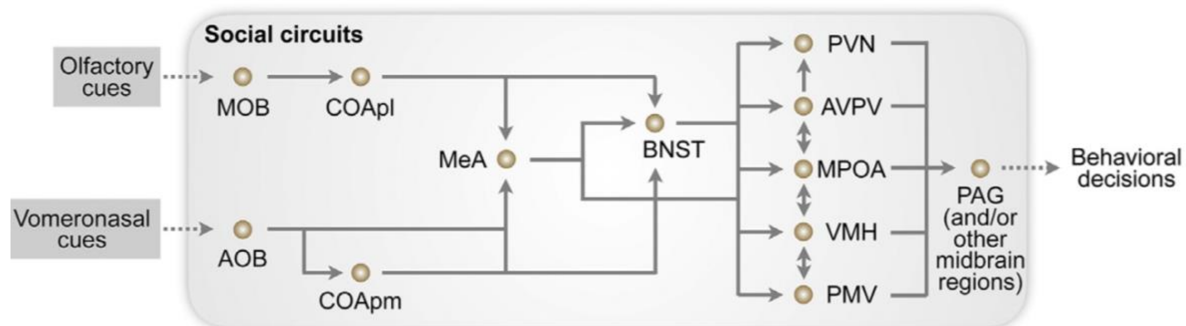


Figure 1.4 An overview of key brain regions and neural circuits involved in social behaviour.

MOB, main olfactory bulb; *AOB*, accessory olfactory bulb; *COApl/pm*, posterolateral and posteromedial cortical amygdala; *MeApd/pv*, posterodorsal and posteroventral medial amygdala; *BNSTpr*, principal nucleus of the bed nucleus of the stria terminalis; *PVN*, paraventricular hypothalamic nucleus; *AVPV*, anteroventral periventricular hypothalamic nucleus; *MPOA*, medial preoptic area; *VMHvl/dm*, ventrolateral and dorsomedial subregions of the ventromedial hypo-thalamic nucleus; *PMV*, ventral pre-mammillary hypothalamic nucleus; *PAG*, periaqueductal gray. *Adapted from Chen and Wong (2018).*

Although, as highlighted above, there are a number regions of the brain thought to play a role in social behaviour generally, many of the same regions are also implicated in the social interactions between mother and infant. The medial preoptic area of the hypothalamus for example has consistently been shown to play a role in parenting,

with lesions to this area and the amygdala culminating in reduced parenting behaviours in rodents such as impaired pup retrieval (Lee et al., 1999). Importantly, the hypothalamus has high expression of a number of receptors known to be modulators of parenting behaviour including oxytocin, estrogen and prolactin (Numan and Insel, 2006). The olfactory bulb also plays an important role in parenting behaviours, primarily in terms of the perception of olfactory cues leading to pup recognition (Numan and Insel, 2006). Removal of the olfactory bulb has been shown to impair maternal behaviour in dams both dams and virgin mice (Gandelman et al., 1971), whilst in neonatal rat pups, olfactory bulbectomy has been shown to impair suckling behaviour (Teicher et al., 1978).

However, as continually highlighted through-out this chapter, the relationship between mother and infant is bidirectional, and these same brain regions also play a role in pup social behaviour designed to elicit maternal care, specifically, ultrasonic vocaliations (USVs). Although discussed in more detail later in this chapter, USVs are high frequency whistles which pups will begin to produce shortly after birth as a means of eliciting maternal care (Branchi et al., 2001, Okabe et al., 2013, Wohr and Scattoni, 2013). Currently, there is very little research into the brain basis of USVs in neonatal mice, however, adult male mice also produce USVs during courtships behaviour, and it may be that the neural basis for the two types of USV are similar.

Although research is limited, a number of studies have identified regions of the brain thought to be assocaited with the production of USVs using methods such as chemical inactivation and eletrical stimulation (Tschida et al., 2019). For example, the periaqueductal grey functions as node for vocal control in a number of species including rodents (Jürgens, 2009), and is thought to play a role in the production of USVs in adult mice as electrical stimulation of this area causes mice to spontaneously emit USVs (Tschida et al., 2019). The same is also true of both the hypothalamus and the amygdala (Schwarting and Wohr, 2012). However, as electrical stimulation of these areas also impact on a number of behaviours, it is likely that USV emission involves a number of brain regions that are yet to be fully identified, and research is yet to explore these findings in the context of neonatal USV emission. Finally, as olfactory bulbectomy has also been shown to reduce USV emission in adult male mice during courtship, the olfactory bulb is also thought to play a role in USV emission (Wysocki, 1982), although again, this remains to be explored in neonatal pups.

In the human body, as in the mouse, *PEG3* is highly expressed in both the placenta, and the adrenal and pituitary glands. In humans, *PEG3* is also seen to be highly expressed in the testes and ovaries. In the human brain, there are again similarities, with *PEG3* having high levels of expession in the hypothalamus, and in addition, the thalamus (Karlsson et al., 2021).

Located on mouse proximal chromosome 7, and human chromosome 19q13.4, the function of *Peg3* is highly conserved across mice and humans, with similar expression patterns across both species (Kuroiwa et al., 1996, Kim et al., 1997). The *Peg3* gene encodes a large zinc-finger protein, and is thought to function as a transcriptional repessor of a number of genes involved in cellular metabolism, and placental hormon production (Thiaville et al., 2013, Lee et al., 2015, Tunster et al., 2018).

Alongside the development of evolutionary theories surrounding the emergence of imprinted genes, much of the early empirical research on the phenotypic role of imprinted genes focused on early development. Targeted loss of function models have established a role for *Peg3* in fetal growth and placental development (Li et al., 1999, Kim et al., 2013, Denizot et al., 2016, Tunster et al., 2018). This was originally observed in the work of Li et al. (1999) on the 129Sv strain, but has since been replicated in numerous studies, including those using different background strains and in other loss of function models (Curley et al., 2004, Kim et al., 2013, Denizot et al., 2016). More recently, loss of function of *Peg3* has also been shown to play a role postnatally, with mutant mice showing impaired suckling, and remaining growth-restricted into adulthood (Curley et al., 2004). Linked to this, loss of function of *Peg3* has also been shown to play a role in metabolism, with mutant mice showing a lower resting body temperature and reduced energy expenditure (Curley et al., 2005). Further, disruption of *Peg3* has also been shown to be associated with leptin resistance, with mutant mice deposisting excess fat in adulthood, despite consuming less food than genetically wild-type mice (Curley et al., 2005). Together, these data highlight the now well established role that *Peg3* plays in both fetal growth and metabolism.

There is also a growing body of evidence to suggest that imprinted genes play a role in social behaviour (Isles et al., 2006, McNamara and Isles, 2014). Loss of the paternally expressed *Growth factor receptor-bound protein 10 (Grb10)* for example has been shown to alter the stability of social behaviour in tests assessing social hierarchies in mice (Rienecker et al., 2020). Whilst twofold over expression of *Cdkn1c* results in abnormal social behaviours and increased instability of social hierarchy in mutant mice housed with WTs (McNamara et al., 2018b). Despite this, outside of the mother-infant relationship, the effects of *Peg3* on both neonatal and adult social behaviour has been largely ignored, with the exception of a characterisation of the olfactory and reproductive abilties of adult mutant mice (see Chapter 4).

The earliest and most critical example of the social relationship is the one between mother and infant, and the role of *Peg3* in the regulation of the mother's behaviour has been well studied.(Li et al., 1999, Curley et al., 2004, Champagne et al., 2009). *Peg3* was one of the first imprinted genes to be associated with the regulation of maternal care behaviour (Li et al., 1999), a phenotype which has been consistently replicated in numerous studies, with the exception of one particular modification (Denizot et al., 2016).

Loss of expression of *Peg3* in dams, has been shown to cause impairment of a number of maternal care behaviours (Li et al., 1999, Curley et al., 2004, Champagne et al., 2009). This was first established by Li et al. (1999) who by targeting the *Peg3* gene on a 129Sv background strain, reported that mutant *Peg3* dams were both slower to sniff and subsequently retrieve their pups during the pup retrieval task, a commonly used measure of maternal behaviour. Further, mutant mothers were slower to demonstrate protective crouching behaviour over their pups and demonstrated poor nest building skills and impaired milk let down (Li et al., 1999). These deficits were later replicated on the same strain by Curley et al. (2004), where *Peg3* mutant dams were again shown to be impaired in their ability both to nest build and retrieve pups compared to WT dams. Mice, like humans, are altricial and are born in an immature state. At birth, mice pups are deaf, blind, have limited motor capabilities and cannot thermoregulate, consequently relying on prompt and efficient maternal care for their survival (Brust et al., 2015). As such, disruptions to normal maternal care in mice is often life-threatening, a consequence reflected in the *Peg3*

knock out (*Peg3KO)* model, with litters from mutant dams showing a drastically decreased likelihood for survival, ranging from 37-92% mortality depending on the study (Li et al., 1999, Curley et al., 2004).

In a study exploring the role of *Peg3* on the behaviour of female mice of different strains, Champagne et al. (2009) demonstrated that despite naturally occuring variations in maternal behaviour, loss of function of *Peg3* resulted in maternal care deficits on both the 129Sv, and the C57BL/6J (B6) strain, though some strain specific effects were observed (Champagne et al., 2009). Specifically, though both strains of mutant dams showed impaired nest-building, *Peg3* mutant dams on the 129Sv background were slower to sniff their pups than WT dams, but did not show any differences in retrieval time. The opposite results were seen in the mutant B6 mice compared to their WT counterparts. For the first time, these deficits were also shown to extend to the frequency of postpartum care, with both mutant strains demonstrating less pup-directed behaviour (licking, grooming and nursing) than WT dams (Champagne et al., 2009); behaviours that when impaired, have long-term adverse effects on offspring development (Kikusui and Mori, 2009). Though only tested on the B6 strain, deficits in maternal behaviour also persisted when, in a cross-fostering experiment, mutant dams were presented with WT pups, suggesting that the deficits in maternal care are due to intrinsic to loss of expression of *Peg3* in the mother.

Peg3 is highly expressed in the adult hypothalamus, and ablation of the *Peg3* gene has been shown to impact both the size and structure of the paraventricular nucleus (PVN) of the hypothalamus, reducing the number of oxytocin-positive neurons in mutant, compared to WT females (Li et al., 1999). Through the secretion of oxytocin, the PVN regulates many aspects of maternal care including nest building, pup-retrieval, food intake and milk-let down (Bridges, 2015). Consequently, the maternal care deficits observed in *Peg3KO* models are hypothesised to be directly due to the impact on this particular neurobiological pathway as a direct consequence of loss of function of *Peg3* (Li et al., 1999, Curley et al., 2004, Keverne and Curley, 2008, Champagne et al., 2009). However, as has already established with other imprinted genes, such as *Phlda2,* the maternal-infant relationship is bi-directional with the fetal genome also regulating maternal behaviour via placental hormones and the placental endocrine system (Creeth and John, 2020).

Peg3 is highly expressed in both the mouse, and human placenta (Jannsen et al., 2016, Tunster et al., 2018). In humans, expression is thought to be localised to the layer of villous cytotrophoblast cells, whilst in mice, expression is much less restricted, with high levels of expression observed in all trophoblast populations (Hiby et al., 2001, Tunster et al., 2018). Functioning antagonisticly to *Phlda2* was which discussed in section 1.4, endocrine lineage analyses of mouse placenta have shown that *Peg3* is required for the proper formation of the spongiotrophoblast and glycogen cell lineages, with loss of expression resulting in a substantial loss of cells of both these lineages. Given that the spongiotrophoblast is the endocrine compartment of the murine placenta, this reduction in cells, also results in reduced expression of a number of placental hormones, inlcuding *prls* (Tunster et al., 2018). In line with this, transcriptional studies have shown that *Peg3* functions as a zinc finger protein, silencing a number of placental hormone genes that are used as markers to identify the endocrine lineages in the placenta (Broad and Keverne, 2011; Thiaville et al., 2013).

Importantly, *Peg3* has also been shown to negatively regulate placental hormone production (Tunster et al., 2018). Specifically, microarray studies have shown loss of function of *Peg3* to be associated with changes in expression of a number of prolactin family members which, as previously mentioned, are essential for maternal care behaviour. Members of the prolactin-like family have been shown to be ectopically expressed in the brain in response to loss of *Peg3* (Kim et al., 2013), whilst pregnancy specific glycoproteins have been shown to be upregulated (Broad and Keverne, 2011). Taken together, these studies indicate that in the mouse placenta, *Peg3* both up and down regulates placental hormones through two distinct mechanisms. Interestingly, the impact of *Peg3* loss of function has sexually dimorphic consequences on endocrine function, with male placenta showing up to 50% loss of spongiotrophoblast cells and 40% glycogen cells, whilst disruption to female placenta was minimal (Tunster et al., 2018).

Given the link between placental hormones and maternal behaviour, these studies suggest that as observed with other imprinted genes (Creeth et al., 2018), *Peg3* expression in the fetally derived placenta, may drive alterations in maternal behaviour. Crucially however, there remains a lack of research measuring the actual levels of placental hormones in circulation, with the research to date looking at gene expression via RNA sequencing and scope, and cell counts.

The majority of behavioural studies targeting *Peg3* have examined the consequences of loss of function of *Peg3* in the adult mouse as opposed to loss of expression in the offspring (Li et al., 1999, Swaney et al., 2007, Champagne et al., 2009). However, Curley et al. (2004), reporting on WT dams raising mutant litters, have shown that when the mutation was in the fetus, WT dams failed to increase their food intake during the last week of pregnancy. In addition, these litters also showed high mortality rates compared to WT dams with WT pups, potentially indicating that loss of *Peg3* in the offspring may result in suboptimal care from WT dams (Curley et al., 2004). To date however, the most substantial evidence that loss of offspring expression of *Peg3* can alter maternal behaviour comes from McNamara et al. (2018a).

In McNamara et al., (2018a), natural matings crossing WT dams with homozygous *Peg3* males were used to generate pregancies where all dams were genetically WT, but the litter was all mutant (*Peg3* $^{(+/-)}$). Using a battery of pre- and postnatal behavioural assessments, as well as RNA-sequencing analysis (RNA-seq), McNamara et al. (2018a) demonstrated that loss of expression in the feto-placental unit affected maternal behaviour in dams, both pre- and postnatally. Prenatally, though dams carrying *Peg3* mutant fetuses showed no deficits in nest building, locomotor activity or anxiety-related behaviour, these dams showed reduced exploratory behaviour when transferred to a novel environment. However, no detectable differences in gene expression in the prenatal hypothalamus or hippocampus were detected at E16.5 via RNA-seq analysis (McNamara et al., 2018a). Postnatally, though no differences were observed in either nest-building or maternal aggression assays, WT dams carrying mutant pups showed deficits in maternal behaviour that were strikingly similar to those observed in dams with loss of function of *Peg3*. Specifically, in McNamara et al. (2018a) dams carrying mutant *Peg3* fetuses were both slower to sniff and retrieve their pups than dams with WT litters (Figure 1.5). Dams of mutant pups were also shown to exhibit increased anxious-like behaviour postnatally, a response which has also been shown to occur in response to low prolactin (Larsen and Grattan, 2012).

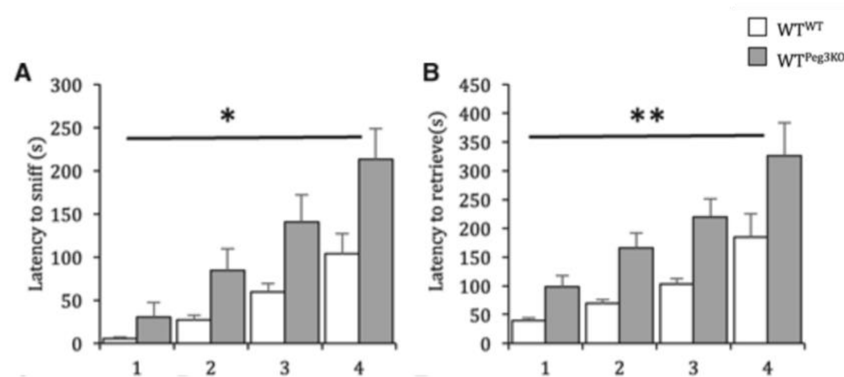


Figure 1.5 Pup retrieval data demonstrating that loss of function of offspring *Peg3* impairs maternal care of WT dams. Exposure to *Peg3KO* pups increases WT dam latency to sniff (**A**) and retrieve their pups (**B**) compared to WT dams of WT litters. Data are mean ± SEM. $*p = .05$, $** p < .01$.
Figure taken from McNamara et al. (2018a)

Taken together, the work of McNamara et al. (2018a) suggests a functional role for loss of expression of offspring *Peg3* in influencing maternal behaviour, particularily in regard to maternal anxiety-like behaviours. Additionally, though subtle, the observed changes in behaviour prenatally, suggest evidence of placental programming. The bi-directional nature of the mother-infant relationship does not stop at birth however, as postnatally, offspring continue to influence their mother's behaviour to establish and consolidate mother-infant attachments and filial bonds (Stoesz et al., 2013). At this stage, infants play a more active role in soliciting maternal care through, in both humans and rodents, a number of social behaviours, but most prominently, vocal communication (Stern, 1997, Lingle et al., 2012). As highlighted earlier, in rodents, ultrasonic vocalisations (USVs) are a primary means of communication, elicited by adult and neonatal mice in a variety of different social contexts (Panksepp et al., 2007, Scattoni et al., 2008, Scattoni et al., 2011). Present from birth, USVs are whistle-like sounds with a frequency of 30-90 kHz (Branchi et al., 2001). USVs are considered to be the earliest communicative behaviour of the mother-pup dyad and have been shown to elicit maternal care provision (Branchi et al., 2001, Okabe et al., 2013, Wohr and Scattoni, 2013) and reduce attacking and cannibalistic behaviours of the dam (Noirot, 1966).

Early USV vocalisation deficits in *Peg3* mutant pups have also been observed by McNamara et al. (2018a), with groups of mutant pups calling on average 42% less than WT pups during maternal seperation immediately prior to the pup retrieval task, a phenotype previously unreported in *Peg3KO* mice. This has two main implications, firstly, the finding of an early USV deficit may implicate *Peg3* in the development of social behaviour. To date, research on social behaviour using the *Peg3KO* model has largely focused on the behaviour of the mother, or specifically on olfactory and reproductive capabilties in adult mice (Li et al., 1999, Curley et al., 2004, Swaney et al., 2007, Swaney et al., 2008, Champagne et al., 2009, Denizot et al., 2016). Though mutant *Peg3* mice have been shown to demonstrate deficits in olfaction, reproduction, and in males, recognising sexually receptive mates (Swaney et al., 2007, Swaney et al., 2008, Champagne et al., 2009), the effect of *Peg3* on social behaviour more broadly, both in neonatal and adult mice remains to be explored.

Secondly, as USVs are a means of neonatal pups eliciting maternal care, this raises the possibilty that the altered postnatal maternal care observed in the dams (McNamara et al., 2018) may be driven by altered USV emissions

rather than placental endocrine insufficiency alone. As such, further research is warranted to ascertain whether the altered maternal behaviour reported both in mutant dams, and in WT dams caring for mutant *Peg3* pups is due to an altered postnatal environment as opposed to abnormal maternal care as a consequence of altered placental functioning.

Together, however, the work of McNamara et al. (2018a) suggests a role for loss of expression of *Peg3* in the offspring in influencing maternal behaviour, particularily maternal anxiety. Reduced expression of *PEG3* has also been shown to be associated with prenatal depression in humans (Janssen et al., 2016), potentially highlighting the use of rodent *Peg3* loss of function models as a model of preclinical mood disorders.

1.6 Translating back to humans

The data outlined in section 1.5 have shown the importance of *Peg3* in a successful pregnancy for both mothers and offspring and have implicated a role for *Peg3* in both anxiety-like behaviour and early offspring communication in rodents. Further, *Peg3* has also been shown to influence maternal behaviour during the perinatal period via regulation of the placental endocrine lineages in rodents. Although care should be taken in the comparison of human and animals, given the high conservation of *Peg3* between mice and humans, this data may also suggest that placental *PEG3* might contribute to depression in human pregnancies by altering the expression of placental hormones.

In humans, there have been few studies assessing the absolute expression levels of *PEG3* in relation to the mother-infant bond, maternal care, and human conditions more generally. However, altered DNA methylation of the *PEG3* promoter region in infant cord blood DNA has been linked to negative affectivity in infants (Fuemmeler et al., 2016). Altered DNA methylation of *PEG3* has also been linked to environmental factors such as maternal antibiotic use (Vidal et al., 2013), folate intake (Dalgaard et al., 2016) and obesity (Vidal et al., 2014), highlighting its sensitivity to environmental exposures in humans.

There is a growing number of studies to suggest that perinatal mental health is associated with differential expression of a number of placental imprinted genes and low birth weight in infants (see Argyraki et al. (2019) for a review). However, the majority of these studies focus on methylation levels in infant cord blood as a proxy for gene expression. For example, infants of mothers with prenatal depression have been observed to show significantly increased DNA methylation levels in cord blood at the *Maternally Expressed gene 3* (*MEG3*) differentially methylated region (DMR) (Liu et al., 2012). Cord blood methylation of *Paternally Expressed gene 1* (*PEG1*) has been positively correlated with maternal psychological stress, a variable strongly associated with maternal depression during pregnancy (Vidal et al., 2014). The standard interpretation of these findings is that depression, or factors associated with stress and / or depression, are inducing these changes in the offspring. However, data from animal models showing that placental alterations influence maternal behaviour may suggest a different relationship (Creeth and John, 2020).

Decreased placental expression of *PEG3* has been shown to be associated with maternal prenatal depression (Janssen et al., 2016). Specifically, a 20-45% reduction in the expression of *PEG3* in term placenta was associated

with prenatal depression in three independent human cohorts (Janssen et al., 2016). Notably, this phenotype was sex-specific, and was only found in male placenta. With the caveat that mice and human placenta have different structures and hormone repertoires (Hemberger et al., 2020), this draws parallels with the phenotypes observed in mouse models of *Peg3* function. In mice, loss of expression of *Peg3* results in a sexually dimorphic placental endocrine phenotype, where male placenta show a substantial loss of the spongiotrophoblast cell lineage and glycogen cells, whilst female placenta were substantially less effected (Tunster et al., 2018). Reflecting this, in Janssen et al. (2016), two out of the three cohorts also showed an association between *hPL* and *PEG3* in male placenta.

In addition to the studies measured above, there is a growing body of evidence to suggest that *Peg3* expression is different in males and females, and that disruption may have a more severe effect in males (Tunster et al., 2018). Faisal et al. (2014) for example reported that in the head of embryonic day 14.5 embryos, *Peg3* is more highly expressed in males, whilst deletion of the *Peg3* demethylated region (DMR) has a greater impact on the survival rates of male pups than female pups with a survival ratio of (male: female 8:31) across 8 litters (He et al., 2016). The growth restriction phenotype observed in *Peg3* mutant mice has been reported to be more pronounced in male pups (Kim et al., 2013), whilst increasing the expression of *Peg3* appears to increase birthweight, but only in female pups (Bretz and Kim, 2018). Maternal malnutrition studies have also shown a sexually dimorphic effect, where placental *Peg3* expression was increased in males but not females (Radford et al., 2012). Despite this, and a number of studies to suggest that males and females exposed to maternal stress *in utero* may have different developmental trajectories, there have been no studies exploring whether these sex differences extend into the postnatal behaviour of *Peg3* mutant mice, and to date, only one study has explored sex differences in infant outcomes in relation to prenatal mood disorders and placental *PEG3* expression.

Based on the findings in Janssen et al. (2016) that both *PEG3* and *hPL* expression were reduced in the placentas of male infants with mothers who were clinically diagnosed and / or self-reporting depression symptoms, the Grown in Wales (GiW) cohort was established to longitudinally explore the relationship between prenatal mood symptoms, placental genomic characteristics, and offspring outcomes (Janssen et al., 2018). The GiW cohort is described in more detail in Chapters 2 and 5.

Recently, the results from Janssen et al. (2016) have been replicated in the larger GiW cohort, again showing an association between prenatal maternal depression symptomology, and reduced placental expression of *PEG3* in male infants, but not females (Sumption, 2020). For the first time, it was also observed that reduced expression of placental *PEG3* was associated with depression symptomology postnatally, crucially, when controlling for depression during pregnancy. Though in rodent models, alterations in anxiety-like behaviour were observed (McNamara et al., 2018a), depression is particularly hard to measure in mice, especially in the context of pregnancy and childbirth. Together, these results suggest that as in mice, placental *PEG3* expression in humans may be driving changes in maternal mood in both the pre- and postnatal period which may contribute to offspring outcomes.

To date, there has only been one study, published by our group, that has explored the association between prenatal maternal mood symptomology, placental *PEG3* expression and offspring outcomes. At 12 months postpartum, a follow up assessment of a subset of mother-infant dyads were recruited and completed both self-report and in-person laboratory assessments scored by researchers. Interestingly, mothers reporting higher prenatal depression and anxiety reported negative characteristics in their infants (increased aggression and negative temperament) only when the child was female. In contrast, independent assessments by researchers demonstrated that males, but not females demonstrated objective impairment in cognitive development, and verbal communication, yet this was not reported by mothers. Despite studies exploring the effects of maternal mood on offspring outcome typically controlling for sex-differences, this supports a growing trend in research to suggest that males exposed to prenatal mental health conditions are more severely affected (Sutherland and Brunwasser, 2018, Savory et al., 2020). Interestingly, in rodents, it has been demonstrated that maternal care varies depending on the sex of the pup, with male pups often preferentially retrieved or groomed more (Moore and Morelli, 1979, Deviterne and Desor, 1990).

A potential explanation for disparity in infant outcomes may be due to societal expectations of an infant's behaviour based on their sex, for example female infants are stereotypically thought to be quieter and more well-behaved than male infants (Koenig, 2018). As prenatal mental health has been associated with altered perception of infant temperament, it may be that when these stereotyped expectations are not met, this manifests as the mother perceiving problems, and subsequently, altering her behaviour to match these perceptions, though objectively, these problems do not exist (Sumption, 2020).

Alternatively, given the sex-specific relationship between *PEG3* expression, and prenatal mood symptomology observed in human studies and the recently established communication deficit in *Peg3KO* pups (McNamara et al., 2018a, Sumption, 2020), it may be that the sex-specific outcomes observed in this study are due to an altered *in utero* environment, due to aberrant expression of placental *PEG3*. However, Sumption (2020) reported only minimal associations between placental *PEG3* and infant outcomes, none of which were associated with the sex-specific outcomes highlighted above.

Though again, care should be taken when translating mouse models to human research, as highlighted, reduced placental *Peg3* expression in mice, has been shown to alter USV emission in mouse pups (McNamara et al., 2018a). Further, Prader-Willi, Beckwith-Wiedemann and Angelman syndrome are all human conditions which share common language deficits and speech problems (Chamberlain and Lalande, 2010, Choufani et al., 2010). In line with this, mouse models of Angelman syndrome featuring silencing of the maternally imprinted gene ubiquitin ligase gene (*Ube3a*) show increased USV emission (Jiang et al., 2010). It may be then, that disruption of placental *PEG3* in humans affects more explicit social communication behaviours in infants, although this remains to be explored.

Though the GiW study utilises the Bayley's language subscale as a means of assessing communication in infants (Bayley, 2009), it has been well established that scores on psychometric tests may not reflect a child's true ability and that behavioural observation should be used alongside these measures for a more accurate representation

(Crais, 2010). As part of the lab assessments for the GiW study, behavioural observations were recorded, however to date, explicit social communication behaviour between mother and infant in these interactions remain to be explored, either in association to infant outcomes, or to placental *PEG3* expression.

Crucially, given the reciprocal nature of the mother-infant dyad, and findings in mouse studies to suggest that offspring loss of function of *Peg3* can alter maternal behaviour (McNamara et al., 2018a), it is also worth exploring the relationship between perinatal mental health, placental *PEG3* expression and maternal social communication. Maternal vocalisations are altered by depression and anxiety (Dib et al., 2019, Lam-Cassettari and Kohlhoff, 2020). However, the tone and quality of maternal vocalisations are also associated independently with infant outcomes, and reciprocally, infant vocalisations have been shown to alter maternal behaviour (Hsu and Fogel, 2003, Gros-Louis et al., 2006). Despite this, there is little evidence exploring the reciprocal effects of altered vocalisations on maternal, or infant outcomes, and no study so far that has examined this in the context of imprinted gene expression.

Together, the above evidence suggest a role for placental *PEG3* expression driving changes in maternal mood and contributing to later life infant outcomes, however, further research is still needed to explore how maternal mood affects both mother and infant vocalisations, and whether placental *PEG3* expression is associated with explicit social communication behaviours in humans.

1.7 Aims

Perinatal maternal mood disorders are among the most consistently disruptive risk factors to the bi-directional mother-infant relationship and have been reliably found to result in adverse outcomes for both mother and infant (Goodman and Gotlib, 1999, Goodman et al., 2011). Based on findings in both animals and humans that link the *Peg3* gene to prenatal maternal mood, and deficits in offspring behaviour, this thesis had two main aims. Firstly, to use the *Peg3KO* mouse model to further explore the reiterative relationship between mothers and their offspring, and the impact of loss of offspring *Peg3* on social behaviour (Chapters 3 and 4). Secondly, to translate this knowledge to a cohort of human mothers and infants from the Grown in Wales study (Chapter 5).

The specific aims of each chapter were as follows:

Chapter 3: To examine the impact of loss of expression of *Peg3* in offspring on the behaviour of WT dams, first by replicating, and then by extending the work of McNamara et al. (2018a). In addition to the original groups used in McNamara et al. (2018a) maternal behaviour was examined for dams carrying and caring for mixed genotype litters, to explore whether the presence of WT pups attenuated deficits in maternal care, and to determine whether dams could discriminate between genetically WT and mutant pups in the pup retrieval task.

Chapter 4: Firstly, to explore the social behaviour of *Peg3* mutant and WT offspring who were raised under conditions of atypical maternal care (Chapter 3). Secondly, this chapter explored whether there were any differences in gene expression in both the hypothalamus and olfactory bulb in *Peg3* mutant mice through

exploratory RNA-sequencing analysis. For both aims, this chapter explored how social behaviour and gene expression was affected by intrinsic loss of function of *Peg3* in mutant mice compared to their WT counterparts. However, inclusion of offspring from mixed genotype litters, also allowed for an exploration of whether changes in behaviour were due to intrinsic loss of *Peg3* expression, or due to an abnormal maternal environment. Finally, given the growing body of evidence to suggest that *Peg3* disruption more severely effects males, sex differences in both behaviour and gene expression were explored.

Chapter 5: Mother-infant interaction was first coded on a micro-analysis event-based basis and was then used to explore the relationship between maternal mood symptoms, mother-infant social behaviour and outcomes in both mothers and infants in the Grown in Wales cohort. Specifically, infant / maternal outcomes at 12 months were explored in relation to maternal / infant verbal and non-verbal social behaviour and measures of maternal mood during pregnancy at 12-months postpartum. In addition, it was further aimed to explore whether these associations differed depending on the sex of the infant. Finally, in an exploratory analysis, the relationships between placental *PEG3* and mother / infant vocalisations were explored.

2

Chapter 2: General Methods

This chapter describes general methods that apply to multiple chapters within this thesis. Methods which were not directly carried out by the researcher but have relevance to and directly inform specific experimental chapters are also detailed here.

2.1 Mouse model and husbandry

2.1.1 *Peg3KO* Mouse Model

The *Peg3KO* transgenic mouse model was used for all animal experiments contributing to this thesis. The strain was originally generated at the Wellcome CRC Institute of Cancer and Developmental Biology at Cambridge University. As described in Li et al. (1999) the *Peg3* mutation was generated by gene targeting embryonic stem cells of the inbred 129S2/SvHSD (129Sv) strain. Specifically, a 4.8 kb IRES-β*geo* selection cassette containing a SV40 polyadenylation site, *lacZ-neo* fusion gene and an internal ribosome entry site, was inserted by homologous recombination into the 5' coding exon of the *Peg3* gene (Figure 2.1). The mouse colony used in this thesis was kindly provided to Professor Ros John by Professor Azim Surani and has subsequently been maintained on a 129Sv background in house at Cardiff University.

Figure 2.1. The mutant *Peg3* locus. A simple schematic illustrating the mutant locus in the *Peg3KO* mouse model. The mutant locus was generated by the insertion of a βgeo selection cassette containing an internal ribosome entry site (green box), lacZ-neo fusion gene (orange arrow) and sv40 polyadenylation (red box) into exon five of the mouse genomic locus. *Adapted from Li et al. (1999).*

2.1.2 Animal Husbandry

All animal studies and breeding were approved by the University of Cardiff ethical committee and performed under a UK Home Office project license (RMJ, PAD270E16). Mice were housed in a regulated holding room (temperature (21°C ± 2) and humidity (50% ± 10%) maintained on a 12-hour light-dark cycle with lights coming on at 06:00 h. All cages (45x12x12 cm), regardless of number of mice, contained the same environmental

enrichment (cardboard tube, chew-stick, nestlet bedding), where enriched chow (Formulab Diet 5008, TestDiet, UK) and tap water were freely available on an *ad libitum* basis. Cages were cleaned out weekly at a regular time to ensure minimal disruption to behavioural testing and animal breeding.

2.2 Breeding schemes

All mice used in this thesis were maintained on a pure 129Sv genetic background and generated from natural mating. As can be seen in Figure 2.2, experimental animals were generated from three separate breeding groups, each requiring a stud of a specific genotype: wild-type (WT), heterozygous or homozygous for the mutant *Peg3* allele (*Peg3*$^{(-/+)}$ and *Peg3*$^{(-/-)}$ respectively).

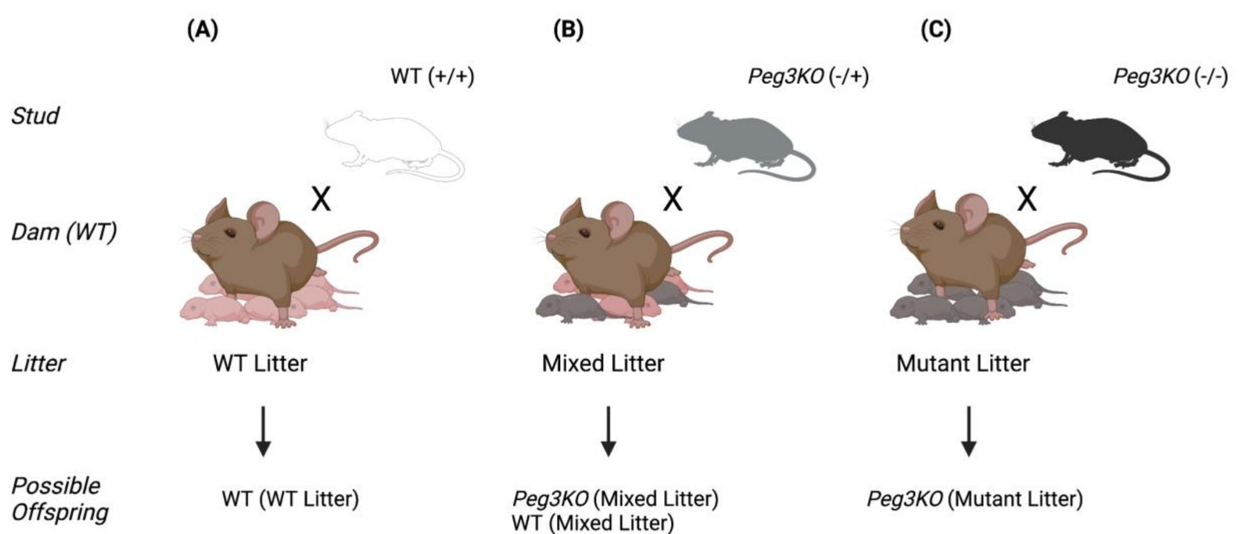


Figure 2.2 Schematic of the breeding design used to generate experimental dams and offspring ***A****) 100% Wildtype litters:* A wild-type (WT) female mated with a WT male produced litters composed of WT pups. ***B****) Mixed genotype litter:* A WT female mated with a heterozygous *Peg3*$^{(-/+)}$ male produced litters with *Peg3* $^{(+/+)}$ (WT $^{(\text{mixed litter})}$) pups and heterozygous mutant *Peg* $^{(+/-)}$ (*Peg3KO* $^{(\text{mixed litter})}$) pups. ***C***) *100% mutant litter*: A WT female mated with a homozygous *Peg3* $^{(-/-)}$ male produced a fully heterozygous litter (*Peg3KO* $^{(\text{mutant litter})}$), all expressing the paternally active copy of the *Peg3KO* mutation. If litter size was larger than 4 and the proportion of mutant pups within the litter was between 45-60%, data from both the dam and their litter were used for analysis and a proportion of litters went on to be used to assess early life social behaviour.

2.2.1 Generation of experimental cohorts.

The three breeding groups used to generate experimental cohorts can be seen in Figure 2.2. Briefly, the breeding groups are as follows: (i) WT females x WT males produced fully WT litters, (ii) WT females x heterozygous *Peg3* $^{(-/+)}$ males produced mixed litters, where offspring were either null for the *Peg3 allele* (*Peg3* $^{(+/-)}$) or WT (WT $^{(+/+)}$); and (iii) WT females x homozygous *Peg3* $^{(-/-)}$ produced a fully heterozygous *Peg3* $^{(+/-)}$ litter, where each pup expressed the paternally active allele of the *Peg3KO* mutation. As *Peg3* is silenced on the maternal allele, paternally inherited heterozygous KO mutation, results in complete loss of function of *Peg3*. To differentiate from pups of the same genotype from the different breeding set-ups; all pups generated from a WT

x WT pairing are referred to as WT $^{(WT\ litter)}$. Pups from mixed litter groups that were null for the *Peg3* allele and consequently were functionally WT are referred to as WT $^{(mixed\ litter)}$, whilst their heterozygous mutant littermates are referred to as *Peg3KO* $^{(mixed\ litter)}$. Finally, to differentiate between the heterozygous pups from both the mixed and fully knock-out litters, pups generated from a WT x *Peg3KO* $^{(-/-)}$ pair are referred to as *Peg3KO* $^{(mutant\ litter)}$ (see Figure 2.2).

The above breeding scheme was used to generate the following experimental cohorts for this thesis;

1. A maternal cohort used to characterise the maternal behaviour of wild-type (WT) dams exposed to different doses of *Peg3 in utero* (Chapter 3).

2. An offspring behavioural cohort (consisting of pups from the maternal cohort), to characterise the social behaviour of pups raised under conditions of atypical maternal care (Chapter 4).

3. Offspring used to generate tissue for exploratory RNA sequencing analysis (RNA-sequencing cohort, Chapter 4).

To generate the experimental cohorts, WT dams obtained from an in-house 129Sv colony (aged between 8-14 weeks) were housed in breeding trios of two females to one male per cage. Successful mating was determined by checking for a vaginal plug daily between 08:00 – 10:30 h. When a vaginal plug was observed, this was considered as embryonic day (E) 0.5. The female mouse was then weighed, and where possible housed with other female mice that had been plugged by a stud of the same genotype. At E16.5, if signs of pregnancy were visible and confirmed by an increase in weight, females were removed from the home cage and singly housed to ensure the biological dam of each litter could be determined. Although single-housed females have been shown to exhibit increased anxiety-like behaviour compared to group-housed females (Kulesskaya et al., 2011), any negative effects were assumed to be consistent across all groups.

Day of birth was designated as postnatal day (P) 0. Dams and their litters remained undisturbed until the first day of testing (P2). At weaning (P21), offspring to be used for further behavioural testing were housed in same-sex cages of four where possible, preferentially keeping mice from the same birth litter together. To maintain consistency between the pre- and postnatal environment, offspring from the mixed litters were, where possible, housed into cages containing two WT $^{(mixed\ litter)}$ pups and two *Peg3KO* $^{(mixed\ litter)}$ littermates. Consistently with previous studies, dams with litter sizes between 4 and 10 live pups were used for analysis (McNamara et al., 2018a), and for the mixed litter group, between 45-65% of the pups were required to be mutant, as identified by genotyping at P14. The mixed litter group was included to allow for an exploration of whether WT pups attenuated maternal phenotypes induced by carrying a mutant litter. Consequently, the inclusion criteria listed above ensured a sufficient number of WT pups were present in the litter.

2.3 Genotyping

Polymerase Chain Reaction (PCR) was used to identify the genotype of all studs and offspring (behavioural and RNA-sequencing cohorts). As dams in the maternal cohort were generated from an entirely WT 129Sv colony, the only possible genotype for these mice were WT, and so no genotyping was performed.

2.3.1 DNA Extraction

Tissue biopsies were taken from offspring at either P14 (ear biopsy) or P6 (tail biopsy, post-dissection) for the offspring behavioural and RNA-sequencing cohort respectively. Following collection, biopsies were digested in lysis buffer (1 M Tris 8.0 pH 8.0 (Sigma), 0.5 M EDTA pH 8.0 (Sigma), 10% SDS (Sigma), 5 M NaCl in water) containing Proteinase K (PK, Sigma UK) to a final concentration of 400 µg/ml. Ear biopsies were digested in 100 µl lysis buffer + 2 µl PK and tail biopsies in 200 µl lysis buffer + 4 µl PK overnight at 55°C.

Following digestion, samples were briefly mixed using a vortex mixer and allowed to cool at room temperature for 20 minutes. Lysates were then diluted at a ratio of 1:10 for ear biopsies, or 1:20 for tail biopsies in 10 mM Tris (pH 8) and heated to 95°C for 20 minutes to inactivate the PK. Dilutions were again vortexed to mix and allowed to cool at room temperature for 20 minutes.

2.3.2 Polymerase Chain Reaction Protocol

Mice were genotyped using two separate PCRs to identify the presence or absence of the mutant *Peg3* allele. Tables 2.1 and 2.2 show the reaction mixes for the WT allele and *Peg3KO* transgene respectively. An additional multiplex PCR for a Y-linked family of genes (*Ssty*) and an autosomal control gene *(Om1a)* was performed for sex typing when necessary to confirm the researcher's visual observations, primarily for those pups used for the generation of brain tissue at P6 for RNA-sequencing. Table 2.3 shows the PCR reaction mix used for sex typing.

Primers were purchased from Sigma, diluted as 100 mM stocks, and stored at -80°C. When needed, a working primer mix was created. For genotyping, 25 µl of the forward primer, 25 µl of the reverse primer and 50 µl of 10 mM Tris pH 8, were combined in a 0.5 ml Eppendorf and stored at -20°C. For sex-typing, 25 µl of each of the four primers were combined (Table 2.3) and stored at -20°C.

All PCRs were carried out in an Applied Biosystems-Veriti 96-well Thermocycler machine and cycling conditions for both genotyping PCRs were as follows: i) 95°C for 3 minutes, ii) 95°C for 30 seconds, iii) 64°C for 30 seconds, iv) 72 °C for 60 seconds, v) repeat ii-iv 34 times, vi) 72°C for 10 minutes, vii) hold at 4°C. The cycling conditions for the sex-typing were: i) 94°C for 4 minutes, ii) 94°C for 45 seconds, iii) 61°C for 45 seconds, iv) 72°C for 45 seconds, v) repeat ii-iv 35 times, vi) 72°C for 5 minutes, vii) hold at 4°C.

Table 2.1 PCR Reaction mix for identification of WT DNA.

Reagent	Volume per reaction (µl)
Distilled H_2O	8.5
10 x Green Dream Taq Buffer (Thermofisher)	1.5
10 mM dNTPs (Thermofisher)	0.3
Primer mix (individual primers from SIGMA) Forward primer: CAAAGCACATCTGACCACTCA (25 µl) Reverse primer: TATGCACACAGCCTCTGCTC (25 µl)	0.6
Betaine, 5 M (Sigma)	3
DreamTaq™ Hot start DNA Polymerase (Thermofisher)	0.1
DNA template	1

Table 2.2 PCR reaction mix for identification of *Peg3KO* expression.

Reagent	Volume per reaction (µl)
Distilled H_2O	8.5
10 x Green Dream Taq Buffer (Thermofisher)	1.5
10 mM dNTPs (Thermofisher)	0.3
Primer mix (individual primers from SIGMA) Forward primer: CGTTGGCTACCCGTGATATT (25 µl) Reverse primer: TATGCACACAGCCTCTGCTC (25 µl)	0.6
Betaine, 5 M (Sigma)	3
DreamTaq™ Hot start DNA Polymerase (Thermofisher)	0.1
DNA template	1

Table 2.3 PCR reaction mix for sex-typing PCR

Reagent	Volume per reaction (µl)
Distilled H_2O	11.5
10 x Green Dream Taq Buffer (Thermofisher)	1.5
10 mM dNTPs (Thermofisher)	0.3
Primer mix (individual primers from SIGMA) *Ssty* Forward primer: CTGGAGCTCTACAGTATGA *Ssty* Reverse primer: CAGTTACCAATCAACACATCAC *Om1a* Forward primer: TTACGTCCATCGTGGACAGCAT *Om1a* Reverse primer: TGGGCTGGGTGTTAGTCTTAT	0.6
DreamTaq™ Hot start DNA Polymerase (Thermofisher)	0.1
DNA template	1

2.3.3 Gel Electrophoresis

On completion of the PCR, 12.5 µl of each PCR product was loaded onto a 1% 1X Tris-acetate EDTA (TAE) agarose gel containing 0.5 µg/ml of SafeView (NBS Biologicals). 10 µl of a 100 bp ladder (PCR Ranger, Norgen) was run alongside the PCR products to determine the size of the product. The gel was run for 25 minutes at 150 V. The gel was then visualised under ultraviolet light using a transilluminator (BioRad).

For the identification of genotype, WT mice were determined by the presence of a WT band at 170 bp and no mutant band (Figure 2.3, sample 7). Mice heterozygous for the *Peg3* mutant allele were identified by the presence

of both a WT band at 170 bp and a mutant band at 695 bp or above (Figure 2.3, samples 1,2,3,6 and 8). Finally, mice homozygous for the *Peg3* mutant allele were identified by the presence of a mutant band at 695 bp or above, and the absence of a WT band (Figure 2.3, samples 4 and 5).

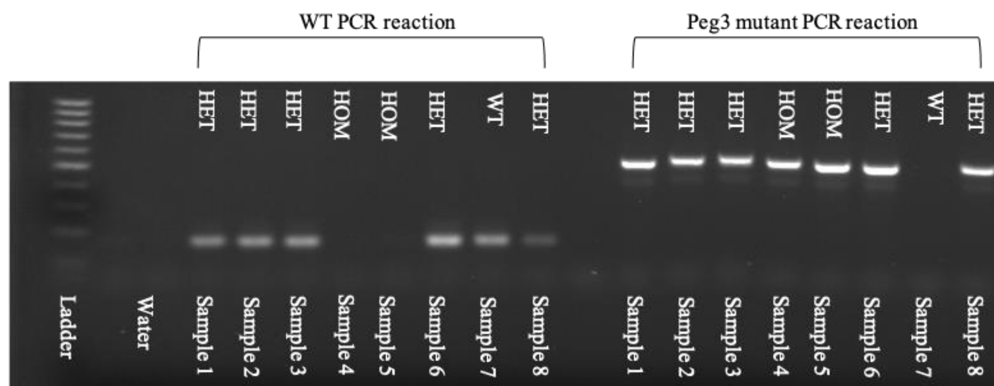


Figure 2.3 Examples of genotyping results. Examples of the bands needed to identify WT mice, and mice either heterozygous or homozygous for the mutant *Peg3* allele across the two PCRs are shown here. HET = heterozygous, HOM = homozygous

When determining the sex of mice, a single band at 245 bp (*Om1a)* was indicative of female sex and an additional band at 343 bp *(Ssty)* indicated male sex (Figure 2.4, sample 5 and samples 1,2,3,4,7 and 8 respectively).

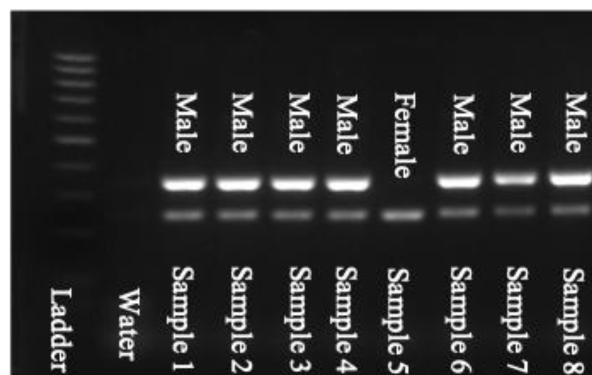


Figure 2.4 Examples of sex-typing results. Amplification of the autosomal control gene (*Om1a)* and Y-linked gene family *(Ssty)*. One band at 245 bp indicated female sex (sample 5) while an additional band at 343 bp indicated male sex.

2.4 Animal Behavioural Methods

All behavioural tests were carried out during the light phase of the light cycle between (08:00-17:00 h) in a dimly lit room (< 30 lux). For each of the tests, with the exception of pup retrieval and isolation induced USVs, the home cage was kept outside of the testing room at all times. All mice were handled either via open hand or tunnel technique to avoid undue stress. Figure 2.5 shows the order in which the tests were carried out for the behavioural cohorts. Details of recording and analysis software can be seen in section 2.4.3

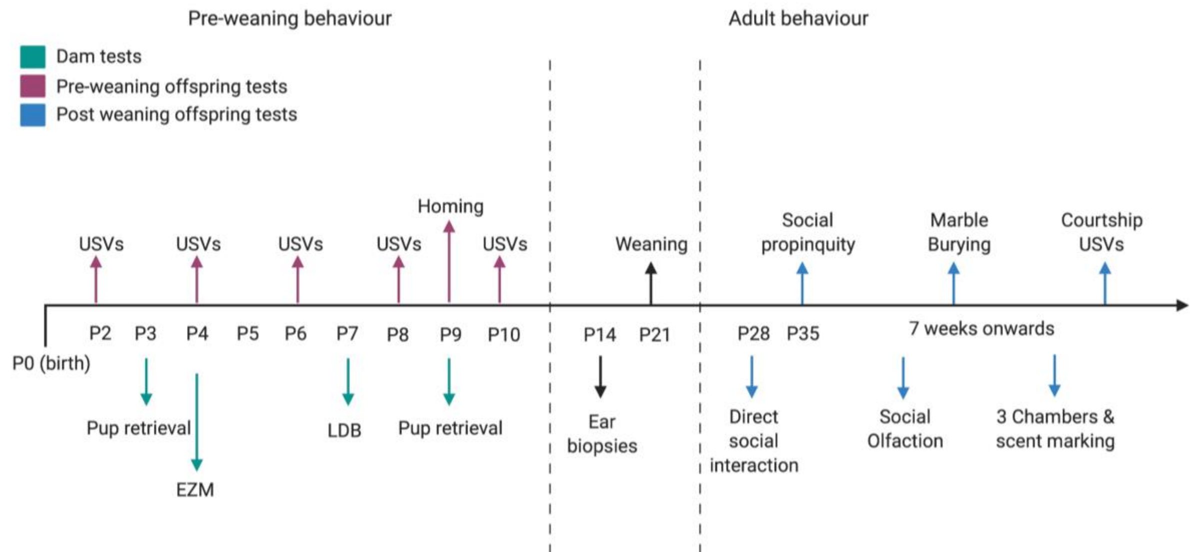


Figure 2.5 Timeline of dam and offspring behavioural tests. The order in which behavioural tests were carried out for all mice.

2.4.1 Maternal Tests

2.4.1.1 Elevated Zero Maze

Adapted from the elevated plus maze, the elevated zero maze (EZM) is a commonly employed method to assess anxiety-like behaviour in mice. The EZM (Figure 2.6) comprised of a 5 cm wide, white acrylic-covered wooden ring-shaped platform, elevated 50 cm from the floor. The ring-shaped platform was divided into four quadrants or arms, two 'open' unenclosed arms and two 'closed' arms, enclosed by opaque, 22 cm high walls.

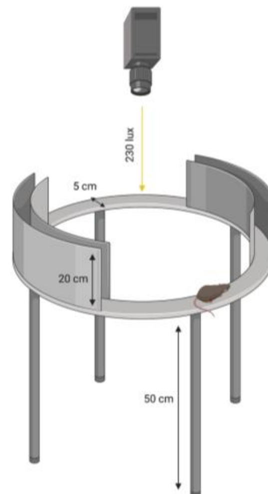


Figure 2.6 The Elevated Zero Maze. The EZM was used as one method of assessing maternal anxiety.

As an "approach-avoidance" task (Tucker and McCabe, 2017), the EZM relies on the conflict between two innate tendencies in mice, the tendency to explore novel areas vs the tendency to avoid potentially dangerous, aversive

areas (Cryan and Holmes, 2005). In the EZM, the aversive area takes the form of the two open arms of the ring-shaped platform.

