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The impact of periconceptional ethanol exposure on maternal and offspring hypothalamic-pituitary-adrenal axis function and associated behaviours in a rat model.

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Abstract

The consumption of alcohol during pregnancy is frequent despite clear guidelines that indicate that abstinence is the safest option to prevent adverse offspring outcomes. These outcomes range from overt craniofacial abnormalities through to outcomes such as mental illness, hyperactivity and social difficulties. Human and animal studies have demonstrated that these neurological outcomes may be due to impaired function of the hypothalamic-pituitary-adrenal axis (HPA) in offspring, resulting in altered basal glucocorticoid tone and disrupted responsiveness to stress. However, little is known of the impact of alcohol consumption around the time of conception, known as the periconceptional period, on offspring HPA function. Therefore, this study aimed to use a well-established rat model of ethanol consumption during the periconceptional period (PC:EtOH) to investigate offspring HPA activity, including behaviours, stress responsiveness and underlying molecular pathways. As alcohol consumption directly alters HPA function, this study also aimed to examine if PC:EtOH exposure impairs maternal HPA activity and related physiological pathways, including renal and metabolic function.

Female Sprague-Dawley rats were treated with PC:EtOH (12.5% v/v EtOH liquid diet) or a control diet from 4 days before conception, until embryonic day (E) 4. Behavioural tests were performed on offspring at three months of age to assess mental illness-like phenotypes (utilising the Forced Swim Test [FST] and Social Interaction [SI] paradigm), and at five months of age, HPA reactivity tests (combined dexamethasone suppression test [DST] and corticotropin-releasing hormone stimulation test [CST] and restraint stress) were performed. In a separate study, basal corticosterone concentrations were measured at 6 months, and adrenal glands were collected for analysis of steroidogenic gene expression. Aged cohorts (12-14 months) were utilised to measure basal plasma corticosterone, followed by the collection of adrenal glands, pituitary glands, hypothalamus and hippocampal tissue for analysis of various steroidogenesis and glucocorticoid signalling genes and pathology. In a separate cohort of aged rats, telemetry was used to asses blood pressure, heart rate and plasma corticosterone concentrations during 30-minute restraint stress.

Maternal hormones (corticosterone, aldosterone), renal function and plasma glucose and lipids were assessed at various stages in gestation. Adrenal glands were collected from dams at E5, E15 and E20 for analysis of steroidogenic gene expression. Placental samples were collected at E20 and genes expression of the glucocorticoid (*Nr3c1*) and corticotrophin hormone receptor (*Crh-r1*) measured.

This study revealed that PC:EtOH exposure resulted in altered offspring behavioural outcomes, including increased depressive-like behaviour in the forced swim test and altered social interaction with a novel rat. Adult offspring also demonstrated HPA hyperactivity, with elevated responses to the DST/CST challenge. Although there was no difference observed in adult offspring, aged PC:EtOH female offspring demonstrated an altered response to restraint, with reduced stress-induced plasma corticosterone and pressor response. Interestingly, PC:EtOH exposure also resulted in reduced basal plasma corticosterone concentrations in adult and aged female but not male offspring. Furthermore, female offspring showed pituitary gland abnormalities and increased mRNA for *Nr3c1* and heat shock protein 90 (*Hsp901a*) in the hippocampus, suggesting altered HPA signalling and regulatory pathways. Adrenal and hypothalamic mRNA expression of genes regulating glucocorticoid production were not overtly altered by PC:EtOH in aged offspring.

PC:EtOH significantly increased plasma corticosterone in the dam prior to mating (E-2). During pregnancy, PC:EtOH resulted in lower concentrations at E5, no differences at E15, and an increase at E20. Only minor changes in the expression of genes which regulate adrenal steroidogenesis were observed in PC:EtOH dams at E5 and E15, with the latter likely to have contributed to the observed increase in plasma corticosterone at E20. PC:EtOH had no impact on metabolic parameters (high and low-density lipoproteins and triglycerides) or renal function (food, water, urinary flow and renal electrolytes) in late gestation. However, placental markers of glucocorticoid exposure were elevated in response to exposure.