Testing took place on P4, in a dimly lit room (230 lux on the open sections of the maze), with the maze directly positioned under a high-definition video recorder. At the beginning of a trial, the dam was placed in an enclosed section of the maze and allowed to freely explore the apparatus for 300 seconds. On completion of the trial, the dam was returned to the home cage, and the maze cleaned with 70% ethanol solution before the next trial took place.

Parameters analysed included time spent in the open and closed arms, latency to enter the open arms, number of crosses between arms and the number of stretch-attend postures, a well-established indicator of anxiety (Lister, 1990, Gilhotra et al., 2015)

2.4.1.2 Light Dark Box

Adapted from (Bourin and Hascoët, 2003), the light-dark box (LDB) test is a well-validated measure of anxiety in rodents (Miller et al., 2011) and is a further example of an "approach-avoidance" task (Tucker and McCabe, 2017). The LDB (Figure 2.7) was comprised of two adjoined acrylic chambers, one white (30x30x30 cm) and one black (15x30x30 cm), separated by a dividing wall with a removable door. A bright lamp (300 lux) was shone directly into the white (anxiogenic) chamber, avoiding the casting of shadows within the white chamber, and ensuring the black chamber was not illuminated.

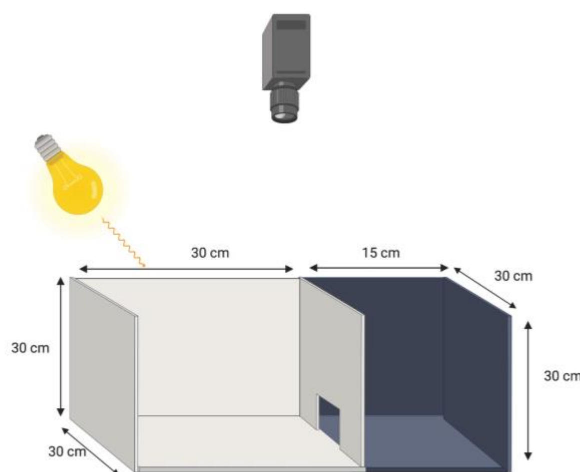


Figure 2.7 Cross-section of the Light-dark box. The LDB was used as one method of assessing maternal anxiety.

Testing took place on P7 under the same conditions as the EZM. At the beginning of each trial the dam was placed in the dark section of the LDB, the door was then removed, and the dam allowed to freely explore each section of the box for 300 seconds. On completion of the trial, the dam was returned to the home cage, and the box cleaned with 70% ethanol solution before the next trial took place.

Parameters analysed included time spent in each chamber, latency to enter the anxiogenic chamber, number of crosses between chambers and frequency of stretch-attend postures.

2.4.1.3 Pup retrieval

Pup retrieval is a commonly used method to assess maternal care in rodents (Weber and Olsson, 2008). Maternal care, including pup retrieval, can be elicited by the ultrasonic vocalisations (USVs) of mouse pups, with the responsiveness of the dam having been previously linked to the number of USVs produced by her pups (D'Amato et al., 2005).

Testing took place between 08:00 and 12:00 h on P3 and P9. Prior to the beginning of each trial both the dam and her litter were removed from the home cage. The dam was placed in a clean, empty cage whilst the litter was placed into a small, lined container on a pre-warmed heat mat to prevent a decrease in body temperature as this is known to affect USV emission (Hennessy et al., 1980). The home cage was then placed into a clear sound attenuating chamber within the testing room. To begin the trial, dams were placed into a sound-attenuating Styrofoam chamber (Figure 2.8A), where USVs were recorded for 180 s using an Avisoft UltraSoundGate 116Hb microphone and accompanying software (Avisoft Bioacoustics e.K., Germany, see section 2.4.3.2). Following this, the dam was returned to the empty cage outside of the testing room.

To record pup USVs, four pups, where possible two males and two females (determined by anogenital distance) were placed equidistant at the opposite end of the cage to the nest, directly under a microphone. USVs from the four pups were simultaneously recorded for 180 s as above. After recording, pups were re-spaced equidistantly and the dam reintroduced to the home cage, indicating time zero (Figure 2.8B). Time taken to sniff and retrieve each pup was recorded as was the latency to perform these behaviours. A successful retrieval was defined as the pup being placed well within the nest. The trial ended when all pups had been successfully retrieved and the dam was crouched over the nest, or after 900 s. Any pups that had not been successfully retrieved were given the maximum score of 900 s. On completion of the trial, the rest of the litter was returned to the home cage, and both the Styrofoam and Perspex chamber were cleaned with 70% ethanol.

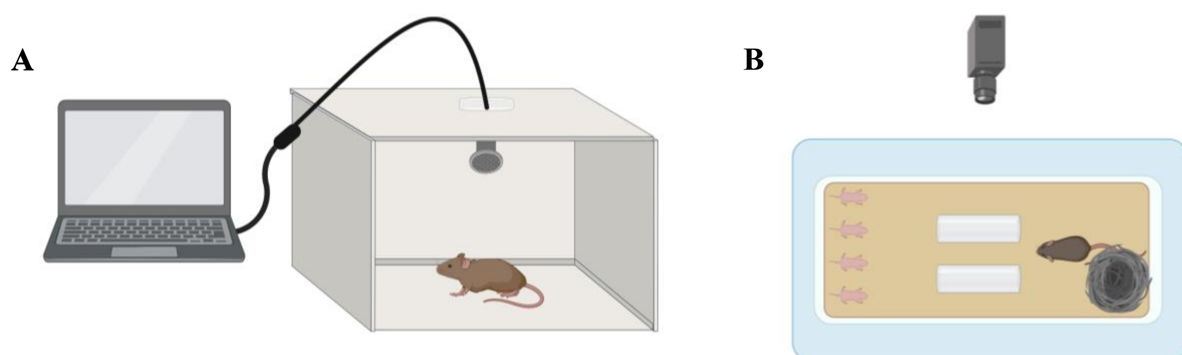


Figure 2.8 Dam USV set up and Pup Retrieval Task. A) Dams were placed in a sound attenuating Styrofoam chamber and USVs were recorded for 180 s. **B)** Following recording, the dam was placed into the home cage and her maternal behaviours recorded.

2.4.2 Offspring Tests

As in previous work (Chourbaji et al., 2011), other aspects of dam behaviour (detailed in Table 2.4), alongside basic timings of pup sniffing, and retrieval were recorded. These behaviours were then assigned as either 'pup-directed' or 'non-directed' and summed to form composite variables as shown in Table 2.4. Composite variables were calculated by summing the time spent engaged in each behaviour making up the composite variable.

Table 2.4 Observed dam behaviour during the pup retrieval task.

Behaviours	Definition	Composite variable category
Exploring	Exploring the home cage, not engaging with pups or the nest, e.g., digging, running around	Non-pup directed
In nest	In the nest but without pups, or can be seen not to be engaged in pup-directed behaviour whilst in the nest	Non-pup directed
Sniff pup	Sniffing, or nose contact with the pup	Pup directed
Retrieval attempt	The dam attempts to move the pup toward the nest, actively picking up the pup in her mouth, or handling the pup with her forepaws whilst moving toward the nest	Pup directed
Groom pup	Dam touches the pup's body with her tongue, or the dam handles the pup's body with her forepaws or nose	Pup directed
Crouching	Dam is in the nest and crouched over pups with little to no movement. If partially obscured by the nest, dam is only assumed to be crouching if pups are present in the nest and there is little movement	Pup directed
Nest build	Collecting and / or handling nesting material with mouth or forepaws	Pup directed
Self-groom	Self-grooming whiskers or body	Non-pup directed
Freeze	Not moving, stationary, whilst not engaging with pups or nest	Non-pup directed

2.4.2.1 Ultrasonic Vocalisations

In addition to group vocalisations during pup-retrieval, USVs were recorded at P2, P4, P6, P8 and P10 individually for each pup in a litter. On each day of testing, each pup was marked with a coloured permanent marker for later identification and was individually placed into an empty plastic petri dish inside a sound-attenuating Styrofoam box. USVs were recorded with an ultrasonic microphone sensitive to frequencies between 10-180 kHz, positioned approximately 25 cm above. Prior to recording, each pup's body temperature was measured using an infra-red thermometer (Fisher-Scientific). After recording, the body temperature was remeasured, the pup was weighed and then was returned to the home cage. Between each pup, the chamber was cleaned using 70% ethanol and then dried.

Parameters analysed included the frequency, mean duration and latency of calls. Sonograms generated from each recording were used to manually identify calls and remove background noise based on previously validated waveform categorisations (Panksepp et al., 2007, Scattoni et al., 2009, Scattoni et al., 2011). Full information on recording equipment, experimental parameters and settings used for acoustic analysis can be seen in Section 2.4.3.2.

2.4.2.2 Homing behaviour

Adapted from (Fiori et al., 2017), homing behaviour is used as a measure of pup olfaction ability by assessing their preference for maternal odour. Testing apparatus comprised of an empty clear container (28x18x18 cm), positioned directly under a high-definition video recorder. The container was divided into three distinct zones; i) the 'home' zone, containing a small petri dish filled with sawdust and bedding from the home cage, ii) the 'clean' zone, containing an identical dish filled with clean bedding and sawdust and iii) an empty 'central' section (Figure 2.9) A paper template placed under the container, featured outlines upon which the dishes were placed to maintain consistency across all trials.

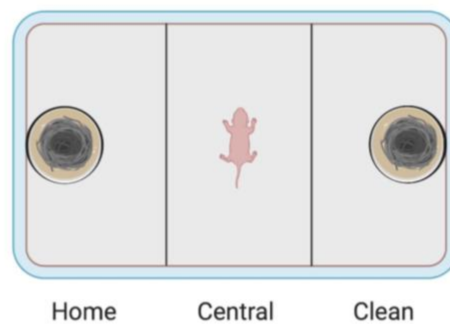


Figure 2.9 The Homing Behaviour Test. The homing behaviour test was used to assess pup olfaction ability and preference for maternal odour.

Testing took place on P9 between 13:00-16:00 h. To begin the trial, a pup was removed from the home cage and placed in the central section of the arena facing away from both the 'home' and 'clean' zones. Pups were then allowed to freely roam the arena for 180 seconds after which the pup was removed to a clean cage warmed by a heat-mat. Prior to beginning the next trial, both sets of bedding and sawdust were replaced, and the arena cleaned with 70% ethanol. Once all pups in the litter had completed the test, the litter was returned to the home cage.

Previous literature has used latency to enter each section, and time spent in each section as an outcome measure of homing ability (Fiori et al., 2017). However, in order to attribute the time spent in each section due to 'homing' as opposed to chance movement, an adaption of time spent within 1 cm of the dishes in each section, and the latency to reach the dish were used in this study.

2.4.2.3 Direct Social Interaction

The direct social interaction test (DSI) was used to measure the tendency of mice to interact with a female mouse of a different strain. Testing took place at four weeks old (P28) before the subjects had reached sexual maturity. Direct social interaction behaviour was observed within an empty Phenotyper cube (30x30x40 cm, Noldus, UK) the sides of which had been obscured with dark-coloured paper to prevent mice from seeing into other cubes.

Testing began when an adult female CD1 'host' mouse was introduced to the box, followed by the test mouse approximately 1 minute later. Their interactions were recorded for 180 s using a camera mounted into the lid of the Phenotyper cube.

The behaviours observed were recorded by the researcher and are comparable to previous works (Yang et al., 2013, Harrison et al., 2020). Table 2.5 shows each observed behaviour and their definitions adapted from Yang et al. (2013). Composite variables subsequently used for analysis were then calculated by summing each of the behaviours included within that variable (Table 2.6).

Table 2.5 Observed behaviours and their definitions during the Direct Social Interaction Test

Behaviours	Definition
Nose-nose sniffing	Sniffing, or nose contact with the host's nose, or around the head or neck
Ano-genital sniffing	Sniffing, or nose contact with the host's ano-genital area
Body sniffing	Sniffing, or nose contact with the host's trunk or limbs
Exploring	Exploring the arena, not engaging with the host mouse, e.g., digging, running around the arena
Following	Walking at the same speed behind the host mouse
Being Followed	The host mouse walking at the same speed behind the test mouse
Grooming	Self-grooming whiskers or body
Immobile	Not moving, stationary whilst not engaging with the host mouse
Being Attacked	Being bitten by the host mouse
Mounting	The test mouse mounts the host mouse

Table 2.6 Composite variables and their component variables used for analysis in the DSI Test

Social Interaction	Non-social behaviour
Nose-to-nose sniffing	Grooming self
Ano-genital sniffing	Immobile
Body sniffing	
Following	
Being followed	
Being attacked	
Mounting	

*As in Harrison et al. (2020) exploring was not included in either social or non-social behaviour.

2.4.2.4 Social Propinquity

Adapted from Tuttle et al., (2017) the social propinquity test is a sociability test in rodents, specifically exploring the tendency for mice to maintain close physical proximity with conspecifics. Three clear Phenotyper cages were placed on a large clear plastic box and illuminated from below to create an aversive arena space. Each cage contained one cardboard tube secured to a wall of the arena which were covered with dark paper to prevent the mice seeing into neighbouring cages on the platform (Figure 2.10).

Testing began when pairs of mice were placed into the arena at the same time. Mice were matched for sex, age, and genotype, and neither cage-mates nor littermates were paired together. Once all three pairs of mice had entered the arenas, they were freely allowed to explore and interact for 1 hour. All trials were recorded by a video recorder mounted in the lid of the Phenotyper cages for later offline analysis. As in Harrison et al. (2020), parameters analysed included latency to first entry and first cohabitation of the cardboard tube. Further, the video recordings were paused at 5-minute intervals and the number of mice occupying the tube was recorded. From this, the approximate percentage of total trial time of the tube spent vacant, singly occupied, or double occupied was recorded. Greater time spent sharing the tube was indicative of greater anxiety-like behaviour (Harrison et al., 2020).

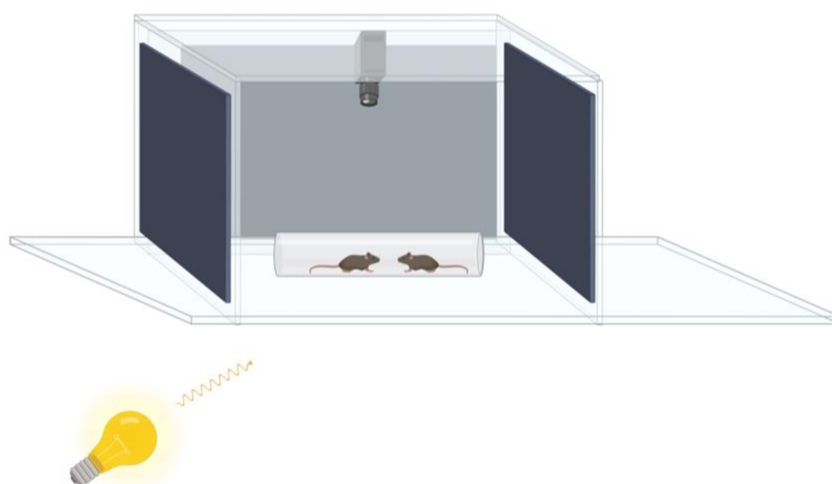


Figure 2.10 The Social Propinquity Arena. The basic set-up of the Social Propinquity test

2.4.2.5 Social Olfaction Test

The social olfaction test is a commonly used test used to assess rodents' ability to detect and discriminate between different odours, in this instance, social odours (Zou et al., 2015). Adapted from Arbuckle et al. (2015), the social olfaction test consists of sequential presentations of different social odours using cotton swabs (Figure 2.11A).

The social odours used in this paradigm were urine samples obtained from the home cages of mice of the opposite sex and a different strain than the test subject mice. To ensure sufficient odour, the cage used for stimuli contained four mice, and had not been cleaned for a minimum of 3 days prior to the day of testing. Cotton buds cut to a

length of 1 cm were used to swab around each of these home cages in a zig-zag fashion and then sealed into two falcon tubes.

For this test, four Phenotyper cages were used as the testing arena. To ensure the arena remained novel after prior behavioural tests conducted in the Phenotyper, light paper was used to obscure the walls of the arena and the floor was covered in clean sawdust. The bottom of a 2.5 cm diameter plastic petri dish (Fisher Scientific, UK) was fixed toward the centre of the arena, within arm's reach of a sliding door at the front of each arena. The lid of the petri dish, with a hole drilled in the middle was then fixed inside the lower portion of the petri-dish inside the arena (see Figure 2.11B).

To begin each trial, subject mice were placed into the arena through the sliding door and allowed to habituate for 5 minutes. As can be seen in Figure 2.11A, the test comprised of 3 trials in which odours are presented sequentially, (water, social odour from cage one, and social odour from cage two), with three different presentations of each odour for two minutes. After habituation, the video recording was started, and the first odour was presented by fixing the cotton swab to the hole in the lid of the petri dish in each arena. At the end of the 2 minutes the recording was stopped, and the odour removed. After an interval of 1 minute the next swab was inserted. On completion of all the trials, mice were returned to the home cage and each arena cleaned with 70% ethanol and lined with fresh sawdust. Ethovision XT version 13 software (Noldus, Tracksys Ltd, UK) was used to detect when the subject's nose was within the perimeter of the odour petri dish and to calculate the latency to first approach, total time spent sniffing, and total number of visits to each odour.

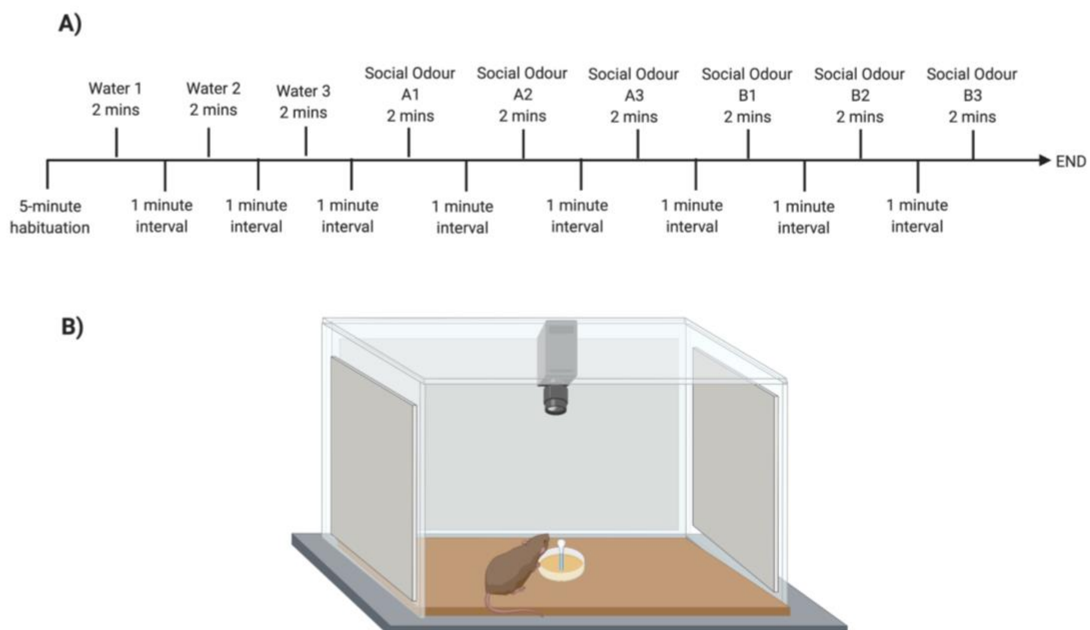


Figure 2.11 Social Olfaction Test. A) The sequential order of stimulus presentation across each trial. **B)** The test arena for the inside the Phenotyper cages.

2.4.2.6 Three Chambers & Scent Marking

The three-chamber paradigm is a commonly used method of assessing sociability in mice (Moy et al., 2004). This thesis features a design and protocol adapted from (Yang et al., 2011) where a large black acrylic box was divided into three equal sized chambers (15x30x30 cm). Each chamber was connected by a 5 mm gap in which removable doors prevented entry between each chamber (Figure 2.12). Whatman chromatography paper (3mm, GE Healthcare Whatman^{TM}, Fisher-Scientific) cut to 14x29 cm was used to line the floor of each chamber to be used in scent marking analysis and to improve contrast between the mouse and the chamber for later automated video analysis.

Prior to beginning the trial, a 'host' CD1 mouse of the opposite sex to the test subject was placed underneath an upturned wire mesh pen holder (10x9 cm, LAAT, China) at the centre of one of the end chambers. An identical pen holder was placed at the other end chamber, and each holder was weighed down with an unmarked tin can, the tops of which were labelled for identification during later video analysis. The chamber in which the host mouse was placed was counter-balanced across trials.

To begin each trial, the test mouse was placed into the middle chamber facing away from the closed doors on either side. The doors were then simultaneously removed, and the test mouse allowed to explore the arena for 180 seconds. On completion of the trial, each mouse was removed, and the chambers cleaned with 70% ethanol.

Parameters analysed included the total length of time spent in each chamber, number of entries and latency to enter each chamber.

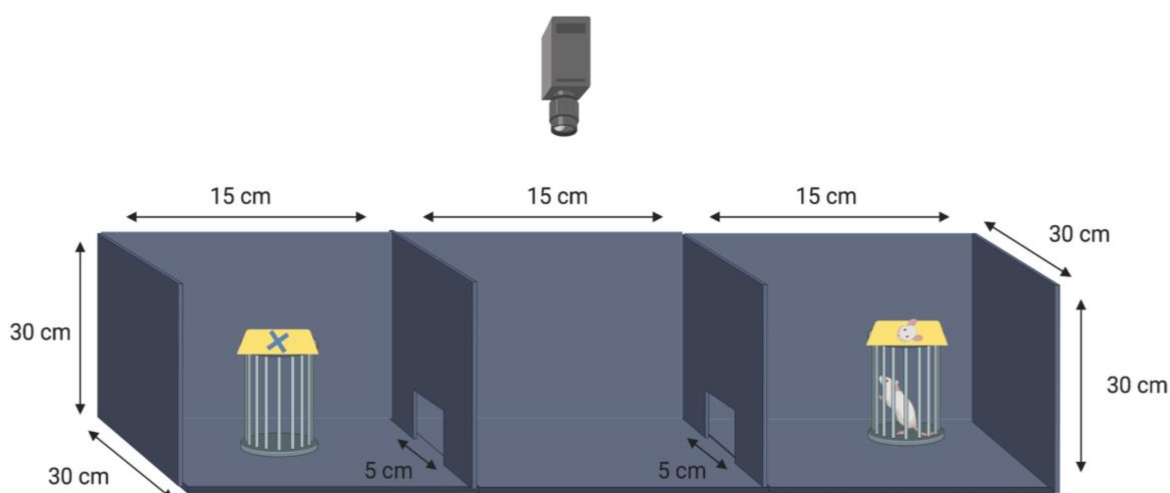


Figure 2.12 The Three Chambers Arena. The arena set-up for the three-chamber paradigm, shown here without paper used for scent marking. Each chamber is labelled for post-test video analysis.

For scent marking, on completion of the trial, marked paper was removed and allowed to dry. Once dry, the outline of any scent marking was traced with a pen under an ultraviolet light (Bio-rad industries). Scent marking was then

scored using a 1 cm^2 overlay and the total number of squares containing a scent mark were counted in each section. Using the work of Rienecker et al. (2020) as a guide, marks covering more than 4 consecutive squares were not included, as this was indicative of urine rather than scent marking. A higher score was indicative of greater scent marking and representative of increased territorial behaviour or social dominance.

2.4.2.7 Marble Burying

Marble burying has been used as a measure of assessing repetitive behaviour and anxiety-like behaviour in mice (Gavioli et al., 2007, Kedia and Chattarji, 2014). Testing apparatus consisted of 8 large clear containers (28x18x 18 cm) arranged in a 2x4 grid, with a high-definition video recorder placed directly above. Each container was filled to a depth of approximately 5 cm with clean sawdust that was evenly distributed to create a flat surface. Twenty identical glass marbles were spaced evenly in a 4x5 grid on the sawdust in each container (Figure 2.13)

Each testing phase began by placing each mouse into an arena ensuring that the mouse was not touching any marbles. A clear lid was then placed on each container, and the mice allowed to freely roam the arena for 30 minutes. At the end of the test, mice were removed from the containers (in the same order in which they were placed) and returned to the home cage. A photo was then taken of the arena for offline scoring. Each test was also recorded for later analysis offline. For each mouse, number of marbles buried up to 60% of their depth was counted. Mice were run through this test twice, with a gap of at least 3 days between each trial. The mean number of marbles buried to a depth of 66% across the two trials was used as the final score for this test.

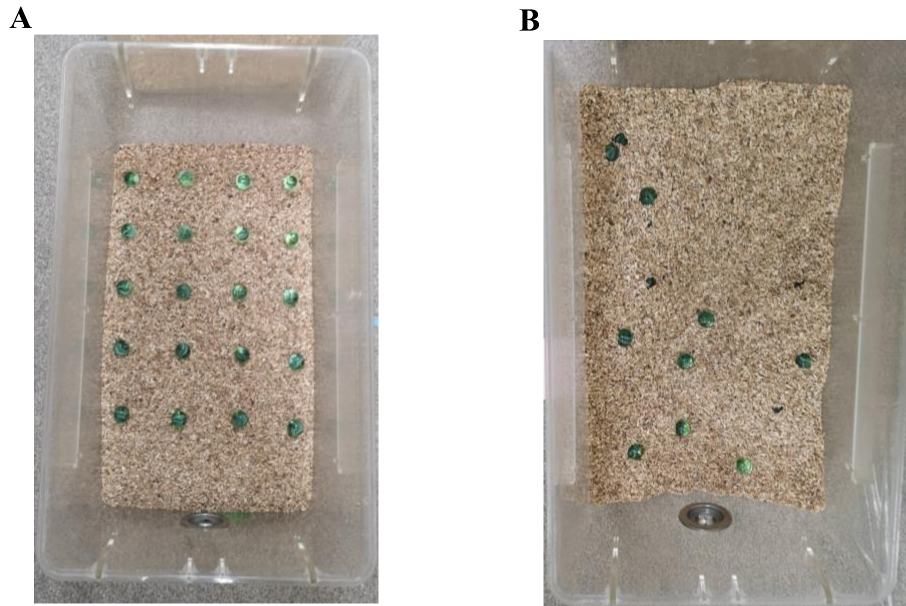


Figure 2.13 Example of the Marble Burying Arena. A) A photograph of a container used for marble burying Twenty glass marbles were arranged in a 5 x 4 grid at the beginning of the experiment. **B**) An example container on completion of the experiment, marbles buried up to 66% of their depth were counted for analysis.

2.4.2.8 Courtship behaviour and USVs

Mice elicit USVs in a variety of social contexts from birth to adulthood. Once sexual maturity has been reached, male mice will emit a number of USVs when exposed to females, a behaviour thought to facilitate courtship behaviours and successful mating (Chabout et al., 2017).

Courtship behaviours and USVs were measured using the three-phase male-female social interaction test adapted from Yang et al. (2013). As in pup retrieval tests (section 2.1.4.3), testing took place inside a large sound-attenuating environmental chamber with a high-definition video recorder placed directly above. An ultrasonic microphone was positioned roughly 15 cm above the testing arena to record USVs.

All female stimuli mice were genetically WT, group housed in cages of 4 and sexually naïve at the time of testing. Only females considered to be in oestrous were used as stimulus mice. Female mice were assessed for oestrous phase on each experimental day by vaginal swabs using a protocol adapted from McLean et al. (2012). Vaginal swabs were collected from each female in the cage using a tapered cotton tipped swab (Superdrug, UK) soaked with room temperature saline solution. The swab was gently rolled and turned 360 ° against the vaginal wall and then removed. Cells were then transferred to a dry glass slide by gently rolling the swab across the slide. Each slide was then air dried, transferred to a glass coplin staining jar and stained with approximately 400 µL of Creysl violet (0.1 g in 100 µL, SIGMA, UK) for 1 minute. Slides were then transferred to a second coplin jar containing distilled H_2O and rinsed for approximately 1 minute. Excess water was removed from the edges of the slides by blotting with tissue paper. Slides were then immediately viewed at 10 x magnification under bright field illumination, if cells were not visible, magnification was increased progressively up to 40 x magnification. The stage of oestrous cycle was then determined based on the presence or absence of cornified squamous epithelial cells, leukocytes, and / or nucleated epithelial cells using. Using Figure 2.14 as a guide, mice were determined to be in oestrous based upon visible inspection of each cytology slide, where the cells present predominantly consisted of cornified squamous epithelial cells.

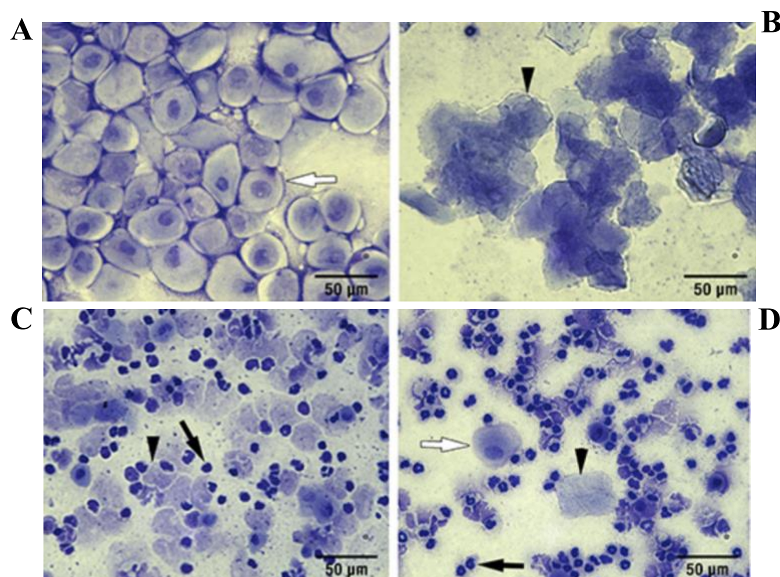


Figure 2.14 Cytology representing each stage of the oestrous cycle in mice A) Proestrus, **B)** Oestrus **C)** Metestrus, **D)** Diestrus. The black arrowheads in B,C, and G point to the presence of cornified squamous epithelial cells. *Taken from McLean et al. (2012)*

Phase one of the test began when a male mouse was removed from the home cage and placed into a clean cage covered with a thin layer of sawdust within the sound attenuating chamber. USVs were then recorded for 1 minute. This phase was not included in the original protocol designed by Yang et al., (2013) but was included in this project to obtain a baseline of male ultrasonic vocalisations prior to being exposed to a female. At the end of phase 1, a female mouse in oestrous was introduced to the cage at the opposite end to the male, USVs and social interactions were recorded for 5 minutes with the experimenter outside of the testing room. Following phase two, the female mouse was then removed and placed into a clean, standard cage outside of the testing room. USVs from the male were then recorded in isolation for a further 3 minutes (phase 3). The final phase began when the female mouse was reintroduced to the testing cage and USVs and interactions were both recorded for a further 3 minutes. On completion of the test, both mice were returned to their respective home cages, the arena cleaned with 70% ethanol and a fresh cage placed into the chamber.

For each test day, male mice that were housed together were all tested on the same day, and each female subject mouse interacted with no more than two male subjects per day, with an interval of at least 60 minutes between tests. Trials were counterbalanced across each day so that males of the same genotype were not consistently exposed to the same females.

2.4.3 Recording and offline analysis

Direct social interaction, social propinquity and social olfaction tests were all carried out in Phenotyper observation cages (30x30x30 cm) with cameras mounted in the lids (Noldus, UK). Tests that were not performed in the Phenotyper cages were recorded by a high-definition video recorder, these included the EZM, LDB, pup retrieval, three-chambers, marble burying and courtship behaviours.

The EZM, LDB and three-chamber tests were all scored offline using Ethovision software (Version XT, Noldus, Netherlands). Pup-retrieval, direct social interaction and behaviour carried out during courtship USVs were manually scored using Behavioural Observation Research Interactive Software (BORIS, (Friard and Gamba, 2016). Finally, marble burying and scent marking were scored by eye by the researcher who was blind to the genotyping of the mice at time of scoring. To check for potential bias, each trial for marble burying, and 20% of both pup-retrieval and scent-marking were also scored by a second researcher.

2.4.3.1 USV Recordings

All vocalisations were recorded using Avisoft Recorder USGH software and an ultrasonic microphone (Avisoft UltraSoundGate 116Hb, Avisoft Bioacoustics, Berlin, Germany) in real time for 3 minutes (isolation induced USVs) or 5 minutes (courtship USVs). As in previous research, recording settings included a sampling rate of 250 kHz; format 16 bit, and a high pass filter set to 15 kHz in order to reduce background noise (McNamara et al., 2018a).

Raven Pro 1.6 software (Bioacoustics Research Program, Cornell Lab of Ornithology, Ithaca, NY) was then used to generate spectrograms with a 256-sample Hann window, 1 frame per second, from which background noise was identified and USVs were manually scored (Figure 2.15)

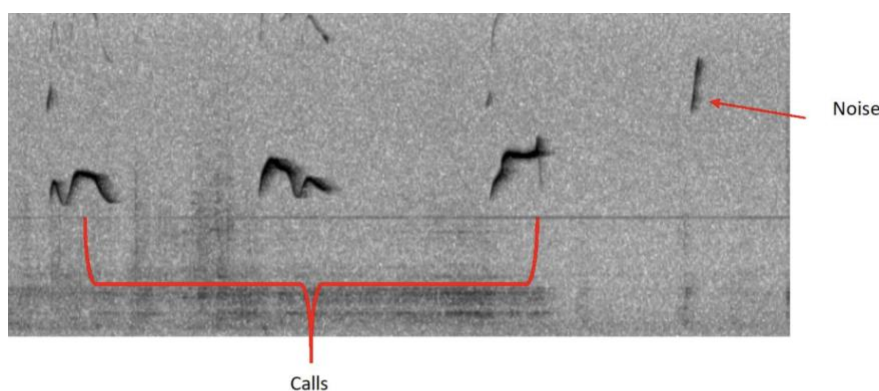


Figure 2.15 Spectrogram from Raven Pro software. A screenshot of a spectrogram showing USVs and background noise picked up in error.

2.5 RNA Sequencing

RNA sequencing was carried out on the hypothalamus and olfactory bulb of P6 mouse pups from the experimental cohort described in section 2.2.1 (Figure 2.16). The following procedure is applicable to both the olfactory bulb and hypothalamic tissues.

2.5.1 Tissue generation

Prior to dissection, pups were sex-typed visually using ano-genital distance. Sex was later confirmed by sex-typing of tail snips (see section 2.16). At P6, pups were culled via the schedule 1 method of decapitation and brains dissected into RNA*later* (Thermofisher, UK). Micro dissected olfactory bulb and hypothalamus tissue were immediately frozen on dry ice in RNAse-free tubes and stored at -80°C until RNA extraction.

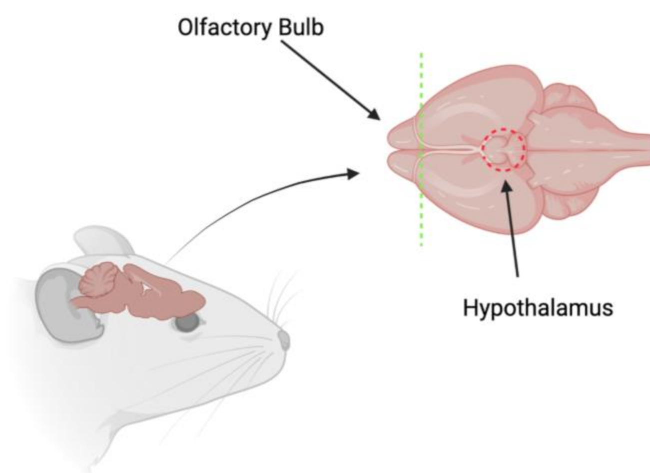


Figure 2.16 Areas of the brain dissected for RNA Sequencing analysis. Mouse brains were dissected out at P6. The olfactory bulb, (demarcated by the green dashed lined) and the hypothalamus (the red dashed line) were then dissected out and frozen at -80°C for later RNA extraction.

2.5.2 Preparation of RNA

RNA was extracted using a GenElute™ Mammalian Total RNA Miniprep Kit (Sigma-Aldrich UK). Reagent volumes were adjusted to reflect the small size of tissue samples as recommended in the kit's original protocol . Tissues were homogenised in 100 µl of Lysis Buffer containing 1 µl 2-Mercaptoethanol using RNA-free pestles (Fisherbrand™, Fisherscientific UK) in a 1.5 ml sterile non-stick microcentrifuge tube (Alpha laboratories UK). The lysate was then pipette mixed and transferred into the GenElute filtration column and centrifuged at 4 °C for 2 minutes at 13,000 rpm (9,069 gs). Following this, 100 µl 70% EtOH was added to the filtered lysate and pipette-mixed to homogenise the suspension. This was transferred to the GenElute DNA binding column, before being centrifuged for 30 seconds at 4°C. Flow-through was discarded and 250 µl of wash solution I (included in the GenElute™ Mammalian Total RNA Miniprep Kit) added to each sample, before being centrifuged for 30 seconds at 4°C and the flow-through discarded.

DNAase for on column digestion was made using a digestion kit (DNASE70-1SET, Sigma-Aldrich UK) containing 700 µl of DNAse digest buffer and 100 µl of deoxynucleuoribose. 80 µl of this master mix was then added to each column before incubating at room temperature for 15 minutes.

Following incubation, 250 µl of wash solution 1 was added to each column, before centrifuging at 4°C for 2 minutes at 13,000 rpm. Flow-through was discarded and 500 µl of wash solution II added (included in the GenElute™ Mammalian Total RNA Miniprep Kit) before being centrifuged as before. This step was repeated twice. Following this, the binding column was transferred into a new collection tube and again centrifuged as before. Finally, each column was transferred into a clean collection tube and 30 µl of elution solution was pipetted onto the column. Samples were centrifuged for 1 minute at 4°C before the final product was transferred to sterile RNAse-free microtubes. Concentration and purity of the RNA was confirmed using a Nanodrop One (ThermoFisher). A A260/A280 ratio of $\geq$ 1.8 and a A260/230 ratio of $\geq$ 2.0 was considered acceptable. Extracted RNA was then frozen at -80°C prior to being sent to the company Genewiz Ltd (Genomics UK, CM22, 6TA) for independent quality checks, library preparation and sequencing.

2.5.3 Library Preparation and Sequencing

Quality checks, library preparation, and sequencing were performed by Genewiz Ltd as follows: RNA samples were quantified using Qubit 4.0 Fluorometer (Life Technologies, Carlsbad, CA, USA) and RNA integrity was checked with RNA Kit on Agilent 5600 Fragment Analyzer (Agilent Technologies, Palo Alto, CA, USA). RNA sequencing library preparation was made using NEBNext Ultra II RNA Library Prep Kit for Illumina following manufacturer's instructions (NEB, Ipswich, MA, USA). Briefly, mRNAs were first enriched with Oligo(dT) beads. Enriched mRNAs were fragmented. First strand and second strand cDNA were subsequently synthesized. The second strand of cDNA was marked by incorporating dUTP during the synthesis. cDNA fragments were adenylated at 3'ends, and indexed adapter was ligated to cDNA fragments. Limited cycle PCR was used for library amplification. The dUTP incorporated into the cDNA of the second strand enabled its specific degradation to maintain strand specificity.

Sequencing libraries were validated using DNA Kit on the Agilent 5600 Fragment Analyzer (Agilent Technologies, Palo Alto, CA, USA), and quantified by using Qubit 4.0 Fluorometer (Invitrogen, Carlsbad, CA). The sequencing libraries were multiplexed and clustered on the flowcell. After clustering, the flowcell was loaded on the Illumina NovaSeq 6000 instrument according to manufacturer's instructions. The samples were sequenced using a 2x150 Pair-End (PE) configuration. Image analysis and base calling were conducted by the NovaSeq Control Software v1.6 on the NovaSeq instrument. Raw sequence data (.bcl files) generated from Illumina NovaSeq were converted into fastQC files and de-multiplexed using Illumina bcl2fastq program version 2.20. One mismatch was allowed for index sequence identification. The fastQC files were then sent to the author for analysis, the pipeline for which is discussed in Chapter 4.

2.6 The Grown in Wales Cohort

Funded by the Medical Research Council, the Grown in Wales (GiW) study was established in 2015 to explore the relationship between prenatal mental health, placental function, and infant outcomes (Janssen et al., 2018).

2.6.1 Recruitment

Between September 1st, 2015, and November 31st, 2016, 355 women were recruited by two trained research midwives at the University Hospital of Wales (UHW) at a presurgical appointment prior to a booked elective caesarean section (ELCS). ELCS was chosen as an inclusion criterion both to maximise the collection of placentas, and to prevent alterations in gene expression as a result of the process of labour (Janssen et al., 2015). Further inclusion criteria were a singleton term pregnancy, absence of fetal anomalies, infections, or diseases and if the mother was aged between 18 – 45. To date, the GiW cohort has been postnatally followed up at four time points; within one week after birth (P1), ten weeks after birth (P2), 12 months postnatally (Y1) and 4 years postnatally (Y4), currently being collected at time of writing (Figure 2.17). For the purpose of this study, only data at recruitment (A1) and the 12-month postnatal follow up (Y1) was used for analysis in Chapter 5, and so data from P1, P2 and Y4 will not be discussed further.

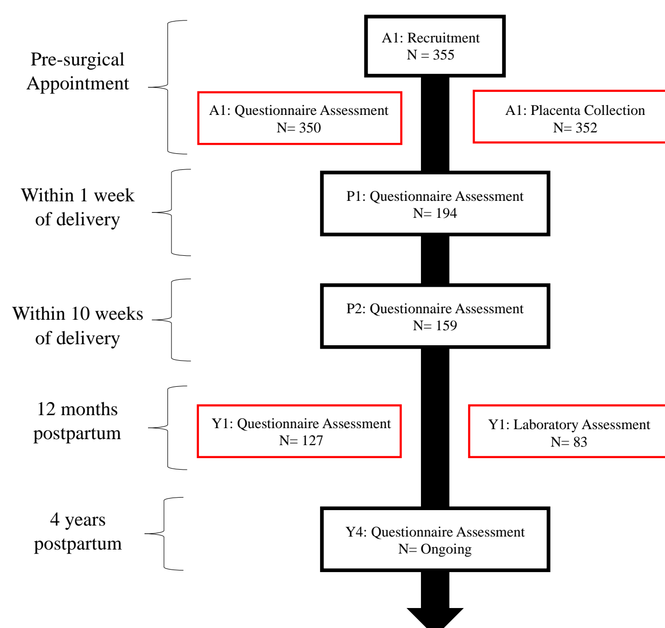


Figure 2.17 The Grown in Wales cohort study design. Summary of recruitment and follow-up timepoints

2.6.2 Grown in Wales Data Collection

The full scope of data collected for the GiW study is described extensively in previous works (Janssen et al., 2018, Savory et al., 2020, Sumption, 2020). Briefly, at the presurgical appointment (A1) where participants were recruited, extensive self-report questionnaires were completed by the mother, and through medical notes were recorded by the research midwife immediately after delivery. Further self-report questionnaires were completed at P1, P2, Y1 and Y4. In addition, at Y1 participants were invited to attend an assessment with their infant in a laboratory session. Table 2.7 shows a list of the measures collected at each time point. As mentioned previously, only data collection pertaining to the analysis presented in Chapter 5 will be discussed in more detail below.

Table 2.7 Measures collected at each time point of the GiW Study

A1 (Pre-surgical appointment)	**P1 (One-week post-delivery)**	**P2 (10 weeks post-delivery)**	**Y1 (12 months postpartum)**	**Y4 (4-year follow-up)**
Questionnaire: Demographics, Lifestyle, Mental health (EPDS & STAI) **Midwife notes** **Biological samples*** Maternal Saliva, Maternal blood serum, Placenta biopsies, Cord blood serum, **Generated from biological samples:* Cortisol, Fatty acids, *Cytokines,* *BDNF,* *Human placental lactogen,* *Placental gene expression*	**Questionnaire:** Mental health (EPDS & STAI)	**Questionnaire:** Mental health (EPDS & STAI)	**Questionnaire:** Demographics, Mental health (EPDS & STAI) CICS, Postnatal Bonding Questionnaire, Infant Behavioural Questionnaire **Lab Assessments** Bayley's scale of Infant Development (III), Lab-TAB components	**Questionnaire:** Demographics, Lifestyle, Mental health (EPDS & STAI), Children's Behaviour Questionnaire, Child Behaviour Checklist, Child-parent Relationship Scale

Abbreviations used: *EPDS:* Edinburgh Postnatal Depression Scale; *STAI:* State-Trait Anxiety Inventory; *BDNF:* Brain derived neurotrophic factor; CICS; Cardiff Infant Contentiousness Scale

2.6.2.1 Participant Questionnaire

At the presurgical appointment, participants were asked to complete an extensive questionnaire split into three sections: demographics, current mental health, and lifestyle, however as the two former sections were the only relevant sections to this thesis, only these will be discussed further. Relevant demographic information for this thesis included maternal age, education, number of previous children and income. Current mental health was measured using two well validated self-report questionnaires designed to assess depression and anxiety symptoms in the general population.

2.6.2.1.1 Edinburgh Postnatal Depression Scale

Developed in 1987 by Cox et al, the Edinburgh Postnatal Depression Scale (EPDS) is the most widely used and one of the most well-validated screening measures for postnatal depression (Boyd et al., 2005). Despite its initial design specifically for detection of postnatal depression, the EPDS has since been validated for use in pregnancy, for mothers beyond the postnatal period and for the antenatal period, although evidence for the latter is limited to

three studies (Cox et al., 1987, Murray and Cox, 1990, Cox et al., 1996, Gibson et al., 2009). The EPDS contains 10 items, which pertain to the respondents' feelings over the last 7 days, for example "*I have looked forward with enjoyment to things'* and "*I have felt sad or miserable"*. Each item is rated on a 4-point Likert scale ranging from 0-3, with a higher score indicative of greater levels of depressive symptoms, with a total possible score of 30 (Cox et al., 1987). Whilst the EPDS can be used as a continuous measure, clinical cut offs can also be used to determine the presence of probable depression. Different EPDS cut off scores have been recommended dependent on the culture or specific population the test has been applied to (Eberhard-Gran et al., Teng et al., 2005, Halbreich and Karkun, 2006). However, the majority of validation studies using community samples, and UK based populations, suggest that a cut off score of 13 and above is optimum for the screening of probable depression (Cox et al., 1987, Murray and Cox, 1990, Shelton and Herrick, 2009). As previously reported, the internal consistency for the present study was determined using Cronbach's alpha and at 0.85 was deemed to be good (Sumption, 2020). The same questionnaire was used at all time points (Figure 2.17 and Table 2.7) and a copy of the EPDS can be seen in Appendix A1.

2.6.2.1.2 State Trait Anxiety Inventory

The State-Trait Anxiety Inventory (STAI) is a commonly used self-report measure of both state and trait anxiety (Spielberger, 1970, Vagg et al., 1980). Trait anxiety refers to the relatively stable personality trait of anxiety defined as an individual's tendency to respond with worry or concern (Saviola et al., 2020). In contrast, state anxiety is more transient, referring to anxiety towards a particular event or moment (Spielberger, 1970). Consequently, only the trait subscale of the STAI was used in the GiW study, as the score of the state anxiety subscale may have been artificially inflated due to the upcoming ELCS. Since its initial publication, the STAI has been validated for use both in pregnancy and the perinatal period (Grant et al., 2008, Meades and Ayers, 2011). The trait subscale of the STAI consists of 20 items, including examples such as "*I am worried"* and "*I feel nervous"*. Each item is rated a 4-point Likert scale ranging from 1-4 resulting in a total possible score of 80, with higher scores indicating greater levels of anxiety symptomology (Spielberger, 1970, Vagg et al., 1980). The STAI is commonly used as a continuous measure and as such there is no validated cut-off score for either of the STAI scales. However, scores of 40 or above are commonly used in community samples to define probable levels of clinical anxiety (Moss et al., 2009). As previously reported, the internal consistency for the present study was determined using Cronbach's alpha and at 0.92 was deemed to be good (Sumption, 2020). The same questionnaire was used at all time points (Figure 2.17 and Table 2.7) and a copy of the STAI can be seen in Appendix A2.

2.6.2.2 Placenta Collection

Placenta collection took place within 2 hours after birth by trained midwives. Of the 355 women recruited for the GiW study, placentas were collected from 352 participants. To account for differential gene expression across the placenta, between 3-5 tissue biopsies were taken per sample (Janssen et al., 2015). 10 mg of each sample was stored overnight in 1 ml RNA*later*, before being dissected into ice cold phosphate buffered saline (PBS) (Life Technologies, UK) and stored at – 80°C prior to RNA extraction.

2.6.2.3 Y1 Questionnaire

At approximately 12 months postpartum GiW participants were invited to complete a further battery of self-report questionnaires, including the EPDS, STAI and general demographic information as at A1. In addition, parents were also asked to complete a number of novel questionnaires including; the Postpartum Bonding Questionnaire (PBQ) (Brockington et al., 2001); the Cardiff Infant Contentiousness Scale (CICS) (Hay et al., 2010), and the Infant Behavioural Questionnaire Revised-Short Form (IBQ-R-SF) (Gartstein and Rothbart, 2003).

2.6.3.2.1 Postpartum Bonding Questionnaire

The PBQ is a self-report screening measure designed to detect issues with bonding early in the mother-child relationship. The PBQ consists of 25 items rated on a 6-point Likert scale ranging from 0-5, with a lower score indicative of better bonding in the mother-infant dyad. Commonly, the PBQ scoring is divided into four scales; general problems with bonding (e.g., "*I feel trapped as a mother"*); rejection and anger ("*I feel angry with my baby"*); infant-focused anxiety ("*my baby makes me feel anxious"*) and risk of abuse. The latter however was not included in the GiW assessment due to the extreme nature of this scale's questions, and its lack of empirical validity (Brockington et al., 2001, Wittkowski et al., 2007). Consequently, scales assessing general bonding problems, rejection and anger, infant focused anxiety and a total PBQ score which assessed overall bonding quality were used for analysis (Chapter 5). The current study demonstrated the internal consistency of the questionnaire to be good, with a Cronbach's alpha of .83 (Sumption, 2020). A copy of the PBQ can be seen in Appendix A3.

2.6.3.2.2. Cardiff Infant Contentiousness Scale

The CICS (Hay et al., 2010) was used as a measure of infant aggression, a variable of interest as early presentation of aggression in infants has been consistently linked to later life behavioural problems. Consisting of 20 items in total, the CICS features 5 keys items assessing infant aggressive behaviour (hitting, biting, temper tantrums and angry moods) and 15 'distractor milestone items' assessing age-appropriate development such as motor and communication skills. Each item is scored on a 3-point Likert scale, ranging from 0-2, with a total sum CICS score. Higher scores are indicative of higher levels of aggression. Internal consistency was demonstrated to be good, Cronbach's alpha = .73 (Sumption, 2020).

2.6.3.2.3 Infant Behavioural Questionnaire Revised-Short Form

Introduced in 1981, the IBQ-R-SF Is one of the most widely used measures to assess infant temperament (Rothbart et al., 1981, Gartstein and Rothbart, 2003, Rothbart et al., 2011). The IBQ-R-SF is a 91-item questionnaire which asks the respondent to report on the frequency of a given behaviour (e.g., crying) over the previous week on a 7-point Likert scale ranging from 1-7. From the 91 items, the IBQ-R-SF measures 14 individual subscales such as 'sadness' and 'soothability' which further load on three main dimensions; Surgency, Negative affect and Regulatory Capacity (Rothbart et al., 2011). For the present study, the average Cronbach's alpha for the 14 subscales was 0.7 (Sumption, 2020).

2.6.4.1 Infant Assessments (Y1)

At approximately 12 months postpartum, GiW participants were invited to attend a laboratory-based assessment with their infants. 83 parent-infant dyads attended the session. All assessments took place in a designated experimental testing room, and the assessments were carried out by two trained researchers unaware of the prenatal mental health scores of the caregivers. All assessments were recorded using a high-definition digital camera for later offline coding. Infant behaviour was initially assessed and coded using the Laboratory Temperament Assessment Battery (Lab-TAB) (Goldsmith and Rothbart, 1996, Planalp et al., 2017) and the Bayley Scales of Infant Development Third Edition (BSID-III) (Bayley, 2009).

2.6.4.1.1 Laboratory Temperament Assessment Battery

The Lab-TAB (Goldsmith & Rothbart, 1996) is a standardised instrument for the early assessment of infant temperament. Specifically, the Lab-TAB uses standardised tasks simulating everyday situations, referred to as episodes, to measure five broad dimensions of infant temperament: joy, fear, anger, activity, and persistence. Six episodes were used in the GiW study: Freeplay, Sustained Attention, Novel Toy, Maternal Separation, Collaboration, and a Joy task. Previous research discussed in Chapter 5 has focused on infant behaviour during the Sustained Attention, Novel Toy and Maternal Separation Task (Savory et al., 2020), whilst data from the Freeplay task is analysed in Chapter 5. As data from the Collaboration and Joy task were not used in this thesis they are not discussed further. Table 2.8 shows the four episodes relevant to this thesis and the variables measured from each task. For this thesis, further, more detailed coding was carried out on the Freeplay task and so this is described in more detail in Chapter 5. Initial coding carried out at Y1 was in line with published Lab-TAB coding (Planalp et al., 2017).

Table 2.8 Lab-TAB Episodes and the variables measured within each task

Episode	Description	Variables measured
Novel Toy	An adaption of the 'Unpredictable Mechanical Toy[1] Task'. Infant was sat in a chair and presented with a novel toy.	Facial Fear, distress, bodily fear, escape, startle, and parent behaviour
Sustained Attention	The infant is presented with a novel object and the duration of their gaze and interaction is measured	Facial interest, duration of looking, gestures, latency to look away, infant affect (positive and negative) and parent behaviour
Freeplay	Mothers were instructed to play with their child as they usually would at home using any toy available.	Maternal intrusiveness, infant responsiveness, infant affect (positive and negative) and maternal affect (positive and negative)
Maternal Separation	Mothers were instructed to leave the room as they usually would at home. The infant was left in the experimental room. One researcher remained behind but did not interact with the infant.	Facial fear, vocal distress, intensity of escape

1Unpredictable Mechanical Toy task originally detailed in Goldsmith and Rothbart (1996)

2.6.4.1.1 Infant Neurodevelopment (Bayley Scales of Infant Development Third Edition)

The BSID-III and its prior versions have been in use for decades as a tool to assess developmental functioning in infants within research and clinical settings (Bayley, 2009, Johnson et al., 2014). In the GiW study, the BSID-III was used to assess age standardised cognition, receptive and expressive language, with the former being assessed following the Lab-TAB assessment and the latter assessed throughout the in-person visit.

2.6.3 Micro-coding

Micro-coding of both the Freeplay and Maternal Separation Task was undertaken to capture more detailed information of the social interaction within the mother-infant dyad from the observational data collected at Y1. All interactions were coded offline, on an event-based basis using The Mental Health Intergenerational Transmission (MHINT) process manual (Costantini et al., 2021) as a coding scheme alongside Noldus Observer XT 14.0 (Noldus, 2021). Whilst specific behaviours of interest are detailed in Chapter 5, Table 2.9 presents all the behaviours available to code from the MHINT process manual.

Table 2.9 Behavioural groups and behaviours available to code from the MHINT process manual

Behavioural Group	Behaviour	Behavioural Group	Behaviour	Behavioural Group	Behaviour
Caregiver Posture	Lying down Lie on side Sit on floor Sit on object Stand up Crawl Crouched down Jump Walk Run Dance NPTC[a] caregiver posture/action	Infant Posture	Lie down Lie on one side Sit on the floor Sit on an object Standing Crawling Jumping Walking Running Held/in hold Try to move in another way NPTC[a] infant posture/action	Visual Attention	Look at infant Look at caregiver 1 Look at caregiver 2 Look at same object/joint attention Look at focus object Look at different object Look at other object Look at object outside of view Look at sibling Look at other person Look at distraction No visual attention NPTC[a] visual attention
Caregiver Vocalisation	Speech Musical Sounds Laugh Nervous laugh Vocal Imitation Bodily sounds Scream Vocal tics Non-verbal sound Silent NPTC[a] caregiver vocalisation	Infant Vocalisation	Laughing Distressed Non-Distress Imitating sounds Babbling First words Screaming Bodily Sounds Silent/none of the above NPTC[an] infant vocalisation	Head Orientation	Vis-a-vis - infant and caregiver Slight (30-90 degree) aversion right Slight (30-90 degree) aversion left Full (90 degree) aversion right Full (90 degree) aversion left Arch aversion Head not in view of infant NPTC[a] head orientation
Caregiver Body Orientation	Body oriented to infant Body oriented to other caregiver Body oriented to sibling Body oriented to other person/object Body oriented to object (focus of the activity) NPTC[a] caregiver body orientation	Infant Body Orientation	Body oriented to caregiver 1 Body oriented to caregiver 2 Body oriented to sibling Body oriented to different person/object Body oriented to object (focus of activity) NPTC[a] code infant body orientation	Facial Expression	Neutral/Alert Positive Smile Negative Disgust Surprise Woe face Mock surprise Face not visible None of the above
Touch Right Hand (R)	Infant touch R Caregiver touch R	Touch Left Hand (L)	Infant touch L Caregiver touch L	Hand Movements	Pointing Reaching

	No infant touch R No caregiver touch R NPTC[a] touch R		No infant touch L No caregiver touch L NPTC[a] touch L		Clapping Waving Gesticulating Stacking attempt Banging Other hand movements No hand movements NPTC[a] hand movement
Caregiver Proximity	Out of reach Within reach Loom NPTC[a] caregiver proximity	Physical Play	Physical play evident No physical play NPTC[a] physical play		

"Behavioural group" refers to an overarching behavioural category, comprised of a subset of mutually exclusive, exhaustive "behaviours"
[a] NPTC = Not possible to code.
Full coding scheme details are detailed in (Costantini et al., 2021)
Table adapted from Burgess et al., (2021) in press.

2.7 Statistical Analysis

All data were analysed using IBM SPSS software (version 27) or in RStudio. Data are presented as mean and standard error of the mean (SEM) unless otherwise stated. All variables were analysed for outliers and normality and unless explicitly stated, all assumptions for each statistical test were met. In Chapter 5, for participants missing less than 20% of data for measures of maternal mental health and infant outcomes, missing data were addressed using participant level mean substitution as in previous research (Savory et al., 2020, Sumption, 2020). Further details of the statistical methods, including sample sizes and exclusion criteria are described in the relevant experimental chapters.

2.7.1 Unified Behavioural Scoring Model

Given the complex, and often multifaceted nature of behavioural traits, it has been recommended that when assessing a given behavioural trait using mutant mouse models, two or more behavioural tests are used to strengthen the interpretation of findings (Bailey and Crawley, 2009). However, this may also lead to an increase in the likelihood of generating type I statistical errors (Shaffer, 1995).

In light of this, the unified behavioural scoring model was introduced by Harrison et al. (2020) as a means of maximising the use of all the data generated in multiple tests, whilst also minimising the risk of both type I and type II errors. In this thesis, as in other research, unified behavioural scoring was used to generate a unified score for anxiety-like behaviours (Harrison et al., 2020, Thornton et al., 2021) and social behaviour (Harrison et al., 2020).

A unified maternal anxiety score was calculated from outcome measures of both the EZM and LDB (Chapter 3) whilst a unified social score was generated from outcome measures of; direct social interaction, social propinquity, three-chamber, and courtship behaviour tests (Chapter 4). Based on existing literature and the work of Harrison et al. (2020), each outcome was assigned as either a positive or negative factor, whereby a positive factor was indicative of greater anxiety / sociability, and a negative factor was indicative of less anxious behaviour /

sociability. Table 2.10 shows the outcome measures used to generate the unified maternal anxiety score in Chapter 3. Tables 2.11 shows the outcome measures used to generate the unified social score in Chapter 4.

Table 2.10 Measures of anxiety used to calculate the unified maternal anxiety score and the direction of their influence.

Test	Measure	Influence	Reference
Elevated Zero Maze	Number of crosses	-ve	(Shepherd et al., 1994, Kulkarni et al., 2007)
	Time in open	-ve	(Pellow et al., 1985, Shepherd et al., 1994, Kulkarni et al., 2007, Rosso et al., 2021)
	Latency to enter open	+ve	(Shepherd et al., 1994, Kulkarni et al., 2007)
	Frequency of stretch-attend posture	+ve	(Lister, 1990, Schulz et al., 2011, Gilhotra et al., 2015)
	Fecal Boli	+ve	(Keers et al., 2012, Seibenhener and Wooten, 2015)
Light Dark Box	Number of crosses	-ve	(Crawley and Goodwin, 1980, Crawley, 1981)
	Time in light	-ve	(Crawley and Goodwin, 1980, Crawley, 1981, Rosso et al., 2021)
	Latency to enter light	+ve	(Crawley and Goodwin, 1980, Crawley, 1981)
	Frequency of stretch-attend posture	+ve	(Gilhotra, Goel, & Gilhotra, 2015; Lister, 1990; Schulz et al., 2011)
	Fecal Boli	+ve	(Keers et al., 2012, Seibenhener and Wooten, 2015)

Table 2.11 Measures of social behaviour used to calculate the unified sociability score and the direction of their influence.

Test	Measure	Influence	Reference
Direct Social Interaction and Courtship Behaviours[1]	Body sniff	+ve	(File and Hyde, 1978, Ikeda et al., 2013, Yang et al., 2013)
	Ano-genital sniff	+ve	
	Nose sniff	+ve	
	Follow	+ve	
	Being followed	-ve	
	Grooming	-ve	
	Immobile	-ve	
	Being attacked	-ve	
	Mounting (Courtship behaviours only)	+ve	
Social Propinquity	Proportion of time in shared occupancy	+ve	(Tuttle et al., 2017, Harrison et al., 2020)
	Proportion of time in single occupancy	-ve	
	Latency to first share	-ve	
Three Chambers	Time spent in occupied chamber	+ve	(Moy et al., 2004, Nadler et al., 2004, Yang et al., 2011)
	Number of crosses into occupied chamber	+ve	
	Latency to enter occupied chamber	-ve	
	Total Area of scent marking[1]	+ve	

[1] This test was only performed using male mice and so contributes to the unified social score in males only.

Outcome measures for each test were normalised to obtain a measure score between 0 and 1 using the following formula:

$$Normalised\ score = \frac{Individual\ score}{Maximum\ score}$$

Where ‘Individual score’ = the value generated by the individual for any given outcome measure, and ‘Maximum score’ = the maximum value generated by any individual for that outcome measure. Any negative

values were assigned a value of 0, and for any latency values where the maximum score was recorded, a score of 1 was assigned. Negative factors associated with a decrease in the measured behaviour were inverted to ensure that a greater score in any measure related to an increase in the specified behaviour. Individual 'test' scores were then calculated as the average of all outcome measures associated with that test (e.g. all anxiety measures of the LDB test). Finally, the scores for each test were averaged to generate a unified behavioural trait score for each mouse, enabling each test to have an equal influence on the final score.

2.8 Figures

Figures from Chapters 1 and 2 detailed in Table 2.12 were created using Biorender.com. All other figures were either created by the author independently of Biorender.com or were adapted from previous work. For the latter, where this is the case, it is explicitly stated in the figure legend.

Table 2.12 Figures created using BioRender.com

Chapter	Figure Number
Chapter 1	Figures 1.2 & 1.3
Chapter 2	Figure 2.2, 2.5 - 2.12 & 2.16

3

Chapter 3: Evaluating the effect of offspring *Peg3* disruption on maternal behaviour

3.1 Overview

This chapter explored how loss of expression of the *Peg3* gene in mouse offspring affects maternal behaviour. First, by replicating, and then by extending the findings of McNamara et al. (2018a).

Maternal disruption of *Peg3* and its behavioural phenotypes have been well characterised in mice, including behavioural deficits such as; increased latencies to crouch over, sniff and retrieve pups; poor quality nest building, and reduced milk let down (Li et al., 1999, Curley et al., 2004, Champagne et al., 2009). Additionally, through its effect on maternal care, disruption of maternal *Peg3* expression has also been found to influence offspring phenotypes, even in genetically WT offspring. Notably, litters generated from *Peg3KO* heterozygous dams crossed with WT sires, show elevated mortality rates even in their WT pups (Li et al., 1999). Further, with the same breeding set-up, the WT offspring, and grand offspring of *Peg3* mutant mothers continue to display reduced levels of maternal care over generations (Curley et al., 2008).

Despite this, and the evidence discussed in Chapter 1, which suggests that an offspring's genetic makeup may influence maternal care behaviour (Creeth et al., 2018, Creeth and John, 2020), there has been little research exploring the effect that loss of expression of *Peg3* in the offspring has on maternal behaviour. The first evidence suggesting that offspring loss of expression may influence maternal care was reported in Curley et al. (2004), whereby both *Peg3* mutant litters raised by WT dams, and WT offspring raised by mutant dams demonstrated high mortality rates, potentially indicative of offspring loss of *Peg3* expression bringing about suboptimal maternal care in WT dams. However, this study was primarily focused on the physiological effects of targeted *Peg3* disruption in both the mother and offspring, and so changes in maternal care behaviour in response to offspring loss of *Peg3* were not assessed fully.

As discussed in Chapter 1, *Peg3* has been shown to play a role in regulating the development of several key placental endocrine lineages important for the programming of maternal behaviour (Creeth et al., 2018, Tunster et al., 2018). Subsequently, McNamara et al. (2018a) reported that loss of expression of *Peg3* in the feto-placental unit affected maternal behaviour in WT dams, both pre- and postnatally. In the work of McNamara et al. (2018a), WT dams with litters consisting solely of heterozygous *Peg3KO* $^{(+/-)}$ pups were studied alongside WT dams carrying 100% WT litters, achieved through crossing WT dams with a homozygous *Peg3KO* male, or WT male respectively. Prenatally, there was little evidence to suggest that exposure to mutant *Peg3* offspring altered the maternal brain, with no significant changes in gene expression in the maternal hippocampus or hypothalamus at E16.5 detected via RNA sequencing. However, in regard to maternal behaviour, while there were no differences in nest-building, anxiety-related behaviour or locomotor activity during pregnancy, pregnant dams carrying *Peg3* mutant fetuses travelled significantly less distance when first transferred to observation cages (novel environment) compared to pregnant dams with WT fetuses (McNamara et al., 2018a). Though subtle, this provided the first evidence that mutant offspring's placental phenotype, induced by loss of *Peg3*, may contribute to the maternal care phenotypes that have previously been observed.

In McNamara et al. (2018a), postnatally, there were no differences observed in the nest building, or maternal aggression tasks. However, dams exposed to *Peg3* mutant pups demonstrated increased anxiety-like behaviour and deficits in maternal care, showing increased latencies to both sniff and retrieve their pups. In addition, mutant pups were found to emit fewer ultrasonic vocalisations (USVs); a form of communication essential for the initiation of maternal care behaviours such as pup retrieval, nursing, and nest building (Champagne, 2004, Scattoni et al., 2009, Wohr and Scattoni, 2013). These findings, summarised in Table 3.1, were taken to suggest that there was evidence for both a pre- and postnatal influence of offspring *Peg3* expression; prenatally, via the placenta; and postnatally, either due to placental programming or due to the presence of mutant pups and their altered USV pattern in the postnatal environment. Whilst the observed postnatal maternal behaviour cannot be specifically attributed to either a prenatal or postnatal influence, this study provided the first evidence that loss of function of *Peg3* in the offspring, may influence maternal behaviour.

Table 3.1 A summary of the behavioural tests and results from McNamara et al., (2018a).

	Behavioural Test	Significant Differences	Result
Prenatal Tests	Activity Monitoring	✓	Dams carrying mutant litters explored a novel arena more than dams carrying WT litters.
	Nest Building	X	
	Elevated Zero Maze	X	
Postnatal Tests	Pup Retrieval	✓	Dams carrying mutant litters were slower to sniff and retrieve pups compared to dams carrying WT litters.
	USVs	✓	Mutant pups called significantly less than WT pups.
	Nest Building	X	
	Maternal Aggression	X	
	Elevated Zero Maze	✓	Dams carrying mutant litters spent significantly less time in the open area of the EZM than dams with WT litters.
	Tail Suspension	X	

In McNamara et al. (2018a) an 'all or nothing' approach was essentially applied in terms of the dam's exposure to *Peg3 in utero*, in that dams were either exposed to 100% mutant or 100% WT litters. While this was successful in uncovering maternal behavioural phenotypes, a graded disruption of *Peg3,* through the introduction of a mixed genotype litter could reveal a more nuanced influence on maternal behaviour as observed with a model based on different doses of the *Phlda2* gene (Creeth et al., 2018). Specifically, the presence of genetically WT offspring in the same litter as *Peg3* mutant pups, may result in attenuated maternal care deficits and anxiety-like phenotypes compared to what has been observed in dams raising 100% mutant litters (McNamara et al., 2018a). Importantly, and again as highlighted in Chapter 1, the mother-infant relationship is a reciprocal process which involves the active solicitation of care by the offspring. As McNamara et al. (2018a) reported that mutant pups were shown to emit fewer USVs than WT pups, the inclusion of the mixed litter group would also allow for an exploration of whether dams preferentially responded to pups unaffected by the disruption of *Peg3* and if they could discriminate between their mutant and WT offspring.

3.2 Aims

In McNamara et al's (2018a) study, WT dams carrying and caring for heterozygous *Peg3KO* litters demonstrated increased levels of maternal anxiety and deficits in maternal care. The primary aim of this chapter was to replicate and extend these original findings by using a more extensive behavioural test battery, and to compare the phenotypes observed across WT dams with 100% mutant litters to WT dams with 100% WT litters. In this study, in addition to the two original groups, a third, intermediate group of dams where only half the litter was mutant, was included. The aim of including this intermediate group was threefold. Firstly, the inclusion of dams with

mixed genotype litters allowed for the exploration of whether dam exposure to both WT and mutant *Peg3* pups would alter maternal behaviour. Specifically, whether the presence of WT pups in a litter attenuated maternal anxiety-like and care phenotypes. Secondly, to assess whether dams could discriminate between their mutant pups, compared to their WT pups in the pup retrieval task. Thirdly, and in relation to the thesis as a whole, one of the main aims was to characterise early life social behaviour in *Peg3KO* mice in the context of the mother-infant relationship. As can be seen in Chapter 4, the inclusion of a mixed litter group allowed for a comparison of WT and mutant pups raised under different maternal environments to their single genotype counterparts (gene x environment). However, prior to those comparisons, the degree to which maternal care is altered for pups in the mixed litter group had to first be established.

3.3 Methods

3.3.1 Generation of maternal cohort

Three experimental groups were generated as described in Chapter 2, Figure 2.2. Briefly, virgin 129Sv WT females (aged 8-13 weeks) were paired with studs that were either WT, heterozygous or homozygous *Peg3KO* males, to generate dams pregnant with 100% WT litters, mixed genotype litters, containing both WT and mutant pups, or 100% mutant litters respectively. Following the observation of a vaginal plug (E 0.5) dams were initially housed with other pregnant females plugged by studs of the same genotype and then singly housed at E16.5. Day of birth was recorded as P0. Inclusion criteria for analysis were that for all groups, litter size was between 4 and 10 pups, and for the mixed litter group, between 45-65% of the pups were required to be mutant, as identified by genotyping at P14.

Table 3.2 shows the number of dams and the genotypes of their litters that were used to assess maternal behaviour. However, due to the loss of 2 pups at P9, resulting in a litter size of 2, one dam in the 100% mutant group was excluded from the pup retrieval analysis at P9.

Table 3.2 Number of dams used to assess maternal behaviour

Dam (WT Litter)	**Dam** (Mixed litter)	**Dam** (Mutant litter)
13	12	12

3.3.2 Behavioural assays

Full details of the behavioural tests used to characterise maternal anxiety and maternal care, and the order in which they were performed are described in section 2.4.1. Table 3.3 shows a comparison of the behavioural tests originally used in (McNamara et al., 2018a), the extended battery of tests used for this thesis, and the time-points at which they were carried out.

Table 3.3. Comparison of the behavioural tests used to assess maternal anxiety and maternal care between McNamara et al. (2018a) and the present thesis.

		McNamara et al., (2018)	*Present Thesis*	
Domain	**Behavioural Test**	**Timepoint**	**Timepoint**	**Additional Variables**
Maternal Anxiety	EZM	P4	P4	Stretch-attend posture, Fecal-Boli
	LDB	Not measured	P7	Stretch-attend posture, Fecal Boli
Maternal Care	Pup-retrieval	P2	P3[b] & P9	Additional timepoint, dam behaviour during retrieval
	Nest Building	P3	Not measured [a]	
	USVs	P2	P3[b] & P9	Additional timepoint, latency to call
	Maternal aggression	P7	Not measured [a]	

[a] These tests were not measured in the present thesis as no significant difference was observed in McNamara et al. (2018a) between dams [(Mutant litter)] and dams [(WT litter)].
[b] Pup-retrieval and USVs were carried out at P3 rather than P2 to avoid multiple testing in one day, as isolation-induced USVs were performed at P2 in this thesis (see Chapter 4).

3.3.3 Statistics

Sample size required for each group was calculated using G*Power (Faul et al., 2007). Although McNamara et al. (2018a) reported using a sample size of n =14 for both groups of dams and a non-pregnant control group consisting of 7 female mice, effect size was not reported for any group across experiments, nor were mean values reported in text. Despite this, Cohen's D as a measure of effect size was calculated using the equation detailed below, from graphs reporting pup retrieval data in McNamara et al., (2018a). In the equation, M_1 and M_2 denote the samples means for group 1 and group 2, and S_p denotes the estimated pooled standard deviation for both groups.

$$D = \frac{M_1 - M_2}{S_p}$$

Using values obtained from the pup retrieval data reported in McNamara et al. (2018a), a Cohen's D value of 0.79 was obtained. Using this, and a required power of $(1 - \beta) = .095$ with a type 1 error rate ($\alpha = .05$), a sample size of 9 animals per group was predicted to be sufficient for one-way Analyses of Variance (ANOVAs) and Analyses of Covariance (ANCOVAs).

As the actual means were not reported in McNamara et al., (2018a), a second power calculation was carried out using a standardised effect size (D) of 0.7 as recommended in Wahlsten (2010) as an appropriate effect size to detect small-moderate effects in animal research. Using these recommended values, a sample size of 10 dams per group was predicted to be sufficient for power (1 - β) = .09 with a type 1 error rate (α = .05) in one-way ANOVAs and Analyses of Covariance ANCOVAs. As such, both power calculations suggested the experiments reported in this chapter were adequately powered.

Differences in litter size between experimental groups were assessed using a Kruskal-Wallis H test due to violations of normality in both the mixed genotype and 100% mutant groups. As a significant difference in litter size was observed between dams carrying 100% mutant and 100% WT litters (Figure 3.1) and as previous literature has demonstrated that litter size can affect maternal care in rodents (König and Markl, 1987, McGuire and Bemis, 2007, Enes-Marques and Giusti-Paiva, 2018) litter size was subsequently included as a covariate in all analyses examining group differences in aspects of maternal care and anxiety-like behaviours.

To analyse litter sizes and mortality scores, the number of pups in a litter were counted at birth (P0) and again on the first day of testing (P2). Although only litters of ≥ 4 were used for the behavioural and sequencing cohort, mortality rates were inclusive of all pups born from the experimental breeding groups (Table 3.4).

Table 3.4 Number of litters in each group used to assess mortality score

WT litters	Mixed litters	Mutant litters
19	41	23

Differences in litter mortality score were analysed using a Kruskal-Wallis H test. Mortality rate at P2 was calculated first by determining the percentage of pups still alive at P2 compared to P0. Adapted from Morello et al. (2020), a mortality score was then calculated where litters with no loss were coded as 0, partial loss was coded as 1, and full litter loss was coded as 2. Mortality score was then used as the dependent variable in this analysis. To explore the survival rates between WT and mutant pups in the mixed genotype litters a Chi-Square Goodness of Fit test was performed.

Differences in maternal behaviour between the groups of dams, for both individual tests and the unified maternal anxiety score (Section 2.7.1), were assessed via a series of one-way Analysis of Covariance tests (ANCOVA) with litter size used as the covariant. Where a significant result was found, post-hoc, pair-wise comparisons were carried out with Bonferroni corrections to determine which groups significantly differed from the others.

Finally, USVs are only one factor which have been shown to influence the manifestation of prompt maternal care, with research to suggest that disruption of gene function or the sex of the pup may alter maternal preference and response time in the pup retrieval task (Moore and Morelli, 1979, Deviterne and Desor, 1990, Bowers et al., 2013a). In light of this, it was further explored whether the genotype, and / or the sex of the pup influenced the dam's pup retrieval at P3 and P9. This was achieved by conducting a 2-way ANCOVA on the average retrieval time for each pup, as opposed to the first or last pup. Average time was used to account for the possibility that within in the mixed litter group, both genotypes may not be represented when analysing the retrieval time for the

first or last pup alone. Consequently, for this analysis, WT $^{(mixed\ litter)}$ and *Peg3KO* $^{(mixed\ litter)}$ pups were analysed as two separate groups as a means of investigating whether dams of mixed litters where able to discriminate between the genotypes and retrieve genetically WT or mutant pups faster on average.

3.4 Results

3.4.1 Litter Size and survival rates

Litter sizes from dams carrying 100% mutant litters were shown to be smaller at P2 ($\chi^2(2)$ = 10.28, *p* = .006, Figure 3.1A), than both dams carrying 100% WT (*p* = .01) and mixed genotype litters (*p* = .04). It was also observed that there were statistically significant differences in median mortality score between litters ($\chi^2(2)$ = 9.53, *p* = .009, Figure 3.1B). Specifically, 100% mutant litters had a statistically significant higher median mortality score than 100% WT litters (*p* = .007). Although initially mixed genotype litters were also seen to have a significantly higher mortality score than WT litters (*p* = .02), this did not survive adjustment for multiple corrections (*p* = .06). Within the mixed litter group, 63 surviving pups at P2 were genetically WT, while 45 were mutant. A chi-square goodness of fit test was carried out to determine if there was an equal proportion of pups from each genotype that survived to P2. The test indicated that at P2 there was no statistically significant difference between the proportion of the two genotypes that survived within the mixed litter group ($\chi^2(1)$ = 3.00, *p* = .083, Figure 3.1C).

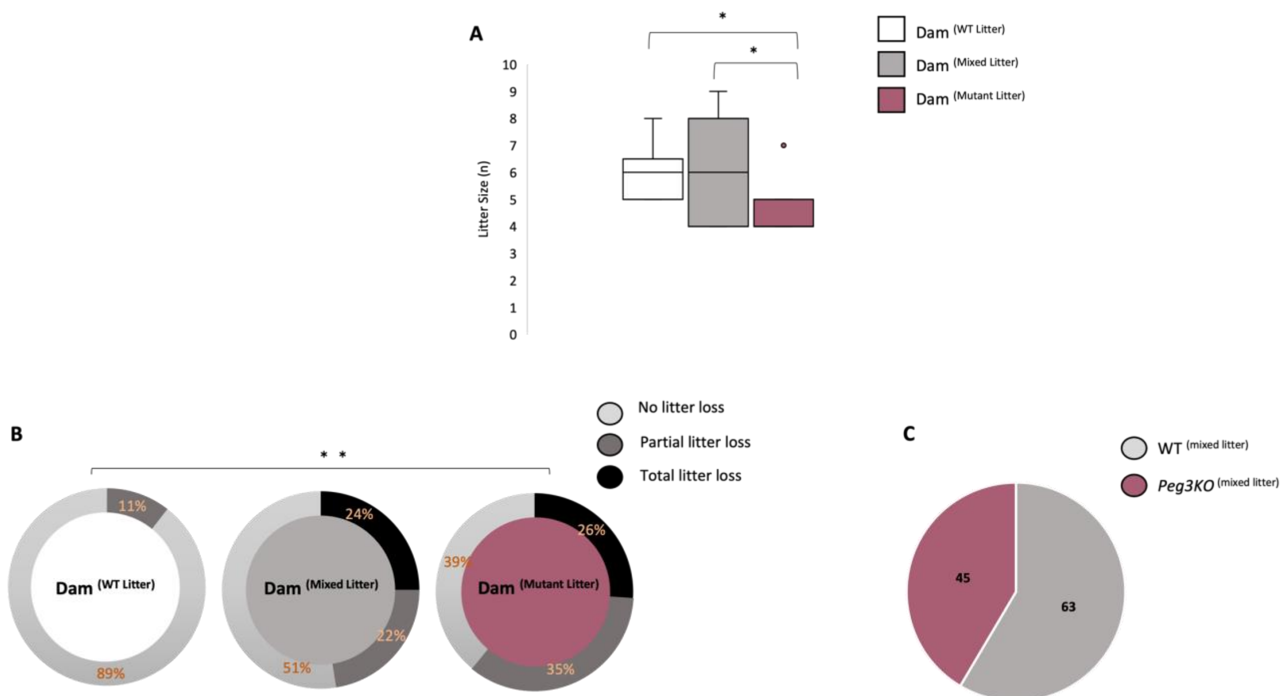


Figure 3.1 Litter sizes and survival rates A) A box and whisker-plot showing the median litter sizes of dams carrying different genotype litters that made up the maternal cohort. Kruskal-Wallis H test determined that there were statistically significant differences between the groups: 100% mutant litters were smaller than both 100% WT and mixed genotype litters. **B**) Percentages of types of litter loss that make up the mortality score for each genotype litter. A Kruskal-Wallis H test determined that dams of mutant litters had a higher mortality score than dams with fully WT litters. **C**) The proportion of the genotypes of pups within the mixed litter genotype that survived to P2. Chi-squared goodness of fit revealed that the difference between the two genotypes was not significant. **A)** shows data calculated from litters used in the behavioural cohorts, whilst **B-C** show data from all litters, not just those used in the behavioural experiments. $*p \leq .05$, $** p \leq .01$

3.4.2 Maternal Anxiety-like Behaviours

3.4.2.1 Elevated Zero Maze (EZM)

After adjustment for litter size, a series of one-way ANCOVAs demonstrated there were no statistically significant differences across the cohorts of dams in terms of; [time spent in the open zone: F(2,34) = .2.87, *p* = .07, Figure 3.2A, latency to enter the open zone: F(2,34) = .85 *p* = .44, Figure 3.2B, and frequency of crosses into the open zone: F(2,34) = 1.00 *p* = .38, Figure 3.2C]. However, there was a statistically significant effect of litter genotype on both the frequency of stretch-attend posturing performed by dams (F(2,34) = 5.18 *p* = .01, Figure 3.2D) and on the total number of fecal boli present at the end of the trial (F(2,34) = .3.61, *p* = .04, Figure 3.2E). Post-hoc analysis revealed that dams of 100% mutant litters showed an increased frequency of stretch-attend posturing, indicative of higher anxiety-like behaviour during the EZM task compared to dams of WT litters (*p* = .01). Conversely, post-hoc analysis also revealed that dams of 100% mutant litters produced fewer fecal boli during the trial than dams of 100% WT litters (*p* = .05). No further statistical differences were found (Figure 3.2).

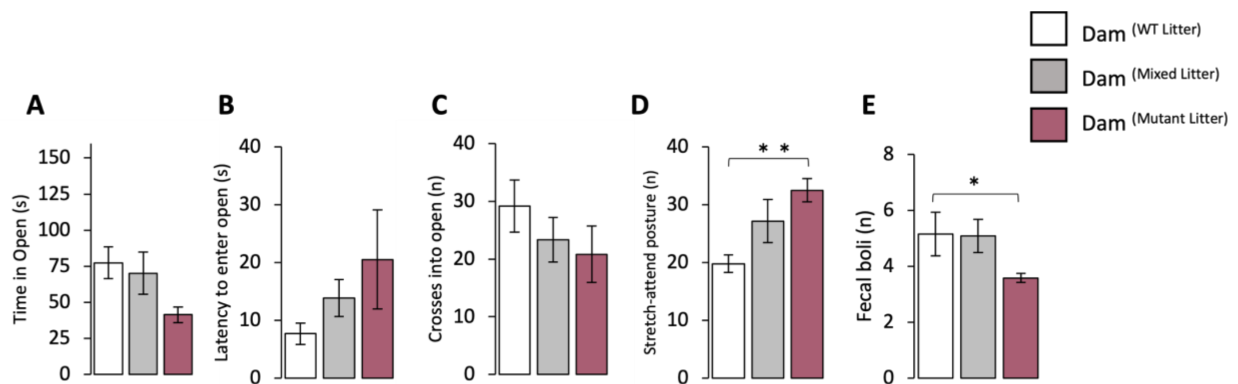


Figure 3.2 Outcome measures on the EZM. **A**) Time in the open section. **B**) Latency to enter open section. **C**) Frequency of crosses into open section. **D**) Frequency of stretch-attend postures. **E**) Total number of fecal boli. One-way ANCOVAs for **D** and **E** showed that dams with fully mutant litters demonstrated statistically greater instances of stretch-attend postures and less fecal boli production than dams of WT litters.
Error bars represent $\pm$ SEM. $*p \leq .05$, $** \ p \leq .01$

3.4.2.2 Light-Dark Box (LDB)

In the LDB, consistent with the results of the EZM, significant differences between the groups of dams in stretch-attend posture frequency were observed (F(2,34) = 4.87 *p* = .01, Figure 3.3D), with dams of 100% mutant litters demonstrating greater frequency of the posture compared to dams with 100% WT litters (*p* =.01). There were also significant differences observed in the time spent in the anxiogenic light section (F(2,34) = 4.87 *p* = .01, Figure 3.3A) and the latency to enter the light (F(2,24) = 4.92, *p* = .01, Figure 3.3B). Post-hoc tests demonstrated that dams with mixed genotype litters spent less time in the light than dams with WT litters (*p* = .04), whilst dams with 100% mutant litters were statistically slower to enter the light than dams with 100% WT litters (*p* = .01). No statistically significant differences were observed between the groups of dams regarding the number of crosses into the light section (F(2,34) = 1.34 *p* = .28, Figure 3.3C), or in the total number of fecal boli (F(2,34) = 1.31, *p* = .28, Figure 3.3E).

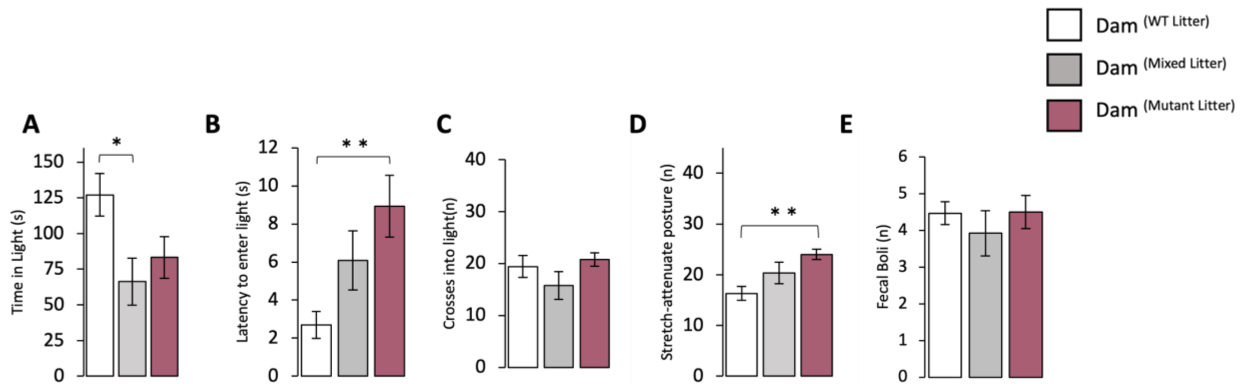


Figure 3.3 Outcome measures for the LDB. **A**) Time in the light section. Dams with mixed genotype litters spent significantly less time in the light than dams with fully WT litters. **B**) Latency to enter light section. Dams with mutant litters took significantly longer to enter the light than dams with WT litters. **C**) Frequency of crosses into light section. **D**) Frequency of stretch-attend postures. Dams with mutant litters displayed significantly more stretch-postures, indicative of higher anxiety, than dams of WT litters. **E**) Total number of fecal boli.
Error bars represent $\pm$ SEM. $*p \geq .05$, $** p \geq .01$

3.4.2.3 Unified Anxiety Score

Combining all anxiety-related outcome measures to generate a unified anxiety score, a one-way ANCOVA demonstrated that litter genotype had a significant effect on maternal anxiety ($F(2,34) = 4.52$, $p = .02$, Figure 3.4). This was driven by dams of 100% mutant litters scoring more highly on the unified anxiety score than dams of WT litters ($p = .02$). When taken together, this data suggests that dams of 100% mutant litters are more anxious than those dams who were not exposed to the mutant *Peg3* pups *in utero*. This is discussed more fully in section 3.5.

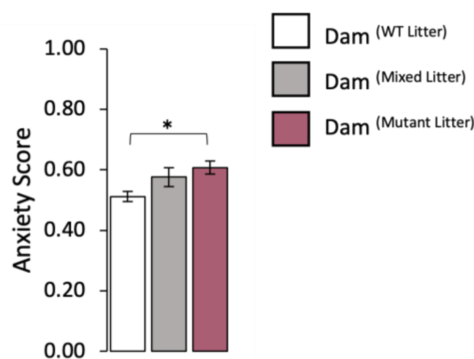


Figure 3.4 Unified Anxiety Score. One-way ANCOVA demonstrated that dams with fully mutant litters scored significantly higher on the unified anxiety score than dams with WT litters. Error bars represent $\pm$ SEM.
$*p \geq .05$

3.4.3 Maternal Care

3.4.3.1 Pup Retrieval

The time taken to sniff and retrieve either the first or last pup in the pup retrieval task is a commonly used measure of maternal responsiveness. In this study, the time taken to sniff and retrieve each of four pups at P3 and P9 was recorded. At each of these timepoints, no significant differences in the time taken for dams to sniff either the first [P3: $F(2,34)= .61$, $p = .55$, Figure 3.5A, P9: $F(2,34) = .81$, $p = .45$, Figure 3.5E] or fourth pup were observed [P3: $F(2,34) = 1.40$, $p = .24$, Figure 3.5C, P9: $F(2,33) = 1.32$, $p = .28$, Figure 3.5G]. This was also true of time taken to retrieve the first [P3: $F(2,34)= 1.30$, $p = .29$, Figure 3.5B, P9: $F(2,33) = .76$, $p = .48$, Figure 3.5F] and fourth pup [P3: $F(2,34)= 1.30$, $p = .29$, Figure 3.5D, P9: $F(2,33) = .76$, $p = .48$, Figure 3.5H].

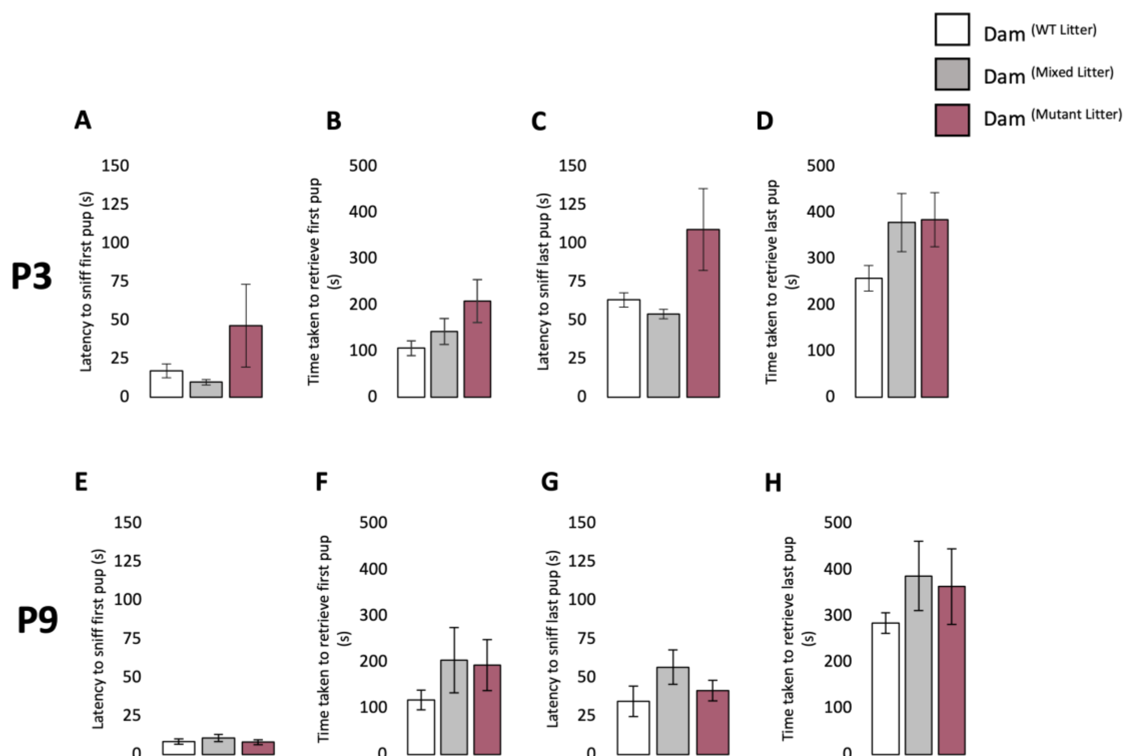


Figure 3.5 Time taken to sniff and retrieve pups during the pup retrieval task at P3 and P9. **A**) Latency to sniff first pup at P3. **B**) Time taken to retrieve first pup at P3. **C**) Latency to sniff last pup at P3. **D)** Time taken to retrieve last pup at P3. **E**) Latency to sniff first pup at P9**. F**) Time taken to retrieve first pup at P9. **G)** Latency to sniff last pup at P9. **H**) Time taken to retrieve last pup at P9. There were no differences between the cohorts of dams in either the time taken to either sniff or retrieve the first or last pup at either timepoint. Error bars represent ± SEM. *At P9, one dam was excluded from the Dam (Mutant Litter) group due to the death of 2 pups prior to the test, leaving the litter too small to be tested.

As in McNamara et al. (2018a), USVs were recorded for three minutes from the group of four pups to be used in pup retrieval, immediately prior to reintroducing the dam to the home cage. At P3, though pups from mutant litters called substantially less on average than pups from WT litters (190 (± 19.98) and 311 (± 49.18) calls respectively), there were no statistically significant differences in pre-pup retrieval call frequency ($F(2,34) = 2.02$, $p = .15$, Figure 3.6C) across any of the groups. This was also true at P9 ($F(2,33) = 1.43$, $p = .25$, Figure 3.6G). As an additional variable, the present thesis also measured latency to call, however, at both P3 and P9, there were no

significant differences in the latency for the litter to call [P3: F2,34) = 1.94 *p* = .16, Figure 3.6D, P9: F(2,33) = .72, *p* =.50, Figure 3.6H]. Similarly to McNamara et al. (2018a), at P3, and the additional timepoint of P9, there were no significant differences in the frequency of dam emitted USVs [P3: F(2,34) = .86, *p* = .43, Figure 3.6A, P9: F(2,33) = 1.90, *p* = .17, Figure 3.6E], nor were there any significant differences in the dam's latency to call [P3: F2,34) = .96, *p* = .40, Figure 3.6F P9: F(2,33) = 2.32, *p* = .12, Figure 3.6H]. Interestingly, dams of mixed litters did not emit any calls at P9.

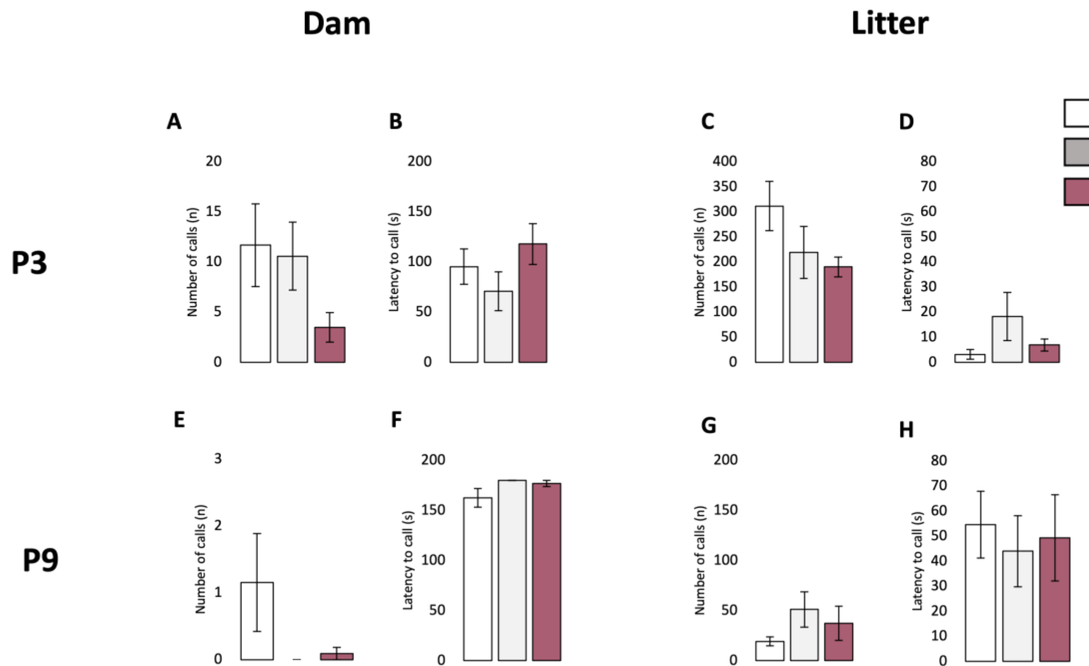


Figure 3.6 Dam and Litter USV recordings at P3 and P9 immediately prior to the pup retrieval task. **A**) Number of calls made by the dam at P3. **B**) Latency of the dam to call at P3 **C**) Number of calls made by four pups at P3. **D**) Latency of four pups to call at P3. **E)** Number of calls made by the dam at P9. **F**) Latency of the dam to call at P9. **G**) Number of calls made by four pups at P9. **H)** Latency of four pups to call at P9. Litter refers to the four pups selected for pup retrieval. No statistically significant differences were observed between any measure at either timepoint. Error bars represent $\pm$ SEM. *At P9, one dam was excluded from the Dam $^{(Mutant\ Litter)}$ due to the death of 2 pups prior to the test leaving the litter too small to be tested.

A two-way ANCOVA was also run to determine whether dams could discriminate between the sex, or in the case of the mixed litter group, the genotype of their pups. No significant interaction between genotype and sex was observed (F(3,140) = 1.73, *p* = .17, Figure 3.7A), nor was there a significant main effect of sex (F(1,142) = 1.94, *p* = .13), however, looking at the average time taken to retrieve pups, a significant main effect of genotype was observed (F(3,140) = 3.55, *p* = .01, Figure 3.7B). Pups from 100% mutant litters were retrieved on average 109 $\pm$ 30.34) seconds slower than those from 100% WT litters at P3 (*p* = .01). Interpreted another way, this demonstrates that on average, dams with 100% mutant litters retrieved their pups significantly slower than dams with 100% WT pups (Appendix A4 demonstrates the results of this analysis with the mixed litter group collapsed across genotype).

At P9, there was no significant interaction effect between genotype and sex on the average time of pups retrieved (F(3,136) = .82, *p* = .49, Figure 3.7C), nor was there any significant main effect of either sex (F(1,142) = 1.94, *p* = .13) or the genotype of the pups (F(1,142) = 1.72, *p* = .19, Figure 3.7D).

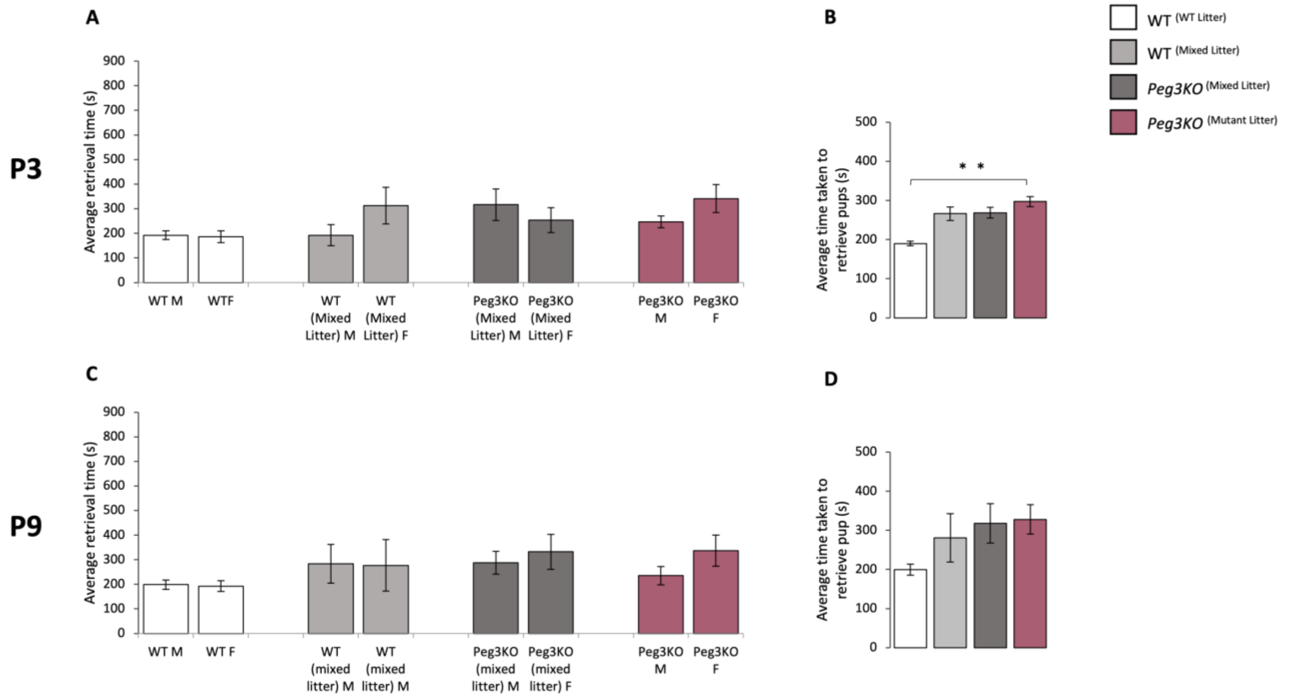


Figure 3.7 Average time taken for dams to retrieve pups of each sex and genotype. A) Average time taken to retrieve each pup based on sex and genotype at P3. A 2-Way ANCOVA demonstrated that there was a significant main effect of genotype, with pups from mutant litters being retrieved significantly slower than pups from WT litters (shown in panel B). **B**) Average time taken to retrieve each genotype of pup at P3. **C**) Average time taken to retrieve each pup based on sex and genotype at P9. **D**) Average time taken to retrieve each genotype of pup at P9. Error bars represent $\pm$ SEM.** $p \leq .01$
[a]At P9, one male and one female Peg3KO (mutant litter) pup were not included in the analysis due to littermate death, rendering the litter too small to be tested.

3.4.3.2 Maternal Behavioural Observation

During the pup retrieval task, at both P3 and P9, a number of dam behaviours were recorded alongside retrieval and sniffing, Tables 3.5 and 3.6 show the average percentage of time engaged in each of the observed behaviours described in section 2.4.1.3 at P3 and P9 respectively.

Table 3.5 Mean percentage of time that dams were engaged in observed behaviours during pup retrieval task at P3. Data are Mean $\pm$ (SEM).

	Exploring	In Nest	Sniff pup	Retrieval Attempt	Groom Pup	Crouching	Nest Build	Self Groom	Freeze
Dam (WT Litter)	46.66 (4.33)	15.37 (2.63)	9.49 (1.57)	11.48 (2.08)	1.88 (0.76)	9.51 (2.86)	4.78 (2.04)	0.00 (0.00)	0.83 (0.83)
Dam (Mixed litter)	55.36 (5.34)	11.08 (1.63)	8.34 (1.35)	10.30 (1.79)	1.20 (0.64)	9.67 (4.17)	2.60 (1.04)	1.23 (0.72)	0.22 (0.16)
Dam (Mutant litter)	56.22 (4.38)	20.97 (3.85)	5.62 (0.57)	10.16 (0.77)	0.00 (0.00)	4.48 (2.19)	1.30 (0.33)	1.14 (1.14)	0.11 (0.07)

Table 3.6 Mean percentage of time that dams were engaged in observed behaviours during pup retrieval task at P9. Data are Mean ± (SEM).

	Exploring	In Nest	Sniff pup	Retrieval Attempt	Groom Pup	Crouching	Nest Build	Self Groom	Freeze
Dam (WT Litter)	65.20	6.34	12.26	13.26	0.00	0.14	2.07	0.73	0.00
	(4.67)	(2.05)	(5.61)	(1.79)	(0.00)	(0.08)	(0.97)	(0.73)	(0.00)
Dam (Mixed litter)	60.81	16.80	4.46	10.41	0.02	1.35	2.96	3.19	0.00
	(4.07)	(2.37)	(0.86)	(1.1.8)	(0.02)	(0.81)	(1.25)	(1.95)	(0.00)
Dam (Mutant litter)	61.74	17.94	6.33	11.48	0.00	0.29	0.50	0.36	0.00
	(4.68)	(3.65)	(0.88)	(1.44)	(0.00)	(0.17)	(0.49)	(0.17)	(0.00)

During P3 pup-retrieval, dams spent the majority of time exploring the arena irrespective of the genotype of their litter, and no significant differences across the groups for the proportion of time engaged in this activity were observed ($F(2,34) = 2.93$, $p = .07$). Nor were there any significant differences in percentage of time spent engaged in any other observed behaviours between the dams, with the exception of time spent sniffing pups ($F(2,34) = 6.34$, $p = .005$) where dams of 100% mutant litters spent significantly less time engaged in sniffing their pups than both dams with WT litters ($p = .01$) and dams of mixed genotype litters ($p = .02$).