This study supports the hypothesis that periconceptional ethanol exposure alters the HPA of the mother and programs sex-specific alterations in offspring in a rat model. Maternal HPA and related physiological changes as a consequence of PC:EtOH is likely to contribute to the HPA hyperresponsiveness, and underlie behavioural outcomes observed in this study. Furthermore, these changes to the HPA may be independent of the adrenal gland, with central regulatory pathways involving the hippocampus altered by PC:EtOH.

This thesis has provided novel and important evidence that alcohol exposure around the time of conception impairs offspring mental-health like outcomes and induces HPA dysregulation. This work reinforces the concept that the maternal stress axis is highly sensitive to perturbations during early pregnancy. As this system is critical in many major physiological pathways, this can have significant long-term disease implications for both the mother and the child, supporting the critical need for education of appropriate health and wellbeing in preparation for pregnancy.

Declaration by author

This thesis *is composed of my original work and contains* no material previously published or written by another person except where due reference has been made in the text. I have clearly stated the contribution of others to jointly-authored works that I have included in my thesis.

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Publications during Candidature

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Abbreviations

Abbreviations used throughout this thesis, listed in alphabetical order.

HSD11b1	11-beta-hydroxysteroid dehydrogenase 1
HSD11b2	11-beta-hydroxysteroid dehydrogenase 2
3B-HSD	3β- hydroxysteroid dehydrogenase
ABS	Australian Bureau of Statistics
ACTH	Adrenocorticotropic hormone
ADHD	Attention Deficit Hyperactivity Disorder
AIHW	Australian Institute of Health and Welfare
ANOVA	Analysis of Variance
AUC	Area under the curve
AUD	Alcohol Use Disorder
CBG	Corticosteroid binding globulin
cDNA	Complimentary DNA
Cl^-	Chloride
CRH/CRF	Corticotropin releasing hormone/factor
CST	Corticotropin-releasing hormone stimulation test
CYP11A1/P450scc	Cytochrome p450 family 11, subfamily a, member 1
CYP11B2	Cytochrome p450, family 11, subfamily B, polypeptide 2,
CYP11B2/P450C18	Aldosterone synthase
CYP17A1/P450c17	Cytochrome p450 family 17, subfamily a, member 1
CYP21A1	Cytochrome p450, family 21, subfamily a, polypeptide 1
DBP	Diastolic blood pressure
Dex	Dexamethasone
DOHaD	Developmental Origins of Health and Disease hypothesis
DST	Dexamethasone Suppression Test
E	Embryonic day
EDTA	Ethylenediaminetetraacetic acid
EtOH	Ethanol
FARE	Foundation of Alcohol Research
FASD	Fetal Alcohol Spectrum Disorder
FBGRKO	Glucocorticoid receptor knock out

FAS	Fetal Alcohol Syndrome
FST	Forced swim test
GDM	Gestational Diabetes Mellitus
GR, *Nr3c1*	Glucocorticoid receptors
HDL	High-density lipoprotein
HPA	Hypothalamic-pituitary-adrenal axis
HPG	Hypothalamic-pituitary-gonadal axis
HR	Heart rate
HSP	Heat shock proteins
IP	Intraperitoneal injection
IUGR	*In utero* growth restriction
IV	Intravenous injection
IVF	*In vitro* fertilisation
K^+	Potassium
LC	Locus coeruleus
LDL	Low-density lipoprotein
MC2R	Melanocortin receptor
MI	Mental Illness
MR, *Nr3c2*	Mineralocorticoid receptors
mRNA	Messenger RNA
NA	Noradrenaline (Norepinephrine)
Na^{2+}	Sodium
NCDMI	Non-Communicable Disease and Mental Illness cluster
NCDs	Non-communicable diseases
NDSHS	National Drug Strategy Household Survey
NHMRC	National Health and Medical Research Council
PAE	Prenatal alcohol exposure
PC:EtOH	Periconceptional ethanol
PN	Post-natal
POMC	Proopiomelanocortin
PVN	Paraventricular nucleus
RAAS	Renin-angiotensin-aldosterone system

SBP	Systolic blood pressure
SI	Social interaction test
StAr	Steroidogenic acute regulatory protein
Trt	Treatment
v/v	Volume per volume
WHO	World Health Organisation
Symbols	
↑	Increase
↓	Decrease
↔	No change
±	Plus and/or minus
Δ	Change in
♂	Male
♀	Female

Chapter One

Literature Review

"It is not right that procreation should be the work of bodies
dissolved by excess of wine,
but rather that the embryo should be compacted
firmly, steadily and quietly in the womb."
- Plato-

1.0 Overview

Medical guidelines around the world generally recommend abstinence from alcohol while pregnant. The 2009 National Health and Medical Research Council of Australia (NHMRC) guidelines clearly state that "When pregnant, or planning a pregnancy, not drinking is the safest option". Although most women attempt to adhere to these guidelines and cease consumption when they become aware of their pregnancy, there is a startling number of women who admit to consumption before pregnancy detection. As alcohol is accepted as a teratogen, with a wide range of research published highlighting adverse outcomes for offspring [1–4], it is concerning that many women may have unintentionally exposed their early embryo to this insult.

A key biological system that is known to be both directly and prenatally influenced by alcohol is the hypothalamic-pituitary-adrenal axis (HPA). This central neuroendocrine system is essential in maintaining homeostasis of a wide range of physiological functions, including cardiovascular, metabolic, mental health and behaviour. As such, numerous human and animal studies have investigated the link between prenatal alcohol exposure (PAE) and alterations to the HPA, with suggestions that this may be the underlying mechanism leading to neurological and cardiometabolic conditions. However, there is little knowledge of the outcomes of alcohol consumption during the periconceptional period, which is defined as the period prior to and including conception, fertilisation and implantation. Furthermore, the investigation of maternal changes associated with PAE has largely been neglected. Therefore, this chapter will firstly explore the epidemiology of Non-Communicable Diseases (NCDs) and alcohol consumption, followed by discussing the specific impacts of alcohol consumption during pregnancy on offspring outcomes and maternal physiology.

1.1 Health burden of Non-Communicable Diseases

NCDs are defined by the World Health Organisation (WHO) as diseases of long duration, which are generally slow in progression, including but not limited to cardiovascular and metabolic disease. NCDs were the lead causes of morbidity and mortality globally in 2012 [5]. In this year, NCDs were responsible for 62% of all deaths and attributed a 54% loss of disability-adjusted life years. Alarmingly, these conditions are projected to be responsible for 70% of all deaths by 2020 [5]. Mental illness is a close second in the cause of morbidity and mortality [6], with a 36%

prevalence rate worldwide and a 13% loss of disability-adjusted life years [6,7]. Due to the burden of these illnesses, the WHO has recently enveloped these conditions into the Non-Communicable Disease and Mental Illness cluster (NCDMI), to ensure focus on addressing these commonly comorbid conditions. This aims to understand risk factors, as well as increasing health care and rehabilitation to prevent premature death and disability. Common risk factors associated with NCDMI include a range of lifestyle factors such as insufficient physical activity, poor dietary regulation and alcohol consumption, which is of interest in this thesis.

1.2 Worldwide alcohol consumption

Worldwide guidelines for alcohol consumption have recommended that for both men and women, no more than two standard drinks (one standard drink is equivalent to 10 grams of ethanol) on any occasion should be consumed [5]. Regardless, alcohol is the most commonly used drug for both adolescents and adults worldwide. Recent data collected in the Australian National Drug Strategy Household Survey (NDSHS) has revealed that 36% of people from 18 to 40 years of age exceeded the recommended 20g of alcohol in a single drinking session, at least once a month [8]. Furthermore, from 2016 – 2017, the Australian Bureau of Statistics (ABS) determined that individuals over the age of 15 drank on average 9.4 litres of pure alcohol per person per year [9]. Similarly, Yusuf *et al.* has demonstrated that Australian men and women are drinking an average of 4-7 drinks per day [10]. Similar consumption levels are observed in many nations including the United States of America and Europe, with the highest consumption worldwide observed in Belarus, Russia, Czech Republic and Lithuania, where consumption reaches 17 litres per person per year [5].