When looking at the composite scores, again fully described in section 2.4.1.3, a statistically significant difference in the percentage of time engaged in pup-directed activity at P3 was observed ($F(2,34) = 8.10$, $p \leq .001$), with dams of 100% mutant litters spending on average 17.6% (± 6.29) and 15.3% (± 4.86) less time engaging in maternal care activities than dams with WT and mixed genotype litters respectively (Figure 3.8A). Mirroring this, there was also a statistically significant difference in non-pup directed behaviour ($F(2,34) = 9.14$, $p \leq .001$) where dams of mutant litters spent 23.1% (± 4.38) and 21.04% (± 7.25) more time engaged in non-pup directed behaviour than dams with WT and mixed genotype litters respectively (Figure 3.7B). Interestingly, despite there being a significant difference in the average time taken to retrieve pups and in time engaged in pup-directed behaviour, there were no statistically significant differences in the frequency of failed retrieval attempts between the dams at P3 ($F(2,34) = .69$, $p = .51$, Figure 3.8C).

At P9, dams still spent the majority of the trial exploring irrespective of genotype, and as at P3, there was no statistically significant differences between the percentage of time that dams spent engaged in exploring ($F(2,33) = .30$, $p = .74$), nor any other of the observed of the observed behaviours. Significant differences in the percentage of time spent engaged in pup directed behaviour were not maintained at P9, ($F(2,33) = 2.09$, $p = .14$, Figure 3.8D) and no statistically significant differences in the time spent engaged in non-pup directed behaviour were observed ($F(2,33) = 1.07$, $p = .35$, Figure 3.8E). As at P3, there were no significant differences in the frequency of retrieval attempts ($F(2,33) = 1.01$, $p = .38$, Figure 3.8F).

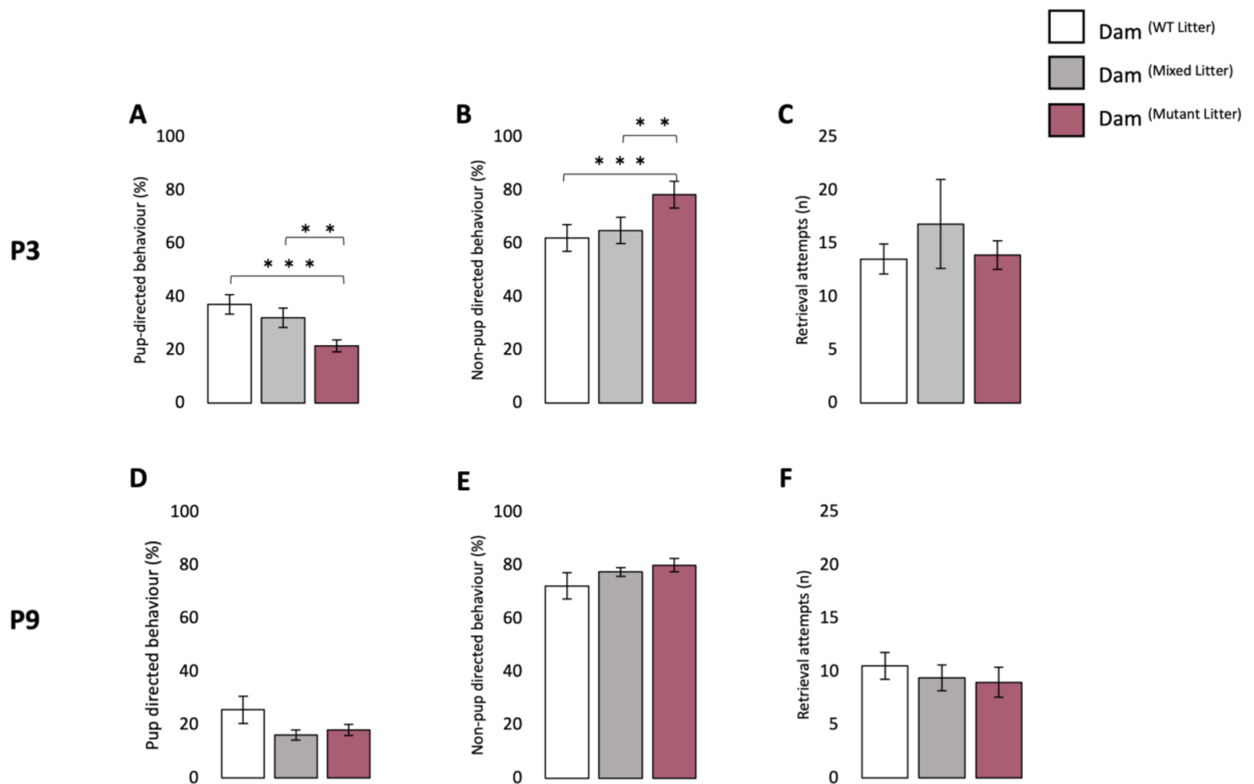


Figure 3.8 Maternal behaviour observation during the pup retrieval task. A) Average percentage of time engaged in pup-directed behaviour at P3. Dams of 100% mutant litters spent a significantly smaller percentage of time engaged in pup directed behaviour than both dams of 100% WT litters and dams with mixed genotype litters. **B**) Average percentage of time engaged in non-pup directed behaviour at P3. Dams of 100% mutant litters spent a significantly greater percentage of time engaged in non-pup directed behaviour than both dams of 100% WT litters and dams with mixed genotype litters. **C**) Average frequency of retrieval attempts at P3. **D**) Average percentage time engaged in pup-directed behaviour at P9. **E**) Average percentage time engaged in non-pup directed behaviour at P9. **F**) Average frequency of retrieval attempts at P9. Error bars represent $\pm$ SEM. ** $p \leq .01$, *** $p \leq .001$.
[a]At P9, one dam was excluded from the Dam (Mutant Litter) due to the death of 2 pups prior to the test rendering the litter too small to be tested.

3.5 Discussion

This chapter investigated the effect of offspring loss of function of *Peg3* on the maternal behaviour of WT dams. Overall, although not consistently observed across each individually measured variable, a battery of assessments and subsequent composite scores replicated results previously observed in McNamara et al. (2018a). Specifically, dams with 100% mutant litters showed greater anxiety-like behaviour and impaired maternal care compared to dams with 100% WT litters. Interestingly, for many behaviours where differences between WT (WT litters) dams and WT (mutant litters) dams were observed, maternal behaviour of dams of the mixed genotype litters did not significantly differ from dams of either single genotype litter, suggesting that the presence of WT pups produces what may be described as an ‘intermediate or attenuated phenotype’ in regard to maternal care and anxiety-like behaviours.

3.5.1 Litter size and survival rates

In the current study, dams carrying 100% mutant litters were found to have smaller litters than dams carrying either 100% WT or mixed genotype litters. It has previously been reported that in litters generated by crossing *Peg3* heterozygous males with either WT or *Peg3* heterozygous dams, that there were no significant differences between litter sizes (Curley et al., 2004). However, as litter size was not reported on in McNamara et al. (2018a), this study is the first to report how exposure to graded disruption of *Peg3* expression may affect the litter size of WT dams, with total loss of *Peg3* expression indicative of smaller litters, but partial loss having no significant effect. Regarding mortality score, the current study demonstrated that 100% mutant litters had a significantly higher mortality score than 100% WT litters when assessing the survival rates of pups from P0 – P2. Although initially, mixed genotype litters also demonstrated a higher mortality score, this did not survive Bonferroni correction. The results shown in this chapter are consistent with those observed in Curley et al. (2004) where mutant offspring raised by WT dams showed a high level of postnatal lethality compared to WT litters. Based on these results, it follows that if reduction of *Peg3* is driving the reduction of litter size in 100% mutant litters, due to direct genetic effects, then fewer mutant pups from the mixed litters should survive to P2, however, although more WT $^{\text{(mixed litter)}}$ pups survived than *Peg3KO* $^{\text{(mixed litter)}}$ pups (63 vs 45), this difference was not seen to be significant. This result should be interpretated with care given that before multiple corrections, mixed genotype litters also demonstrated a significantly higher mortality rate than WT litters, but the lack of significant difference in the survival rates between the two genotypes within the mixed litter group may be subtle evidence for the presence of WT pups attenuating the severity of phenotype observed in the fully mutant group. To test this more formally, a comparison between the proportion of *Peg3KO* $^{\text{(mixed litter)}}$ and *Peg3KO* $^{\text{(mutant litter)}}$ pups that survived to P2 should be made, however as the pups lost between P0 and P2 were not genotyped, it is not possible to accurately calculate the proportion of mutant pups from either group that did not survive.

3.5.2 Maternal Anxiety-Like Behaviours

In the first instance, at P4, dams were tested on the EZM, however, there were no significant differences in the dam's behaviour on the three traditional measures of anxiety-like behaviour used in this test: time spent in the open section; latency to enter the open section; and the frequency of crosses (Bailey and Crawley, 2009). There were however significant differences observed both in the frequency of stretch-attend postures, and the total number of fecal boli produced during the trial. Dams with 100% mutant litters performed more stretch-attend posturing compared to dams with WT litters, suggestive of greater anxiety-like behaviour. Stretch-attend posturing, though often referred to as a measure of anxiety, is also cited as a measure of active risk assessment, which occurs in response to an 'approach-avoid' conflict in mice (Mackintosh and Grant, 1963). Stretch-attend posturing may therefore reflect the general motivation of the mice to explore a potentially threatening environment, rather than an indicator of anxiety alone. As motivation is a commonly reported problem in 129sv mice, the current thesis could benefit from further experiments exploring the activity levels of the mutant dams compared to WT controls on a different background strain. However, as stretch-attend posture frequency has been shown to decrease in response to anxiolytic drugs (Molewijk et al, 1995, Kaesermann, 1986), and increase in response to anxiogenic treatments (Grewal et al, 1977, Rey et al., 2012), for the current study, as in previous literature (Holly et al., 2016) stretch-attend posturing has been used as a measure of anxiety.

Interestingly, dams of 100% mutant litters also produced significantly less fecal boli than dams of WT litters, conversely suggesting lower levels of anxiety-like behaviour. Although historically conceptualised as a measure of anxiety in mice (Hall, 1934), fecal boli production is suggested not to be a particularly robust measure of anxiety, as it may relate to other behaviours such as fear, general motor abilities or interest in a novel environment (Ramos et al., 1997, Ramos, 2008, Bailey and Crawley, 2009). As such, production of fewer fecal boli in this test may be more indicative of reduced movement or motivation to explore in the WT $^{(mutant\ litter)}$ group, as opposed to a reduction in anxiety-related behaviour.

Due to the multi-faceted nature of behavioural traits, it is recommended to use multiple measures to capture any behavioural phenotype, as one test in isolation may not be sensitive enough to detect subtle differences in the measured behaviour (Belzung and Griebel, 2001, Ramos, 2008). Maternal anxiety was therefore also assessed using the LDB at P7. As with the EZM, results were not consistent across each measure of the test, however, significant behavioural differences were still observed between the groups of dams. Specifically, dams who had carried 100% mutant litters demonstrated an increased latency to enter the light section of the arena and exhibited greater numbers of stretch-attend postures than dams of WT litters. Interestingly, in the LDB, dams with mixed genotype litters spent less time in the light section than dams with WT litters, the only measure in which this group demonstrated any statistically significant differences in maternal anxiety-like behaviour.

Differences in the anxiety profile from measures within the EZM and LDB are not uncommon and although there are no studies looking at maternal anxiety in the 129Sv strain using both the EZM and LDB, different anxiety profiles in 129Sv adult virgin mice have been observed using these two measures (Harrison et al., 2020). It has been suggested that although both the EZM and LDB measure anxiety-like behaviours generally (Ramos et al., 1997), the psychological aspects of the different tests (e.g., aversion to light vs aversion to open spaces / falling) may result in partially idiosyncratic results (Ramos, 2008), which may reflect why the results in the current study were not the same in both tests. To provide a more robust assessment of maternal anxiety a unified maternal anxiety score was generated by combining the scores from all anxiety-related measures within the EZM and LDB as described in Section 2.7.1. Using the unified score, dams of 100% mutant litters displayed a higher maternal anxiety score than dams with WT litters, suggesting that the maternal anxiety phenotype observed in McNamara et al. (2018a) was replicated in the current study.

3.5.3 Maternal Care

Pup retrieval is a commonly used measure to assess maternal care through the domain of maternal responsiveness. Whilst there are some strain specific differences (Champagne et al., 2009, Denizot et al., 2016), disruption of *Peg3* in both the mother and offspring has been shown to be associated with poor performance in the pup retrieval task in terms of increased time to display prompt maternal care, including the sniffing and retrieval of pups (Li et al., 1999, Champagne et al., 2009, McNamara et al., 2018a).

In contrast to McNamara et al. (2018a) who demonstrated that WT dams caring for *Peg3KO* pups showed increased latency to both sniff and retrieve their pups, in this study, the delay in latency to sniff and retrieve the

pups was not statistically significant. Whilst not significant in this study, a similar pattern of delay was evident between the two studies, with WT (mutant litter) dams taking on average 29.8 (± 26.91) and 45.8 (± 46.59) seconds longer to sniff and retrieve their first pup, respectively, compared to WT dams with WT litters. Interestingly, when assessing maternal anxiety, WT (mixed litter) dams appeared to demonstrate an intermediate phenotype.

All dams across all trials demonstrated nose-to-body sniffing of each pup, suggesting that the lack of significant differences between dams was not due to issues with any of the groups recognising their pups. Importantly, while it should be noted that the 129Sv strain does not always show consistently poor maternal care in response to disruption of *Peg3* as measured by the pup retrieval task (Champagne et al., 2009), it is likely that the discrepancy in results between the current study and McNamara et al. (2018a) is due to the difference in timepoint at which the task was initially performed.

In McNamara et al's (2018a) study, pup retrieval was performed at P2, whereas in the current study, pup retrieval was initially performed at P3. One of the main aims of this thesis was to characterise the early life social behaviour of *Peg3KO* pups, which involves a developmental assessment of USVs from P2-P10 (Chapter 4). Consequently, for the current study, to avoid multiple tests on one day, the pup retrieval test took place on P3 rather than P2 (see Table 3.3). As neonatal mice are born blind, deaf and without the ability to thermoregulate, prompt maternal care when separated from the nest is essential for their survival, however as mice begin to develop, the requirement for as prompt maternal care decreases (Brust et al., 2015). Although there was only one day difference between when the pup-retrieval test was carried out in the two studies, the difference in maternal responsiveness required between P2 and P3 dramatically decreases with pups beginning to develop hair and open their ears at P3 (Okon, 1970, Theiler, 1972). This may also explain why no differences in pup retrieval at P9 were observed, as in addition to having developed hearing and grown fur by P9, neonatal pups have greater motor capabilities than at P3.

Pup retrieval is not a passive process for neonatal pups, who from birth emit USVs to elicit maternal care, including retrieval when displaced from the nest (König and Markl, 1987, Champagne et al., 2007). Although McNamara et al. (2018a) observed that pups from 100% mutant litters emitted significantly fewer USVs immediately prior to the pup retrieval task than WT pups, no statistically significant difference was observed between any of the groups in the current study. The pattern of the results is consistent across both studies, with both groups of litters that had mutant pups present calling less than the 100% WT litters. Whilst the lack of a significant difference may again be due to the timepoint at which pup retrieval was carried out, it may also be a result of methodological differences. Notably, in the current study, rather than being tested in the home cage as in McNamara et al. (2018a), USVs were recorded within an isolation chamber to reduce background noise. Despite this, the average number of USVs emitted in each group was almost twice that of McNamara et al. (2018a) which suggests that the disturbance caused from moving the pups to the isolation chamber and back may have influenced USV emission, as in addition to maternal isolation, USVs may be induced by stress and a reduction of body temperature (Elwood and Keeling, 1982).

As previously mentioned, although USV emission has been extensively linked to eliciting maternal care (Scattoni et al., 2009), USV call rate is not the only factor to affect pup retrieval. Previous research has shown that although

pup weight and developmental stage affects retrieval preference in rats in the early postnatal stages, by P9, males are retrieved quicker (Deviterne and Desor, 1990). Further, in mice, pup genotype has been shown to effect maternal preference during the retrieval task as early as P4 (Bowers et al., 2013a). Although no preference for sex was seen at either timepoint, it was observed that at P3, pups from 100% WT litters were retrieved quicker than pups from 100% mutant litters when looking at average time retrieved as opposed to the time taken for the first or last pup to be retrieved. Whilst the time taken to retrieve the first or last pup is most commonly used as an indicator of the quality of maternal responsiveness, as previously highlighted in this experiment, average time to retrieve *each* pup was used to ensure that both sexes and all genotypes were represented when analysing maternal responsiveness. Appendix A5 demonstrates that the difference between the average time and time to retrieve the last pup, and by proxy complete the trial was not due to a difference in retrieval time between each pup retrieved. Consequently, despite having adequate power to detect a small-moderate effect of litter genotype (Section 3.3.4) on retrieval time of the last pup, it is likely that the significant result using average retrieval time is a result of an increased sample size, and statistical power from using each pup in the analysis.

One of the main aims of this chapter was to determine whether dams could discriminate between mutant pups and WT pups within the same litter. While dams were slower to retrieve mutant pups in 100% mutant litters versus all WT litters, when looking at average retrieval time, WT $^{\text{(mixed litter)}}$ dams showed no statistical difference in the retrieval time between their mutant or WT pups. This data can be interpreted as subtle evidence that disruption of offspring *Peg3* prenatally programs deficits in maternal care via, as discussed in Chapter 1, the placental deficits in this model. Alternatively, the maternal care deficit in the pup retrieval task may still be a result of the USV deficit in *Peg3KO* $^{\text{(mixed litter)}}$ pups, but the dam cannot distinguish which pups are calling less during the retrieval task. An important caveat to the latter, however, is that USVs have not been individually characterised for either *Peg3KO* $^{\text{(mixed litter)}}$ or WT $^{\text{(mixed litter)}}$ mice, the assumption being that *Peg3KO* $^{\text{(mixed litter)}}$ pups will show the same deficit as *Peg3KO* $^{\text{(mutant pups)}}$. As recent research has shown that the presence of mutant pups can alter WT littermate behaviour and vice versa (Kalbassi et al., 2017, Harrison et al., 2021), it may be that the pups in the mixed litter may not behave as expected based on the phenotypes observed in single genotype litters. This is discussed further in Section 3.5.4.

As in previous studies, maternal behaviour was also recorded during the pup retrieval task (Li et al., 1999). Dams of 100% mutant litters were found to spend significantly less time engaged in pup directed behaviour than both dams of WT and mixed genotype litters. Previous research has suggested that poor pup retrieval is not predictive of poor postnatal maternal care more broadly (Curley et al., 2008) and there have been similar instances of research failing to see differences in pup retrieval behaviour but observing differences in pup orientated behaviour when exploring the effect of offspring gene dosage on maternal behaviour (Creeth et al., 2018). During pup retrieval there is a conflict between the time spent retrieving each pup, and the time spent nurturing the pups that already have been retrieved and care of the dam herself. As WT $^{\text{(mutant litter)}}$ dams spent less time engaged in pup-directed activity than dams with WT litters, this further supports the work of McNamara et al. (2018a) suggesting reduction in offspring *Peg3* expression disrupts the provision of postnatal maternal care. WT $^{\text{(mutant litter)}}$ dams also displayed significantly less pup-directed activity than WT $^{\text{(mixed litter)}}$ dams, which may suggest that the presence of WT pups in the litter attenuate the maternal care deficits observed in the WT $^{\text{(mutant litter)}}$ dams. However, it should be noted

that the allocation of maternal care towards a particular genotype was not considered within the mixed litter group, due to the difficulty of distinguishing between mutant and WT pups when in the nest. Therefore, it may be that though dams did not discriminate between pups based on USVs, other factors may play a role and more care was given to the WT pups during this task. Future research would benefit from investigating this further. Finally, although no differences in maternal care were found at P9, this is not unsurprising, given that maternal behaviour is more intense at earlier timepoints in rodents (Champagne et al., 2007).

3.5.4 Limitations

Originally, the goal of this chapter was to provide the foundation for an extensive cross-fostering study, with the aim of disentangling where the influence of reduced offspring *Peg3* expression is most disruptive to maternal care behaviour, prenatally, via the placental endocrine system, or postnatally, due to the behavioural phenotype of the offspring. By cross fostering the 100% mutant litters to WT dams who had carried and given birth to 100% WT litters, this would mean that one group of dams would have only prenatal exposure to offspring with loss of expression of *Peg3* and the other, predominately postnatal exposure. Behavioural characterisation of both the dams, and offspring would then have aided in teasing out whether the influence of *Peg3* disruption is more severe pre- or postnatally.

However, due to the time constraints imposed by Covid-19, and the difficulties in breeding the experimental cohorts, this was unable to be carried out. Consequently, the biggest limitation to this study, and the work of McNamara et al. (2018a) remains the lack of a cross-fostering study. Further studies would therefore benefit from the inclusion of an extensive cross-fostering experiment to isolate the pre- and postnatal influence of *Peg3* and to fully explore whether the dams of mixed litters display attenuated phenotypes.

The lack of clear differences between the mixed genotype and single genotype groups may also be due to the fact that *Peg3* has a sexually dimorphic placental endocrine phenotype, where the placenta of males is more severely affected (Tunster et al., 2018). Consequently, if females are less severely affected, then only a quarter of the offspring in the mixed litter group would effectively be exerting an influence on maternal behaviour through the expression of *Peg3*. Ideally, mixed litters would have only been included for behavioural analysis if there was an equal distribution of both sex and mutant pups, however, as can be seen in Table 3.4, the number of mixed genotype litters required to generate enough pups for the three experiments was more than double that of the other two groups, and so, whilst optimum, this was not feasible given time, money, or the ethical constraints of breeding so many mice.

As discussed earlier, a limitation of the inclusion of the mixed genotype litter within this study is that despite research suggesting that within mixed genotype litters the behaviour of mutant pups may alter the behaviour of WT pups and vice versa (Kalbassi et al., 2017, Harrison et al., 2021), the behaviour of either genotype pup within the WT $^{(mixed\ litter)}$ group has not been individually characterised. This is problematic, as without a characterisation of the behaviour in these groups, we do not know whether the WT $^{(mixed\ litter)}$ pups exhibit 'normal' behaviour, or whether the *Peg3KO* $^{(mixed\ litter)}$ pups show the same deficit observed in mutant pups from single genotype litters.

As such, we cannot say for certain a) whether WT pups within the mixed litters do attenuate the observed maternal phenotypes and b) whether the influence of offspring *Peg3* disruption has more of an impact on maternal care prenatally *in utero,* or postnatally through the offspring's altered behaviour. Consequently, the behaviour of both pups within the mixed litter genotype was characterised in the following chapter (Chapter 4).

Finally, although the reasons for using a different time point to McNamara et al. (2018a) for the pup retrieval task have been explained previously, for more accurate comparisons between the two studies, it would be beneficial to repeat the pup retrieval task at the same timepoint, with a separate behavioural cohort. However again, due to time and financial constraints this was not possible.

3.6 Summary

The key findings from this chapter are presented in Table 3.7

Table 3.7 Summary of key findings from Chapter 3

Analysis	Key Findings
Maternal Anxiety	• WT dams, carrying mutant *Peg3* litters demonstrated significantly increased anxiety-like behaviour compared to WT controls with WT litters. • WT dams, carrying mixed genotype litters which include *Peg3* mutant offspring, demonstrated impairments in anxious behaviours compared to WT controls, though to a lesser degree than dams carrying fully mutant litters.
Maternal Care	• WT dams, carrying mutant *Peg3* litters were significantly slower to retrieve their pups on average, and engaged in less pup directed activity than WT controls with WT litters • WT dams, carrying mixed genotype litters spent significantly less time engaged in pup directed behaviour than WT controls. Potentially indicative of impaired maternal care, albeit to a lesser extent than WT dams carrying 100% mutant litters. • WT dams did not appear to distinguish between the sex or genotype of their pups during the pup retrieval task.

The first aim of this chapter was to replicate the maternal anxiety-like phenotype and maternal care deficit observed in dams of 100% mutant litters compared to WT dams with WT litters in McNamara et al. (2018a). Using both unified and composite scores, overall, the current study has replicated these phenotypes, albeit with some caveats in that each individual measure did not consistently reflect this. Notably, although for many of the individual measures, particularly those on the pup retrieval task (Figure 3.5 B,C,D) and USVs (Figure 3.6 A,C) the difference between dams [(mutant litter)] dams and dams [(WT litter)] were not statistically significant at the alpha level of p = .05, the pattern of data does indicate that there is a disruption to both maternal care and USV emission in the 100% mutant litters. However, this may be masked by the presence of the third group (mixed genotype litter) in the analyses, which is apparent when looking at the unified scores.

The second aim of this chapter was to characterise the maternal behaviour of dams [(mixed litter)] to assess whether the presence of WT pups in a litter attenuated maternal anxiety-like and maternal care phenotypes. In many tests where differences were observed between dams of single genotype litters, dams of mixed litters did not demonstrate differences compared to either group. In addition, dams of mixed litters spent a greater percentage of time spent engaged in pup-directed activity compared to dams of 100% mutant litters. Together, this provides some evidence to suggest that the presence of WT pups in the mixed genotype litters do attenuate, to an extent, the behaviour of WT dams. Finally, the last aim of this chapter was to explore whether dams of mixed litters could discriminate between the genotypes of their pups, since mutant pups from heterozygous litters had previously been observed to emit fewer USVs. Evidence from the pup retrieval task suggests that this is not the case, as in dams with mixed litters, there were no significant differences in the latency to sniff or retrieve WT versus mutant pups in dams with mixed litters.

4

Chapter 4: The influence of *Peg3* disruption on social behaviour in mice

4.1 Overview

In Chapter 3, it was demonstrated that WT dams carrying litters consisting of *Peg3KO* pups showed increased levels of maternal anxiety, and decreased levels of maternal care, compared to WT dams with WT litters. It was also shown that WT dams carrying mixed genotype litters, and consequently exposed to half the levels of offspring *Peg3* gene disruption, demonstrated an 'intermediate' form of atypical maternal care. Though it has been well established that atypical maternal care affects a myriad of offspring outcomes, including social behaviour, this remains unexplored in the *Peg3KO* model.

This chapter investigates the social behavioural phenotypes of *Peg3KO* mice raised under the atypical conditions of maternal care explored in Chapter 3. For the first time, this chapter characterises social behaviour of *Peg3KO* mice from pre-weaning (P2) through to early adulthood. Importantly, by including offspring from mixed genotype litters who were also raised under atypical maternal care conditions (Chapter 3), this chapter began to explore whether changes in behaviour were due to intrinsic loss of *Peg3* expression, or due to the abnormal pre- and postnatal maternal environment.

The quality of maternal care is well established to have long-lasting effects on later life behaviour for offspring, impacting social and sexual behaviours (Cameron et al., 2008, Bondar et al., 2018), and the quality of maternal care provision to the next generation (Curley et al., 2008). Disruption of *Peg3* has been extensively linked to maternal care deficits (Li et al., 1999, Curley et al., 2008, Champagne et al., 2009, Chiavegatto et al., 2012, McNamara et al., 2018a), and to deficits in social behaviour, though with the latter, this research has almost exclusively focused on olfaction recognition and sexual behaviour in adult mice (Swaney et al., 2007, Swaney et al., 2008, Champagne et al., 2009). For example, male *Peg3*$^{(+/-)}$ mice have been shown to demonstrate deficits in

the ability to discriminate between sexually receptive and unreceptive females and have also shown reduced improvement in sexual behaviour with experience (Swaney et al., 2007, Swaney et al., 2008). Female *Peg3*$^{(+/-)}$ mice show deficits in the ability to discriminate between the urine of different strains (Champagne et al., 2009), and are slower to enter puberty than their WT counterparts.

Research into characterising social behaviour more broadly in the *Peg3KO* model is lacking. Studies exploring the effects of *Peg3* disruption in neonatal mice have mostly focused either on survival rates, physiological effects such as weight gain or suckling behaviour, or the effects of this disruption in the mutant mother (Li et al., 1999, Curley et al., 2004). Only one study to date has explored the effects of loss of *Peg3* on social behaviour in early life (McNamara et al., 2018a). Although in the context of maternal responsivity, as discussed in Chapter 3, McNamara et al. (2018a) found that fully mutant *Peg3* litters emitted significantly fewer USVs than pups from WT litters during the pup retrieval task. As the dams in this study were genetically WT, this provided the first evidence that disruption of *Peg3* in the offspring may produce early life social deficits. Importantly however, the sex of the pups was not accounted for, a factor which has previously been shown both to influence maternal responsivity and USV call emission in previous research (Moore and Morelli, 1979, Deviterne and Desor, 1990).

The behaviour of the offspring is just one lens through which to explore the effects of both gene disruption and atypical maternal care, as exposure to these factors may not manifest behavioural effects and instead result in changes at the level of the transcriptome alone. Many studies have identified tissues in which *Peg3* is highly expressed, with the hypothalamus, hippocampus, placenta, and ovaries being the most prominent (Li et al., 1999, Kim et al., 2013). In addition, due to *Peg3*'s role as a transcription factor, there have been a number of studies investigating its downstream target genes, implicating *Peg3* in the regulation of fetal growth, maternal care, and the development of placental endocrine lineages (Broad and Keverne, 2011, Kim et al., 2013, Thiaville et al., 2013, He et al., 2016, Tunster et al., 2018). However, few studies have explored differential gene expression changes in response to loss of expression of *Peg3* beyond the embryonic stage. Further, as in the behavioural studies, little research has explored whether male and female mice are affected in the same way by *Peg3* disruption.

Broadly, in WT mice a number of imprinted genes have been observed to be more highly expressed in males than in females, including *Peg3* (Faisal et al., 2014), although this was only observed in embryo heads at E14.5, and not at E10.5 or in neonate brains at P5. Consistently, there is evidence to suggest that *Peg3* disruption produces sexually dimorphic physiological and placental endocrine phenotypes (Kim et al., 2013, Tunster et al., 2018). Notably, female mutant mice are slower to enter puberty (Curley et al., 2005) and loss of function of *Peg3* in mouse pups consistently results in both lighter fetuses and placenta (Li et al., 1999, Curley et al., 2004, Kim et al., 2013, Denizot et al., 2016), phenotypes which are more pronounced in male mutant mice (Kim et al., 2013). Further, at the molecular level, using RNA scope technology, Tunster et al. (2018) reported that male mutant *Peg3* placenta show a substantial loss of the spongiotrophoblast and glycogen cell lineages, whilst in females, this loss was less severe.

One study utilising genome wide analysis (via microarray) reported differential gene expression in E14.5 embryonic heads and placentas between *Peg3* mutant and WT pups from a mixed genotype litter, generated by crossing a WT female and *Peg3* heterozygous male (Kim et al., 2013). In the embryo head, but not the placenta, affected genes clustered onto two pathways. Firstly, one involved with lipid metabolism consistent with previous research linking *Peg3* to growth rate regulation, and secondly, one linked to placenta specific gene families, suggesting that *Peg3* is involved in the transcriptional control of these gene families. The same study also demonstrated a sexually biased effect of *Peg3* disruption, whereby the up-regulation of placenta-specific gene families was more apparent in females than males. Although this is one of few studies exploring genome-wide effects of the disruption of offspring *Peg3*, there are a number of limitations to Kim et al.'s. (2013) study. Firstly, replicates for the microarray analysis were only obtained from one litter. 'Litter-effects', where all test subjects are obtained from the same litter, are a common problem in rodent research. Studies have shown that litter effects can account for up to 61% of the variation in a number of outcomes (Lazic and Essioux, 2013). This is particularly true in gene expression analyses. Animals from the same litter are often considered to be 'technical replicates' as opposed to 'biological replicates' given their similarity to each other, and so do not accurately reflect the biological variation in the population, leading to incorrect inferences about the data (Blainey et al., 2014). Secondly, previous research has shown that within mixed genotype litters, even genetically WT pups can be affected at both the brain and behavioural level by atypical maternal environments induced by placental endocrine insufficiency (Harrison et al., 2021). As such, comparing mutant pups with WT pups from mixed genotype litters as in (Kim et al., 2013) does not necessarily provide an accurate control group when exploring the effect of intrinsic loss of function of *Peg3*.

In summary, despite *Peg3* disruption being extensively linked to deficits in maternal care, and both male and female adult mutant mice showing deficits in social olfactory ability, the effect of loss of function of *Peg3* on early life social behaviour is yet to be fully characterised. Secondly, though some gene expression analyses exploring the effect of *Peg3* disruption have been carried out, the majority of these have been at the embryonic stage of development, have not included robust controls, or have used microarray as opposed to next generation RNA sequencing. Consequently, this chapter aimed to address these issues, characterising social behaviour from P2 to adulthood, and conducting exploratory RNA-sequencing analysis on the hypothalamus and olfactory bulb in neonatal (P6) pups. These regions were chosen as they have been shown to demonstrate *Peg3* expression in adult mice and have been implicated in both maternal care and aspects of social behaviour (Li et al., 1999, Kim et al., 2019). P6 was chosen as the time-point to reflect the typical peak of USV emission trajectory (Scattoni et al., 2009), (the easiest measure of social behaviour in neonatal pups) and to allow for exposure to the postnatal maternal environment.

4.2 Aims

This chapter aimed to explicitly characterise the social behaviour of *Peg3KO* pups from P2 through to early adulthood. Secondly, this chapter aimed to examine gene expression differences in the hypothalamus and the olfactory bulb in *Peg3KO* mice through exploratory RNA-sequencing analysis.

For both the behavioural and sequencing experiments, this chapter explored how social behaviour and gene expression was affected by intrinsic loss of function of *Peg3* in mutant mice compared to their WT counterparts. To enable a more nuanced comparison, this chapter also explored the differential effects of both offspring disruption of *Peg3* and the effect of an atypical maternal environment, by including mutant and WT pups raised in mixed litters. Inclusion of the mixed litter group, in addition to mutants raised in single genotype litters, and fully WT controls, allowed for an assessment of whether behavioural and transcriptional changes were due to the adverse pre- and postnatal environment caused by placental endocrine insufficiency, rather than the specific gene change of reduced *Peg3* expression. Finally, the effect of *Peg3* disruption in male and female mice was explored in a similar way, at both the behavioural and gene expression level.

4.3 Methods

4.3.1 Generation of Experimental Cohorts

Four experimental groups were generated using the offspring of dams used in Chapter 3 as described in Chapter 2. For ease of interpretation, the mating set up each that group was generated from, the genotype, and the nomenclature used to differentiate between the groups are redefined in Table 4.1.

Table 4.1 Details of experimental groups and their generation

Nomenclature	Parents (dams x stud)	Test mouse genotype
WT $^{(\text{WT litter})}$	WT x *Peg3KO* $^{(+/+)}$	WT
WT $^{(\text{mixed litter})}$	WT x *Peg3KO* $^{(-/+)}$	*Peg3KO* $^{(+/+)}$
Peg3KO $^{(\text{mixed litter})}$	WT x *Peg3KO* $^{(-/+)}$	*Peg3KO* $^{(+/-)}$
Peg3KO $^{(\text{mutant litter})}$	WT x *Peg3KO* $^{(-/-)}$	*Peg3KO* $^{(+/-)}$

As in Chapter 3, inclusion criteria for analysis included pups obtained from litters containing between 4 and 10 pups and, for the mixed litter group, between 45-65% of the pups were required to be mutant, as identified by genotyping at P14. Table 4.2 shows the number of mice used to assess preweaning social behaviour.

Table 4.2. Number of mice used to assess preweaning social behaviour

	WT	WT $^{(\text{mixed litter})}$	*Peg3KO* $^{(\text{mixed litter})}$	*Peg3KO* $^{(\text{mutant litter})}$
Male	41	18	20	25
Female	39	17	20	28
Total	80	35	40	53

Due to the time constraints of age matched testing, only a subset of mice were taken forward to assess post-weaning offspring social behaviour (Table 4.3). As described in Chapter 2, at weaning, offspring to be used to assess post-weaning social behaviour were housed in same sex cages of four, preferentially keeping mice from the same birth litter together. To maintain consistency in the prenatal environment, offspring from the mixed litters were, where possible, housed into cages containing two WT $^{(\text{mixed litter})}$ pups and two *Peg3KO* $^{(\text{mixed litter})}$ littermates. Where this was not possible, mice in the mixed litter group were housed so there was at least one mouse of each genotype in the cage.

Table 4.3. Number of mice used to assess post-weaning social behaviour

	WT	WT (mixed litter)	*Peg3KO* (mixed litter)	*Peg3KO* (mutant litter)
Male	16	16	13	14
Female	20	15	13	14
Total	36	31	26	28

4.3.2 Behavioural Assays

Full details of the behavioural tests used to characterise both pre- and post-weaning social behaviour and the order in which they were performed are described in Section 2.4.1

4.3.3 RNA Sequencing

Hypothalamus and olfactory bulb tissues were dissected at P6 as described in Chapter 2. Table 4.4 shows the experimental groups and number of biological replicates per group. To avoid litter effects, pups from single genotype litters were obtained from 6 separate litters, 1 male and 1 female from each, whilst 12 litters were used to generate the mixed litter pups from which brain regions were dissected.

Table 4.4. Experimental groups and number of biological replicates per group for both the hypothalamus and olfactory bulb.

	WT	WT (mixed litter)	*Peg3KO* (mixed litter)	*Peg3KO* (mutant litter)
Male	6	6	6	6
Female	6	6	6	6
Total	12	12	12	12

Figure 4.1 shows the analysis pipeline for all sample types. Reads were first trimmed to remove any low-quality base calls and any adapter sequences using Trimgalore (Krueger et al., 2021). Quality checks were then implemented in FastQC (Andrews, 2010). All samples passed initial quality checks as assessed by per base sequence quality, per sequence quality score and per sequence GC content generated in the FastQC report. Reads were then mapped to the mouse reference genome (GRCm39) using STAR (Dobin and Gingeras, 2015). Finally, read counts were generated using featureCounts (Liao et al., 2013), allowing genomic features to be allocated to mapped reads using the mouse Ensembl gene annotation (GRCm.39.104). Differential gene expression was performed using the Limma Bioconductor software package with voom weighting (Ritchie et al., 2015). The Benjamini Hochberg correction was used to correct for multiple testing and identify genes that were differentially expressed between groups ($p_{adj} < .05$). Following this, Gene Ontology and MGI Mammalian Phenotype enrichment analyses (Ashburner et al., 2000, Smith and Eppig, 2009) were conducted using EnrichR (Chen et al., 2013).

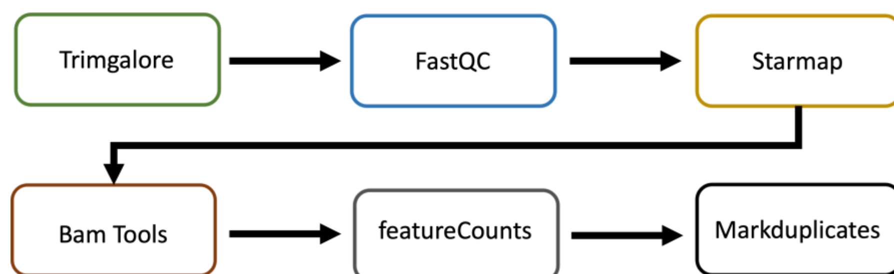


Figure 4.1 Pre-processing pipeline for RNA-Sequencing analysis

4.3.4 Statistics

Initially, a series of two-way ANOVAs were planned to analyse the effect of genotype and sex on a number of social behaviours. Power analyses were performed using the National Centre for the Replacement, Refinement and Reduction of Animals in Research Experimental Design Assistant (EDA) (Percie du Sert et al., 2017). As recommended by Wahlsten (2010), a standardised effect size (d) of 1.0 was used in order to detect moderate effects, whilst a value of 1.0 was also used as the estimate of variability as recommended by the EDA given the lack of availability of reported variability values (SD) in previous literature. Based on these calculations, a sample size of 23 mice per group was predicted to be sufficient for power ($1 - \beta$) = .09 with a type 1 error rate ($\alpha = .05$) using two-way ANOVAs. However, due to time constraints imposed by COVID-19, the required sample size for each group was not generated for this experiment (Table 4.3). In light of this, given previous literature in both mice and human studies suggesting that disruption of *Peg3* impacts males and females differently (Kim et al., 2013, Faisal et al., 2014, Janssen et al., 2016, Tunster et al., 2018, Sumption, 2020), each sex was treated as a distinct population and a series of one-way ANOVAs were conducted for each sex as in Savory et al. (2020). Power calculations for one-way ANOVAs exploring the effect of genotype subsequently predicted that a sample size of 12 mice per group would be sufficient to detect small-moderate effects at a type 1 error rate of $\alpha = .05$. By using one-way instead of two-way ANOVAs, the current study was unable to draw conclusions as to whether males are more severely affected by *Peg3* disruption than females explicitly, but instead compared how the effects of *Peg3* disruption manifested in each sex.

Differential gene expression was analysed in two ways. Firstly, for both the hypothalamus and olfactory bulb, replicates were initially analysed together. Secondly, given previous research suggesting evidence of sex differences in the impact of *Peg3* disruption on gene expression (Kim et al., 2013, Faisal et al., 2014), replicates were then divided into male and female groups and were analysed separately to explore if the same genes were affected in males and females. Finally, for both the combined, and sex-specific analyses, enrichment analyses were carried out using Gene Ontology (Ashburner et al., 2000, GeneOntology Consortium., 2021) and Mammalian Phenotype Ontologies (Smith and Eppig, 2009) to explore which pathways, if any, differentially expressed genes mapped to. Given the exploratory nature of these analyses, where less than 100 differentially expressed genes (DEGs) were found using the criteria of $p_{adj} < .05$, inclusion criteria for enrichment analyses were extended to include genes that were differentially expressed at the level of $p_{non\text{-}adj} < .05$.

4.4 Results: Pre-weaning behaviour

4.4.1 Preweaning weight

At P2, weight data for female WT $^{(mixed\ litter)}$ and *Peg3KO* $^{(mutant\ litter)}$ pups were not normally distributed, nor were data for either sex in the WT and WT $^{(mixed\ litter)}$ groups at P4. Despite this, due to the robustness of ANOVAs to violations of normality (Maxwell, 1980, Maxwell et al., 2017), a two-way mixed ANCOVA was run to explore the effect of genotype on weight at P2-P10 in both male and female pups, with litter size as a covariate. Greenhouse-Geisser correction values are reported due to a violation of Mauchly's assumption of Sphericity.

In males, a significant age*genotype interaction was observed [age*genotype: $F(7.36, 238.06) = 8.80$, $p < .001$, Figure 4.2]. A main effect of genotype was observed on the weight of male pups at P2 [Genotype: $F(3,100) = 7.97$ $p < .001$], P4: $F(3,100) = 3.59$ $p < .016$], P8: $F(3,99) = 9.26$, $p < .001$], and P10: $F(3,99) = 10.91$ $p < .001$] but not P6 ($F(3,100) = 2.54$ $p = .06$). Post-hoc analyses demonstrated that mutant males from both single genotype and mixed genotype litters were significantly lighter than WT male pups from single genotype litters at P2 ($p < .001$ across both comparisons). At P4, mutant males from the mixed genotype litters were significantly lighter than WTs from the same litter ($p = .03$). Whilst at both P8 and P10, mutant males from mixed genotype litters were consistently statistically lighter than males in every other group ($p < .001$ across each group at both P8 and P10, Figure 4.2A).

In females, a similar pattern of results were shown, with. a significant age*genotype interaction also being observed [age*genotype: $F(6.95, 227.14) = 10.67$, $p < .001$, Figure 4.2B]. A significant effect of genotype on the weight of female pups at all time points with the exception of P6 was also observed, [P2: $F(3,100) = 23.30$ $p < .001$], P4: $F(3,100) = 15.96$ $p < .001$, P6: $F(3,100) = 1.76$, $p = .16$, P8: $F(3,99) = 14.87$, $p < .001$, and P10: $F(3,99) = 15.46$, $p < .001$]. At both P2 and P4, mutant females from both single genotype and mixed genotype litters weighed significantly less than WTs from single genotype and mixed litter genotypes ($p < .001$ across each comparison). Whilst at P8 and P10, mutant females from the mixed genotype litters only, were significantly lighter than females from every other group ($p \leq .001$ across all comparisons).

In summary, while *Peg3KO* pups, regardless of sex or presence of WT pups in the litter, were lighter than WT controls early on, at later ages, *Peg3KO* $^{(mutant\ litter)}$ pup's growth had caught up with controls. However, mutant mice from mixed genotype litters did not show the same catch-up growth.

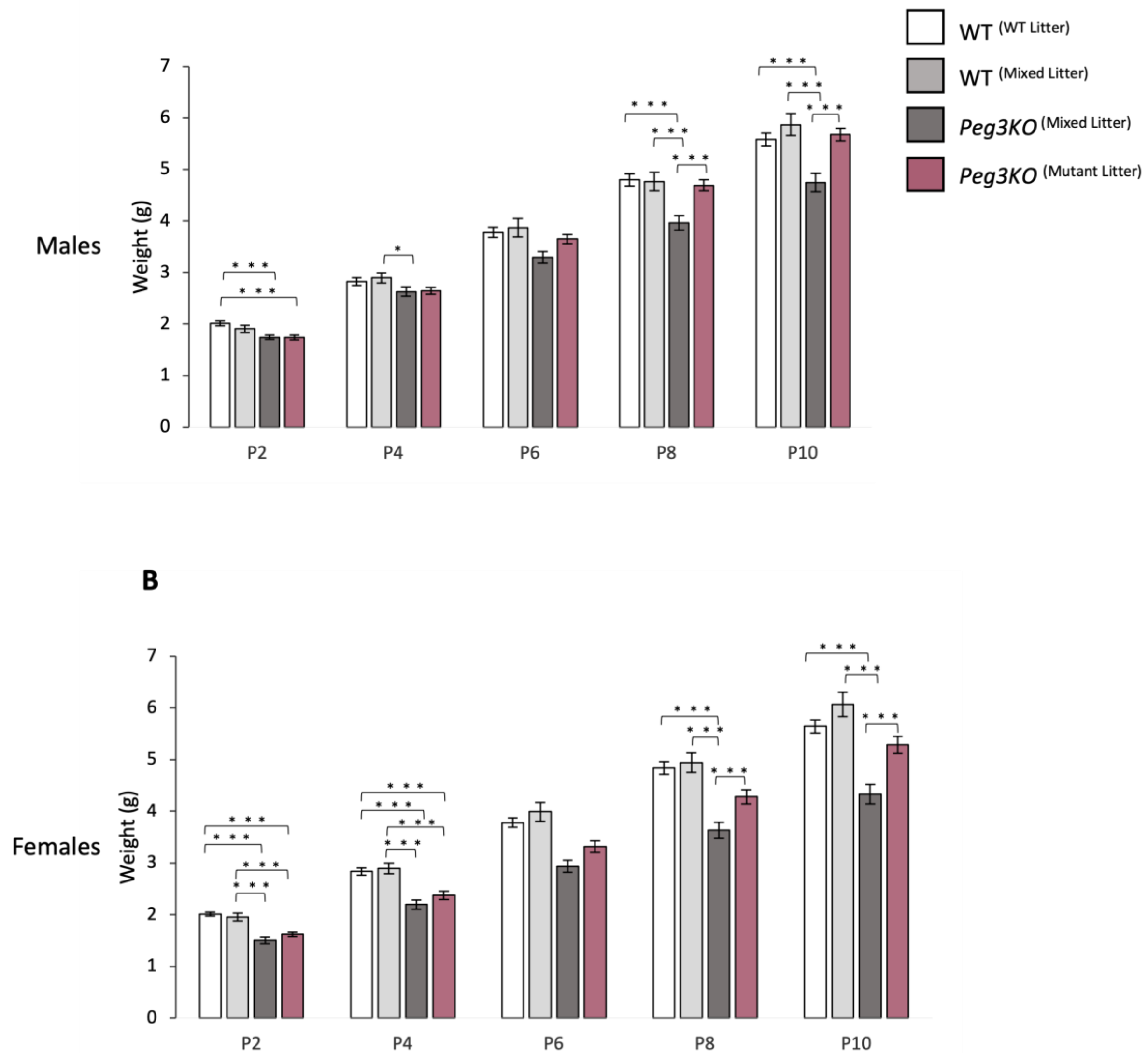


Figure 4.2 Pre-weaning weight data from P2-P10. A) Data from male pups. 2-Way ANCOVA demonstrated a significant age*genotype interaction. At P2, mutant males from single genotype and mixed genotype litters were significantly lighter than WT males. At P4, mutant males from mixed genotype litters were lighter than WT males from the same group. At both P8 and P10, mutant males from the mixed genotypes were significantly lighter than males from all other groups. **B**) Data from female pups. At P2 and P4, mutant females from both the single genotype and mixed genotype litters were significantly lighter than WTs from both groups. At both P8 and P10, mutant females from mixed genotype litters were significantly lighter than all females from all other groups. In summary, mutant mice irrespective of litter type were lighter than WT controls at earlier timepoints, but at later timepoints mutant mice from single genotype litters displayed catch up growth, whilst mutant mice from mixed litters did not. Error bars are $\pm$ SEM. * $p < .05$, *** $p \leq .001$

4.4.2 Isolation induced USVs

In addition to recording USVs for groups of pups in the home cage as part of the pup retrieval task, individual isolation induced USVs were recorded at P2, 4, 6, 8 and 10 in a sound attenuating chamber. Two-way mixed ANCOVAs, with Greenhouse-Geisser correction values, demonstrated that for both males and females, there was a significant interaction effect between timepoint and genotype on the number of calls [Males: timepoint*genotype ($F(11.09, 365.99) = 2.23$, $p = .023$), Females: timepoint*genotype ($F(3.64, 356.93) = 3.81$, $p = .012$)]. In males, although initially there was a significant effect of genotype at P2, P4 and P6, only the effect at

P4 (F(3,100) = 4.51, p = .005, Figure 4.3A) and P6 (F(3,100) = 5.01, p = .003, Figure 4.3A) remained significant after correcting for multiple comparisons. As can be seen in Figure 4.3A, at P4, mutant males from single genotype and mixed genotype litters called significantly fewer times than WT males, with *Peg3KO* $^{(mutant\ litter)}$ males making 79.89 ($\pm$ 18.60, p = .019) fewer calls and *Peg3KO* $^{(mixed\ litter)}$ males making 87.63 ($\pm$ 22.88, p = .014) fewer calls on average compared to WT males across the 3-minute trial. At P6 however, the only significant difference was found between WT males and *Peg3KO* males from single genotype litters with *Peg3KO* $^{(mutant\ litter)}$ calling on average 116.20 ($\pm$ 15.76, p = .002) times fewer across trials than WT males.

In females, the only significant effect of genotype was seen at P2 (F(3,100) = 3.810, p = .012, Figure 4.3C) with WT females calling on average 51.8 ($\pm$ 12.47) times more frequently than *Peg3KO* $^{(mutant\ litter)}$ females (p = .01). There was also a significant main effect of timepoint (age) in both males (F(3.69, 365.99) = 27.14, p <.001) and females (F(3.64, 356.93) = 27.49, p <.001). The general pattern of USV emission showed a reduction in all groups by P10 (see Figure 4.3), supporting previous reports by Scattoni et al., (2009, 2011).

In terms of the latency to call, there was a significant interaction observed between timepoint and genotype in males (F(6.31, 211.45) =2.21, p = .04). However, in males, differences in latency were only observed at P4 where there was a main effect of genotype (F(3,100) = 4.51, p = .01, Figure 4.3B) with both groups of mutant males taking longer to call than WT controls (p = .014 and p = .019 for *Peg3KO* $^{(mutant\ litter)}$ and *Peg3KO* $^{(mixed\ litter)}$ males respectively). Whilst a main effect of genotype was also seen at P2 (F(3,100) = 3.21 p = .026, Figure 4.3D) , no comparison survived post hoc corrections. As with number of calls, there was a significant effect of timepoint on the latency to call in males, with the latency to call significantly increasing across timepoints (age). This was also the case in females, (F(2.29, 219.59) = 42.59, p <.001). However, in females, no significant main effect of genotype was observed (F(3,100) = 2.03, p = .115). Notably, these results were independent from the change in the pup's body temperature from pre- to post-testing which was included as a covariate for all analyses (see Appendix A6 for the average change in pup body temperature across each timepoint).

When looking at the total number of calls averaged across each timepoint tested, one-way ANCOVA demonstrated that there was a significant main effect of genotype in males (F(3,100) = 5.31, p = .002), with *Peg3KO* $^{(mutant\ litter)}$ males emitting significantly fewer calls in total across P2-P10 than WT males (p < .011, Figure 4.3E). In females, no significant differences between the groups were observed (F(3,100) = .83, p = .67, Figure 4.3F).

To summarise, over the 5 time-points, *Peg3KO* $^{(mutant\ litter)}$ male pups were shown to vocalise significantly less than WT controls. However, when considering each timepoint individually, this deficit was restricted to P4 and P6 for *Peg3KO* $^{(mutant\ litter)}$ male pups and P4 for *Peg3KO* $^{(mixed\ litter)}$ male pups. This social deficit was not apparent in *Peg3KO* $^{(mutant\ litter)}$ nor in *Peg3KO* $^{(mixed\ litter)}$ female pups.

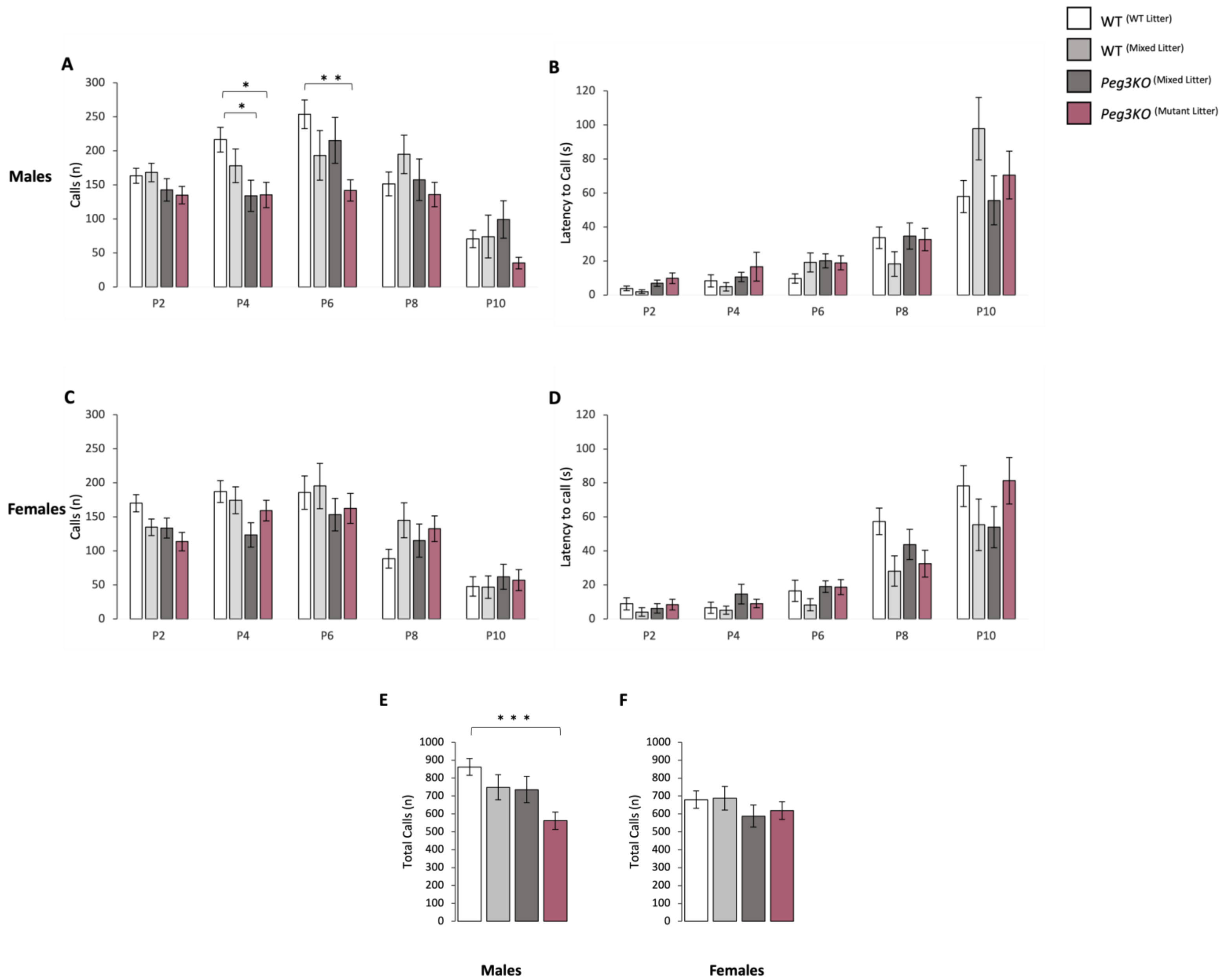


Figure 4.3 Data from isolation induced USVs across P2-P10 in male and female pups. Data is presented from isolation induced USVs in Males (**A,B,E)** and Females (**C,D,F**). **A and C)** Number of calls at each timepoint. Two-way mixed ANCOVA demonstrated that at P4 mutant males from single genotype and mixed genotype litters called significantly less than WT male controls. At P6 mutant males from single genotype litters persisted in making significantly fewer calls than WTs from single genotype litters. **B and D)** Latency to call at each timepoint. There were no significant differences observed in either males or females. **E and F)** Averaged calls across each timepoint. *Peg3KO* (mutant litter) males called less than WT on average across all timepoints, with no further significant differences observed in females. Error bars are ± SEM.

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

4.4.3 Homing behaviour

No differences in the total time spent within 1 cm of the home bedding was observed in either males (F(3,99) = 2.02, *p* = .12, Figure 4.4A) or females (F(3,99) = 4.81, *p* = .70, Figure 4.4E) in any genotype. Likewise, there were no observed differences in the latency to enter this area in either males (F(3,99) = 1.85, *p* = .14, Figure 4.4C) or females (F(3,99) = .82, *p* = .49, Figure 4.4G). The same was also true of the time spent within 1 cm of the clean bedding: [males: F(3,99) = 1.18, *p* = .32), Figure 4.4B, females: F(3,99) = .71, *p* = .55, Figure 4.4F], and the latency to enter this zone: [males: F(3,99) = .18, *p* = .91, Figure 4.4D, females: F(3,99) = .38, *p* = .77, Figure 4.4H]. Overall, there were no significant differences observed in this test.

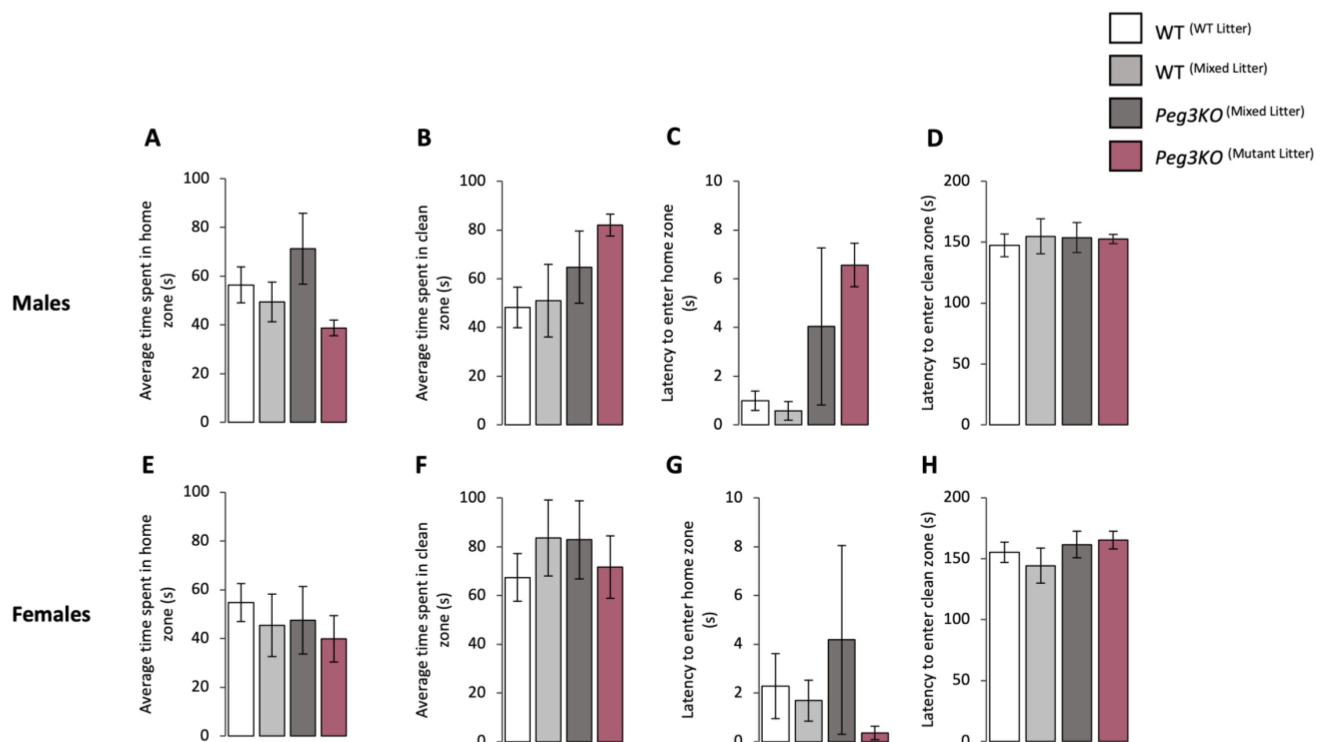


Figure 4.4. Results from the Homing Behaviour test. Data is presented from the homing behaviour task across males(**A-D**) and females (**E-H**). **A, E** show the average time in within 1 cm of home bedding. **B, F,** show the average time within 1 cm of the clean bedding. **C, G** show the average latency to enter the home bedding proximity and **D,H,** show the average latency to enter the clean bedding proximity. No significant group differences were found across any of the parameters measured. Error bars are ± SEM.

4.5 Results: Post-weaning behaviour

4.5.1 Post-weaning weight

Recordings of post-weaning weight begun at 4 weeks, prior to the direct social interaction test and were carried out weekly until the completion of testing. Two-way mixed ANCOVAs were run, reporting Greenhouse-Geisser values due to a violation in the assumption of sphericity. In both males (F(9.63, 176.58) = 21.70, *p* <.001 Figure, 4.5A) and females (F(6.42, 124.21) = 5.94, *p* <.001, Figure 4.5B) there was a significant interaction between

genotype and timepoint (age) on post-weaning weight. Table 4.5 shows the results for the main effect of genotype at each age in both males and females, and the resulting significant post-hoc comparisons.

Table 4.5 Simple main effects of genotype on weight across the testing period in both male and female mice.

	Males				**Females**			
Timepoint	**Main effect of genotype**			**Significant post-hoc results**	**Main effect of genotype**			**Significant post-hoc results**
	F (3,55)	**Mean Square**	***p***	**Result**	**F (3,58)**	**Mean Square**	***p***	**Result**
Week 4	25.05	17.19	< **.001**	*Peg3KO* (mixed litter) significantly lighter than all other groups (***p* <.001**)	55.02	34.82	< **.001**	*Peg3KO* (mixed litter) significantly lighter than all other groups (***p* <.001**)
Week 5	30.42	19.99	< **.001**	*Peg3KO* (mixed litter) significantly lighter than all other groups (***p* <.001**)	55.09	33.12	< **.001**	*Peg3KO* (mixed litter) significantly lighter than all other groups (***p* <.001**)
Week 6	41.29	26.29	< **.001**	*Peg3KO* (mixed litter) significantly lighter than all other groups (***p* <.001**)	55.77	34.44	< **.001**	*Peg3KO* (mixed litter) significantly lighter than all other groups (***p* <.001**)
Week 7	36.35	29.86	< **.001**	*Peg3KO* (mixed litter) significantly lighter than all other groups (***p* <.001**)	56.28	39.00	< **.001**	*Peg3KO* (mixed litter) significantly lighter than all other groups (***p* <.001**)
Week 8	46.35	41.75	< **.001**	*Peg3KO* (mixed litter) significantly lighter than all other groups (***p* <.001**)	61.18	40.44	< **.001**	*Peg3KO* (mixed litter) significantly lighter than all other groups (***p* <.001**)
Week 9	53.03	52.43	< **.001**	*Peg3KO* (mixed litter) significantly lighter than all other groups (***p* <.001**)	76.49	41.13	< **.001**	*Peg3KO* (mixed litter) significantly lighter than all other groups (***p* <.001**) *Peg3KO* (mutant litter) significantly heavier than WT (mixed litter) (***p* = .009**)
Week 10	70.69	63.02	< **.001**	*Peg3KO* (mixed litter) significantly lighter than all other groups (***p* <.001**)	75.90	49.48	< **.001**	*Peg3KO* (mixed litter) significantly lighter than all other groups (***p* <.001**) *Peg3KO* (mutant litter) significantly heavier than WT (mixed litter) (***p* < .004**)
Week 11	69.18	78.18	< **.001**	*Peg3KO* (mixed litter) significantly lighter than all other groups (***p* <.001**)	74.85	50.87	< **.001**	*Peg3KO* (mixed litter) significantly lighter than all other groups (***p* <.001**) *Peg3KO* (mutant litter) significantly heavier than WT (mixed litter) (***p* < .003**)
Week 12	67.31	89.41	< **.001**	*Peg3KO* (mixed litter) significantly lighter than all other groups (***p* <.001**)	64.98	46.56	< **.001**	*Peg3KO* (mixed litter) significantly lighter than all other groups (***p* <.001**) *Peg3KO* (mutant litter) significantly heavier than WT (mixed litter) (***p* < .011**)
Week 13	75.35	115.02	< **.001**	*Peg3KO* (mixed litter) significantly lighter than all other groups (***p* <.001**)	63.31	44.84	< **.001**	*Peg3KO* (mixed litter) significantly lighter than all other groups (***p* <.001**) *Peg3KO* (mutant litter) significantly heavier than WT (mixed litter) (***p* < .016**)
Week 14	73.46	125.94	< **.001**	*Peg3KO* (mixed litter) significantly lighter than all other groups (***p* <.001**)	126.18	58.70	< **.001**	*Peg3KO* (mixed litter) significantly lighter than all other groups (***p* <.001**) WT significantly heavier than both WT (mixed litter) (***p* <.003**)

In summary, consistent with their weight observed in the pre-weaning tests (Figure 4.2), at 4 weeks, and at each timepoint going forward, *Peg3KO* (mixed litter) mice, irrespective of sex, were significantly lighter than mice in all other groups. However, in females, from 9 weeks onward *Peg3KO* (mutant litter) females showed increased weight gain and were significantly heavier than female WT (mixed litter) mice. As shown in Figure 4.5, *Peg3KO* (mutant litter) males maintained their catch-up growth into adulthood.

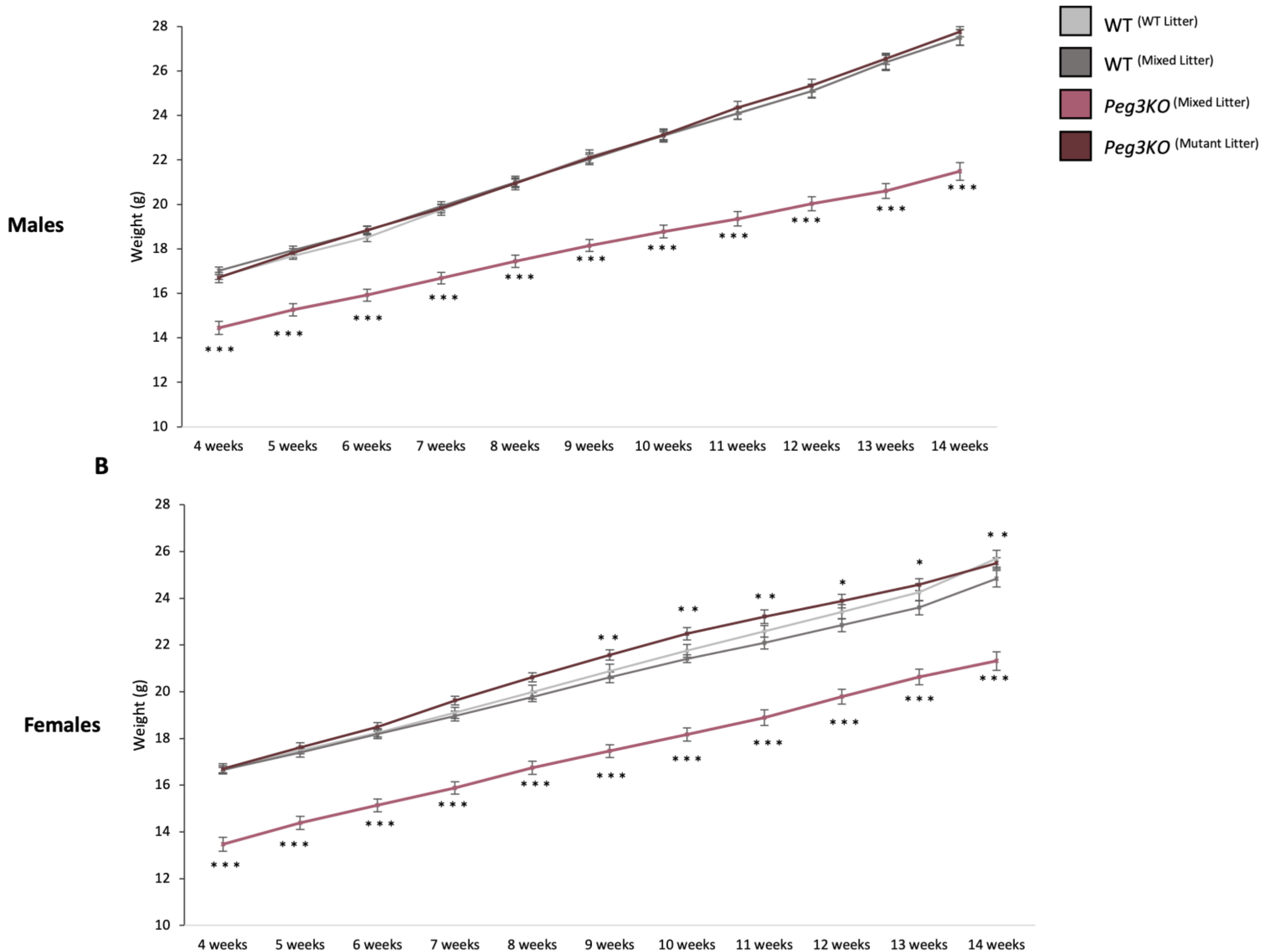


Figure 4.5. Post-weaning weight data from 4-14 weeks. Two-way mixed ANCOVAs demonstrated a significant genotype*age interaction in both males (**A**) and females (**B**). **A)** In males, *Peg3KO* (mixed litter) mice were consistently significantly lighter at each timepoint ($p < .001$). This was also true in females (**B**), however, in addition, from 9 – 13 weeks, *Peg3KO* (mutant litter) females were also significantly heavier than WT (mixed litter) mice. At 14 weeks, WT females were significantly heavier than WT (mixed litter) females. In both males and females, main effects of time showed that regardless of genotype, each group was significantly heavier in the following week. Error bars are ± SEM. *$p < .05$ **$p < .01$ ***$p < .001$

4.5.2 Direct Social Interaction

Across the direct social interaction test, females consistently showed no effect of genotype on both social ($F(3,58) = 2.08$, $p = .113$, Figure 4.6C) and non-social behaviour ($F(3,58) = 2.82$. $p = .05$, Figure 4.6D). In males however, significant differences were observed in both instances [social behaviour: $F(3,55) = 1.22$ $p = < .01$, Figure 4.6A, non-social behaviour: $F(3,55) = 2.83$, $p = .05$, Figure 4.6B]. *Peg3KO* $^{\text{(mutant litter)}}$ males and *Peg3KO* $^{\text{(mixed litter)}}$ males engaged in less social behaviour than WT males ($p <.001$). Whilst *Peg3KO* $^{\text{(mixed litter)}}$ males also engaged in less non-social behaviour, than WT males ($p <.04$).

In summary, mutant males from both mixed, and single genotype litters displayed less time engaged in social behaviour than WT males from single genotype litters. No significant differences were observed between groups of female mice.

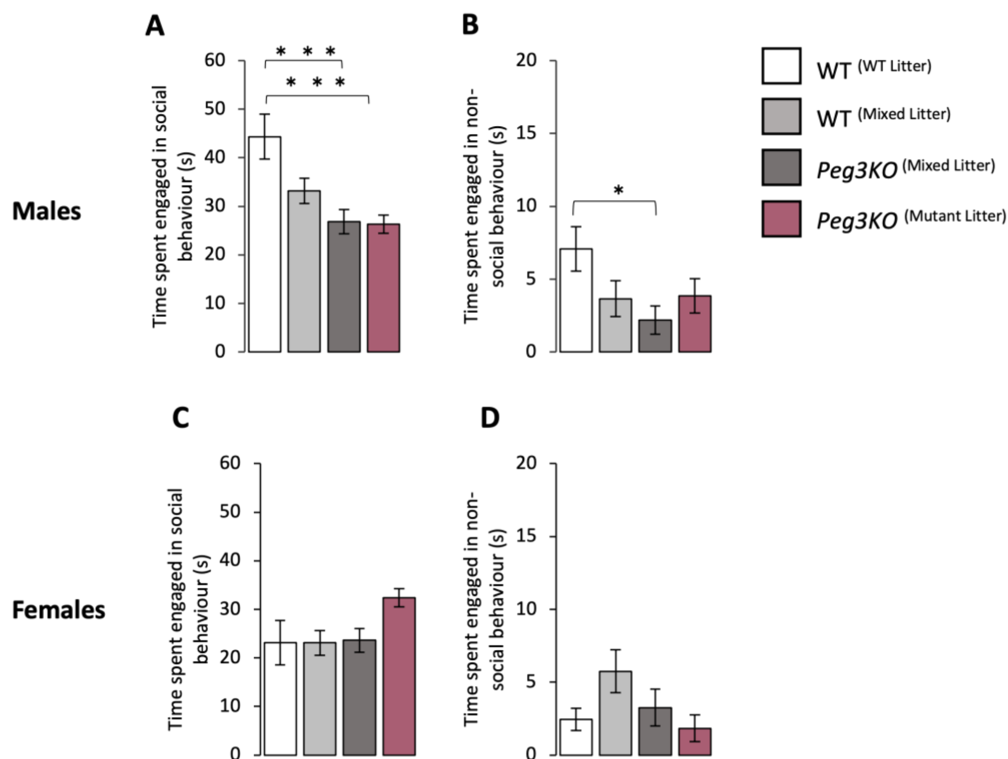


Figure 4.6. Results from the Direct Social Interaction test. Data is presented from the direct social interaction task across males (**A-B**) and females (**C-D**). **A,C** show the average time spent engaged in social behaviour. **A)** A one-way ANOVA showed that mutant males from both single and mixed genotype litters spent significantly less time engaged in social behaviour than WT males from a single genotype litter. **B,D** show the average time engaged in non-social behaviour. **C)** mutant males from mixed genotype litters spent less time engaged in non-social behaviour than WT males from single genotype litters. Data are mean ± SEM. * $p < .05$, *** $p < .001$

4.5.3 Social Propinquity

There were no differences observed in the latency to share the tube in either males ($F(3,26) = 1.17$, $p = .34$, Figure 4.5A) or females ($F(3,28) = .32$, $p = .87$, Figure 4.7D). Whilst genotype did not have a significant effect on the percentage of time that the tube was occupied by both mice in females ($F(3,28) = 1.79$, $p = .17$, Figure 4.7E), in males, significant differences were observed, ($F(3,26) = 4.62$, $p = .01$, Figure 4.5B), with *Peg3KO* (mixed litter) mice spending on average 36.59% (± 15.76, $p = .02$) more time sharing a tube than WT mice from single genotype litters. In contrast, significant differences in both males ($F(3,26) = 6.57$, $p = .002$, Figure 4.7C) and females ($F(3,28) = 4.26$, $p = .01$, Figure 4.7F) were observed in the time that neither mouse inhabited the tube, with a greater time spent sharing the tube, indicative of anxious behaviour. In males, *Peg3KO* (mixed litter) mice spent a smaller proportion of time in the tube than both WT controls ($p = .02$) and *Peg3KO* (mutant litter) males ($p = .04$). The same was also true of WT (mixed litter) males compared to WT controls ($p = .03$) and *Peg3KO* (mutant litter) males ($p = .04$). In contrast, in females, the only significant difference was observed between WT and *Peg3KO* (mixed litter) mice, with *Peg3KO* (mixed litter) females spending a smaller proportion of the trail with the tube fully vacant that WT females ($p = .02$). Overall, these findings suggest that though mutant male mice from mixed litters were more social than WT males from WT litters, males from both genotypes in the mixed litter group were more anxious than males from single genotype litters.

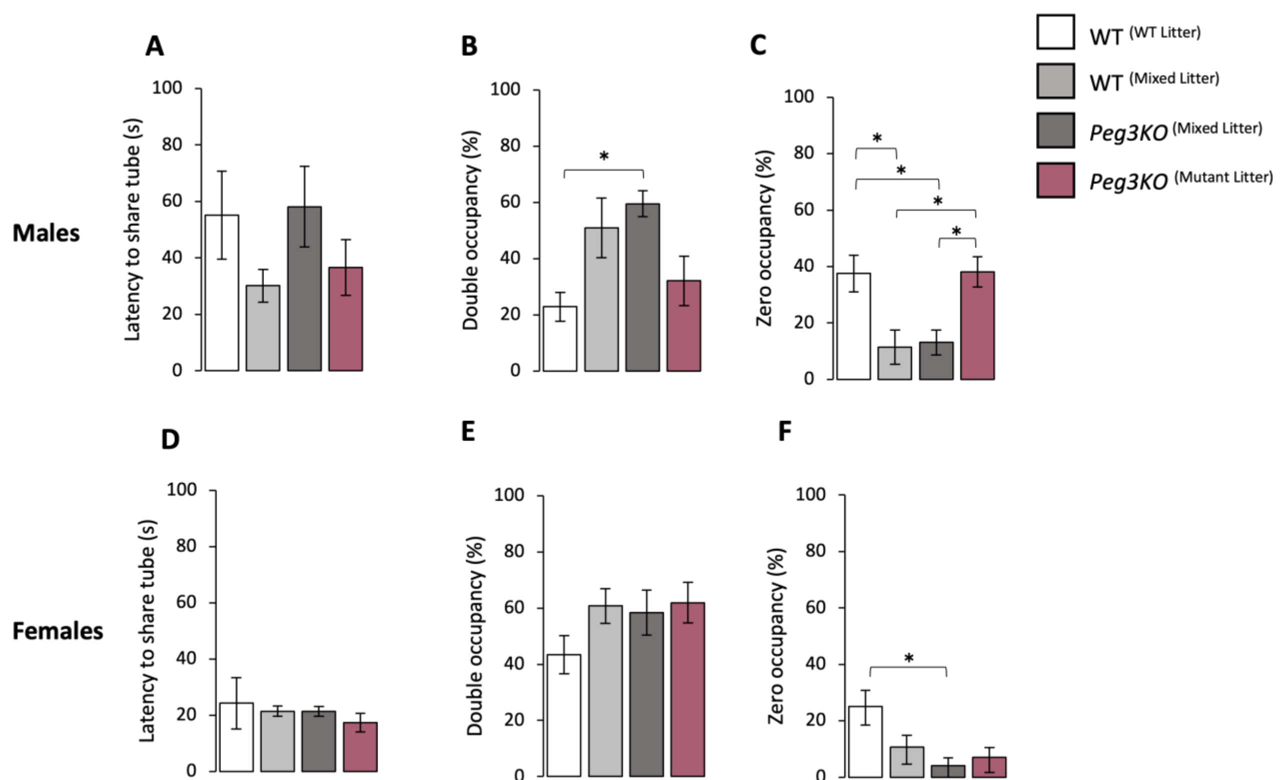


Figure 4.7 Results from the Social Propinquity test. Data is presented from the Social Propinquity test across males(**A-C**) and females (**D-F**). All data was analysed using One-Way ANOVAs. **A, D** show the latency to share the tube. There were no significant differences across males or females. **B, E** show the percentage of the trial that the tube was double occupied. **B)** Pairs of *Peg3KO* (mixed litter) mice spent a significantly greater percentage of the trial sharing the tube than WT males. **C, F** show percentage of the trial that pairs of mice spent with the tube vacant. **C)** In males both genotype of mice from mixed litters spent a smaller percentage of time with the tube unoccupied than males from single genotype litters. **F)** Female mutant mice from mixed litters spend a smaller percentage of the trial with the tube unoccupied than WT female mice. Error bars are are mean ± SEM. * $p < .05$.

4.5.4 Social Olfaction Test

To determine whether mice could discriminate between two social stimuli, comparisons were made between the final presentation of the first urine type (A3) and the first presentation of the second urine type (B1), see Figure 4.8. In males, there was an initial significant effect of genotype on both the differences in number of visits to ($F(3,55) = 3.33$, $p = .029$, Figure 4.8A), and time spent sniffing ($F(3,55) = 2.99$, $p = .029$, Figure 4.8B) the novel social odour (stimulus B1). However, with Bonferroni corrections, these effects did not persist, and no significant differences were observed between groups. The same was also true of the time spent sniffing in females ($F(3,58) = 3.18$, $p = .031$, Figure 4.8E), but there was no significant effect of genotype on the frequency of visits in females ($F(3,58) = 1.23$, $p = .31$, Figure 4.8D). Whilst objectively these results suggest that all groups have issues in discriminating between social odours, it is more likely that there was a problem with the way in which the test was carried out. This is supported by previous research showing no deficits in this test in WT mice of the same background strain (Harrison et al., 2020). Consequently, results from the social olfaction test were not included in the unified social score detailed in Section 4.6.8. Further, when looking at the differences in time spent sniffing between water and a novel social odour, repeated measures ANOVA demonstrated that there was a significant difference across all groups. Both males ($F(1,55) = 244.93$, $p < .001$, Figure 4.8C) and females ($F(1,58) = 95.94$, $p < .001$, Figure 4.8G), spent more time sniffing the social odour than water, suggesting that the results are not due to problems with olfaction generally, although this is discussed further in section 4.8.

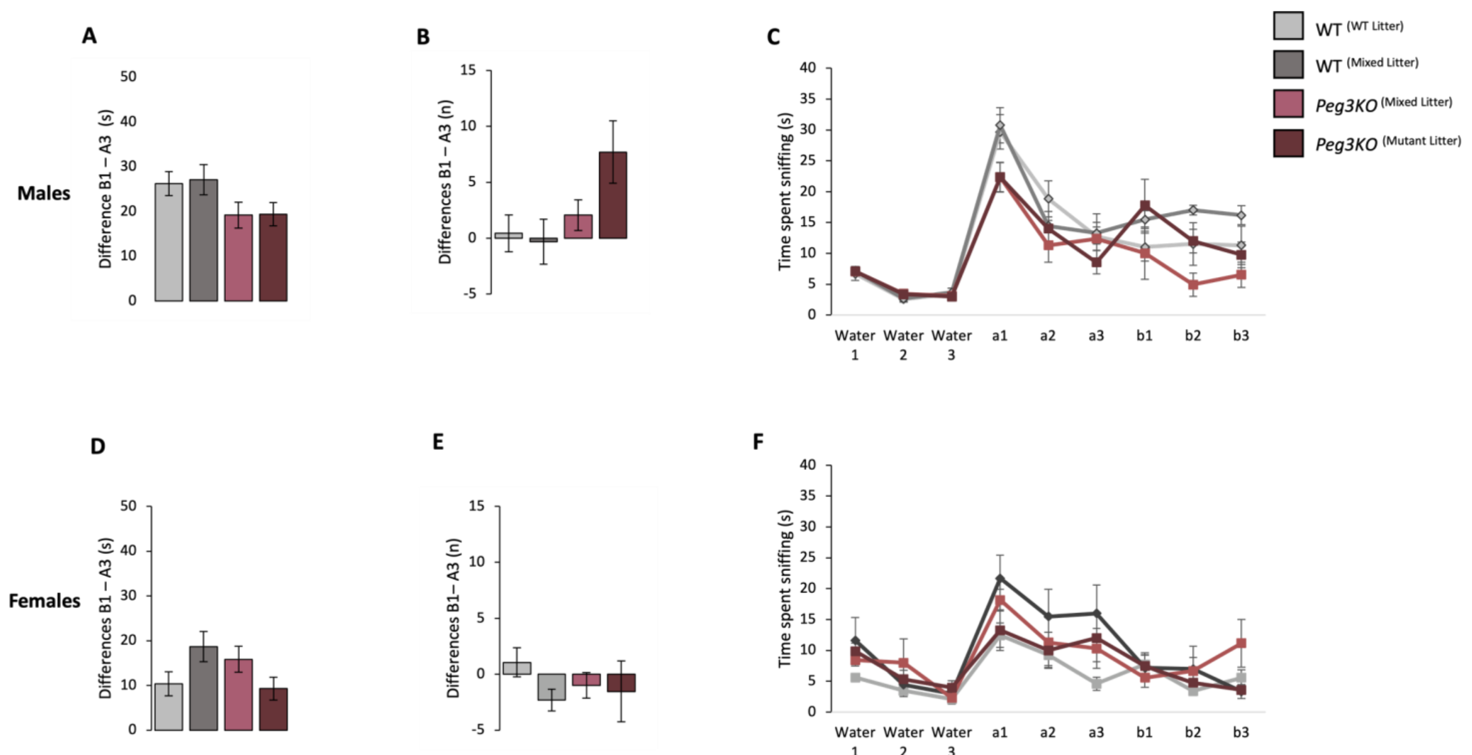


Figure 4.8 Results from the Social Olfaction test. Data is presented from the Social Olfaction test across males (**A-C**) and females (**D-F**). **A, D** shows the difference in time spent sniffing the first presentation of the second urine stimulus compared to the third presentation of the first urine stimulus, whilst **B, E** show the difference in number of visits. There were no significant differences across males or females. **C, F** show the duration of time spent sniffing each stimulus across each presentation. Error bars are are mean ± SEM.

4.5.5 Marble Burying

In both males and females, a significant effect of genotype on the number of marbles buried was observed in males and females [$F(3,55) = 5.46$, $p = .002$, Figure 4.9A, females: $F(3,58) = 3.22$, $p = .029$, Figure 4.9B]. In males, WTs from single genotype litters buried more marbles than mutants from single genotype litters ($p = .003$) and mixed genotype litters ($p = .021$). In females however, no group differences survived adjustment for multiple corrections.

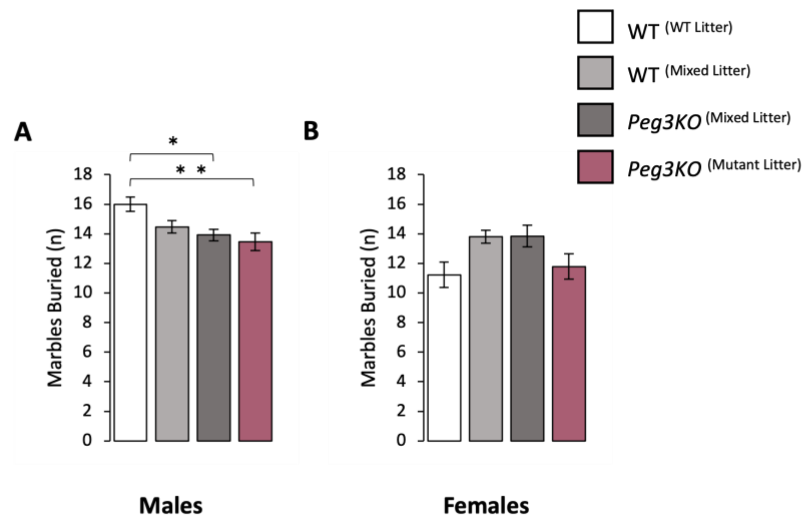


Figure 4.9 Results from the Marble burying test. Data shows the number of marbles buried to 66% of their depth in trials with male (**A**) and female (**B**) mice. One-way ANOVAs showed that there was a significant effect of genotype in males, with WT males burying on average more marbles than mutants from both single and mixed litter genotypes. Whilst there was an effect observed in female mice, this did not survive Bonferroni corrections. Error bars are are mean ± SEM. * $p < .05$. ** $p < .01$

4.5.6 Three-Chambers Test and Scent Marking

During the three-chamber test no effects of genotype were observed on the time spent in the occupied chamber in either males or females [males: $F(3,55) = 2.55$, $p = .65$ Figure 4.10B, females: $F(3,58)$ $p = .20$, Figure 4.10E]. Whilst there was a significant effect of genotype in males on the latency to enter the occupied chamber ($F(3,55) = 3.63$, $p = .02$, Figure 4.10A], this did not survive Bonferroni correction. No significant differences in the latency to enter the occupied chamber were observed in females ($F(3,58) = .08$, $p = .97$, Figure 4.10D]. There was however a significant difference in the number of times female mice crossed into the chamber containing the unfamiliar host mouse ($F(3,58) = 3.57$, p = .02, Figure 4.10F) ,with WT $^{(mixed\ litter)}$ females crossing more times than *Peg3KO* $^{(mixed\ litter)}$ ($p = .006$) and *Peg3KO* $^{(mutant\ litter)}$ females ($p = .03$). As there were no differences in the duration of time spent in the occupied chamber however, this is likely explained by different levels of exploratory activity or anxiety in the single genotype litters, rather than an increased interest in the host mouse. In males, there were no differences in the number of crosses, ($F(3,55 = .77$, $p = .52$, Figure 4.10C).

Scent marking behaviours were also assessed in males during the three-chamber test, with a significant effect of genotype observed ($F(3,55) = 9.63$, $p < .001$, Figure 4.10G). WT males from single genotype litters exhibited greater scent marking than all other genotypes [WT (mixed litter): $p = .01$, *Peg3KO* (mixed litter): $p < .001$, and *Peg3KO* (mutant litter): $p < .001$]. These findings suggest that mutant males, and males from mixed genotype litters, demonstrate less territorial behaviour compared to WT controls.

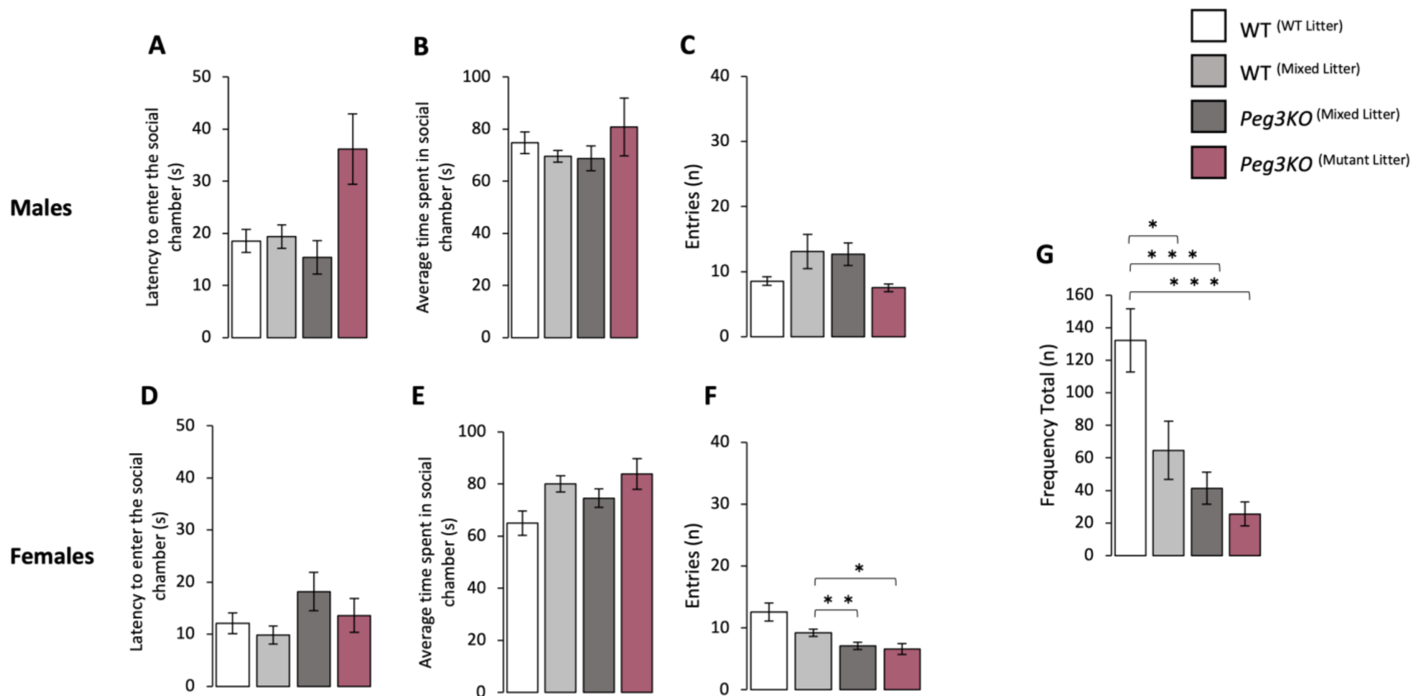


Figure 4.10 Results from the Three-Chambers and Scent Marking test. Data is presented from the Three-chambers test across males (**A-C**) and females (**D-F**) and scent marking (**G**) which was carried out on males alone. All data was analysed using one-way ANOVAs. **A, D** show the latency to enter the social chamber. **B, E** show the total duration spent in the social chamber. **C, F** show the number of entries made into the social chamber. **F)** In females, WT (mixed litter) mice made a greater number of crosses into the social chamber than both mutants from mixed genotype and single genotype litters. **G)** Shows the total of 1 cm^2 containing a scent mark during the three-chamber trial. On average, WT males scent marked a greater area than all other groups. Error bars are are mean ± SEM. * $p < .05$. ** $p < .01$. *** $p < .001$.

4.5.7 Courtship USVs and behaviours

Courtship USVs were tested in two phases. For both phases, no significant effect of genotype on: latency to call; [Initial: $F(3,55) = 1.87$, $p = .15$, Figure 4.11A, Reunion: $F(3,55) = .50$, $p = .68$, Figure 4.11D], number of calls [Initial: $F(3,55) = .71$, $p = .55$, Figure 4.11B, Reunion: $F(3,55) = .79$, $p = .50$, Figure 4.11E], or mean duration of call [Initial: $F(3,55) = 1.24$, $p = .30$, Figure 4.11C, Reunion: $F(3,55) = 2.00$, $p = .23$, Figure 4.11F] was observed.

During the initial and reunion phases, the behaviour of the male mouse was also recorded. In both phases there was no significant differences in: time spent engaged in social behaviour [Initial: $F(3,55) = .74$ $p = .53$, Figure 4.12A, Reunion: $F(3,55) = .12$, $p = .95$, Figure 4.12C] or time spent engaged in non-social behaviour [Initial: $F(3,55) = 1.60$ $p = .20$ Figure 4.12B, Reunion: $F(3,55) = .71$, $p = .55$, Figure 4.12D). In summary, there were no significant differences observed in courtship behaviours, or USVs across any of the groups.

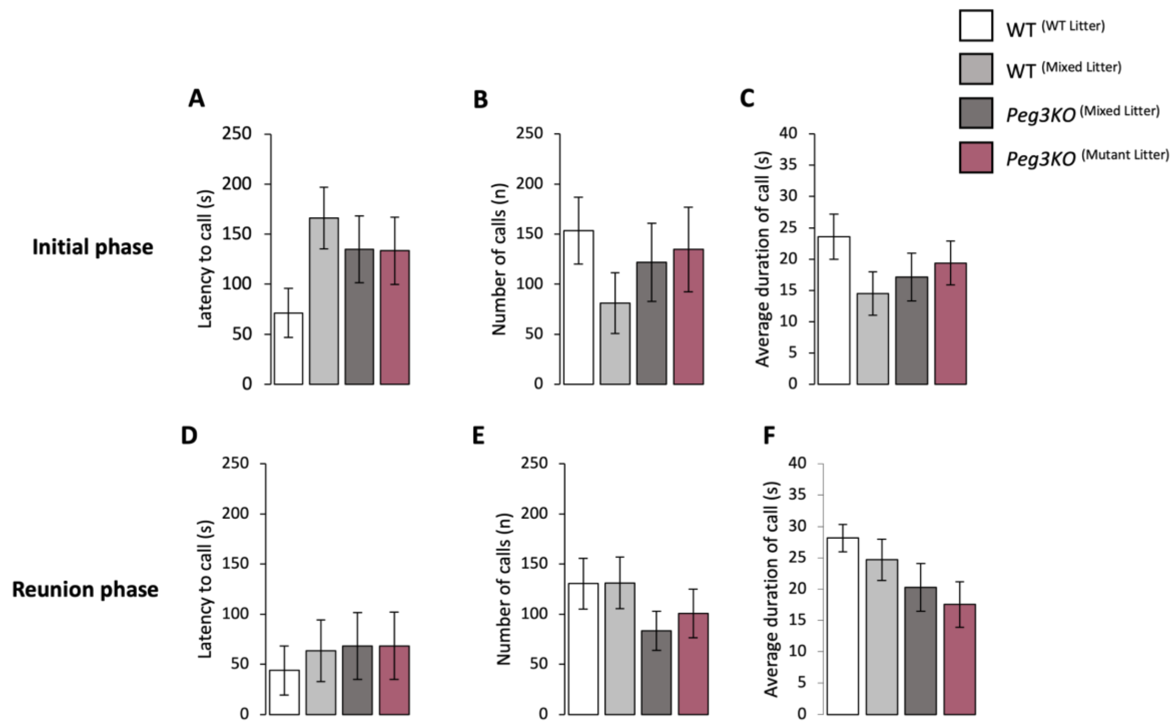


Figure 4.11. Results from Courtship USVs. Male mice USV parameters observed during interaction with a female WT mouse in oestrus. **A, D** show the latency to call in the initial and reunion phase. **B, E** show the total number of calls in both phases and **C, F** show the mean duration of call in each phase. One-way ANOVAs demonstrated that there was no effect of genotype on any of the USV parameters in either phase. Error bars are are mean ± SEM.

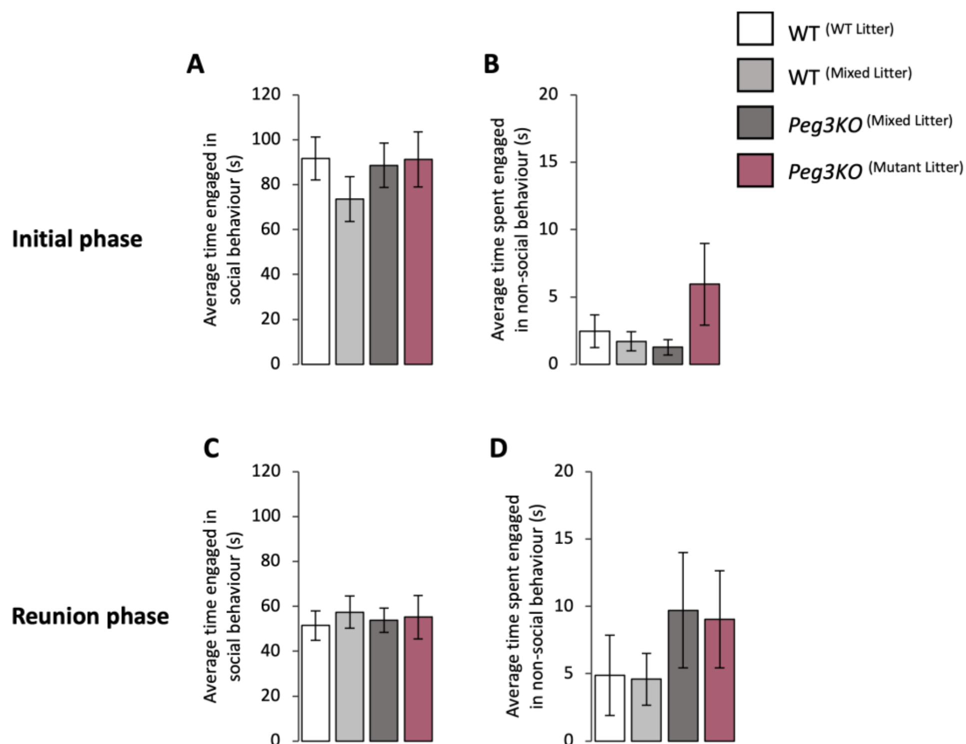


Figure 4.12 Results from Courtship Behaviours. Composite behaviors of male mice during interaction with a WT female mouse in oestrus. **A, C** show the time spent engaged in social behaviour, while **B, D** show the time spent engaged in non-social behaviour during the initial and reunion phase. One-way ANOVAs demonstrated that there was no effect of genotype on social behaviour in either phase. Error bars are are mean ± SEM.

4.5.8 Unified Social Score

Described in section 2.7.1, a unified social score was created to provide an overall measure of sociability using measurements from the direct social interaction; social propinquity, three chambers, scent marking, and courtship tests, with the latter two tests used only in the generation of the unified social score for males. Whilst there was no significant differences in sociability in females ($F(3,58) = .201$, $p = .123$, Figure 4.13B), significant differences in male sociability were observed ($F(3,55) = 15.15$, $p < .001$), Figure 4.13A). Specifically, all male groups, including WT males within mixed litters, showed reduced sociability as compared to WT controls from single genotype litters ($p < .001$ in each comparison).

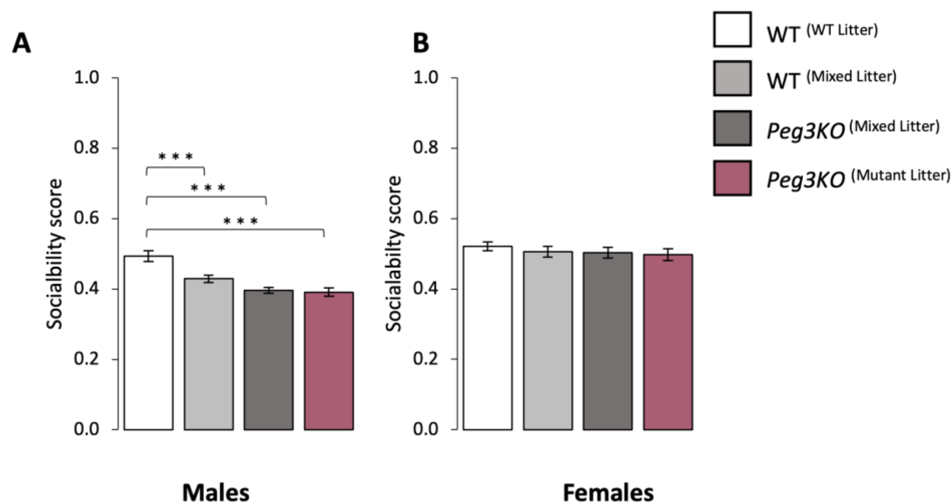


Figure 4.13 The Unified Social Score. One-way ANOVAs demonstrated that there was a significant effect of genotype on post-weaning unified sociability in males (**A**) but not in females (**B**). Mutant males, from both single, and mixed genotype litters, and WT males raised in mixed litters, scored significantly less on the unified social score than WT controls, suggesting deficits in post-weaning social behaviour. Error bars are are mean ± SEM. *** $p < .001$

4.6 Results: RNA-Sequencing

4.6.1 Hypothalamus

Principal component analysis (PCA) of all samples together and with the sexes presented separately can be seen in Figure 4.14. As seen in Figure 4.14, there was one outlier identified which belonged to a WT (WT litter) female sample, however removal of this sample did not affect the results, and so it was included in the analysis.