Alcohol Use Disorder is one of the most common substance use disorders worldwide, with 4.9% of the population with this diagnosis [5]. The Global Status Report on Alcohol and Health released by the World Health Organisation in 2014 suggests that alcohol is within the top five risk factors for disease, disability and death worldwide [5] and is associated with a number of NCDMI outcomes. The NDSHS revealed that more than 3 million Australians are at risk of alcohol-related disease or injury during their lifetime, particularly those within the 18 to 29 age bracket [8]. Gao *et al.* recently compiled data from the Victorian Health System in 2010, showing that 5 554 deaths and 157 132 hospitalisations could be directly attributed to alcohol consumption

(Table 1. 1) [11]. These statistics demonstrate the prevalence of alcohol consumption and illustrate the consequences for both individual and societal health and disease.

Table 1. 1: The per cent (%) of hospitalisations and deaths associated with alcohol consumption during 2010, within the Victorian (Australia) Health System [5].

Health Condition	Hospitalisations		Death	
	Men (n =101 425)	**Women (n =55 707)**	**Men (n =3 467)**	**Women (n =2 087)**
Cancer	5%	9%	25%	31%
Cardiovascular	7%	6%	13%	34%
Digestive	7%	5%	16%	11%
Infectious	5%	7%	4%	6%
Neuropsychiatric	30%	41%	7%	6%
Injury	47%	32%	37%	12%

1.2 Sexual dimorphism of alcohol effects

Traditionally, research investigating the adverse effects of alcohol consumption on health and wellbeing have focused on males, however as shown in Table 1. 1, women are also at a significant risk of adverse health outcomes [5]. Although women do drink less than men, recent epidemiological studies have shown that the gap between male and female alcohol consumption levels is closing, particularly within the child-bearing years [10,12,13]. It is suggested that this is due to men reducing their consumption, as well as a response to societal changes regarding gender equality [14–17]. However, women are considerably more susceptible to the toxic effects of alcohol, due to a slower metabolism as a result of less water volume and reduced activity of alcohol metabolising enzymes [12,18]. Frezza *et al.* determined that the activity of alcohol dehydrogenase is significantly less in women than in men, with negligible levels in those women who drank at high levels [19]. This results in the majority of alcohol reaching the liver, increasing intoxication levels and susceptibility to widespread tissue damage [18,19]. Alcohol-induced hypertension and stroke are often observed within both sexes[20–23]. However, it has recently been shown that women have a greater susceptibility to alcohol-induced cardiovascular outcomes, neurological damage, and sex-specific cancers [24–26].

Evidence shows that alcohol consumption significantly contributes to the development of female breast cancer, but not in the rarer male breast cancer [16,27]. Hamajima *et al.* performed an analysis of 53 epidemiological studies to demonstrate that for every additional 10 grams of alcohol consumed there was a 7.1% increased risk of developing breast cancer [28]. A study carried out in New Zealand has revealed a 55% increase in the risk of breast cancer in Maori women who have at least one weekly binge (greater than 40 grams of alcohol). Interestingly, in this study, these women were more likely to have estrogen or progesterone positive breast cancers, drawing attention to the close link of alcohol and the endocrine system [29].