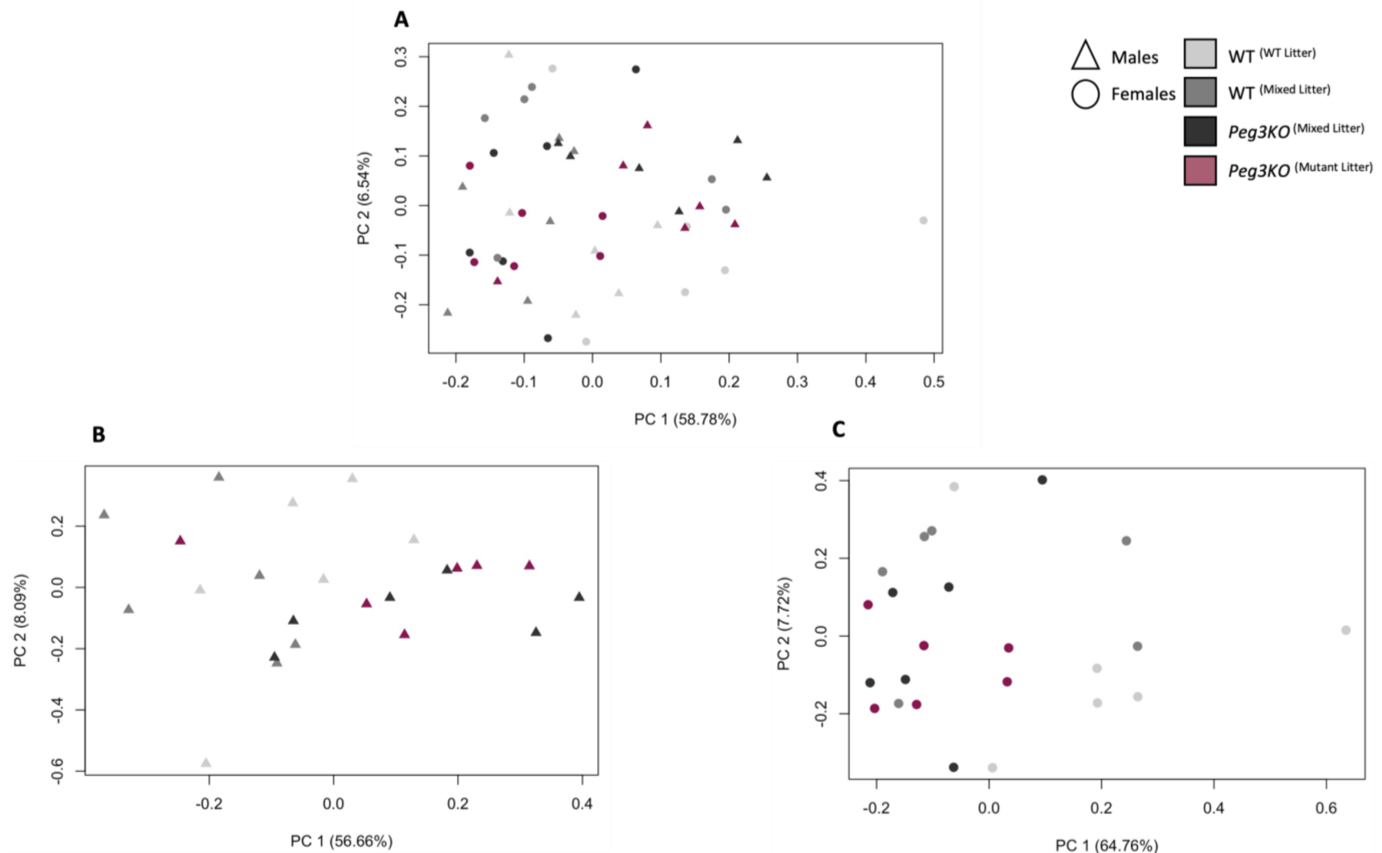


Figure 4.14 Principal Component Analysis (PCA) plot of hypothalamus samples plotted by gene expression. A) PCA plot including all samples. **B**) PCA plot for male samples. **C**) PCA plot for female samples.

4.6.1.1 *Peg3KO* (mutant litter) VS WT

To explore differential gene expression induced by intrinsic loss of function of *Peg3*, differential gene expression was compared between *Peg3KO* (mutant litter) and WT mice. When controlling for sex, there were only 3 differentially expressed genes that survived multiple corrections in the hypothalamus of *Peg3KO* (mutant litter) compared to WT mice (Table 4.6). Given the low number of DEGs after multiple corrections, using the criteria outlined in section 4.3.3 of a non-adjusted p value of $< .05$, 1,997 genes were taken forward for Gene Ontology and MGI Mammalian Phenotype enrichment analysis.

Results of the GO enrichment analysis showed that the nominally expressed gene set was significantly enriched for a number of Biological, Molecular and Cellular GO terms as well as a number of lethality and embryonic

growth annotations in the MGI Mammalian Phenotype gene set enrichment analysis. For each database, the three most significant functional categories with the largest gene counts are presented in Figure 4.15.

Table 4.6 Differentially Expressed Genes in *Peg3KO* (mutant litter) mice compared to WT mice

DEG	Log2 Fold Change	*p* value	Adjusted *p* value	B
Fndc1	0.53	1.31e-06	**0.01**	4.99
Lft80	0.29	1.54e-06	**0.01**	4.86
DEAF1	-0.19	3.91e-06	**0.01**	4.79

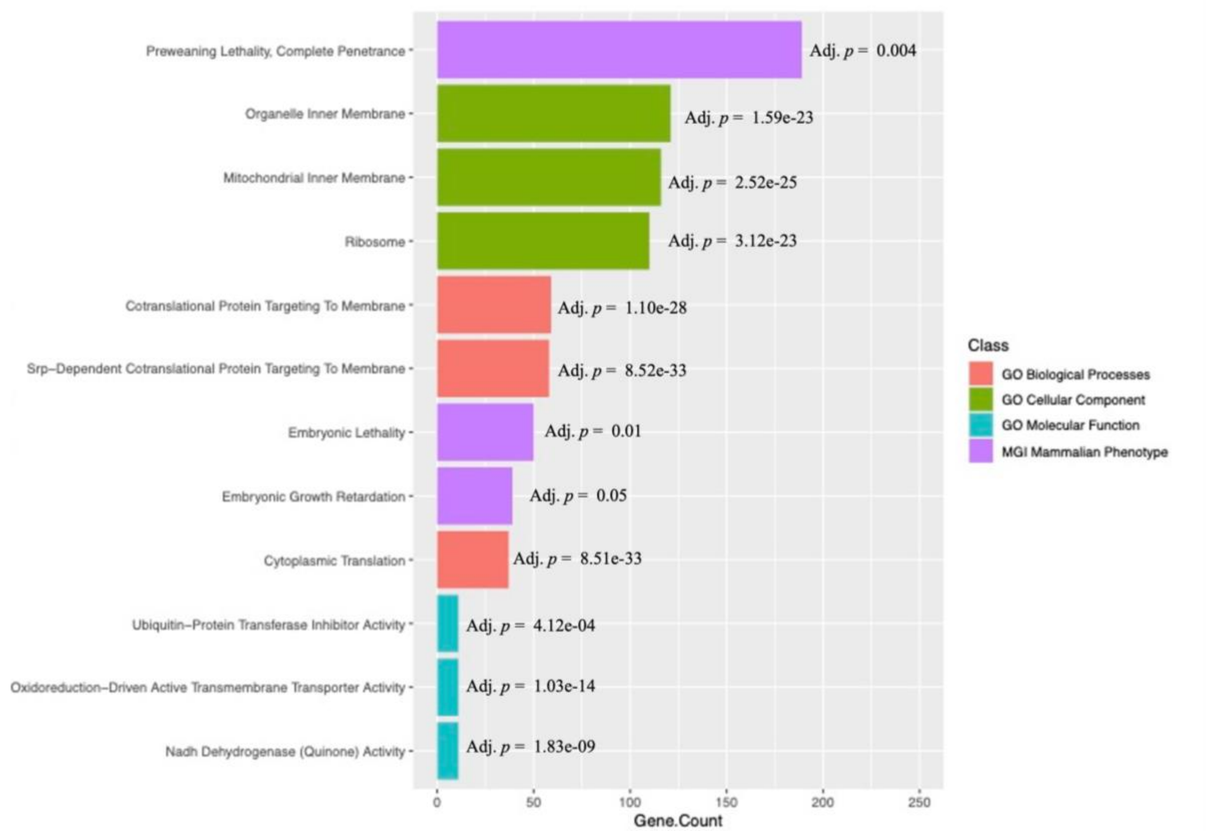


Figure 4.15 Gene Ontology and MGI Enrichment Analysis: *Peg3KO* (mutant litter) and WT mice. Enrichment analyses were carried out on the nominally significant gene set (non-adjusted $p < .05$) between *Peg3* (mutant litter) and WT mice, controlling for sex. The bar chart shows the top significant enrichment terms, alongside the number of genes contributing to each term. Different colours represent a different class of enrichment categories.

When analysing separately by sex, in males, there were no differentially expressed genes between male *Peg3KO* (mutant litter) and WT mice. In females however, *C1s2,* a gene associated with calcium ion binding was significantly downregulated in *Peg3KO* (mutant litter) females (Table 4.7).

Table 4.7 Differentially Expressed Genes female mice when comparing *Peg3KO* (mutant litter) to WT mice

Sex	DEG	Log2 Fold Change	*p* value	Adjusted *p* value	B
Females	*C1s2*	-1.16	5.92e-06	**0.001**	-2.35

Using the non-adjusted *p* value of < .05, 392 nominally differentially expressed genes were taken forward for enrichment analyses in males and 1,047 were selected in females. The top result from each enrichment class for males can be seen in Table 4.8, however, no enrichment terms survived multiple corrections.

Table 4.8 Results from enrichment analyses (*Peg3KO* (mutant litter) vs WT males)

Class	Enrichment Term	Gene Count	*p* value	Adjusted *p* value
GO Biological Process	Peptidyl-lysine methylation (GO:0018022)	3	0.003	0.799
GO Cellular Component	Recycling endosome membrane (GO:0055038)	4	0.006	0.826
GO Molecular Function	G protein-coupled receptor activity (GO:0004930)	12	0.004	0.54
MGI Mammalian Phenotype	Ectopic neuron (MPP:001723)	2	0.001	0.13

In females, a number of significant enrichment terms across both GO and MGI analyses were observed, the majority of which were associated with metabolic activity. Table 4.9 shows the top enrichment terms in each class.

Table 4.9 Results from enrichment analyses (*Peg3KO* (mutant litter) vs WT females)

Class	Enrichment Term	Gene Count	*p* value	Adjusted *p* value
GO Biological Process	Axonemal dynein complex assembly (GO:0070286)	11	1.33e-11	**4.26e-08**
	Aerobic electron transport chain (GO:00195646)	14	2.61e-10	**2.77e-07**
	Mitochondrial ATP synthesis (GO:0042775)	18	3.46e-10	**2.76e-07**
GO Cellular Component	Mitochondrial inner membrane (GO:0005743)	74	9.01e-12	**2.61e-09**
	Mitochondrial membrane (GO:0031966)	53	1.65e-11	**2.62e-09**
	Organelle inner membrane (GO:0019866)	60	6.51e11	**7.04e-09**
GO Molecular Function	Oxidoreduction-drive active transmembrane transporter activity (GO:0015453)	18	3.72e-09	**2.33e-05**
	NADH dehydrogenase (quinone) activity (GO0050136)	8	9.94e-06	**2.07e-03**
	NADH dehydrogenase (ubiquinone) activity (GO:0008137)	8	9.94e-07	**2.07e-02**
MGI Mammalian Phenotype	Abnormal respiratory motile cilium morphology (MP:0011050)	6	7.83e-05	0.19

In summary, only a handful of significant DEGs were observed between *Peg3KO* (mutant litter) and WT mice. Using nominally differentially genes ($p_{non\text{-}adj}$ < .05), enrichment analysis revealed a number of significant enrichment terms associated with metabolic activity in female, but not male mutant mice. While this is interesting given that behavioural changes were observed in males, and not females, this should be interpreted with care given the use of the non-adjusted alpha level.

4.6.1.2 WT (mixed litter) VS WT

WT mice from both mixed and single genotype litters were compared to evaluate the contribution of an atypical maternal care environment and a shared postnatal environment with mutant mice on the effect of gene expression. When controlling for sex, *Stxbp4*, a gene associated with metabolic activity was seen to be up regulated in WT mixed litter mice (Table 4.10).

Table 4.10 Differentially Expressed Genes in WT (mixed litter) mice compared to WT mice

DEG	Log2 Fold Change	*p* value	Adjusted *p* value	B
Stxbp4	0.20	3.96e-01	**0.04**	3.20

For enrichment analysis, due to the low number of DEGs, the criteria of a non-adjusted alpha level ($p < .05$) was used, consequently 139 genes were selected for enrichment analysis. GO enrichment analyses determined that this nominally significant gene set was significantly enriched for a number of biological processes (see Figure 4.14), most notably, dopaminergic neuron differentiation (GO term: 0071542).

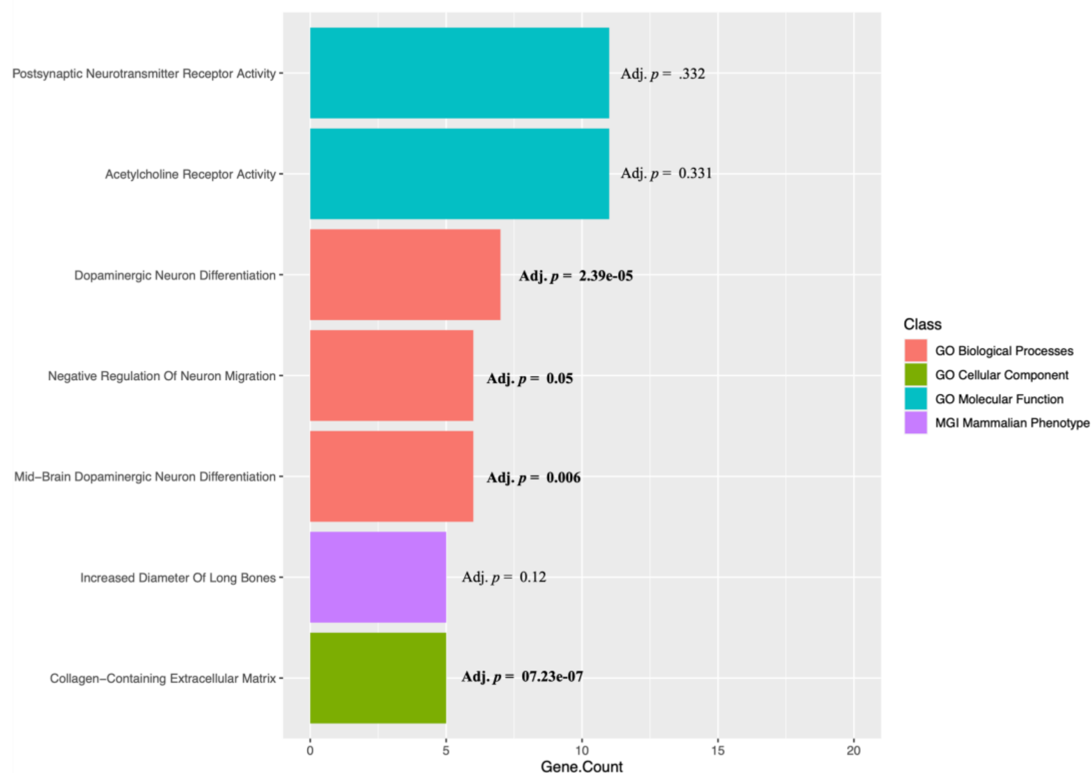


Figure 4.16 Gene Ontology and MGI Enrichment Analysis: WT (mixed litter) and WT mice Analyses was carried out on the nominally significant gene set (non-adjusted $p < .05$) between *Peg3* (mutant litter) and WT mice, controlling for sex. Bar chart shows the top significant enrichment terms, alongside the number of genes contributing to each time. Different colours represent different classes of enrichment categories.

When analysing separately by sex, no DEGs were observed in either males or females. Despite this, 454 and 2,065 genes were taken forward for gene enrichment analysis in males and females respectively, using the criteria of a non-adjusted alpha level ($p < .05$). Interestingly, as when comparing *Peg3KO* (mutant litter) to WT males, no

enrichment term reached statistical significance, with the exception of an annotation associated with a protruding tongue phenotype (Table 4.11). In females, a similar pattern was observed in the gene set derived from *Peg3KO* (mutant litter) females compared to WT females, where the same enrichment terms in both cellular components and molecular functions were significantly enriched, again associated mainly with metabolic activity (Table 4.12).

Table 4.11 Results from enrichment analyses (WT (mixed litter) vs WT males)

Class	Enrichment Term	Gene Count	*p* value	Adjusted *p* value
GO Biological Process	Fatty-acyl-CoA biosynthetic process (GO:0046949)	3	0.004	0.77
GO Cellular Component	Catenin complex (GO:0030126)	4	0.03	0.96
GO Molecular Function	Mannosyltransferase activity (GO:0000030)	5	1.12e-04	0.051
MGI Mammalian Phenotype	Protruding tongue (MP: 0009908)	5	9.96e-06	**0.01**

Table 4.12 Results from enrichment analyses (WT (mixed litter) vs WT females)

Class	Enrichment Term	Gene Count	*p* value	Adjusted *p* value
GO Biological Process	Aerobic electron transport chain (GO:0019646)	22	6.74e-17	**2.66e-13**
	Mitochondrial ATP synthesis (GO:0042775)	44	1.99e-16	**2.66-e13**
	Mitochondrial Respiratory chain complex (GO: 0032981)	22	2.12e-12	**2.35e-09**
GO Cellular Component	Mitochondrial inner membrane (GO:0005743)	101	1.10e-10	**8.96-e09**
	Mitochondrial membrane (GO:0031966)	131	1.18e-19	**1.611e-17**
	Organelle inner membrane (GO:0019866)	192	1.15e-20	**2.36e-18**
GO Molecular Function	Oxidoreduction-drive active transmembrane transporter activity (GO:0015453)	12	1.59e-11	**1.39e-08**
	NADH dehydrogenase (quinone) activity (GO0050136)	12	1.32e-09	**3.85e-07**
	NADH dehydrogenase (ubiquinone) activity (GO:0008137)	11	1.32e-09	**3.85e-07**
MGI Mammalian Phenotype	Preweaning lethality, complete penetrance (MP:0011100)	194	5.49e-06	**0.02**

Overall, when analysing samples together, enrichment analysis on genes significant at the non-adjusted alpha level demonstrated that there was enrichment of two dopaminergic ontologies associated with dopamine neuron differentiation. When separating by sex, no DEGs were observed, however enrichment analysis on genes significant at the non-adjusted alpha level, demonstrated a number of significant enrichment terms associated with metabolic activity in female mutant mice from mixed litters, but not males.

4.6.2.3 *Peg3KO* (mutant litter) VS *Peg3KO* (mixed litter)

Mutant mice from both mixed and single genotype litters were compared to evaluate whether the presence of WT pups in the pre- and postnatal environment attenuated any phenotypes present in *Peg3KO* (mixed litter) mice. However, when controlling for sex, no DEGs were observed. 411 genes significant at the non-adjusted alpha level

(p <. 05) were taken forward for enrichment analyses, however, no enrichment terms survived multiple corrections. Table 4.13 shows the top significant term for each enrichment analysis.

Table 4.13 Results from enrichment analyses (*Peg3KO* $^{(mutant\ litter)}$ vs *Peg3KO* $^{(mixed\ litter)}$ mice)

Class	Enrichment Term	Gene Count	*p* value	Adjusted *p* value
GO Biological Process	Sulfate transport (GO:0008272)	4	2.42e-04	0.31
GO Cellular Component	tRNA Methyltransferase (GO:0043527)	2	0.005	0.65
GO Molecular Function	Sulfate transmembrane transporter activity (GO:0015103)	3	0.001	0.39
MGI Mammalian Phenotype	Abnormal sensory capabilities/reflexes	4	0.002	0.70

As in the comparison of both the genetically WT groups, when comparing both groups of mutant mice, there were no DEGs in either males or females. In males, enrichment analyses were carried out on 531 nominally differentially expressed genes, whilst in females, 310 genes were analysed. Significant enrichment terms for both males and females are presented in Tables 4.14.

Table 4.14 Results from enrichment analyses (*Peg3KO* $^{(mutant\ litter)}$ vs *Peg3KO* $^{(mixed\ litter)}$ males and females)

	Class	Enrichment Term	Gene Count	*p* value	Adjusted *p* value
Males	GO Biological Process	Myelination (GO:0042552)	15	4.30e-07	**0.001**
		Gluconeogenesis (GO:0006094)	15	1.02e-06	**0.001**
	GO Molecular Function	NADP binding (GO:0050661)	9	3.87e-05	**0.02**
Females	GO Molecular Function	tRNA methyltransferase activity (GO:0016423)	12	3.67e-05	**0.01**

4.6.2 Olfactory Bulb

Figure 4.17 shows the PCA plots for all olfactory bulb samples presented together, and for each sex presented separately. As with the hypothalamus, although outliers were observed in both male and female WTs, removing these samples from the analysis did not alter the results, and so they were included in all relevant analyses.

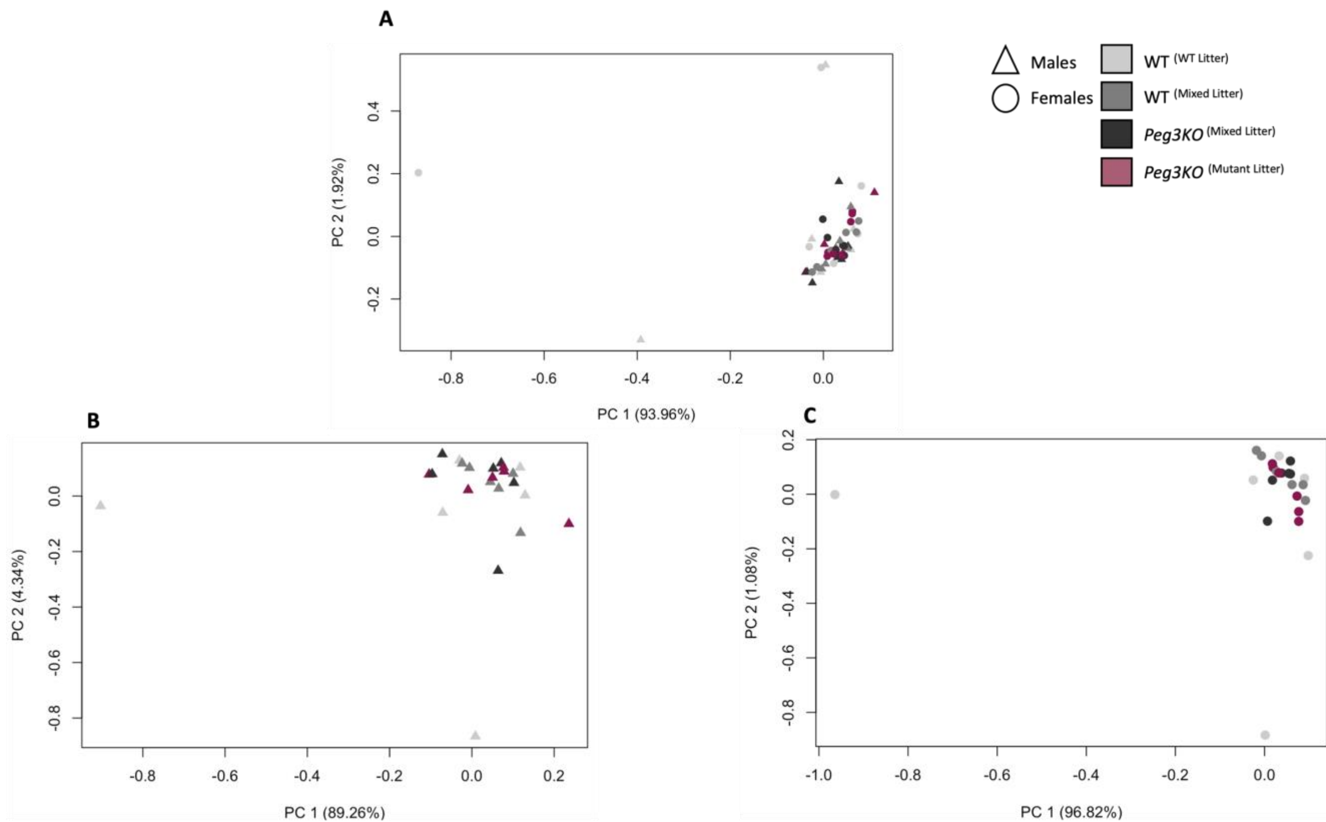


Figure 4.17 Principal Component Analysis (PCA) plot of olfactory bulb samples plotted by gene expression. **A)** PCA plot for samples controlling for sex. **B**) PCA plot for male samples. **C**) PCA plot for female samples.

4.6.2.1 *Peg3KO* (mutant litter) VS WT

As with the hypothalamus, differential gene expression analysis was first run controlling for sex before being run separately. In all analyses, both when controlling for sex and analysing separately, no DEGs, were observed at the Benjamini Hochberg adjusted alpha level.

Using the criteria of a non-adjusted alpha level ($p < .05$), an absence of DEGs was also observed both when controlling for sex, and also when looking at males separately. In females, only three genes were found to be significantly expressed at the non-adjusted level. Consequently, enrichment analyses were unable to be carried out. Nominally significant differentially expressed genes for this analysis can be seen in Appendix A7.

Interestingly, on further inspection, in the olfactory bulb, *Peg3* was not observed to be differentially expressed between mutant and WT mice.

4.6.2.2 WT (mixed litter) VS WT

No DEGs were observed both when controlling for sex, or when looking at males or females. At the non-adjusted criterion, no genes were nominally significant both when controlling for sex, or between female WT (mixed litter) and WT mice. At the non-adjusted level, only one gene (*Acta2)* was significantly up regulated in the olfactory bulb of male WT (mixed litter) mice, and so no enrichment analyses were able to be carried out.

4.6.2.3 *Peg3KO* (mutant litter) VS *Peg3KO* (mixed litter)

No DEGs were observed both when controlling for sex, or when looking at males or females. This was also true for all analyses at the non-adjusted level, and so no enrichment analyses were carried out.

In summary, regarding gene expression in the olfactory bulb, no DEGs were observed at the level of $p_{adj} < .05$, and too few DEGs were nominally significant at the non-adjusted level to carry out enrichment analysis across any comparisons.

4.7 Discussion

In this study, neonatal USV deficits were observed only in *Peg3KO* (mutant litter) males, suggesting a direct role for loss of *Peg3* function in the development of typical social behaviour. However, post-weaning unified social scores show that alterations in social behaviour were apparent in *Peg3KO* (mutant litter) and *Peg3KO* (mixed litter) males, but also in WT littermates sharing their abnormal pre- and postnatal environment. As such, though deficits were greater in male mutants from single genotype litters, these results suggest that the atypical environment shared between males in the mixed genotype litters may play a role in driving these behavioural changes, either through an altered *in utero* environment, or postnatally, due to atypical maternal care. As the same deficits were not observed in female mice, these findings lend further support to previous research suggesting that disruption of *Peg3* has a sexually dimorphic phenotype (Tunster et al. 2018, He et al. 2016, Kim et al. 2013). These findings however were not reflected at the level of gene expression, where, with the exception of hypothalamic *Peg3* expression between mutant mice and WT controls, no clear differences in differentially expressed genes were observed when looking at males and females separately.

4.7.1 Pre-weaning Behaviour

Characterisation of isolation induced USVs suggest a previously unreported early life social deficit in *Peg3KO* (mutant litter) males. Despite not observing significant USV differences between groups of pups in Chapter 3, when individually testing pups, we observed that *Peg3KO* (mutant litter) males called significantly less than WT males at both P4, P6 and when looking at a composite score for USV emission across development. In females, there was no significant effect of genotype. USV emission typically follows a 'U-shaped' developmental trajectory, peaking around day 6-8, as can be seen in the WTs in this study, although this can vary depending on the strain of mouse (Scattoni et al., 2011). However, USV emission in the male *Peg3KOs* (mutant litter) followed a flattened trajectory, increasing only negligibly before dropping off at P10. Given the association between USV emission and the

elicitation of maternal care, a significant deficit at P6, the peak of calling, may indicate a greater functional impact of USV emission on maternal care in the *Peg3KO* group. Interestingly, this deficit was not attributed to a drop in body temperature during testing, a factor which has been shown to effect USV emission (Ehret, 2005).
It is also interesting to note, that with the exception of P4 in males, mutants from the mixed litter group did not appear to statistically differ from WT controls. Equally, there was a general pattern of results to show that in comparison to their single genotype counterparts, *Peg3KO* $^{\text{(mixed litter)}}$ males tended to call more than *Peg3KO* $^{\text{(mutant litter)}}$ males, whilst WT $^{\text{(mixed litter)}}$ males called less than WT controls. While the spectographic qualities of USVs are thought to be innate (Kikusui et al., 2011, Mahrt et al., 2013), it has been suggested that in adult mice, social experience is necessary to learn the appropriate context in which to emit USVs (Screven and Dent, 2019). Although this has not been formally tested in neonatal mice, one potential explanation for the lack of call deficit in *Peg3KO* mice in mixed litters may be that when raised in an environment with WT littermates, they learn to call more frequently and appropriately than *Peg3KO* males raised in exclusively mutant littters.

Consistent with previous research reporting that intrinsic loss of *Peg3* expression is associated with *in utero* growth restriction (Li et al., 1999, Kim et al., 2013, Denizot et al., 2016), the current study reported that regardless of sex, at P2, mutant mice were significantly lighter than WT controls. However, for the first time this study reports that though *Peg3KO* $^{\text{(mutant litter)}}$ mice exhibit catch up growth, *Peg3KO* $^{\text{(mixed litter)}}$ mice remain growth restricted into adulthood. Currently, the existing literature suggests that *Peg3* mice are intrinsically growth restricted due to loss of function of *Peg3*. However, results from the weight data in this study suggest that extrinsic growth restriction is also present. Extrinsic growth restriction of *Peg3KO* $^{\text{(mixed litter)}}$ mice has also been observed in previous work, where the weight differences between *Peg3KO* $^{\text{(mixed litter)}}$ and WT $^{\text{(mixed litter)}}$ mice were thought to be attributed both to *in utero* and postnatal competition for maternal resources, in addition to the placental deficits seen in the *Peg3KO* model (Curley et al., 2004). However, this is the first study to systematically follow up *Peg3* mutant mice on the 129Sv background strain, from neonates to adults, which may explain why studies have not observed catch up growth in *Peg3KO* mice previously. Importantly, this has implications for the interpretation of any later life behaviours, as any observed differences between the mixed litter genotypes may be a product of an altered *in utero* or postnatal environment, or a consequence of growth restriction. Interestingly, it also worth noting that previous research has shown that catch-up growth in some instances has been shown to elicit worse outcomes than remaining growth restricted (Mikaelsson et al., 2013) which may be a further possible interpretation as to why *Peg3KO* $^{\text{(mutant litter)}}$ males, but not *Peg3KO* $^{\text{(mixed litter)}}$ males demonstrate USV deficits.

4.7.2 Post-weaning Behaviour

Given the large number of tests carried out in this study, a unified behavioural scoring system was used to assess atypical social behaviour, consolidating measures from across the various behavioural tests. This approach has previously been used in studies focused on assessing the impact of direct genetic alteration versus an atypical environment (Harrison et al., 2021) and also helps to avoid potential issues with multiple testing given the large number of behavioural tests used in this study.

Using this approach, it was shown that *Peg3KO* (mutant litter) males also demonstrated deficits in social behaviour post-weaning, consistent with the neonatal USV deficits observed in this group. In addition, social deficits were also observed post-weaning in both *Peg3KO* (mixed litter) and WT (mixed litter) males, who share not only a litter, but also, an abnormal pre- and postnatal environment. Crucially, the observation of these deficits in WT littermates, suggest that the behavioural changes across males are not solely driven by intrinsic loss of *Peg3* function, but rather are the result of an abnormal pre- and postnatal environment, likely driven by placental endocrine insufficiency.

Importantly, there are a number of limitations to this interpretation, firstly as mentioned in the previous section, it is difficult to disentangle whether differences within the mixed litter group are due to the altered *in utero* environment, or the atypical postnatal environment which includes both impaired maternal care, and littermates with altered behaviour. It may be for example, that an abnormal *in utero* environment explains both the behavioural and physiological weight differences observed between the groups, for example, disruption of placental hormones as a consequence of placental endocrine insufficiency would impact nutrient supply to both WT and mutant mice, potentially impacting later life growth and behaviour. An examination of fetal weight would aid in beginning to disentangle whether this hypothesis is true, however, though McNamara et al. (2018a) reported on the *in utero* weights of *Peg3KO* mice compared to WT controls and found no differences, to date no studies have reported on the *in utero* weight of mutant or WT pups from mixed litters.

Secondly, it is well established that social behaviours are shaped by experience (Sachser et al., 2013), and as discussed in regard to USVs, it cannot be ruled that the mutant offspring in the mixed litter groups, are altering the behaviour of their WT littermates or vice versa. Similar findings have been reported previously, where the social behaviour of WT mice has reportedly been altered by the presence of littermates lacking the Neuroligin-3 (*Nlgn3)* gene (Kalbassi et al., 2017). Notably, this study reported similar sexually dimorphic outcomes, whereby male, but not female social behaviours were affected.

However, in spite of these limitations, recent research has shown that altered expression of an imprinted gene, and resultant placental insufficiency, is enough to modify offspring behaviour (Mikaelsson et al., 2013, Harrison et al., 2021) and as such, the most likely explanation for the social deficits observed in this study remains that they are a consequence of an altered pre- and postnatal maternal environment, as a result of placental endocrine insufficiency. Though as discussed in Chapter 6, this interpretation still warrants further investigation.

This conclusion however does not explain the lack of early life social deficits in *Peg3KO* (mixed litter) males. One possible explanation may be that pre- and post-weaning social behaviours are used to elicit different outcomes, with pre-weaning social behaviours used to seek maternal care, and post-weaning to interact appropriately with conspecifics. Though there has been much research into the social behaviour of mice at different ages (Shoji et al., 2016, Shoji and Miyakawa, 2019), there has been little research systematically examining social behaviours in the same mice over time. However, there is some evidence to suggest that social behaviours may reduce with age (Peleh et al., 2019, Shoji and Miyakawa, 2019). USV emission, even in genetically WT mice, has been shown to change over time, with call rates varying depending on the age of the mouse, and social stimuli (Shoji and

Miyakawa, 2019). This may also go some way in explaining the lack of USV deficit observed in *Peg3KO* (mutant mice) during the courtship test, though to date, there have been no studies explicitly exploring whether USV frequency in the neonatal period is associated with those produced in a reproductive context in adulthood. However, given the previously reported alterations in sexual responses in *Peg3KO* males (Swaney et al., 2007) and the deficits in the social interaction observed in the current study, the lack of an observed courtship deficit remains surprising.

Regardless, this is the first study to demonstrate a male specific USV deficit in *Peg3KO* mice in the neonatal period, and male specific social deficits in the post-weaning stage of life. Results from the post-weaning behaviour present further evidence to support research suggesting that males may be impacted to a greater extent by early-life adversity (O'Connor et al., 2002b, Glover and Hill, 2012, Sutherland and Brunwasser, 2018). Relevant to the *Peg3* model, maternal anxiety and depression have also been linked to males being at greater risk for a number of detrimental outcomes, including poor educational achievement (Kurstjens and Wolke, 2001), increased risk of ADHD (Glover and Hill, 2012) and increased risk of internalizing behavioural problems (de Bruijn et al., 2009). Interestingly, in humans, we have reported that mother's prenatal mental health symptoms are associated with delays in language development and emerging temperamental difficulties in males, but not female infants (Savory et al., 2020), although this is yet to be explored in the context of *PEG3* expression in a study with adequate sample size.

4.7.3 RNA-Sequencing

This analysis set out to determine genome-wide transcriptional changes in two regions of the brain associated with social behaviour, the hypothalamus and olfactory bulb, in P6 mice exposed to varying levels of atypical maternal care and *Peg3* disruption, with a focus on sex-specific responses. Though there were some changes in the neonatal social behaviours of *Peg3KO* males, these changes were not reflected in the transcriptomes of the olfactory bulb or hypothalamus from P6 male mice. Interestingly, across all analyses, when exploring sex-specific differences in gene expression, with the exception of the downregulation of *Peg3* in comparisons between mutant and WT mice, no significantly differentially expressed genes were observed. Given the role of *Peg3* as a transcription factor and its regulation in a number of downstream target genes (Kim et al., 2013, Thiaville et al., 2013, Lee et al., 2015), this result is surprising, and may suggest that the current study was underpowered to detect expression differences at this level. Alternatively, previous research has utilised microarray, as opposed to RNA-sequencing, which due to the use of predefined target genes, requires a less conservative approach to statistical correction.

It should also be noted however that behavioural changes do not necessarily indicate transcriptome changes, and the results of the current study should be interpreted with care, given the use of nominally expressed gene sets. Further, previous research looking at sex-specific differences in the expression levels of imprinted genes have suggested that sexual differentiation of imprinted genes happens *in utero* in the brain of developing embryos (Faisal et al., 2014) rather than the neonatal timepoint used in this study.

In the hypothalamus, when analysing the sexes together, 4 differentially expressed genes were observed between *Peg3KO* (mutant litter) and WT (WT litter) mice, including *Peg3*. Interestingly the gene *Deformed Epidermal Autoregulatory Factor 1 Homolog* (*DEAF1)* was one of the genes significantly downregulated in *Peg3KO* (mutant litter) mice. In mice, conditional knock-out of *DEAF1* has been associated with increased anxiety-like behaviour and perinatal lethality, the latter of which is consistent with the elevated mortality scores observed in the *Peg3KO* (mutant litter) mice in Chapter 3. In humans, *DEAF1* has been associated with neurodevelopmental disorders, speech delay and clinical depression (Lemonde et al., 2003, Rajab et al., 2015, Chen et al., 2017), which is consistent with research to suggest that disruption of *PEG3* expression in humans is associated with higher levels of depression and anxiety. Although conclusions cannot be drawn from single genes it was interesting to note the parallels between the phenotypes of this gene and those of the current model.

Though few significant DEGs were identified, enrichment analyses on nominally differentially expressed genes were carried out to explore the underlying mechanisms that may be affected by loss of *Peg3* expression. A number of GO terms that were nominally enriched when comparing single genotype mutant groups to WT groups fall under the umbrella of metabolic activity which, despite the catch-up growth in *Peg3KO* (mutant litter) mice, is consistent with the early life growth restriction observed in this model. Consistent with this, the top MGI terms that were nominally enriched were associated with embryonic growth restriction and preweaning lethality, which is reflected both in the weight data at P2, the mortality score in the *Peg3KO* (mutant litter) group in Chapter 3, and previous literature (Li et al., 1999, Kim et al., 2013).

Though there were no significant DEGs or enriched pathways when comparing the two mutant groups, when comparing WT (mutant litter) to WT (WT litter) mice, enrichment of two dopaminergic ontologies tentatively suggest evidence of altered dopaminergic neuron differentiation which can be attributed to environmental effects, rather than that of *Peg3* disruption. Disruption of dopamine receptors has been shown to be associated with decreased USV emission (Curry et al., 2013). Although the results of this comparison should be interpreted with care given the use of nominally differentially expressed genes, this is consistent with the atypical maternal care of the mixed litter dams observed in Chapter 3.

When looking at the olfactory bulb, there were no significant differentially expressed genes across any of the groups. At the adjusted alpha level, this also included *Peg3* when comparing mutant and WT mice. Whilst shown to be highly expressed in the olfactory bulb of adult mice (Li et al., 1999, Ramos et al., 2013) there is limited research exploring the extent to which *Peg3* is expressed in the olfactory bulb of neonatal mice. Of the two studies reporting expression, Kuroiwa et al. (1996) observed low *Peg3* expression in the neonatal olfactory bulb, while at P0, Gray et al. (2004) failed to observe any expression. In the current study, averaged read counts show that *Peg3* was expressed in the olfactory bulb, albeit at lower levels than the hypothalamus. These discrepant findings suggest that further research would benefit from exploring the varying levels of *Peg3* expression in different tissues throughout the lifespan, potentially utilising single-cell RNA-sequencing to explore the possibility that loss of *Peg3* results in fewer *Peg3* positive cells. Alternatively, as *Peg3* was observed to be differentially expressed at the non-adjusted level, it may be that running the analyses through less stringent differential expression analysis software would yield the expected result, however limma + voom, the software used in the current study, has

consistently been reported as one of the most balanced software options when considering, precision, sensitivity, and accuracy (Costa-Silva et al., 2017).

4.7.4 Limitations

One of the main limitations of this study remains, as discussed in section 4.3.3, the lack of power to explore a sex by genotype interaction. Consequently, though the current study explored how the effects of *Peg3* loss of function impacted the social behaviour of males and females independently, it was unable to draw conclusions as to whether males were more severely affected than females. Previous research has suggested that the impact of *Peg3* results in a sexually dimorphic phenotype whereby males are more severely affected (Kim et al., 2013, Faisal et al., 2014, Janssen et al., 2016, Tunster et al., 2018, Sumption, 2020), in conjunction with this, the current study shows that the development of social behaviour in male, but not female *Peg3KO* mice, is disrupted. Consequently, further research should aim to explore this further, with adequate power to more accurately test whether the social behaviour in males is more severely affected than in females. In the same vein, given that post-weaning social deficits were observed in males, but not females in this study, future research could explore more male specific social behaviour, such as social dominance behaviours. This is particularly important as the significant differences seen between the groups of males in figure 4.17 were driven by the male specific tests, specifically courtship behaviours, and scent-marking, as removal of these tests resulted in the loss of significant differences between the male groups. Recently, it has been shown that *Peg3* adult males show reduced social dominance behaviours compared to their NTG littermates (Tanaka et al., 2022), which provides preliminary support for the idea that male *Peg3* mutant mice may show specific social impairments and emphasises the need to explore this further.

A further limitation of this study is that not all post-weaning tests were incorporated into the unified sociability score. Firstly, the social olfaction test was excluded from the unified scoring model as all mice, including WT $^{(WT\ litter)}$ did not seem to be able to discriminate between the two novel urine samples. As previous research has not found 129Sv WT mice to have olfaction deficits, (Harrison et al., 2020) and as the current study showed that all mice could distinguish between water and the first social stimuli presentation, this suggests that the problem was with the second novel social stimuli. The current study used urine as social stimuli from C57BL6/J (B6) mice, with the two novel odours taken from two different cages containing the same number of mice. Past research using this paradigm tends to use urine from a different strain to the host mouse, however the same 'stimuli' strain is generally used to generate both novel stimuli as in Harrison et al. (2020) where both novel stimuli were taken from different cages of CD1 mice. As B6 mice are highly inbred, showing high levels of genetic homogeneity it may be that the novel stimuli were too similar to produce the expected result in WT mice. Ideally, this behavioural paradigm would have been repeated with a separate cohort using urine from different strains of mice for each stimulus presentation. However, due to time and financial restraints this wasn't possible in the current study.

In a similar vein, a further limitation was that the same strain and genotype of mouse was used in the social propinquity test, which given that both genotypes in the mixed litter group spent significantly more time sharing the tube, may have biased the test in favour of the mixed litter mice given that they had prior experience of sharing their environment. In the future, this test should be repeated using a conspecific of a different strain, to aid in

delineating whether mice from mixed genotype litters show a preference for social proximity to an unfamiliar conspecific as opposed to an aversive environment.

Finally, a limitation of this experiment, is its use of a global loss of function model of *Peg3*. As highlighted in Chapter 1, *Peg3* is expressed in many areas of the body and brain. Loss of function of *Peg3* generally has not been widely researched, with the majority of research to date being focused on maternal behaviour and the growth and development of offspring, both pre, and postnatally (Li et al., 1999, Curley et al., 2004, Curley et al., 2005, Kim et al., 2013, Denizot et al., 2016, McNamara et al., 2018a, Tunster et al., 2018). In addition to the decificits in body weight, postnatal lethality and maternal care highlighted in the previous chapters, more generally, loss of function of *Peg3* has been associated with altered p53 signaling in apoptosis, and disregulation of mitochondrial genes and oxytocin receptors in the brain (Relaix et al., 2000, Thiaville et al., 2013). Given that *Peg3* is completely knocked out in the model used, it may be this results in deficits which would alter the social behaviour in mice, however, previous literature has not reported any deficits that would impair social behaviour directly, such as general motility deficits, or general sensory ability, and globally, the nervous system phenotype has been found to be functioning normally (Li et al., 1999).

Importantly, with a global loss of function model, it is difficult to ascertain the extent to which loss of *Peg3* in specific brain areas may impact on the behaviour of *Peg3* mutant mice. For example, many of the impairments associated with global loss of function of *Peg3,* such as impaired milk let down, and poor maternal care in dams are thought to be due to a reduction of oxytocin producing neurons in the hypothalamus (Li et al., 1999). On the other hand, phenotypes relating to energy homeostasis, such as pubertal delay, excess fat deposits and growth restriction (Curley et al., 2005) are thought to associated with loss of expression of *Peg3* in the hippocampus. It is unlikely that the behaviours shown by *Peg3* mutant mice in this chapter is solely driven by loss of *Peg3* in a specific brain region, as, as highlighted in Chapter 1, social behaviours are associated with a number of brain regions and neural networks. Further, though maternal care deficits observed in this model may be partially explained by loss of *Peg3* in the hypothalamus (Li et al., 1999), it is unlikely that this is the sole cause for the observed maternal phenotypes, as mammary gland development, and sexual reproduction are both normal in mutant mothers, despite this behaviours being linked to the hypothalamus (Lightman, 1992). Despite this, though conditional knockouts of the *Peg3* model do not yet exist, they would be useful for future research to determine the extent to which these specific brain regions contribute to the altered behaviour of *Peg3* mutant mice.

4.8 Summary

Table 4.15 highlights the key findings from this chapter, whilst the results are summarised below.

Table 4.15 Summary of Chapter 4 results

Analysis	Key Finding
Pre-weaning Behaviour	• *Peg3KO* (mutant litter) males show an early life USV deficit compared to WT controls • *Peg3KO* (mutant litter) mice although growth restricted at P2 demonstrate catch up growth to WT controls by P10 • *Peg3KO* (mixed litter) mice remain growth restricted throughout adulthood and do not show catch up growth
Post-weaning Behaviour	• *Peg3KO* (mutant litter) males show social deficits compared to WT male controls • Both WT and mutant *Peg3* male mice raised in mixed genotype litters displayed impairments in post-weaning social behaviour compared to WT mice raised in WT litters. • No significant differences in social behaviour were observed in female mice.
RNA-sequencing	• No significant differences in gene expression were observed in either the hypothalamus or olfactory bulb across any group

For the first time, this chapter demonstrated that loss of *Peg3* expression results in early life USV deficits specifically in male mutant mice raised in single genotype litters. Unified scoring of later life social behaviour demonstrated not only deficits in mutant males from single genotype litters, but also mutants and their WT littermates raised in mixed litters. As these behaviours were observed in male mutants from both single, and mixed genotype groups, and in male WT littermates, the behavioural changes were attributed the adverse pre- and postnatal environment induced by placental endocrine insufficiency though this warrants further investigation. As these deficits were only observed in male, but not female mice, this chapter lends further support to the suggest that loss of expression of *Peg3* has a sexually dimorphic phenotype. Finally, we did not see transcriptional changes in any group in either the olfactory bulb or hypothalamus at P6.

5

Chapter 5: Perinatal Mental Health and Mother-Infant Social Behaviours

5.1 Overview

It has been well established that exposure to stressors during gestation can be detrimental to both pregnancy and to fetal and infant health, with recent studies suggesting that exposure to prenatal stresses *in utero* can be persistent enough to span generations (Bowers and Yehuda, 2020). Perinatal mental health has been cited as one of the most disruptive factors to the mother-infant relationship and is among the most well-replicated risk factors for a host of negative infant outcomes (Goodman and Gotlib, 1999).

The most common mental health conditions perinatally are depression and anxiety, with prevalence rates of perinatal anxiety ranging between 13-21% depending on the population (Fairbrother et al., 2015), and recent reports suggesting a worldwide prevalence of 11.9% for perinatal depression (Woody et al., 2017). There is a substantial body of evidence demonstrating that perinatal depression is associated with increased risk for a number of adverse infant outcomes including impaired cognitive, emotional, and behavioural development (Field, 2010, 2011, Goodman et al., 2011). Although less widely researched, there is also a growing body of evidence to support the detrimental impact of perinatal anxiety on the same infant outcome domains (Barker et al., 2011, Davis and Sandman, 2012, Savory et al., 2020). As mentioned in Chapter 1, it is difficult to disentangle the effects of depression and anxiety given their high comorbidity, however there is a growing body of evidence to suggest that the conditions have distinct trajectories, the severity of which increases when the conditions are comorbidly presenting (Penninx et al., 2011). Studies using the Avon Longitudinal Study of Parents and Children (ALSPAC) cohort have highlighted evidence of distinct trajectories for perinatal depression and anxiety. In a study of over 3,000 mother-infant dyads, prenatal depression was associated with increased externalising difficulties and a decrease in verbal IQ score, whilst prenatal anxiety was associated only with increased internalising difficulties (Barker et al., 2011). Postnatally, maternal depression showed a more 'general effect' on a number of infant outcomes, whilst postnatal anxiety was again associated with an increase in internalising difficulties, with an effect twice as large as the effect of maternal depression on the same measure (Barker et al., 2011). Regarding the severity of comorbid depression and anxiety, mothers demonstrating both high depression and anxiety scores have been shown to smile less and show more intrusive behaviours than those scoring highly on each condition

independently (Heinisch et al., 2019). In conjunction with this, infants with comorbid high scoring mothers have also been shown to express more distress than infants with mothers presenting with high depression or anxiety scores alone (Field et al., 2005). Importantly, these negative associations are not only observed in mothers with a clinical diagnosis, but also are apparent in infants of mothers with subclinical symptomology, suggesting the relationship may act on a spectrum, or be subject to a number of mediating variables (Stein et al., 2014).

Notably, the period in which the onset of symptoms occur has been shown to manifest different developmental trajectories both for the mother and for the infant (Barker et al., 2011, Putnam et al., 2017, Wikman et al., 2020). The onset of anxiety symptoms in the first 8 weeks postpartum for example, have been shown to be more severe than the onset of those prenatally (Putnam et al., 2017). In regard to infant developmental trajectories, prenatally, exposure to maternal depression in the second, but not the first or third trimester has been associated with altered brain morphology in children (Lebel et al., 2016). In a UK cohort, infants were more likely to be diagnosed with depression at 18 when their mothers suffered from prenatal depression, independently of maternal depression symptoms that manifested postnatally (Pearson et al., 2013). In the same study, maternal education was seen to moderate the association between symptoms of maternal postnatal depression and offspring depression, but not prenatal depression, suggesting that different mechanistic pathways underlie the relationship between offspring outcomes and pre- and postnatal exposure to maternal mental health conditions (Pearson et al., 2013, Stein et al., 2014). In light of this, it is important for future research to consider assessing maternal mental health symptomology at multiple time points (Stuart-Parrigon and Stuart, 2014).

A further potential confounder in exploring the relationship between perinatal mental health and infant outcomes is infant sex. Sex differences in both fetal and placental development have consistently been reported in healthy pregnancies, and it is well established that exposure to *in utero* stressors lead to sex differences in infant mortality, cortisol responses, and psychopathology risk patterns (Bale, 2011, 2016, Ostlund et al., 2016, Hodes and Epperson, 2019). Yet despite this, only a limited number of studies separate analysis by fetal sex, instead controlling for this variable, and potentially obscuring any sex-specific findings (Savory et al., 2020). Evidence for sex-specific outcomes as a result of prenatal stressors has largely been based on animal models, which have suggested males to be more severely affected by prenatal and early postnatal exposure to altered maternal behaviour. For example, Iturra-Mena et al. (2018) demonstrated that when pregnant rat dams were subject to stress, only their male offspring showed postnatal increased anxiety and depression-like behaviours. Similarly, in Chapter 4, it was shown that only male mouse pups who had been exposed to 'anxious' dams and atypical maternal care demonstrated impaired social behaviours.

Human research that has explored sex differences in infant outcomes in response to perinatal depression has largely demonstrated similar findings. In a Finnish cohort, postnatal depression was shown to be associated with low social competence in boys, but not girls (Korhonen et al., 2012). Importantly, this also reinforces the suggestion that the timepoint at which maternal symptomology is assessed is important, as this effect was only observed postnatally. However, another study has also shown that prenatal depression may affect males more severely, with prenatal depression showing associations with impaired motor skills and affect regulation in males, but not females (Gerardin et al., 2010). However, there are discrepancies in the human literature. Kersten-Alvarez

et al. (2012) for example has shown that postnatal depression was associated with low social competence and verbal IQ in girls but not boys. More recent research, however, suggests that males and females show different susceptibilities to risk in response to exposure to perinatal mental health conditions, but the consequences may be more long term for males (Braithwaite et al., 2020). However, given the lack of research in this area, more research is needed to fully understand the sex-specific associations between perinatal mental health and infant outcomes.

Recently, it has been demonstrated that the manner in which infant outcomes are reported is also important, as maternal mood symptoms have been shown to be associated with sex-specific differences in maternally reported infant outcomes (Savory et al., 2020). In this study, in addition to completing a battery of maternal report questionnaires, a sub-set of the GiW mother-infant dyads attended an in-person laboratory assessment one year after birth (Chapter 2). However, there were distinct differences in the relationship between infant outcomes and prenatal maternal mood when infant outcomes were measured via maternal report or assessed independently. When considering maternally reported outcomes, prenatal depression and anxiety were associated with impaired bonding, negative temperament, and worse behaviour in female infants, but not males. Whilst in contrast, when using independently rated assessments, the associations were seen between language development and temperament in males, with only minimal associations to female characteristics (Savory et al., 2020). Poor maternal mental health has been linked both to impairments in mother-infant bonding and to negative infant temperament (Davies et al., 2021). As such, it has been suggested that the discrepancies between measures in Savory et al. (2020) may, due to societal expectations of girls and boys, be due to mothers perceiving female infant behaviour as more problematic, particularly when confounded by the effect of poor prenatal maternal health (Savory et al., 2020, Sumption, 2020). However, the mechanisms linking exposure to perinatal maternal mood symptomology and sexually dimorphic outcomes are still largely unknown.

Recent studies have found evidence for a mediating effect of the quality of mother-infant interaction in the relationship between maternal depressive symptoms and adverse infant outcomes (Burt et al., 2005, O'Leary et al., 2021). However, these studies tend to focus on broad domains of interaction, such as parenting quality, rather than specific manifestations of these broader constructs. Social behaviours between mother and infant, both verbal and non-verbal, are among the cornerstones of infant development, laying the foundation for both social-emotional, and language development (Murray et al., 1993). Though the research is limited, studies have also shown an association between perinatal mental health, and maternal communication, with one study finding that 80% of mothers with depression are either withdrawn, or intrusive in both verbal and non-verbal interactions with their infants (Defelipe et al., 2019). Whilst other studies have shown that mothers with depression vocalise less to their infants (Lovejoy et al., 2000). Mothers scoring highly on depression measures have also been shown to use less infant-directed speech (Lam-Cassettari and Kohlhoff, 2020), a key component of responsive communication, and an important factor in the development of language for infants (Ramírez-Esparza et al., 2017). Although maternal anxiety has been explored substantially less, there is evidence that mothers with anxiety also show increased levels of intrusive communication (Crugnola et al., 2016). However, there remains a lack of research exploring the links between vocalisation, perinatal mental health, and infant outcomes, and little, if any research exploring sex-specific outcomes in this relationship.

Research into the relationship between perinatal mental health and the social communication between mother and infant is also often limited by the methods of data collection or coding. For example, as in Savory et al. (2020), many studies utilise methods of coding which score via 'time-bins' where a behaviour is only recorded at a given instance in incremental time windows, for example, every 30 seconds, as was used in the original scoring of the GiW laboratory assessments using the Lab-TAB coding scheme. Subsequently, subtle behaviours and the full repertoire of social behaviours may not be adequately captured. Further, many studies focus on only global behaviour categories, and so may unwittingly group unrelated behaviours, hiding or emphasising differences that do not exist (Weinberg and Tronick, 1994). Finally, many studies focus solely on the either the mother, or infant, neglecting the reciprocal relationship between the two.

As highlighted in Chapter 1, the mother-infant relationship is reciprocal, and though mothers with perinatal mood disorders have been shown to engage less with their children, children of depressed mothers have also been shown to be more difficult to engage, be less likely to maintain social interactions and be more temperamentally difficult than children with mothers not scoring highly on depressive symptomology measures (Jameson et al., 1997, Tronick and Reck, 2009). This altered behaviour, may in turn, negatively affect the mother, culminating in a negative cycle of mother-infant interaction which may further contribute to maternal mood symptomology. Whilst this relationship can be difficult to disentangle, consideration of whether specific maternal or infant social behaviours as mediators in the relationship between perinatal mental health and infant outcomes may shed light on whether these variables play a role in this relationship (O'Leary et al., 2021).

Importantly, not all children exposed to perinatal depression and anxiety suffer adverse consequences, suggesting that gene by environment interactions play a role in this variation, and may potentially also explain sexually dimorphic outcomes such as those reported in Savory et al. (2020). One potential mechanism for this is via an altered *in utero* environment, specifically through altered functioning of the placenta (Creeth and John, 2020). As highlighted in Chapter 1, the placenta is a fetally derived organ where sex-dependent differences in gene expression and hormone production naturally occur (Murphy et al., 2006, Clifton, 2010, Bale, 2016). *PEG3* is an imprinted gene expressed in the placenta which our group has recently shown to be associated with prenatal depression, but only in mothers of male infants, across 4 independent cohorts, including the GiW cohort (Janssen et al., 2016, Sumption, 2020). In the GiW cohort, placental *PEG3* expression has also been shown to be negatively associated with infant positive affect in the sustained attention task (Sumption, 2020), whilst conversely, another study has shown that methylation at the *PEG3* promoter region is positively associated with negative affectivity (Fuemmeler et al., 2016). Taken together, these studies suggest placental *PEG3* expression as a candidate in linking perinatal mental health and adverse infant outcomes. Notably, in mouse models, *Peg3* expression has been associated with early life social deficits in male, but not female mouse pups (Chapter 4) and also been associated with increased anxiety-like behaviours in dams (Chapter 3 and McNamara et al. (2018a)). Consequently, it may be that placental *PEG3* expression is also associated with either maternal, or infant social behaviours, though this remains to be explored.

5.1.1 Aims

The over-arching aim of this chapter was to explore the relationship between mother-infant interactions, pre- and postnatal maternal mental health and placental *PEG3* expression, using previously collected data from mother-infant dyads in the Grown in Wales cohort, and newly generated vocalisation and communication data from the same mother-infant dyads, using a novel, micro-analysis, coding scheme.

Firstly, this chapter aimed to explore the relationship between both maternal and infant vocalisations and perinatal mental health. Secondly, for the first time, this chapter aimed to explore the relationship between placental *PEG3* expression and vocalisations in humans. As sexually dimorphic outcomes have been observed both in response to *PEG3* expression, and independently in the relationship between perinatal mental health and infant outcomes, the impact of fetal sex was also explored.

Finally, in light of research highlighting a mediating role for mother-infant interaction in the relationship between perinatal mental health and adverse infant outcomes, this chapter aimed to explore whether the relationship between prenatal mental health and maternally reported outcomes in Savory et al. (2020) were mediated by specific aspects of maternal or infant social behaviour.

5.2 Methods

Methods for recruitment of the Grown in Wales (GiW) cohort are detailed in Chapter 2, for ease of interpretation, the nomenclature for collection times are redefined in Table 5.1, however only time points A1 and Y1 are of interest to the current study.

Table 5.1 Data collection time points

Time-Point	Reference
Day of pre-surgical appointment	A1
Within one week of birth	P1
Ten weeks after birth	P2
One year after birth	Y1

One year postpartum was chosen as the time to conduct in person laboratory assessments of the mother-infant dyads, due to the wide range of available tests available at the timeframe, including the 'maternal separation, or 'strange situation' test (Ainsworth and Bell, 1970; Goldsmith and Rothbart, 1996, the gold standard assessment for infant attachment which is designed to be carried out at 12 months, and due to the expertise of the developmental psychologist associated with the GiW study with this age range (Janssen, 2015). The implications of this choice of time-point is discussed in section 5.4.

5.2.1 Participants

As described in Chapter 2, participants from the GiW cohort at A1 were invited to attend an in-person laboratory assessment at 12 months postpartum. Participant demographics and exclusion criteria are described in section 5.3.1.

5.2.2 Setting and Task

As described in Chapter 2, mother-infant dyads were assessed in an experimental testing room with tasks recorded via a digital camera for later offline analysis. The focus of this experiment was on the social behaviour of both mother and infant during the Freeplay task. The Freeplay task was the first task undertaken during the laboratory assessments, where mothers were instructed to play with their child as they usually would at home, using any of the toys present in the room. Researchers were instructed not to interact with the mother or child. The duration of the task was 5 minutes.

5.2.3 Coding of social behaviours

Social behaviours of both the mother and infant were micro-coded on a frame-by-frame basis using the Mental Health Intergenerational Transmission (MHINT) coding manual (Costantini et al., 2021), and Observer XT software (Noldus). Tables 5.2 and 5.3 show the verbal and non-verbal behaviours that were coded for during the Freeplay task. Total duration and the frequency of each behaviour were automatically generated by Observer XT and were both used as variables for analysis.

Table 5.2 Vocalisation behaviours, their definition, and the percentage of mother-infant dyads in which the behaviour was present during the Freeplay task (N).

Vocalisation	Definition	N (%)
	INFANT VOCALISATIONS	
Distress	Infant's tone conveys sad, scared, fussy or angry affect	19 (28%)
Non-Distress	Infant's tone conveys positive or neutral affect	68 (100%)
All Vocalisation	All infant vocalisation	68 (100%)
	MATERNAL VOCALISATIONS	
All Vocalisation	All vocalisations made by the mother	68 (100%)
Infant-directed Speech	Maternal speech directed to infant	67 (99%)
Other-directed	Maternal speech directed to a subject other than the infant	48 (71%)
Adult Register	Words or speech directed to the infant characterised by normal intonation patterns	66 (97%)

Infant Register	Words or speech directed to the infant characterised by high pitch	61 (90%)
Positive tone	Mother's tone conveys positive, warm, and happy affect	68 (100%)
Negative Tone	Mother's tone conveys distressed, sad, angry, upset, or disgusted affect	10 (15)%
Neutral tone	Caregiver's tone lacks a clear affect	68 (100%)
Command	The mother orders the infant to do something	58 (85%)
Question	The mother is asking something	65 (96%)
Statement	Making a statement about something in the environment	64 (94%)
Acknowledgement	Description or validation of the infants' actions	61 (90%)
Odd content*	A vocalisation not aligned with the context of the task e.g., swearing, lying	0
Praise of Infant	Verbal expression of positive evaluation	42 (62%)
Criticism of Infant*	Verbal expression of negative evaluation	3 (4%)
Verbal Intrusiveness*	Caregiver verbally interrupts infants' action	8 (12)
Teaching*	The mother is communicating in such a way to pass along knowledge	0
Humour	A vocalisation expressed with the purpose of being funny e.g., a joke, rhyme etc	17 (25%)
Laugh	Laughing	59 (87%)
Maternal Imitation	Caregiver imitates infant vocalisations	34 (50%)
Encouragement	Caregiver positively attempts to draw the infant's attention toward focus	65 (96%)
Discouragement	Caregiver negatively attempts to draw the infant's attention away from focus	44 (65 %)

*Vocalisations coded in less than 10 mother-infant dyads were excluded from analysis

*Definitions adapted from (Costantini et al., 2021)

Table 5.3 Non-verbal behaviours, their definition, and the percentage of mother-infant dyads in which the behaviour was present during the Freeplay task (N).

Behaviours		N (%)
	INFANT BEHAVIOURS	
Caregiver-directed play	Infant is playing with, or trying to play with the caregiver	45 (66%)
Solitary play	Infant is playing alone	57 (84%)
Oriented to Caregiver	The infant's shoulders and legs are turned in the direction of the caregiver	67 (99%)
	MATERNAL BEHAVIOURS	
Infant directed play	Caregiver is playing with, or trying to play with the infant	65 (96%)
Oriented to Infant	The mother's shoulders and legs are turned in the direction of the caregiver	66 (97%)
Loom	While within reach, the caregiver intentionally moves their face to within 30 cm of the infant's face either cheek to cheek or face to face	39 (57%)
Within reach of Infant	Caregiver is within their own arm's length of infant	68 (100%)

*Definitions adapted from (Costantini et al., 2021)

Each behaviour was coded individually by a researcher blind to the mental health scores of mothers and the placental *PEG3* expression of the infant. 20% of videos were independently coded by an additional researcher in order to calculate inter-rater reliability. Cohen's Kappa values for each measure, and for overall reliability between coders are presented in Table 5.4. Values of .61-.80 demonstrate substantial and acceptable agreement between coders, whilst a value of > .81 is deemed as excellent, demonstrating near perfect agreement (McHugh, 2012).

Table 5.4 Inter-rater reliability statistics of video coding

Measure	Cohen's Kappa
Vocalisations	0.91
Play	0.87
Orientation	0.87
Proximity	0.94
Overall	0.90

5.2.4 Questionnaire Measures

Details of the questionnaires used at each time-point are extensively described in Chapter 2. However, for ease, the names of the questionnaires, their broad focus of assessment, and the timepoint at which they were assessed are recapped in Table 5.5.

Table 5.5 Questionnaires used for assessment

Questionnaire	Target Measure	Timepoint
Edinburgh Postnatal Depression Scale (EPDS)	Depressive symptoms	A1 & Y1
State-trait Anxiety Inventory (STAI)	Anxiety symptoms	A1 & Y1
Postpartum bonding questionnaire (PBQ)	General bonding problems (PBQ 1) Rejection and anger (PBQ 2) Infant-focused anxiety (PBQ 3)	Y1
Cardiff Infant Contentiousness Scale (CICS)	Infant aggression	Y1
Infant Behavioural Questionnaire Revised-Short Form (IBQ-R-SF)	Infant temperament: surgency, negative affect and regulatory capacity	Y1

5.2.5 Placental gene expression

Placental gene expression data was generated by Sumption (2020) and methods are fully described in Sumption (2020). Briefly, placentas were collected within 2 hours of delivery by trained research midwives and were both weighed and assessed for abnormalities. Three to five biopsies of villous tissue were taken from the maternal side of the placenta, midway between the cord and distal edge, within these confines, the sample sites were random to account for differential gene expression across the placenta (Janssen et al., 2015, Sumption, 2020). To reduce contamination of the maternal decidual cells, the cotyledon surface was removed. Following collection, biopsies were washed in PBS (Life Technologies, UK) to remove amniotic fluid, and were then stored in RNA*later* (Sigma, UK). Biopsies were then combined to make a pooled sample for RNA extraction. Full details of RNA extraction can be seen in Appendix A8.

Once extracted, RNA concentration was determined using a Nanodrop ND-1000 spectrophotometer and a Qubit Assay Kit (ThermoFisher), with a minimal acceptable RIN value of 7. RNA was then stored at -80°C until needed.

Gene expression data was calculated using quantitative PCR (qPCR) by Sumption (2020). Although Sumption (2020) calculated gene expression data for a number of genes, only placental *PEG3* data was used in the current study and was available from 43 placentas. Primer sequences for *PEG3* were taken from Feng et al. (2008) and can be seen in Table 5.6, alongside the primer sequences for YWHAZ and SDHA which were used as reference genes based on their use in previous studies on human placental tissue (Meller at al. 2005, Drewlo et al. 2012).

Table 5.6 Primers used for qPCR analysis of *PEG3* gene expression

Target Gene	Primer Sequence	Reference
PEG3	Forward: CTCACAACACAATCCAGGAC Reverse: TAGACCTCGACTGGTGCTTG	(Feng et al., 2008)
SDHA[1]	Forward: GAACATCGGAACTGCGACTC Reverse: CCTCTGCTCCGTAGATGGTC	(Janssen 2015)
YWHAZ[1]	Forward: TTCTTGATCCCCAATGCTTC Reverse: AGTTAAGGGCCAGACCCAGT	(Sumption 2020)

[1] *used as a reference gene*

The protocol for qPCR quantification of *PEG3* expression is detailed in Appendix A8. Gene expression was calculated and expressed as -ΔCT, normalised to the two reference genes, where the target gene expression is relative to the geometric mean of the reference gene expression. ΔCT values were multiplied by -1 so that higher ΔCT values represented higher concentrations. Values of ΔCT placental *PEG3* expression of the samples used in this analysis is presented in Table 5.7.

Table 5.7 Placental *PEG3* Expression from dyads who attended the in-person assessments

Sex	N	Gene expression (-ΔCT)
All Samples	43	1.75
Male	20	1.79
Female	23	1.72

5.2.6 Statistics

The aim of this chapter was to explore extensively characterised social behaviours of mother-infant dyads from the GiW cohort in relation to maternal mental health, infant outcomes and placental *PEG3* expression data that has previously been collected and analysed in both Savory et al. (2020) and Sumption (2020). As such, for consistency, and due to the same relationships being analysed in the context of new social behaviour data, the current chapter used the same statistical procedures as both Savory et al. (2020) and Sumption (2020).

Normality was assessed using both Shapiro-Wilk and Kolmogorov tests in conjunction with visual inspections of histograms. However, all data with the exception of some demographic variables met the assumption of normality. Where slight variations of skew or kurtosis were observed, data was still analysed via parametric tests following guidelines to suggest regression analyses are robust to minor deviations of normality, and that transformation of data may lead to less reliable or transparent findings (Knief and Forstmeier, 2021).

Continuous scales of all measurements were used rather than categorical cut-offs to maximise statistical power. To initially assess the relationship between maternal mental health and both mother and infant social behaviour, partial correlations controlling for infant age at assessment were utilised. To determine the potential influence of infant sex, partial correlations were repeated separately for male and female infants. As sex differences were a main focus of this chapter, only relationships significant at $p < .05$ when separating for infant sex were taken

forward for analysis at the regression level, whereby a series of multiple hierarchical regression analyses were then conducted controlling both for infant age, and maternal parity.

Maternal parity has consistently been shown to affect early mother-infant relationships and has been associated with maternal depression (Fish and Stifter, 1993, Myers and Emmott, 2021). Other maternal demographic variables have been associated with maternal mental health and the mother-infant relationship, such as maternal education (Fergusson and Woodward, 1999, Carr and Pike, 2012, Pearson et al., 2013), and maternal age at birth (Ragozin et al., 1982, Fergusson and Woodward, 1999, Camberis et al., 2016). However, due to the small sample size in the current study, additional confounding variables were not added to the regression models without comprising the validity of the model (Labovitz, 1965). Consequently, as in previous research with the GiW cohort, separate analyses were run on pre-and postnatal data to differences in infant outcomes at each timepoint (Savory et al., 2020, Sumption, 2020).

The same statistical procedures were followed when assessing the relationship between placental *PEG3* expression and maternal and infant vocalisations.

As highlighted in section 5.1, if prenatal maternal mental health was predictive of any social behaviour variable, a further aim of this study was to examine whether these behaviours mediated the relationships between prenatal maternal mental health and maternally reported infant outcomes one year postpartum that were observed in Savory et al. (2020). Prior to running mediation analyses however, regression analyses were first carried out on maternal mental health data and maternally reported outcomes to ensure these findings were replicated in the current sample. Although data from both studies were taken from GiW participants who had attended the laboratory assessment sessions, additional exclusion criteria for this study meant that the sample size in this chapter was slightly different to that reported in Savory et al. (2020) (section 5.3.1).

If these results were replicated, and social behaviours were found to be significantly associated with prenatal mental health scores, significant variables would be taken forward for mediation analysis using guidelines set out by Baron and Kenny (1986) and the conceptual template shown in Figure 5.1 For a mediation effect to be present three assumptions must be met:

1. The independent variable must significantly predict both the mediator and dependent variable.
2. The mediator must significantly predict the dependent variable
3. There must be a significant relationship between the dependent and independent variables, with the addition of the mediation factor.

If these assumptions are not met then a mediation effect cannot be present (Baron and Kenny, 1986).

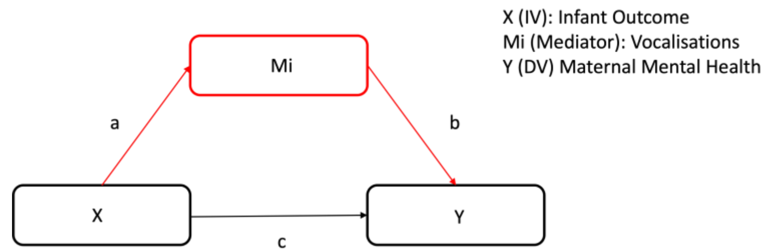


Figure 5.1 Conceptual template for mediation analysis

5.3 Results

5.3.1 Participant Characterisation

Demographics for the 68 mother-infant dyads who took part in the assessments and whose video recordings were able to be analysed are presented in Table 5.8. Initially, 83 participants were recruited for laboratory analysis as in Savory et al. (2020), however for the current experiment, 15 participants were excluded. 3 participants were excluded due to technical difficulties resulting in loss of video data for the assessment, 4 were excluded to due to excessive interaction from an individual other than the mother during the Freeplay task, 5 further dyads were excluded due to the infant being off camera for more than 50% of the task, 2 further dyads were excluded due to the mother not having undergone an elective caesarean, and finally 1 dyad was removed as the father attended the assessment and not the mother. Of the dyads whose data was useable, A1 maternal mental health scores were available for 55 mothers, and Y1 scores were available for 61.

Table 5.8 Participant demographic information

Demographics	**Mother-Infant Dyads (n = 68)**
Categorical Variables	***%***
Highest Education Level	
Left before GCSE	0.00
GCSE or vocational	13.43
A-level	8.96
University	41.79
Postgraduate	35.82
Parity	
Nulliparous	20.59
Multiparous	79.41
Fetal Sex	
Male	52.94
Female	47.06
Continuous Variables	***Mean***
Maternal age at birth (years)	34.47
Infant age at assessment (months)	12.65
A1 EPDS	7.09
A1 STAI	34.13
Y1 EPDS	6.64
Y1 STAI	35.68

5.3.2 Maternal perinatal mental health and infant social behaviours

Partial correlations were performed for both A1 and Y1 depression and anxiety scores against both the frequency and duration of infant vocalisation (Tables 5.19 and 5.10) and the frequency and duration of non-verbal social behaviours (Appendix A9)

Table 5.9 Perinatal mental health and frequency of infant vocalisations during the Freeplay task. Data is R_s; *p* value

FREQUENCIES												
	A1						Y1					
	EPDS			STAI			EPDS			STAI		
	All	Male	Female	All	Male	Female	All	Male	Female	All	Male	Female
Infant Distress	-.11; .43	-.19; .35	-.12; .53	-.09; .53	-.07; .75	-.12; .55	-.25; .49	-.09; .66	-.31; .11	-.12; .37	-.09; .67	-.19; .33
Infant Non-Distress	-.07; .60	-.20; .34	.09; .64	.12; .37	.14; .49	.10; .61	-.01; .92	-.06; .78	.06; .77	.09; .50	.15; .47	-.003; .99
Any Infant Vocalisation	-.12; .43	-.21; .35	.06; .76	-.09; .54	.14; .50	.07; 72	-.12; .92	-.06; .76	-.03; .87	-.12; .37	.14; .49	-.06; .77

[1] Data is R_s; :Spearman's rank correlation coefficient
[2] Controlling for infant age at time of assessment

Table 5.10 Perinatal mental health and the duration of infant vocalisations during the Freeplay task. Data is R_s; *p* value

DURATION												
	A1						Y1					
	EPDS			STAI			EPDS			STAI		
	All	Male	Female	All	Male	Female	All	Male	Female	All	Male	Female
Infant Distress	-17; .21	-.22; .29	-.16; .42	-.17; .21	-.19; .36	-.17; .39	-.27; .05	-.29; .17	-.30; .12	-.20; .14	-.22; .28	-.20; .30
Infant Non-Distress	-.01; .94	-.14; .51	-.01; .97	.04; .79	.25; .21	.03; .87	.05; .70	.003; .99	.08; .67	.17; .22	.12; .56	.24; .21
Any Infant Vocalisation	-.02; .86	-.24; .24	-.02; .92	.02; .87	.10; .65	.02; .91	.03; .83	-.16; .43	.06; .76	.15; .26	-.03; .88	.23; .24

[1] Data is R_s; :Spearman's rank correlation coefficient
[2]Controlling for infant age at time of assessment

5.3.2.1 Prenatal mental health and infant vocalisation

When assessing prenatal EPDS and STAI scores, there were no significant associations observed with either the frequency or duration of infant vocalisations, either when analysing all participants together, or split by infant sex (Tables 5.9 and 5.10).

5.3.2.2 Postnatal mental health and infant vocalisation

When assessing maternal mental health postnatally, there were no significant associations between any domain of infant vocalisations, either when analysing participants together, or when stratifying by sex. (Table 5.10).

5.3.2.3 Perinatal mental health and infant non-verbal social behaviour

There were no significant correlations between frequency or duration of infant non-verbal social behaviour and measures of prenatal or postnatal maternal mental health (Appendix A9).

5.3.3 Maternal perinatal mental health and maternal social behaviours

Partial correlations were also performed for both A1 and Y1 depression scores against both the frequency and duration of maternal vocalisations (Tables 5.11, and 5.12) and non-verbal social behaviours during the Freeplay task (Appendix A10).

Table 5.11 Perinatal mental health and frequency of maternal vocalisations during the Freeplay task. Data is R_s; *p* value

FREQUENCY												
	A1						**Y1**					
	EPDS			**STAI**			**EPDS**			**STAI**		
	All	*Male*	*Female*	*All*	*Male*	*Female*	*All*	*Male*	*Female*	*All*	*Male*	*Female*
All Vocalisations	-.19; .17	-.01; .97	-.34; .07	-.12; .40	.02; .96	-.24; .23	-.13; .33	.06; .78	-.32; .10	-.05; .70	.05; .81	-.11; .57
Infant-Directed speech	-.26; .05	-.22; .28	-.30; .12	-.19; .15	-.16; .44	-.22; .27	-.15; .26	-.02; .92	-.29; .13	-.13; .35	-.15; .46	-.06; .76
Other-directed speech	-.12; .39	-.11; .62	-.19; .33	.04; .78	.07; .75	-.07; .74	-.08; .59	-.10; .65	-.10; .62	-.07; .60	-.01; .97	-.24; .22
Adult Register	-.23; .10	-.12; .56	-.32; .10	-.04; .78	-.07; .74	.01; .97	-.06; .68	.07; .75	-.21; .28	-.01; .96	-.09; .66	.09; .65
Infant Register	.05; .72	.17; .40	-.05; .79	-.02; .91	.16; .44	-.13; .51	-.08; .58	.01; .95	-.11; .57	.03; .82	.22; .27	-.05; .80
Positive tone	-.21; .12	-.20; .33	-.21; .29	-.26; .05	-.19; .35	-.33; .09	-.22; .11	-.08; .69	-.33; .09	-.16; .24	-.06; .78	-.21; .29
Negative Tone	**.28; .04**	**.46; .02**	-.06; .78	**.33; .01**	**.43; .03**	.13; .52	**.31; .02**	**.52; .01**	-.19; .34	.21; .12	.36; .07	-.04; .85
Neutral tone	-.01; .94	.19; .35	-.27; .16	.11; .44	.20; .32	-.02; .93	.70; .61	.15; .46	-.04; .85	.09; .52	.12; .57	.07; .71
Praise	-.09; .50	-.11; .60	-.07; .73	-.15; .28	-.16; .43	-.14; .48	-.16; .25	-.05; .83	-.33; .09	-.05; .72	-.06; .77	-.05; .81
Command	-.01; .93	.20; .33	-.26; .18	-.06; .64	.01; .94	-.16; .42	.01; .97	.14; .43	-.20; .31	-.004; .98	.06; .77	-.05; .82
Question	-.004; .97	.31; .13	-.25; .20	-.03; .84	.23; .25	-.23; .25	.08; .55	**.39; .04**	-.16; .42	.14; .32	.31; .12	.06; .77
Statement	-.09; .53	.15; .48	-.34; .08	.06; .65	.24; .23	-.14; .47	-.11; .43	-.003; .99	-.21; .27	.04; .76	.11; .59	.01; .97
Acknowledgement	-.12; .39	-.14; .48	-.11; .57	-.14; .30	-.20; .32	-.04; .83	-.21; .12	-.19; .35	-.23; .24	-.12; .38	-.22 .27	-.03; .88
Humour	.14; .30	.30; .13	-.02; .91	.08; .53	.18; .38	-.05; .80	.07; .63	.11; .58	-.02; .92	.05; .73	.19; 37	-.13; .52
Laugh	-.04; .80	-.11; .58	.05; .81	-.08; .54	-.19; .37	.04; .85	-.01; .93	.04; .85	-.09; .67	-.05; .74	.02; .92	-.15; .45
Encouragement	**-.27; .04**	**-.44; .02**	-.11; .57	-.20; .15	-.23; .17	-.03; .87	**-.31; .02**	-.38; .06	-.25; .20	-.23; .08	-.33; .10	-.03; .90
Discouragement	-.17; .22	-.21; .30	-.14; .48	.10; .32	-.13; .54	-.14; .47	-.15; .27	-.14; .50	-.20; .31	-.13; .35	-.12; .57	-.16; .42
Maternal Imitation	-.07; .63	-.08; .71	-.04; .84	.18; .20	.27; .19	-.02; .91	.02; .89	-.05; .82	.09; .65	.11; .43	.17; .41	-.03; .88

[1] Data is R_s :Spearman's rank correlation coefficient
[2] Controlling for infant age at time of assessment
[3] Correlations significant at the level of *p = .05* are reported in **bold**.

Table 5.12 Perinatal mental health and duration of maternal vocalisations during the Freeplay task. Data is R_s; *p* value

	DURATION											
	A1						**Y1**					
	EPDS			***STAI***			***EPDS***			***STAI***		
	All	*Male*	*Female*	*All*	*Male*	*Female*	*All*	*Male*	*Female*	*All*	*Male*	Female
All Vocalisations	**-.36; .01**	-.17; .40	**-.57; .001**	-.24; .07	-.16; .42	**-.42; .03**	-.17; .07	-.09; .67	**-.48; .01**	-.17; .20	-.12; .56	-.30; .12
Infant-Directed speech	**-.38; .003**	-.28; .17	**-.50; .01**	**-.33; .01**	-.26; .19	**-.47; .01**	-.21; .16	-.09; .65	**-.39; .04**	-.20; .14	-.21; .30	-.22; .26
Other-directed speech	-.12; .36	-.11; .58	-.15; .42	.-07; .60	.02; .93	.11; .57	-.12; .39	-.08; .71	-.19; .33	-.08; .57	-.05; .80	-.16; .41
Adult Register	**-.32; .05**	-.23; .26	**-.44; .02**	-.17; .20	-.25; .28	-.13; 51	-.16; 25	-.08; .71	-.31; .10	-.12; .38	-.22; .27	-.05; .78
Infant Register	-.09; .52	.11; .61	-.30; .12	-.08; .57	.15; .46	**-.36; .05**	-.11; .41	-.02; .94	-.22; .26	-.03; .85	.17; .39	-.21; .27
Positive tone	**-.29; .03**	-.25; 22	-.34; .07	**-.30; .02**	-.23; .25	**-.45; .02**	-.22; .10	-.10; .60	**-.38; .04**	-.20; .14	-.14; .97	-.30; .12
Negative tone	.16; .23	.30; .14	-.07; .72	.21; .13	.27; .18	09; .64	**.26; .05**	**.44; .02**	-.20; .31	.14; .29	.25; .20	-.05; 78
Neutral tone	-.14; .31	.10; .62	-.35; .06	-.03 .82	.09; .70	-.04; .82	-.06; .66	-.01; .98	-.14; .47	-.02; .89	-.004; .98	-.07; .70
Praise	-.21; .37	-.14; .49	-.11; .57	-.14; .31	-.16; .43	-.17; .37	-.18; .19	-.05; .82	**-.37; .05**	-.03; .81	-.06; .77	-.06; .75
Command	-.09; .52	.09; .65	-.33; .08	-.16; .23	-.09; .64	-.30; .10	-.06; .66	.02; .94	-.19; .32	-.07; .61	-.04; .86	-.13; .51
Question	-.11; .40	.14; .42	-.32; .09	-.11; .40	.05; .82	-.26; .17	-.07; .60	.22; .28	-.31; .10	.02; .89	.11; .60	-.06; .76
Statement	-.12; .39	.10; .62	-.34; .07	.11; .41	.20; .32	-.02; .90	-.08; .53	.05; .80	-.27; .15	-.3; .81	.12; .57	-.09; .65
Acknowledge	-.21; .11	-.26; .19	-.21; .28	-.21; .11	-.27; .17	-.17; .37	-.26; .05	-.27; .17	-.28; .14	-.18; .17	-.31; .11	-.09; .63
Humour	-.06; .67	**.45; .02**	-.06; .74	.14; .31	.28; .15	.01; .98	.06; .65	.20; .32	-.09; .64	.11; .41	.32; .11	-.08; .68
Laugh	-.06; .67	-.13; .52	.01; .95	-06; .64	-.19; .36	.07; .71	-.07; .63	-.04; .83	-.12; .55	-.07; .61	-.05; .80	-.14; .48
Encourage	-.10; .46	-.26; .19	-.04; .85	-.04; .76	-.10; .60	.10; .61	-.12; .37	-.13; .52	-.12; .55	-.06; .66	-.13; .53	.04; .84
Discourage	-.19; .15	-.23; .24	-.22; .25	-.15; .26	-.15; .44	-.26; .18	-.13; .32	-.15; .46	-.19; .31	-.13; 34	-.14; .50	-.25; .19
Maternal Imitation	-.05; .73	.03; .88	-.11; .59	.22; .10	**.41; .03**	.03; .87	.03; .80	.17; .40	-.11; .57	.01; .95	.20; .31	-.20; .29

[1] Data is R_s: Spearman's rank correlation coefficient
[2] Controlling for infant age at time of assessment
[3] Correlations significant at the level of *p = .05* are reported in **bold**

5.3.3.1 Prenatal mental health and maternal vocalisation

For frequencies, when analysing all samples together, prenatal depression scores showed a weak positive correlation with negative tone, and a weak negative correlation with encouragement. Prenatal anxiety scores were also moderately associated with the frequency of negative tone (Table 5.11). For duration, prenatal depression scores were negatively associated with all maternal vocalisations, infant directed speech, use of the adult register and positive tone. Prenatal anxiety scores were also negatively associated with the duration of infant directed speech and positive tone (Table 5.12)

When analysing separately by infant sex, sex-specific associations were observed. In mothers with male infants, prenatal depression scores were positively associated with duration of humour. Whilst in mothers of females, a number of negative associations were observed, specifically prenatal depression scores were significantly negatively associated with the duration of any vocalisation, infant-directed speech, and adult register speech.

Consistently, in mothers with female infants, prenatal anxiety scores were also significantly associated with the duration of all vocalisations and infant-directed speech, but also additionally, infant register and positive tone. In mothers with males, prenatal anxiety scores were only associated with the duration of maternal imitation (Table 5.12). There were no significant associations with maternal prenatal health and the frequency of vocalisations in mothers with females, however in mothers of males, both prenatal depression and anxiety were positively associated with frequency of negative tone. Whilst depression scores were also associated with decreased frequency of encouragement in mothers with male infants (Table 5.11).

Significant associations were further analysed in multiple linear regressions controlling for infant age and maternal parity. At the regression level, the association between maternal EPDS scores and the duration of adult register speech was no longer significant ($F(3,27) = 2.64$, $p = .07$), nor was the relationship between prenatal STAI scores and duration of speech in the infant register ($F(3,27) = 3.62$, $p = .06$) in mothers with female infants. In mothers with male infants, the relationship between prenatal depression and frequency of positive tone ($F(3,26) = .37$, $p = .78$) and maternal encouragement ($F(3,26) = .92$ $p = .45$) did not persist at the regression level, nor did the relationship between prenatal anxiety scores and frequency of positive tone ($F(3,27) = .69$, $p = .57$). However, all other significant partial correlations remained significant at the regression level when separating by infant sex. Adjusted R^2 values, regression coefficients and confidence intervals for each significant predictor variable are presented in Table 5.13.

Table 5.13 Multiple regression results for prenatal maternal mental health and maternal vocalisations

Infant Sex	Measure	Vocalisation	B	*p*	95% CI	ΔR^2
Females	*EPDS (A1)*	All Vocalisations (D)	-.05	**.002**	-.09,-.02	.28
		Infant directed (D)	-.05	**.01**	-.09, -.01	.21
	STAI (A1)	All Vocalisations (D)	-.05	**.04**	-.11, -.003	.24
		Positive tone (D)	-.07	**.02**	-.12, -.01	.27
Males	*EPDS (A1)*	Humour (D)	1.92	**.01**	.51, 3.33	.19
	STAI (A1)	Maternal Imitation (D)	2.95	**.02**	.60, 5.30	.25

[1]D: Duration, F: Frequency.

[2]B = Coefficient, CI: Confidence Interval, ΔR^2: Adjusted R squared

[3]Controlling for infant age at time of assessment and maternal parity

In summary, maternal depression scores at term, were significantly associated with reduced duration of all maternal vocalisations generally, and more specifically, with reduced duration of infant directed vocalisations associated with A1 depression scores in mothers of female infants only. Prenatal depression scores from mothers of male infants, however, were significantly associated with increased duration of humour in the Freeplay task. Prenatal anxiety scores also demonstrated specific associations, with higher scores on the STAI being indicative

of a reduction in the frequency of maternal vocalisations generally, but also a reduction in positive tone in mothers of females. Whilst in mothers of males, prenatal anxiety was associated with increased maternal imitation.

5.3.3.2 Postnatal mental health and maternal vocalisation

When analysing participants together, significant associations were observed between postnatal EPDS scores and the frequency of negative tone, and encouragement (Table 5.11). Postnatal depression scores were also significantly associated with the duration of negative tone (Table 5.12). When separating the samples by sex, postnatal depression scores were positively associated with the both the duration and frequency of negative tone, and the frequency of questions in mothers of males. In mothers with female infants, a number of significant negative associations were observed between postnatal EPDS and the duration of maternal vocalisations, specifically; all maternal vocalisations, infant-directed speech, positive tone, and praise. No vocalisations were seen to be significantly associated with postnatal anxiety.

Significant partial correlations were then taken forward for multiple regression analysis as previously noted. The majority of relationships significant at the correlational level did not persist at the regression level. In mothers of males, the relationship between postnatal depression and the duration of negative tone and frequency of questions did not persist at the regression level [negative tone: $F(3,25) = 2.17$, $p = .12$, questions: $F(3,25) = 1.76$, $p = .18$]. In mothers of female infants, duration of infant speech [infant directed: $F(3,26) = 2.58$, $p = .07$, positive tone: $F(3,26) = 2.41$, $p = .09$ and praise: $F(3,26) = 2.34$, $p = .10$] also did not remain significant.

The only relationships to persist at the regression level were the positive association between the frequency of negative tone and postnatal depression scores in mothers of male infants, and the negative association between any vocalisation and postnatal depression scores in mothers of female infants (Table 5.14).

Table 5.14 Multiple regression results for postnatal maternal mental health and maternal vocalisations

Infant Sex	Measure	Vocalisation	B	*p*	95% CI	ΔR^2
Females	*EPDS (A1)*	All Vocalisation (D)	-.05	**.01**	-.08, .01	.21
Males	*EPDS (Y1)*	Negative tone (F)	4.05	**.01**	1.35, 6.76	.02

[1]D: Duration, F: Frequency.

[2]B = Coefficient, CI: Confidence Interval, ΔR^2: Adjusted R squared

[3]Controlling for infant age at time of assessment and maternal parity

To summarise, maternal depression scores 12 months postpartum were significantly associated with negative tone in mothers of males, whilst mothers of females showed a negative association between maternal vocalisation and postnatal depression. The higher the depression score, the greater frequency of negative tone used by the mothers of boys, whilst higher EPDS scores were indicative of fewer overall vocalisations during the Freeplay task in mothers of female infants.

5.3.3.3 Perinatal mental health and maternal non-social behaviour

There were no significant associations between maternal mental health either pre- or postnatally with any maternal non-verbal social behaviour (Appendix A10).

5.3.4 Placental *PEG3* expression and Infant social behaviour

Placental *PEG3* data was available for 43 infants (20 male). Exploratory analyses were carried out on the relationship between placental *PEG3* expression on both infant and maternal social behaviour.

5.3.4.1 Placental *PEG3* and infant vocalisation

Partial correlations between placental *PEG3* expression and infant vocalisations in the Freeplay task demonstrated that there were no significant associations between placental *PEG3* and any domain of infant vocalisation (Tables 5.15 and 5.16), both when analysing all samples together, and when separating by sex.

Table 5.15 Placental *PEG3* and frequency of infant vocalisations. Data is R_s; *p* value

FREQUENCY			
	All	*Males*	*Females*
Infant Distress	-.09; .56	-.04; .88	-.10; .68
Infant Non-Distress	-.20; .22	-.11; .68	-.30; .19
Any Infant Vocalisation	-.22; .17	-.12 .68	-.35; .13

[1] Data is R_s; :Spearman's rank correlation coefficient
[2] Controlling for infant age at time of assessment

Table 5.16 Placental *PEG3* and duration of infant vocalisations. Data is R_s; *p* value

DURATION			
	All	*Males*	*Females*
Infant Distress	-.06; .74	-.06; .83	-.10; .66
Infant Non-Distress	-.07; .66	-.44; .08	-.04; .87
Any Infant Vocalisation	-.07; .64	-.36; .15	-.05; .84

[1] Data is R_s; :Spearman's rank correlation coefficient
[2] Controlling for infant age at time of assessment

5.3.4.2 Placental *PEG3* expression and infant non-verbal social behaviour

No significant associations were observed between placental *PEG3* expression and any domain of infant non-verbal social behaviour (Appendix A11).

5.3.4.3 Placental PEG3 expression and maternal vocalisation

The relationship between placental *PEG3* expression and maternal vocalisations variables were also explored. Though *PEG3* expression was associated with the frequency of all maternal vocalisation and maternal positive tone, this did not persist when separating samples by sex (Table 5.17). When considering duration of vocalisations, analysing samples together demonstrated significant positive associations between any vocalisation, and positive tone, and infant directed speech. When separated by sex, these associations appeared to be driven by mothers of females, with the exception of infant directed speech which was not significantly associated with male or female infants (Table 5.18).

Table 5.17 Placental *PEG3* expression and frequency of maternal vocalisation. Data is R_s; *p* value

FREQUENCY			
	All	*Males*	*Females*
All Vocalisations	**.38** **.02**	.40; .12	.41; .06
Infant-Directed speech	.26; .11	.21; .42	.35; .12
Adult Register	.18; .28	.06; .84	.27; .23
Infant Register	22; .17	.25; .33	.21; .36
Positive tone	**.40;** **.01**	.43; .08	.42; .05
Negative tone	.16; .33	.13; .62	.11; .62
Neutral tone	.17; .30	.20; .45	.20; .38
Praise	.01; .97	-.27; .29	.21; .29
Humour	-.03; .88	-.31; .23	.14; .55
Laugh	.10; .90	-.06; .81	.21; .37
Encouragement	.01; .97	-.06; .82	-.05; .85
Discouragement	-.05; .80	-.36; .16	.27; .23
Maternal Imitation	-.23; .15	.02; .93	-.44; .05

[1] Data is R_s; :Spearman's rank correlation coefficient
[2] Controlling for infant age at time of assessment.
[3]Correlations significant at the level of *p = .05* are reported in **bold**

Table 5.18 Placental *PEG3* expression and duration of maternal vocalisation. Data is R_s; *p* value

DURATION			
	All	*Males*	*Females*
All Vocalisations	**.37; .02**	.17; .50	**.42 .05**
Infant-Directed speech	**.33; .04**	.15; .57	.39; .08
Adult Register	-.15; .35	-.15; .57	-.18; .48
Infant Register	.17; .30	.05; .84	.21; .17
Positive tone	**.38; .01**	.07; .80	**.47 .03**
Negative tone	.12; .48	.13; .62	.05; .83
Neutral tone	-.15; .37	.23; .39	-.32; .16
Praise	.11; .50	.03; .90	.22; .35
Humour	.18; .27	.16; .55	.26; .24
Laugh	.01; .93	.02; .93	.22; .32
Encouragement	-.07; .68	.04; .87	-.19; .40
Discouragement	.08; .68	-.34; .18	.31; .17
Maternal Imitation	-.13; .44	-.28; .29	-.05; .85

[1] Data is R_s; :Spearman's rank correlation coefficient
[2] Controlling for infant age at time of assessment
[3] Correlations significant at the level of *p = .05* are reported in **bold**

As before, significant partial correlations were taken forward for multiple linear regression, controlling for both infant age at assessment and maternal parity. Although the relationship between all maternal vocalisations ($F(3,19) = 2.26$, $p = .12$) did not persist at the regression level, placental *PEG3* expression was significantly associated with a greater duration of positive tone used by mothers of females in the Freeplay task (Table 5.19).

Table 5.19 Multiple regression results for placental *PEG3* expression and maternal vocalisations

Infant Sex	Measure	Vocalisation	B	*p*	95% CI	ΔR^2
Female	*PEG3* expression	Positive Tone (D)	25.88	.02	5.59, 46.17	.22

[1]D: Duration, F: Frequency.

[2]B = Coefficient, CI: Confidence Interval, ΔR^2: Adjusted R squared

[3]Controlling for infant age at time of assessment and maternal parity

5.3.4.4 Placental *PEG3* expression and maternal non-verbal social behaviour

There were no significant associations between placental *PEG3* and maternal non-verbal social behaviour. Results from partial correlations are detailed in Appendix A12.

5.3.5 Mediation analysis for the relationship between Maternal Mental health, maternally reported infant outcomes and vocalisations.

Prior to exploring mediation analyses, to ensure the same results were seen in the current sample as in Savory et al. (2020), regression analyses were carried out to establish the relationship between prenatal maternal mental health scores and a number of maternally reported outcomes in the current sample. Table 5.21 shows the outcomes measures that were significantly associated with maternal mental health symptoms at term in Savory et al. (2020). In the cohort of dyads used for this analysis, we did not observe significant associations between prenatal depression symptoms and PBQ 3, or negative affect as measured by the IBQ-R-SF in mothers of female infants. Nor did any relationship between IBQ-SF-R factors and prenatal anxiety symptoms persist. These discrepancies are likely due to a reduced sample size in the current study. In line with Baron and Kenny (1986), only significant outcome measures were taken forward for mediation analysis. As only 'all maternal vocalisations' were seen to be significantly associated with both prenatal depression and anxiety at term (Table 5.12), this was taken forward as the potential mediator variable for mothers of females, whilst maternal imitation was taken forward as the only vocalisation variable significantly associated with prenatal depression in mothers with male infants.

Table 5.20 Associations between prenatal mental health scores and maternally reported outcomes at 12 months postpartum.

Infant Sex	Mental Health Measure	Outcome Measure	B	*p*	95% CI	ΔR²
Females	*EPDS*	PBQ 1 (Bonding problems)	**.79**	**<.001**	**.37, 1.22**	**.43**
		PBQ 3 (Infant focused anxiety)	.58	.26	-.45, 1.61	.14
		CICS (infant aggression)	**.85**	**.01**	**.20, 1.51**	**.30**
		IBQ-SF-R (Negative affect)	1.99	.08	-.23, 4.25	.18
		IBQ-SF-R (Sadness)	**1.84**	**.02**	**.31, 3.37**	**.26**
		IBQ-SF-R (Distress)	**1.89**	**.04**	**.11, 3.68**	**.22**
		IBQ-SF-R (Soothability)	**-3.09**	**<.001**	**-4.75, -1.43**	**.42**
	STAI	IBQ-SF-R (Sadness)	2.13	.07	-7.90, 4.10	.31
		IBQ-SF-R (Distress)	.2.01	.14	-.68, 4.70	.28
		IBQ-SF-R (Soothability)	-2.40	.09	-5.26, .45	.29

Males	*EPDS*	IBQ-SF-R (Approach)	**-3.74**	**-6.24, -1.24**	**.01**	**.32**

[1]B = Coefficient, CI: Confidence Interval, ΔR^2: Adjusted R squared

[2]Correlations significant at the level of *p = .05* are reported in **bold**

[3]Controlling for infant age at time of assessment and maternal parity

[4]Adapted from Savory et al. (2020).

As previously noted in section 5.2.5 however, variables must meet three assumptions to qualify for mediation analysis (Baron and Kenny, 1986). Whilst Table 5.14 demonstrates that the assumption that the mediator (vocalisations) predicts the dependent variable (maternal mental health) is met, Table 5.21 demonstrates that for each outcome variable, the second major assumption is violated, in that the independent variable does not significantly predict the mediator variable. Consequently, in the current sample, maternal vocalisations do not mediate the relationship between maternal mental health at term and maternally reported infant outcomes at 12 months reported in Savory et al. (2020).

Table 5.21 Regression Results for testing the assumption that infant outcomes (Independent variable) predicts vocalisations (Mediator)

Infant Sex	Independent Variable	Mediator Variable	B	p	95% CI	ΔR²
Female	PBQ -1	Maternal Vocalisation (D)	-.4.28	.10	-.97, .91	.08
	CICS		-2.63	.49	-10.37, 5.1	-.01
	IBQ-SF-R (sadness)		12.10	.15	-28.68, 4.78	.09
	IBQ-SF-R (Distress)		16.09	.09	-34.71,2.53	.11
	IBQ-SF-R (Soothability)		4.78	.47	-16.35, 25.91	.01
Males	IBQ-SF-R (Approach)	Maternal Imitation (D)	-3.63	.79	-31.51, 24.23	.02

[1]D: Duration,

[2]B = Coefficient, CI: Confidence Interval, ΔR^2: Adjusted R squared

3Controlling for infant age at time of assessment and maternal parity

5.3.6 Summary of Results

Table 5.22 shows a summary of the main results from this Chapter.

Table 5.22 Summary of Chapter 5 Results

Analysis	Findings
Prenatal mental health, infant vocalisation, and social behaviours	No significant associations
Postnatal mental health, infant vocalisation, and social behaviours	No significant associations
Prenatal mental health, maternal vocalisation, and social behaviours	Associations predominately observed in the mothers of female infants. • Overall vocalisation (STAI and EPDS) • Infant directed speech (EPDS) • Positive Tone (STAI) Prenatal mental health in mothers of male infants was positively associated with; • Humour (EPDS) • Maternal imitation (STAI)
Postnatal mental health, maternal vocalisations, and social behaviours	Associations were observed between postnatal depression and maternal vocalisations in both mothers of girls (overall vocalisations) and boys (negative tone)
Placental *PEG3* expression and maternal or infant social interaction behaviours	Minimal number of associations Positive association with positive tone in mothers with female infants
Vocalisations as a mediator between prenatal mental health and maternally reported infant outcomes	No significant observations

5.4 Discussion

This chapter aimed to explore the relationship between mother-infant social interactions, pre- and postnatal maternal mental health and placental *PEG3* expression. The key finding from these analyses was that prenatal depression and anxiety symptoms were predominately associated with maternal vocalisations in mothers of girls, but not boys. Though some associations were observed in mothers of boys, these were fewer in a number, and were predominately positive associations, in contrast to those seen in mothers with girls. Placental *PEG3*

expression did not appear to be driving this relationship and was not clearly associated with any measure of social communication behaviour.

5.4.1 Perinatal Mental Health

In the current study, both prenatal anxiety and depression scores were negatively associated with general vocalisation in mothers of girls, but not boys. Though it is well established that both pre- and postnatal depression and anxiety can negatively affect the quality of the mother-infant relationship, there is little research surrounding vocalisation behaviour specifically, and the limited studies that exist have largely focused on postnatal symptomology (see Lovejoy et al. (2000)). Though there are some inconsistencies in the literature, these studies largely support the idea that mothers scoring highly for depressive symptomology in the postpartum period, show less vocal interaction with their infants (Field, 1984, Field et al., 1988, Fleming et al., 1988, Defelipe et al., 2019, Lam-Cassettari and Kohlhoff, 2020), a finding which is also reported in the current study. Although recently it has been reported that women with both prenatal depression and anxiety direct less vocalisations to their fetuses (Hernandez-Reif et al., 2018), there is little, if any, research exploring the relationship between prenatal depression and anxiety on maternal vocalisation in the postpartum period. As such, the current study represents one of the first to show an association between prenatal depression and anxiety scores and maternal vocalisations post-pregnancy.

Consistently with sex differences that were reported in response to prenatal maternal mood in Savory et al. (2020), the current study predominately observed associations between prenatal maternal mood and maternal vocalisations in mothers of girls, and not boys. In addition to general vocalisations mentioned above, prenatal depression was seen to be negatively associated with the duration of infant directed speech, whilst prenatal anxiety was negatively associated with maternal positive tone. Of the maternal vocalisation characteristics, infant-directed speech is perhaps the most well-studied, though it remains under-researched compared to global domains of maternal behaviour. Mothers with self-reported postnatal depression have been shown to demonstrate less infant-directed speech at 3-4 months (Bettes, 1988), whilst other studies have shown similar results in depressed mothers with infants ranging from 3-14 months (Kaplan et al., 2001, Porritt et al., 2014, Lam-Cassettari and Kohlhoff, 2020). Though none of these studies have explored the effect of infant sex, studies of non-depressed mothers have shown that mothers are more likely to use increased infant directed speech when interacting with their daughters, but not sons (Kitamura and Burnham, 2003, Clearfield and Nelson, 2006).

Reduced maternal positive affect is a consistently reported outcome of both pre- and postnatal depression (Lovejoy et al., 2000, Dib et al., 2019), and mothers with postnatal depression and anxiety have been shown to use less positive affect in mother-infant interactions than non-depressed mothers (Stevenson-Hinde et al., 2013, Lam-Cassettari and Kohlhoff, 2020). However, there are limited studies exploring positive tone in vocalisations explicitly. Whilst Campbell (2021, unpublished) have also reported that postnatal maternal anxiety is negatively associated with maternal positive tone, infant sex was controlled for in this analysis.

The results of the current study draw parallels with those reported in Savory et al. (2020), where as highlighted in section 5.1, prenatal maternal mood scores were associated with maternally reported adverse infant temperament,

bonding and aggression in female infants, while few associations were observed in male infants. Importantly, these relationships were not observed in independent infant measures assessed by researchers. In the current study, prenatal maternal mood scores were negatively associated with select maternal vocalisations, only in mothers of female infants. Though difficult temperament and increased crying has been shown to alter maternal social responses to infants, particularly in the context of maternal mood disorders (Jameson et al., 1997, Baker and McGrath, 2011), there were no statisitical difference in either overall, or distress vocalisations between male and female infants in the current study (Appendix A13). In Savory et al. (2020), the observed sex differences on maternally reported measures were hypothesised to be due to maternal mood compounding violations of expectation bias. Specifically, as female children are expected to be 'easier', if these gendered expectations are not met, mothers scoring highly on maternal mood symptomology may perceive there to be difficulties which are not present, and rate their mother-infant bond more poorly. This may also explain the sex differences in maternal vocalisation in the current study. Prenatal maternal mood symptomology has been shown to reduce mother's responsiveness to infant cues (Lovejoy et al., 2000), and impaired maternal bonding has been shown to influence maternal perception of infant's social skills (Joas and Möhler, 2021). As such it may be that if mothers of females perceive their daughters to be more difficult, they engage less positively and consistently. As similar numbers of males and females were analysed, this suggests that the sample size does not explain the lack of significant findings in mothers with male infants.

In contrast, prenatal mental health was positively correlated with mothers' use of humour and imitation in mothers with boys. To date, there has only been one unpublished study exploring the relationship between humour and maternal mental health, though only postnatal mental health scores were reported. Based on observations recorded via headcams in a home setting, Campbell (2021, unpublished) also observed a positive association between maternal use of humour and depression symptoms. It has previously been established that there are four types of humour, two of which are maladaptive (Braniecka et al., 2019). One limitation of the coding scheme used in this study is that it did not distinguish the type of humour used by mothers, therefore it may be that mothers of boys were more likely to use maladaptive humour, which has previously been associated with depressive symptoms, or use humour as a coping mechanism (Martin and Ford, 2018).

Multiple factors have been demonstrated to mediate the relationship between prenatal depression and anxiety and infant outcomes, including exposure to glucocorticoids, maternal age, and socioeconomic status (Fergusson and Woodward, 1999, O'Keane et al., 2011, Stein et al., 2014). However, the quality of parenting remains the most salient predictor of infant outcomes (Stein et al., 2014), and global domains of parenting, such as maternal sensitivity have been shown to mediate the relationship between maternal mental health and infant outcomes (van Doorn et al., 2016). Social communication between mother and infant is the corner stone of successful parenting and a healthy mother-infant bond (Johnson, 2013), and recently studies have shown that the frequency and duration of maternal vocalisations at 12 months, but not infant vocalisations, are predictive of later life psychopathology in infants at 7 years (Allely et al., 2013). Based on this, and previous research demonstrating that more specific maternal behaviours, rather than global domains of parenting, can act as mediators in the relationship between prenatal mental health and infant outcomes (Rodrigues-Palucci et al., 2020), it was hypothesised that maternal vocalisations may mediate the significant relationships between prenatal mental health

and maternally reported infant outcomes observed in Savory et al. (2020). However, we did not find this to be the case across any of the significant relationships observed in Savory et al. (2020).

The lack of mediation may be explained in a number of ways, firstly it may be that maternal vocalisation genuinely does not mediate the relationship between maternal mental health and the specific infant outcomes measured in this study. Secondly, it may be that other, more specific aspects of maternal speech may mediate this relationship. Mind-minded speech for example reflects a mother's ability to accurately recognise her infant's intentions and mental state (Meins, 2013), and has been shown to be impaired in mothers with depression (Pawlby et al., 2010). Third, it may be that the mediating effect of maternal vocalisation on the relationship between maternal mood and infant outcomes is age specific. The current study investigated an infant sample (12 months), however, it may be that as in Allely et al. (2013), maternal vocalisations do not affect infant outcomes until a later age, though longitudinal studies would be needed to test this hypothesis. Finally, an explanation of this null finding may be due to recruitment bias in the current sample. The sub-set of mothers that attended laboratory assessments were relatively well-educated, with over 50% holding an undergraduate degree or higher, and reported having a high level of family income. As maternal education has been shown to influence the quality of mother-infant interaction (Pearson et al., 2012, Pearson et al., 2013), including aspects of vocalisation (Campbell, 2021, unpublished) it may be that a mediation effect is seen in samples more representative of the general population.

5.4.2 Postnatal Mental Health

As previously mentioned, consistent with previous research (Field, 1984, Field et al., 1988, Fleming et al., 1988, Defelipe et al., 2019, Lam-Cassettari and Kohlhoff, 2020), the current study reported a negative association between postnatal depression and overall maternal vocalisations. However, in the current study, this was only observed in mothers of girls, and not boys. The same association was observed with prenatal mood symptomology, in line with previous research to suggest that prenatal depression can be more severe and have more long-lasting consequences than postnatal onset (PACT Consortium, 2015, Putnam et al., 2017), although this could not be formally tested in the current study due to small sample size (section 5.4.4).

5.4.3 Placental *PEG3* expression

Placental expression of the imprinted gene *PEG3* has recently been linked to prenatal depression in human studies (Janssen et al., 2016, Sumption, 2020), and both anxious-like behaviour, and impaired early life social interaction in mice (Chapters 3,4 and McNamara et al. (2018a). However, in the current study, with the exception of a positive association with positive tone in mothers of girls, there were no clear associations between placental *PEG3* expression and any domain of maternal or infant social behaviour. In line with Sumption et al. (2020), there was also no association between placental *PEG3* expression and prenatal mental health observed in this subsample of GiW participants (Appendix A14). However, as noted in Sumption et al. (2020), this is likely due to the dramatically reduced sample size in the number of mother-infant dyads who took part in the laboratory assessments compared to those recruited at A1. In the same vein, in the current study, gene expression data was only available for 43 infants, and so it is likely the current study was underpowered to detect any associations.

Similarly, limited results were also reported in Sumption et al. (2020), who explored the relationship between placental *PEG3* expression and infant outcomes assessed by researchers on a battery of assessments during the laboratory visits. Specifically, expression was negatively associated with infant positive affect in the sustained attention task, though no differences between males and females were apparent. In contrast, in another study, increased methylation (a proxy for decreased expression) was associated with negative affectivity in infants (Fuemmeler et al., 2016), though this disparity is likely explained by the use of a different tissue type, and the mode of delivery of the participants. Together, results from Sumption et al. (2020) and the current study do not suggest a relationship between *PEG3* expression and infant or maternal outcomes, however, further investigation using a larger cohort is necessary given the small sample size utilised in the current study.

5.4.4 Limitations

Though the use of the MHINT coding scheme and micro-analysis of social behaviour is a strength of the current study over traditional, event-based scoring, one of the main limitations of the current study is that tests were performed in a laboratory, as opposed to a homebased environment. Recent research has shown that both mother and infant behaviour vary based on the location of observation, and the situation (Maas et al., 2013). Interaction quality between mother and infant has found to be highest in face-to-face situations in clinics or labs, and the lowest whilst engaged in free play at home (O'Brien et al., 1989, Van Bakel and Riksen-Walraven, 2002), suggesting that both mother and infant behaviour is altered when observed by researchers in a clinical setting. A recent study has utilised the same MHINT coding scheme and method of micro-analysis in investigating the relationship between mother-infant vocalisation and maternal mood using headcam recordings obtained in the home environment (Campbell, 2021. unpublished). Although results from this study are as of yet unpublished, this presents an avenue for future research in the GiW cohort, to explore whether the same associations between maternal mood and social behaviour are observed in naturalistic interaction within the home.

It is well established that both prenatal depression and anxiety are risk factors for postnatal mental health (Goodman and Gotlib, 1999, Heron et al., 2004), and this has also been shown previously in the GiW cohort (Sumption, 2020). Though depression and anxiety are highly correlated, and prenatal maternal mood is predictive of postnatal mood symptomology, there is also evidence to suggest that the effects of pre- and postnatal mental health, and depression and anxiety, on infant outcomes are distinct (O'Connor et al., 2002a, Penninx et al., 2011). The current study observed that while there was some overlap in the outcomes associated with prenatal depression and anxiety scores, the majority of outcomes were only related to one measure of maternal mood. In addition, the association between prenatal depression and overall vocalisation persisted postnatally. However, given the small sample size of the current study, time-point was unable to be added to the regression models, and so no conclusions can be made as to which time point had the most influence on outcomes, or whether these findings can be attributed solely to depression or anxiety given their high comorbidity. Future research, using a larger sample size, could explore these associations, including these variables in regression models, or by running hierarchical regression models, adjusting for both additional demographic variables known to effect social interaction such as maternal education, and for depression and anxiety symptoms separately.

Similarly, a further limitation is that this thesis was only able to look at data from timepoint A1 (pre-ELCS) and Y1 (12-months after birth) to assess the mother-infant interaction. As mentioned in section 5.2, this timepoint was chosen as it is often cited as the 'gold-standard' timepoint for assessing infant attachment. A particular advantage of studying maternal-infant behaviour at this timepoint, is that by 12 months, infants exhibit a much wider repertoire of reciprocal behaviours, including turn-taking and a larger number of vocal responses (Rutter and Durkin, 1987), therefore allowing for a more in-depth assessment of mother-infant interaction. Although self-report questionnaire was collected at P1 (one week postpartum) and P2 (ten weeks postpartum), due to a lack participant response maternal mood data at these timepoints were unable to be used. The lack of an earlier timepoint is a limitation to the current study, as mother-infant interaction even as early as ten minutes after birth is sufficient to predict interaction behaviours at 12-months (Britton et al., 2001).

Finally, a limitation of this study, and of the GiW cohort as a whole, is the inclusion criteria of birth via elective caesarean, limiting the translation of these findings to other modes of delivery. Importantly however, a number of studies have shown there to be no differences in rates of postnatal depression, or mother-infant bonding between mothers who underwent elective caesareans versus vaginal delivery (Forti-Buratti et al., 2017, Poojari et al., 2019, Yoshida et al., 2020).

5.5 Summary

To summarise, this chapter reported on the relationship between perinatal mental health, mother-infant interaction and placental *PEG3* expression in the GiW cohort. The key finding was the relationship between prenatal mental health scores and maternal vocalisation in observed predominately in mothers with female infants. Despite research previously linking placental *PEG3* expression to maternal depression, this did not appear to drive any of the relationships observed in the current study, potentially due to small sample size. Finally, in this cohort, infant vocalisation or non-verbal social behaviour was not seen to be associated with pre- or postnatal mental health in mothers.

6

Chapter 6: General Discussion

6.1. Overview

Successful social interactions are integral to the development and survival of mammals (Seebacher and Krause, 2017). Of all the social interactions, the reciprocal relationship between mother and infant is the most important, as the quality of maternal care, if disrupted, can have long-lasting detrimental impact on both brain and behaviour in offspring (Champagne and Curley, 2005, Curley et al., 2008, Stein et al., 2014). Maternal mental health conditions, in particular depression and anxiety during the perinatal period are among the most disruptive to the mother-infant bond, exposure to which is commonly associated with adverse outcomes in infants including low birth weight and impaired social development, amongst others (Goodman and Gotlib, 1999, Goodman et al., 2011, Slomian et al., 2019). A number of these associations, show sexually dimorphic effects, for example, males exposed to prenatal anxiety were 5.6% more likely to meet criteria for externalising disorders than females (Hicks et al., 2019). Whilst in other studies, males are often at a greater risk for impaired cognitive development and increased risk of prematurity (Field, 2011, Sutherland and Brunwasser, 2018). The placental programming hypothesis suggests placental endocrine insufficiency, as a result of aberrant imprinted gene expression, as a mechanism underlying these associations (Creeth and John, 2020).

Loss of expression of the imprinted gene *Peg3* in mice has been shown to produce a more severe placental phenotype in males, and in humans, reduced expression of placental *PEG3* in male placenta has been linked to prenatal depression (Janssen et al., 2016, Sumption, 2020). In mice, loss of function of *Peg3* in dams has been associated with impaired maternal care (Li et al., 1999, Curley et al., 2004, Curley et al., 2008, Champagne et al., 2009), whilst loss of function in the fetus has been shown to elicit increased anxiety-like behaviours in dams, and impaired early-life social behaviours in the offspring (McNamara et al., 2018a). Despite this, mother-offspring interaction in this model has not been well characterised, nor has social behaviour broadly. In addition, in humans, little research has explored maternal social communication in relation to perinatal maternal health or infant outcomes, whilst only one study has explored infant outcomes in relation to *PEG3* expression (Sumption, 2020). Based on this, the overarching aims of the current thesis were firstly, using a *Peg3KO* model, to explore how loss of function of offspring *Peg3* affects the mother-infant relationship in mice, and how this is in turn affects offspring social behaviour in mice. Secondly, to translate this knowledge to a subset of mother-infant dyads from

the GiW study and explore the relationship between perinatal mental health, mother-infant social communication and placental *PEG3* expression.

6.2. Main Findings

The work described in this thesis has been extensively discussed independently in each experimental chapter, and the main findings from these are displayed in Table 6.1. This chapter will serve as a general overview of the results, and consider the implications, limitations, and future directions for the work detailed in this thesis.

Table 6.1 Summary of the key findings from each experimental chapter

Chapter	Key Findings
Chapter 3: "Evaluating the effect of offspring *Peg3* disruption on maternal behaviour in mice"	• WT dams, carrying *Peg3KO* litters demonstrated increased anxiety-like behaviour and deficits in maternal care. • WT dams, carrying mixed genotype litters which include *Peg3* mutant offspring, demonstrated impairments in anxious behaviours and maternal care, though to a lesser degree than dams carrying 100% mutant litters. • WT dams did not appear to distinguish between the sex or genotype of their pups during the pup retrieval task.
Chapter 4: "The influence of *Peg3* disruption on social behaviour in mice"	• Male *Peg3KO* mice raised in single genotype litters showed early USV deficits. • Post-weaning, both WT and *Peg3KO* male mice raised in mixed genotype litters displayed impairments in social behaviour compared to WT mice raised in WT litters. • Despite behavioural changes, no clear differences in gene expression in either the hypothalamus or olfactory bulb were observed using RNA-sequencing analysis.
Chapter 5: "Perinatal mental health and mother-infant social behaviours"	• Prenatal maternal mood was negatively associated with maternal vocalisations in mothers of girls, but not boys. • Placental *PEG3* was not associated with infant, or maternal social behaviours.

6.2.1 Findings from the use of Mouse Models

It is well established that the mother's behaviour and genotype can influence the phenotype of their offspring, however, the reciprocal effects of offspring behaviour and genotype on maternal behaviour has been much understudied (Potter et al., 2019). This reciprocal relationship is particularly important when considering the association between maternal mood symptomology and adverse offspring outcomes, as the mechanisms underlying this relationship are still largely unknown.

Chapter 3 replicated the work of McNamara et al. (2018a), and using a more extensive test battery, demonstrated that loss of expression of *Peg3* in the offspring, resulted in impaired maternal care, and increased levels of anxiety-like behaviour in WT dams. It is important to note that though reduced *Peg3* expression has been linked to maternal mood sympomology in both humans and mice, the specific domains of symptomology differ across species. In mice, placental *Peg3* expression has only been associated with heightened anxiety-like behaviour (McNamara et al., 2018a). Whilst in humans, placental *PEG3* expression has been linked to prenatal depression in four independent human cohorts (Janssen et al., 2016, Sumption, 2020). One study has explored the relationship between placental *PEG3* expression and prenatal anxiety and found no association, though this was in a very small subset of participants from the GiW cohort, meaning no clear conclusions could be drawn (Sumption, 2020).

However, the complexity of depression and its lack of functional biomarkers makes measuring depression in animals difficult, with research tending to focus on one particular symptom such as anhedonia (Söderlund and Lindskog, 2018). As such, given the high comorbidity between maternal depression and anxiety in humans (Field et al., 2010), and the lack of perinatal anxiety research generally, it is useful to measure maternal anxiety-like behaviours in mice as a proxy measure for maternal mood symptomology (Tarantino et al., 2011).

There are also differences in the time-points of mood symptomology measures between the mouse, and human studies. Notably, in mice, expression has been associated with increased anxiety-like behaviour *postnatally,* as in the current study and in McNamara et al. (2018a). Whilst in humans, reduced placental *PEG3* expression was associated with *prenatal* depression symptomology (Janssen et al., 2016, Sumption, 2020). Though McNamara et al. (2018a) assessed anxious behaviour in dams during gestation and found no difference between dams carrying mutant fetuses and dams carrying WT fetuses, both McNamara et al. (2018a) and the current study lacked a pre-pregnancy baseline assessment and so subtle alterations may have been missed. Further, there are number of ethical limitations in assessing depressive behaviour in pregnant or lactating mice, as the majority of 'depression' tests rely on exposing the dam to physical stressors such as foot shocks or restraint stress (Weinstock, 2017). Recently however, a novel model system has been suggested which would allow for the assessment of prenatal depressive symptomology in mice (Scarborough et al., 2021). Rather than physical stressors, in this model, the degree of depression like-behaviours are induced via social isolation prior, and during pregnancy, resulting in increased anxiety-like behaviour and increased social interaction deficits in dams, reflective of human behaviour during depressive episodes (Scarborough et al., 2021). It may be interesting for future research to use this novel model to ascertain the relationship between placental *Peg3* expression and prenatal 'depression' in mice, though experimental groups would need careful consideration in order to determine the effects of social isolation vs placental endocrine insufficiency as a consequence of aberrant *Peg3* expression in the offspring.

Following on from Chapter 3, Chapter 4 explored the social behaviour in both WT and *Peg3* mutant offspring raised under the varying atypical conditions of maternal care observed in Chapter 3. Inclusion of the mixed litter group, in addition to mutants raised in single genotype litters, and fully WT controls, allowed for an assessment of whether behavioural changes were due to the adverse pre- and postnatal maternal environment caused by placental endocrine insufficiency, rather than the specific gene change of reduced *Peg3* expression. Deficits in neonatal USVs observed only in male mutant mice from single genotype litters suggested a direct role for loss of *Peg3* function in the development of social communication behaviour. However, unified scoring from adult tests instead suggested that genetically reduced expression of *Peg3* drives changes in social behaviour, not only in mutant mice carrying the genetic modification, but also in WT littermates sharing the abnormal pre- and postnatal environment. Though deficits were greater in male mutants from single genotype litters, as mutant and WT males from mixed genotype litters both showed social impairment compared to controls, intrinsic loss of function of *Peg3* could not solely be driving the social deficits observed in Chapter 4.

Two previous studies have shown that altered expression of an imprinted gene, and resultant placental insufficiency, is enough to modify offspring behaviour (Mikaelsson et al., 2013, Harrison et al., 2021). Though Mikaelsson et al. (2013) demonstrated that mice null for the placenta specific P0 transcript of *Igf2* demonstrated increased reactivity to anxiety inducing stimuli, this was observed in transgenic mice compared to WT littermates alone. More recently, Harrison et al. (2021) have shown, in an elevated expression of *Phlda2* model, that both genetically modified offspring, and their WT littermates show similar behavioural and transcriptional alterations compared to WT controls. Crucially, the same model has also been shown to alter behaviour in WT dams (Creeth et al., 2018). The results from these studies, together with the results of Chapters 3 and 4, suggest that one of the wider implications of this thesis is that it provides further support for the placental programming hypothesis. Specifically, as social impairments were observed in male mutants from both single and mixed genotype litters, and in their male WT littermates, these impairments were likely a consequence of wider adversities caused by reduced placental *Peg3* expression and subsequent placental endocrine insufficiency, including an altered *in utero* environment, and impaired postnatal maternal care.

This interpretation is not without its caveats, however. Firstly, in mammals, social behaviour is the result of both genetics and experience, with evidence suggesting that even behaviours such as pup USVs can be modified by experience rather than being innate (Grimsley et al., 2011, Chabout et al., 2012). As such, it cannot be ruled out that the mutant offspring in the mixed litter groups, are altering the behaviour of their WT littermates. However, given that USV deficits were only observed in mutants raised in single genotype litters, this seems unlikely, and a more reasonable suggestion would be that the altered postnatal behaviour is a product of the atypical maternal care observed in Chapter 3, brought about by placental endocrine insufficiency.

Secondly, as highlighted in Chapter 3, this study is still unable to completely disentangle whether the altered maternal behaviour of dams carrying mutant pups is due to placental endocrine insufficiency and subsequent aberrant maternal programming, or an altered postnatal environment due to altered social behaviour of the *Peg3KO* pups. Given the reciprocal nature of the mother-infant bond and evidence to show that offspring USV

emission can alter maternal behaviour (D'Amato et al., 2005, Okabe et al., 2013), future research should endeavour to conduct an extensive cross-fostering experiment to further disentangle this, and to provide more concrete evidence for the placental programming hypothesis from the *Peg3KO* model (Creeth and John, 2020).

In addition to observing general social deficits in *Peg3KO* mice, one of the most striking findings from this thesis, is that these deficits were observed only in male, and not female mice. Consequently, adding to the growing body of literature to suggest that males may be more vulnerable to the disruption of *Peg3.* Though previous research has shown that survival rates (Kim et al., 2013, He et al., 2016), and placental endocrine dysfunction (Tunster et al., 2018) show more severe phenotypes in males, this is the first study to show that a sexual dimorphism also extends to a behavioural phenotype in this *Peg3KO* model.

6.2.2 Findings from the Grown in Wales cohort

The GiW study is particularly important as many human cohorts focus exclusively on postnatal mental health. Though relatively small in sample size, the GiW study was the first cohort in South Wales to consider rates of prenatal anxiety and depression and it is one of only a few human cohorts to consider both pre- and postnatal mental health. Inclusion criteria meant that all participants delivered by ELCS, and as such, translatability of these findings to natural births is limited, however the high prevalence of prenatal depression (14%) and anxiety (28%) in this cohort, and in other cohorts (Heron et al., 2004) reinforces the importance of studying these disorders and the impact they have on the mother-infant relationship.

In Chapter 5, drawing parallels to the work in mice explored in Chapters 3 and 4, maternal-infant social communication behaviours at 12 months postpartum were explored in relation to maternal mood symptomology and placental *PEG3* expression. Perhaps unsurprisingly given the low number of placental samples available for those infants who attended the laboratory sessions, placental *PEG3* expression was not clearly associated with any aspect of maternal or infant social behaviour in either males or females. Further, in the sub-selection of GiW participants used for Chapter 5, neither pre- or postnatal mental health scores were associated with *PEG3* expression, although again this is likely due to the smaller sample size and a reduced availability of postnatal questionnaire data due to participant drop out.

Though historically, studies of perinatal mental health have either controlled for sex, or focussed solely on male infants, increasingly, there is a growing body of literature that when separating analyses by fetal sex, observe sexually dimorphic infant outcomes (Kott and Brummelte, 2019). Findings from the GiW cohort have demonstrated that male infants, independent of *PEG3* expression, showed impairments in language development (Savory et al., 2020), and that placental *PEG3* expression is associated with prenatal depression in mothers of boys, but not girls (Janssen et al., 2016). These findings, coupled with findings from mouse models (Tunster et al., 2018, Kim et al., 2013), including the results from Chapter 4, suggest a sexually dimorphic effect of *Peg3* disruption, with males potentially being more vulnerable to these effects. As such, it was assumed that male infants would vocalise less and / or that mother's interactions with males in the Freeplay task would be affected more greatly than those with girls. However, this was not the case, and though Chapter 5 showed no difference between

male and female infants' frequency of vocalisations, prenatal maternal mood symptomology was negatively associated with a number of maternal vocalisations directed at girls, but not boys.

These findings draw parallels with previous work from the GiW study, in which prenatal mood scores were associated with poor bonding and increased adverse infant temperament in female infants, but not males, despite these differences not being observed independently by researchers (Savory et al., 2020). Maternal mood symptomology has been associated with negative distortion of the maternal perception of infant behaviour (Najman et al., 2001, Foreman and Henshaw, 2002, Mäntymaa et al., 2006), and in turn negative perception of infants have been shown to result in altered infant behaviour, with research suggesting that caregivers, based on their perceptions, will act in such a way to elicit the behaviour they expect from their infants (Mebert, 1991). In the case of a negative perception, this leads to increased negative temperament from the child, and a coercive interaction style between mother and infant (Lee and Bates, 1985). Crucially, negative infant temperament has been established as a risk factor for later life psychiatric symptoms up to three decades later (Teerikangas et al., 1998, Tang et al., 2020). From a clinical perspective, it is therefore important to distinguish risk factors which contribute to the mother's perception of their infant. Findings from Savory et al. (2020) and Chapter 5, suggesting that mothers with maternal mood symptomology both perceive and act differently toward female infants, may therefore suggest a need for intervention strategies to be tailored differently for mothers with female infants. This is particularly important given research suggesting that girls are at a higher risk of internalising disorders, and report having more arguments with their mother's than boys (Gutman and Codiroli McMaster, 2020), both of which have been associated with early life mother-infant interactions (Kim and Smith, 1998).

To summarise, despite observing sex-specific outcomes in mothers of female, but not male infants, as might have been expected, Chapter 5 contributes to the growing body of literature observing sex differences in infant outcomes in response to perinatal depression. Highlighting the need for future studies to stratify samples by sex. Furthermore, given that sex-specific trajectories alter with infant age (Hops, 1995), future research should continue to explore infant outcomes at different ages. This is currently already underway within the GiW cohort, as data at 4 years postpartum is currently being collected.

6.3. Limitations and Future Directions

Several limitations and ideas for future research have been discussed throughout this chapter, however they will be summarized here. Firstly, a prominent limitation throughout this thesis has been sample size, though this was most apparent in Chapters 4 and 5. In Chapter 4, the limited sample size due to animal facility shut down during the Covid-19 pandemic meant that the experiments in this chapter were not adequately powered to conduct 2-way ANOVAs. As such, direct comparisons surrounding the severity of *Peg3* disruption on males and females could not explicitly be made, and instead only comparisons of how the effects of *Peg3* disruption manifested in each sex could be drawn. Future research should therefore aim to replicate the findings in Chapter 4, using a larger sample size.

Sample size was also an issue in Chapter 5, particularly in regard to placental *PEG3* expression measurements not being available for all samples, which may potentially explain the lack of relationship observed between *PEG3*

expression and mother-infant communication measures. In the same vein, as highlighted both in Chapter 1 and Chapter 5, comorbidity of depression and anxiety have been shown to elicit different trajectories of infant outcomes compared to the presence of only one condition (Field et al., 2010). However, the limited sample size of the GiW cohort meant that only limited numbers of mothers scored highly on both the EPDS and STAI. A larger sample size, with greater numbers of mothers scoring highly on both measures, and measures individually, would allow for better separation of the two prenatal disorders, and an exploration for how comorbidity differentially effects infant outcomes. As highlighted in Chapter 5, a larger sample size would also allow for a formal statistical assessment of which timepoint, pre- or postnatally, maternal mood symptomology had the greatest effect on infant outcomes.

An additional limitation of Chapter 5 is the use of self-report mental health questionnaires. Though useful in terms of ease of administration and low-cost, the gold-standard of mental health assessment would have been via DSM (American Psychiatric Association, 2013) diagnosis by qualified medical professionals. However, given the financial costs associated with this, the use of self-report questionnaires is commonly used in the majority of human cohort studies such as GiW and the ALSPAC cohort.

A limitation consistent across this thesis is the homogenous nature of the experimental cohorts. In Chapters 3, and 4, the 129Sv mouse strain was used. Although the *Peg3KO* model used in this thesis was originally created on the background of the 129Sv strain, most research has explored the effects of *Peg3* disruption on a C57BL/6J background. Critically, behavioural differences in maternal care and olfactory ability have been observed in *Peg3* dams across the two different strains (Champagne et al., 2009), as such, future research should explore whether the same deficits observed in Chapters 3 and 4, are also present on the C57BL/6J background.

Similarly, whilst the homogenous nature of the GiW cohort may be seen as a strength in that it limits variation, it is also a limitation. The GiW cohort is predominately comprised of White women (91%), and whilst the South Wales population is predominately White, this proportion is higher than the area from which mothers were recruited, which had a White population of 84% in 2016 (Welsh Government Statistics, 2016). Further, of the mother-infant participants used for data analysis in Chapter 5, only 2 were non-Caucasian. This is particularly important as women from ethnic minorities are at greater risk for developing perinatal mental illness (Watson et al., 2019), and different cultures have been shown to differ in overall rate of maternal-infant vocalisation (Bornstein et al., 2015). Further, women from different ethnicities have also been shown to vary in expression levels of imprinted genes (King et al., 2015). Consequently, an important caveat to the human findings from this thesis is that they may not translate to other ethnicities, and future research would benefit from replicating the work from Chapter 5 in different populations.

Finally, despite obvious differences between mice and humans, mouse models remain essential to understand mechanisms occurring in the perinatal period, primarily due to the ethical constraints of such research in humans, and as such, the use of both mouse and human models in this thesis is a considerable strength. However, questions still remain about the translatability of such models.

As highlighted in Chapter 1, though there are core similarities between the cell types, structure and mechanisms underlying the development of both human and mice placenta, the gross architecture, and hormonal repertoire are substantially different (Carter and Enders, 2004). In regard to *Peg3*, in humans, expression of placental *PEG3* is found only in the villous cytotrophoblast (Hiby et al., 2001), whilst in mice, expression is more widespread across all of the placental lineages (Tunster et al., 2018). Further, in Janssen et al. (2016), human placental *PEG3* was reduced by a maximum of 40%. However, in mice, the nature of the *Peg3KO* model means that *Peg3* expression is globally absent. These differences may therefore explain why we do not see comparable phenotypes in the social behaviour of mice and humans, or a relationship between placental *PEG3* expression and social behaviour in Chapter 5.

As consistently highlighted throughout this thesis, and as demonstrated in Chapter 4, research suggests that disruption of *Peg3* results in sexually dimorphic phenotypes, and that males may be more severely impacted by *Peg3* disruption (Kim et al., 2013, He et al., 2016, Bretz and Kim, 2018, Tunster et al., 2018). As mouse pregnancies consist of mixed-sex litters, it may be that the more modest phenotype observed in female *Peg3* placentas attenuate the severe phenotypes observed in males. However, this presents a problem in translating the impact of the placenta on maternal behaviour to human studies, as human pregnancies are overwhelmingly singleton (Easter et al., 2018). Recent technological advances, however, have allowed for the generation of single sex litters in mice (see Douglas and Turner (2020) for a review), which presents an interesting avenue for future research, potentially allowing *Peg3* functionality studies to model pregnancies more akin to human pregnancies. This, combined with the cross-fostering study recommended in section 6.2.1 would be integral to further research exploring the relationship between *Peg3* expression, maternal behaviour, and infant outcomes.

6.4 Concluding Remarks

This thesis contributes to a number of different areas under the overarching theme of exploring the relationship between maternal mood symptomology, mother-infant communication and placental *Peg3* expression. This thesis provides further evidence that offspring loss of function of *Peg3* influences the maternal behaviours of WT dams, likely as a result of placental endocrine insufficiency. It also contributes to the growing body of literature showing a sexually dimorphic effect of *Peg3* disruption, showing for the first time, a male specific deficit in social behaviour. Crucially, as this deficit was observed in both mutant and WT littermates, this thesis also provides further tentative evidence for the placental programming hypothesis. Finally, although no associations were observed between mother or infant social communication behaviour and placental *PEG3* expression in humans, results from this thesis serve as further evidence of sexually dimorphic outcomes in response to prenatal depression. Prenatal maternal mood symptomology was related to different aspects of social behaviour in mothers of male, and female infants, with maternal mood symptomology predominately negatively associated with specific vocalisation behaviours in mothers with female, but not male infants.

Appendix

A1. EPDS Questionnaire

DIRECTIONS: Please UNDERLINE the answer which comes closest to how you have felt in the **past week**, not just how you feel today. Here is an example already completed:
I have felt happy:
Yes, all the time
Yes, most of the time
No, not very often
No, not at all
This would mean: I have felt happy most of the time in the past few days.
Please complete the other questions in the same way. Do not take too long over it and make sure you answer all the questions.

IN THE PAST WEEK

1. I have been able to laugh and see the funny side of things:
   As much as I always could
   Not quite so much now
   Definitely not so much now
   Not at all

2. I have looked forward with enjoyment to things
   As much as I ever did
   Rather less than I used to
   Definitely less than I used to
   Hardly at all

3. I have blamed myself unnecessarily when things went wrong
   Yes, most of the time
   Yes, some of the time
   Not very often
   No, never
4. I have been anxious or worried for no good reason
   No, not at all
   Hardly ever
   Yes, sometimes
   Yes, very often

5. I have felt scared or panicky for no very good reason
   Yes, quite a lot
   Yes, sometimes
   No, not much
   No, not at all

6. Things have been getting on top of me
   Yes, most of the time I haven't been able to cope at all

Yes, sometimes I haven't been coping as well as usual
No, most of the time I have coped quite well
No, I have been coping as well as ever

7. I have been so unhappy that I have had difficulty sleeping
   Yes, most of the time
   Yes, sometimes
   Not very often
   No, not at all

8. I have felt sad or miserable
   Yes, most of the time
   Yes, quite often
   Not very often
   No, not at all

9. I have been so unhappy that I have been crying
   Yes, most of the time
   Yes, quite often
   Only occasionally
   No, never

10. The thought of harming myself had occurred to me
    Yes, quite often
    Sometimes
    Hardly ever
    Never

A2. STAI Questionnaire

DIRECTIONS: A number of statements which people have used to describe themselves are given below. Read each statement and then tick in the appropriate box on the right to indicate how you **generally** feel. There are no right or wrong answers. Do not spend too much time on any one statement but give the answer which seems to describe how you **generally** feel, even before pregnancy.

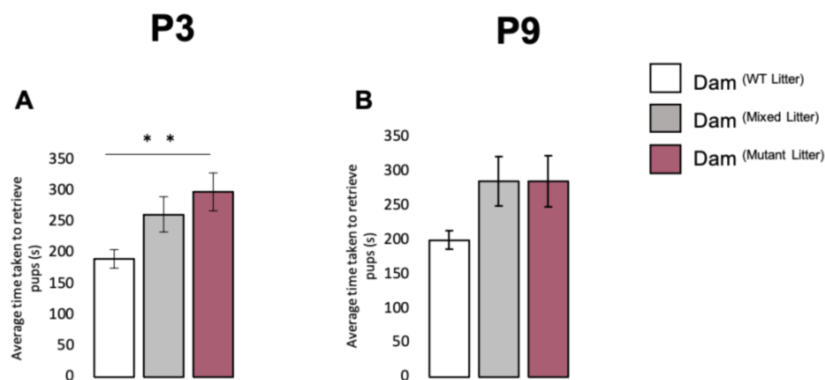
	In general….	***Almost never***	***Sometimes***	***Often***	***Almost always***
1.	I feel pleasant				
2.	I feel nervous and restless				
3.	I feel satisfied with myself				
4.	I wish I could be as happy as others seem to be				
5.	I feel like a failure				
6.	I feel rested				
7.	I am "calm, cool and collected"				
8.	I feel that difficulties are piling up so that I cannot overcome them				
9.	I worry too much over something that really doesn't matter				
10.	I am happy				
11.	I have disturbing thoughts				
12.	I lack self-confidence				
13.	I feel secure				
14.	I make decisions easily				
15.	I feel inadequate				
16.	I am content				
17.	Some unimportant thought runs through my mind and bothers me				
18.	I take disappointments so keenly that I can't put them out of my mind				
19.	I am a steady person				
20.	I get in a state of tension or turmoil as I think over my recent concerns and interests				

A3. Postpartum Bonding Questionnaire

DIRECTIONS: Please indicate how often the following are true for you. There are no 'right' or 'wrong' answers. Choose the answer that seems right in your recent experience.

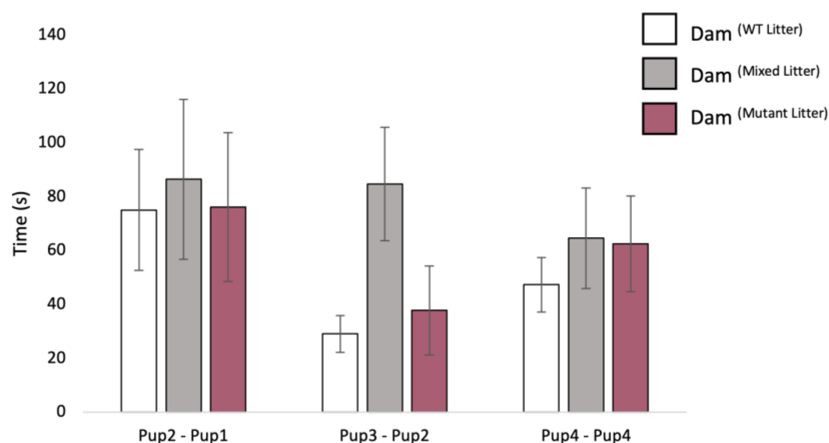
		Never	Rarely	Sometimes	Quite Often	Very Often	Always
1	I feel close to my baby						
2	I wish the old days when I had no baby would come back						
3	I feel distant from my baby						
4	I love to cuddle my baby						
		Never	Rarely	Sometimes	Quite Often	Very Often	Always
5	I regret having this baby						
6	The baby does not seem to be mine						
7	My baby winds me up						
8	I love my baby to bits						
9	I feel happy when my baby smiles or laughs						
10	My baby irritates me						
11	I enjoy playing with my baby						
12	My baby cries too much						
13	I feel trapped as a mother						
14	I feel angry with my baby						
15	I resent my baby						
16	My baby is the most beautiful baby in the world						
17	I wish my baby would somehow go away						
18	My baby makes me feel anxious						
19	I am afraid of my baby						
20	My baby annoys me						
21	I feel confident when caring for my baby						
22	My baby is easily comforted						

A4. Average time taken for cohorts of dams to retrieve pups



Appendix 4. Average time taken for cohorts of dams to retrieve pups. A) Average time taken to retrieve pups in each group at P3, with the mixed litter group collapsed by genotype. The one-way ANOVA was significant (F((2,145) = 4.91, p = .009), with Bonferroni corrections demonstrating that dams with fully mutant litters retrieved their pups on average, significantly slower than dams with fully WT litters (p = .008). There were no differences between the mixed litter and either of single genotype groups. **B)** Average time taken to retrieve pups in each group at P9, with the mixed litter group collapsed by genotype. There were no statistically significant differences at this timepoint.

A5. Difference in the retrieval time between each pup during the pup retrieval task at P3



Appendix 5. Difference in retrieval time between each pup during the pup retrieval task at P3. There was no statistically significant differences between the time it took dams to retrieve each pup in the pup retrieval at P3. This suggests that the significant differences in average retrieval time compared to the lack of significant different observed when looking at time to complete the pup retrieval task is not due to a difference in the rate of retrieval between each pup.

A6. Change in body-temperature during isolation induced USVs

Appendix A6 shows the mean values for average change in pup body temperature, pre – post isolation induced USV testing.

Table A6: Average change in body temperature from pre- to post testing during isolation induced USVs data are mean ± SEM

		P2	P4	P6	P8	P10
Male	WT	2.34 (0.17)	1.87 (0.16)	1.43 (0.14)	1.12 (0.13)	1.25 (0.15)
	WT (Mixed litter)	2.02 (0.26)	2.17 (0.27)	1.42 (0.16)	1.07 (0.17)	2.07 (0.58)
	Peg3KO (Mixed litter)	2.46 (0.20)	1.92 (0.22)	1.74 (0.19)	1.38 (0.20)	0.84 (0.16)
	Peg3KO (Mutant litter)	2.57 (0.23)	1.92 (0.25)	1.35 (0.15)	1.06 (0.12)	1.19 (0.17)
Female	WT	2.29 (0.18)	1.84 (0.15)	1.40 (0.17)	1.27 (0.12)	0.9 (0.14)
	WT (Mixed litter)	2.69 (0.31)	3.94 (1.83)	1.9 (0.25)	0.94 (0.27)	0.96 (0.16)
	Peg3KO (Mixed litter)	2.45 (0.23)	1.91 (0.18)	1.79 (0.17)	1.6 (0.21)	1.00 (0.11)
	Peg3KO (Mutant litter)	3.18 (0.23)	2.07 (0.24)	1.59 (0.20)	1.08 (0.18)	1.09 (0.15)

A7. Nominally significant differentially expressed genes in the olfactory bulb between *Peg3KO* (mutant litter) and WT mice

Appendix A7 shows the nominally significant differentially expressed genes in the olfactory bulb between *Peg3KO* $^{\text{(mutant litter)}}$ and WT mice.

Table A7. Nominally significant differentially Expressed Genes in the olfactory bulb between *Peg3KO* $^{\text{(mutant litter)}}$ and WT mice

Analysis	DEG	Log2 Fold Change	*p* value	Adjusted *p* value	B
Combined	*Peg3*	-0.79	1.20e-04	0.99	-2.07
Male	*Peg3*	-0.71	0.006	0.99	-3.76
Female	*Peg3*	-0.87	0.001	0.99	-3.39
	Rpl7-ps10	0.67	0.044	0.99	-4.46
	7SK	2.26	0.033	0.99	-4.73

A8. RNA Extraction from Placental Tissue

The following protocols have been adapted from Sumption (2020).

RNA Extraction

Placenta samples were pulverised and 1 ml TRIzol (Life Technologies) was added prior to incubation at room temperature for 5 minutes. 200 µl of chloroform (Sigma) was then added to each sample before incubating for a further three minutes after which samples were centrifuged at 4°C for 15 minutes. The aqueous RNA phase was then transferred into a fresh tube before 500 µl of isopropanol (Sigma) and 1 µl of GlycoBlue™ (ThermoFisher, UK) was added to precipitate and stain the RNA respectively. Tubes were mixed by inversion and incubated for ten minutes at room temperature. Tubes were then centrifuged for 10 minutes at 12000 g at 4°C.

Supernatant was discarded leaving the pellet, which was washed in 75% ethanol. The tubes were centrifuged at 7500 g at 4°C for 5 minutes prior to the ethanol being discarded and the pellet left to dry before resuspension in 30 µl RNase free water. RNA concentration was determined using a Nanodrop ND-1000 spectrophotometer and a Qubit RNA BR Assay Kit (ThermoFisher). RNA quality was assessed using a TapeStation (Agilent Technologies, USA). RNA was stored long term at -80°C.

To remove genomic DNA contamination, extracted RNA was treated with a DNase. 1 µl of RNase-Free DNase 10X Reaction Buffer (Promega UK) and 2 µl of RNase-Free DNase (Promega) were added to 2 µg of RNA. Nuclease-free water was then added to total 10 µl. Samples were incubated at 37°C for 30 minutes. 1 µl of RQ1 RNase-Free DNase Stop Solution (Promega) was then added to each tube and left at 65°C for 10 minutes to stop the reaction and inactive the DNase.

DNase treated RNA samples were reverse transcribed to generate cDNA for use in qPCR 10 µl of RNA was combined with 1 µl of Random Primers (Promega), 1 µl of dNTPs (ThermoFisher) and 1 µl of RNase free water. This mix was left to incubate at 65°C for 5 minutes. 4 µl of 5X First-Strand Buffer (Invitrogen, UK), 1 µl of 0.1 M DTT and 1 µl of RNaseOUT (Invitrogen) were added to each sample along with 1 µl of SuperScript III Reverse Transcriptase (Invitrogen). Samples were then incubated at 25°C for 5 minutes, then at 50°C for 60 minutes. The reaction was terminated by heating to 70°C for 15 minutes. cDNA samples were diluted 1:5 in 10 mM Tris (pH 8.0, Sigma) before storage at -20°C.

qPCR Protocol

Quantitative qPCR (qPCR) analysis, cDNA was diluted (1:20) in 10mM Tris (pH 8.0, Sigma). 7.5 µl qPCR mastermix was then added to 2 µl of diluted cDNA in each well of a 384 well qPCR (ThermoFisher) plate.

qPCRwas performed using a QuantStudio™ 5 Real-Time PCR System, 384-well machine in a 9.5 µl reaction. The reaction contained 2 µl of cDNA (diluted 1 in 100) and 7.5 µl of mastermix .Reference genes *YWHAZ* and *SDHA* were run alongside the PEG3 on each plate. qPCR was performed under the

following conditions: i) 3 minutes at 95°C, ii) 20 seconds at 95°C, iii) 20 seconds at 53°C, iv) 20 seconds at 72°C, and v) 20 seconds at 75°C. Steps ii-v were repeated 40 times. The melt curve was executed at 60°C to 95°C, read every 0.5°C and held for 2 seconds.

A9. Maternal perinatal mental health and infant non-verbal social behaviour

Appendix A9.A and A9.B show partial correlations for both A1 and Y1 depression and anxiety scores for both the frequency and duration of infant non-verbal social behaviours.

Table A9.A Perinatal mental health and frequency of infant non-verbal social behaviour during the Freeplay task **.** Data is R_2; *p* value

FREQUENCIES												
	A1						Y1					
	EPDS			STAI			EPDS			STAI		
	All	Male	Female	All	Male	Female	All	Male	Female	All	Male	Female
Caregiver-directed play	.18; .19	.14; .50	.21; .28	-.15; .27	.35; .08	.01; .97	**.28; .04**	.30; .13	.30; .12	.18; .18	.33; .09	.06; .75
Solitary play	-.06; .68	.02; .93	-.14; .46	-.07; .63	-.17; .40	.05; .81	.04; .77	.004; .99	.10; .60	-.03; .85	-.07; .71	.04; .89
Oriented to Caregiver	.05; .72	-.03; .88	.17; .39	.21; .12	.14; .49	.23; .23	.05; .72	.06; .79	-.02; .92	.16; .23	.10; .63	.14; .47

[1] Data is $R_{s;}$:Spearman's rank correlation coefficient
[2] Controlling for infant age at time of assessment

Table A9.B Perinatal mental health and duration of infant non-verbal social behaviour during the Freeplay task. Data is R_2; *p* value.

DURATION												
	A1						Y1					
	EPDS			STAI			EPDS			STAI		
	All	Male	Female	All	Male	Female	All	Male	Female	All	Male	Female
Caregiver-directed play	-.14; .29	-.07; .72	-.25; .20	-.04; .78	.21; .29	-.24; .20	.04; .76	.14; .47	-.04; .83	.08; .54	.28; .16	-.07; .72
Solitary play	.02 .91	.09; .67	-.04; .84	.03; .85	-.07; .73	.14; .48	.11; .41	.07; .74	-.04; .83	.06; .68	-.03; .87	.17; .38
Oriented to Caregiver	.07; .61	.03; .87	.07; .70	-.02; .89	.17; .40	-.11; .58	.15; .25	.20; .33	.18; .35	.05; .72	.19; .35	-.01; .97

[1] Data is $R_{s;}$:Spearman's rank correlation coefficient
[2] Controlling for infant age at time of assessment

A10. Maternal perinatal mental health and maternal non-verbal social behaviours

Appendix A10A and A10.B show partial correlations for both A1 and Y1 depression and anxiety scores for both the frequency and duration of maternal non-verbal social behaviours.

Table A10.A Perinatal mental health and frequency of maternal non-verbal social behaviour during the Freeplay task. Data is R_2; *p* value

FREQUENCIES												
	A1						Y1					
	EPDS			STAI			EPDS			STAI		
	All	Male	Female	All	Male	Female	All	Male	Female	All	Male	Female
Infant directed play	.20; .13	.15; .46	.30; .11	.10; .45	.06; .76	.20; .29	.07; .57	.11; .60	.08; .66	.15; .91	.01; .97	.10; .62
Oriented to Infant	.06; .68	-.15; .46	.28; .14	.03; .80	-.16; .44	.23; .23	.07; .62	-.16; .42	.34; .07	.14; .30	-.15; .45	.46; .05
Loom	-.07; .62	-.06; .76	-.11; .58	-.19; .16	-.05; .81	-.33; .08	.08; .57	-.15; .45	-.14; .48	.02; .91	-.09; 65	-.08; .67

Within reach of Infant	-.04; .75	-.05; .79	-.06; .76	-.18; .19	-.09; .65	-.35; .06	.04; .77	.10; .64	-.06; .77	-.04; .76	.06; .79	-.04; .84

[1] Data is R_s; :Spearman's rank correlation coefficient
[2] Controlling for infant age at time of assessment

Table A10.B Perinatal mental health and duration of maternal non-verbal social behaviour during the Freeplay task. Data is R_2; *p* value

DURATION												
	A1						Y1					
	EPDS			STAI			EPDS			STAI		
	All	Male	Female	All	Male	Female	All	Male	Female	All	Male	Female
Infant directed play	-.13; .34	-.30; .12	-.01; .97	-.17; .22	-.22; .27	-.03; .87	-.09; .49	-.16; .44	.002; .99	-.22 .11	-.18; .33	-.19; .34
Oriented to Infant	-.04; .76	.13; .51	-.22; .25	.001; .99	-.01; .97	-.04; .83	-.02; .87	.10; .62	-.21; .29	-.07; .57	.02; .94	-.25; .18
Loom	-.05; .71	-.13; .53	-.07; .72	-.13; .34	-.09; .67	-.17; .39	-.09; .53	-.16; .43	-.09; .65	-.08; .57	-.13; .53	-.08; .70
Within reach of Infant	-.11; .44	-.16; .44	-.06; .78	-.17; .22	-.16; .42	-.04; .85	-.09; .49	-.08; .70	-.26; .17	-.22; .11	-.23; .26	-.28 .15

[1] Data is R_s; :Spearman's rank correlation coefficient
[2] Controlling for infant age at time of assessment

A11. Placental *PEG3* expression and Infant non-verbal social behaviour

Appendix A11.A and A11.B show partial correlations between placental *PEG3* expression and the frequency and duration of infant non-social behaviour.

Table A11.A Placental *PEG3* expression and the frequency of infant non-verbal social behaviour. Data is R_2; *p* value

FREQUENCY			
	All	*Males*	*Females*
Caregiver-directed play	-.18 .27	-.41; .09	-.09; .68
Solitary play	-.14; .39	.15; .56	-.20; .39
Oriented to Caregiver	-.31; .06	-.51; .05	-.15; .60

[1] Data is R_s; :Spearman's rank correlation coefficient
[2] Controlling for infant age at time of assessment.

Table A11.B Placental *PEG3* expression and the duration of infant non-verbal social behaviour. Data is R_2; *p* value

DURATION			
	All	*Males*	*Females*
Caregiver-directed play	-.13; .43	-.06; .81	-.15; .50
Solitary play	-.19; .22	.09; .72	-.28; .28
Oriented to Caregiver	.12; .45	.29; .19	-.25; .32

[1] Data is R_s; :Spearman's rank correlation coefficient
[2] Controlling for infant age at time of assessment.

A12. Placental *PEG3* expression and Maternal non-verbal social behaviour

Appendix A12.A and A12.B show partial correlations for placental *PEG3* expression and Maternal non-verbal social behaviour.

Table A12.A Placental *PEG3* expression and the frequency of maternal non-verbal social behaviour. Data is R2; *p* value

FREQUENCY			
	All	*Males*	*Females*
Infant directed play	-.05; .75	.01; .97	-.11; .62
Oriented to Infant	-.08; .61	-.23; .36	.01; .95
Loom	-.24; .12	.03; .92	.-.42; .05
Within reach of Infant	-.12; .47	.12; .62	-.23; .30

[1] Data is $R_{s;}$:Spearman's rank correlation coefficient
[2] Controlling for infant age at time of assessment.

Table A12.B Placental *PEG3* expression and the duration of maternal non-verbal social behaviour. Data is R2; *p* value

DURATION			
	All	*Males*	*Females*
Infant directed play	**.35; .03**	.14; .59	.47; .05
Oriented to Infant	**.36; .02**	.41; .09	.34; .12
Loom	.06; .71	-.01; .98	.09; .68
Within reach of Infant	.16; .32	.27; .29	,06; .80

[1] Data is $R_{s;}$:Spearman's rank correlation coefficient
[2] Controlling for infant age at time of assessment.

A13. Differences in vocalisations between male and female infants

One-way ANOVAs demonstrated that there were no significant differences between males and female infants in overall ($F(1,66) = 2.17$, $p = .15$) or distress vocalisations $F(1,66) = 1.14$, $p = .29$.

A14. Placental *PEG3* expression and Perinatal mental Health

Appendix A14 shows partial correlations for placental *PEG3* expression and perinatal mental health scores. No significant associations were observed.

Table A14. Placental *PEG3* expression and Perinatal mental health. Data is R2; *p* value

Placental *PEG3* Expression			
	All	*Males*	*Females*
A1 EPDS	**.83; .61**	.29; .26	-.01; .98
A1 STAI	**-.04; .83**	-.03; .92	.01; .98
Y1 EPDS	.10; .54	.24; .35	.03; .89
Y1 STAI	.00; .99	.18; .50	-.11; .62

[1] Data is $R_{s;}$:Spearman's rank correlation coefficient
[2] Controlling for infant age at time of assessment.

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