Sexual dimorphism is also observed in alcohol-induced brain damage. Imaging studies reveal that the corpus callosum and intracranial spaces are smaller in alcoholic women compared to both the brains of women who did not consume alcohol, as well as alcoholic and non-alcoholic men [30,31]. However, alcohol-induced mental illness is observed in both men and women, with varying symptoms. Men are more likely to experience disinhibiting traits such as impulsivity and risk-taking compared to women [16,32–34], whereas women are three times more prone than men to affective disorders such as depression and anxiety [16,35]. Surveys have also revealed that suicide attempts are more likely to occur in women who drink, while men are more likely to experience suicidal thoughts [36,37]. Although these outcomes will influence the lives of both men and women who consume alcohol, this is highly relevant to female emotionality and behaviours relating to family structure and child-bearing.

1.3 Alcohol consumption during pregnancy

The sexual dimorphism and severity of outcomes for women is an essential focus for this thesis, as many women of childbearing age (18 – 23 years of age) are drinking five or more drinks per occasion, at least once a week [38]. Recent statistics reveal that up to 75% of women consume alcohol when planning a pregnancy [39]. This may seem surprising; however, over the years, conflicting advice has been provided to women from medical, government and media sources regarding consumption during pregnancy. In just 2001, Australian guidelines stated that two standard drinks per day and no more than seven per week were permissible in pregnancy, which was revised from the 1992 recommendation of abstinence [40]. However, there is much-varied advice (Table 1. 2), which has undoubtedly caused significant confusion for pregnant women. In 2009, the NHMRC restated their earlier advice that during pregnancy, abstinence is

the safest option [41,42]. However, a survey performed by the Foundation of Alcohol Research and Education in 2018 revealed that only 46% of Australian women were advised by a health professional during their pregnancy to modify their alcohol intake [41,43]. Of these women, fewer than 15% adhere to the current guidelines presented by the NHMRC [43]. For this reason, it is not surprising that such a high number of women admit to drinking at some point during pregnancy. This suggests a widespread public health message was and still is required.

Given that 50% of all pregnancies are unplanned [44,45], and the fact that many women will cease alcohol consumption once they become aware of their pregnancy, estimates suggest that up to 30% of women may have consumed alcohol after conception but prior to pregnancy recognition [44,46]. As such, alcohol consumption around conception is likely to be the most common exposure during pregnancy and therefore is the focus of this thesis. Very little evidence is available on the impact of this early alcohol exposure on offspring outcomes. In contrast, numerous studies have investigated gestational alcohol exposure and its effects on fetal development and childhood outcomes. As there may be overlapping pathological mechanisms which contribute to disease outcomes regardless of the timing of exposure, the impact of consumption throughout pregnancy on offspring outcomes must first be discussed.

Table 1. 2: The policies for alcohol consumption during pregnancy, in 2007 across Australia [47].

Sources	Abstinence	Drinking level	Other recommendations and information
		Federal	
NHMRC (2001)	Consider	≤ 2 per day ≤ 7 per week	• Consume drinks slowly over two hours. • Do not become intoxicated. • Avoid the first trimester.
Australian Government, Department of Health and Aging	Consider	≤ 2 per day ≤ 7 per week	• Do not become intoxicated. • Evidence of adverse outcomes following moderate alcohol consumption is not clear.
Ministerial Council on Drug Strategy National Clinical Guidelines	Ideal	≤ 2 per day ≤ 7 per week	• No level has been determined as completely safe. • All women need to communicate their consumption levels and be advised of the risks associated.
		State	
The Australian Capital Territory	Not stated	Not stated	• ACT Drug and Alcohol Office stated that advice to women varies across health service providers.
Queensland Health	Consider	Reduce consumption	• It is recommended to reduce or cease alcohol consumption. • No level of consumption is recommended.
NSW Health	Safest	Low levels may be harmful	• Binge drinking, particularly in the first trimester is harmful. • No safe level has been determined.
NSW Health (Centre for Drug and Alcohol)	Safest	Moderate consumption (2 drinks/day, 3-4 times per week) may be harmful	• Heavy drinking is dangerous during pregnancy.
South Australian Department of Health	Ideal	Not recommended	• Reduce alcohol consumption during the planning of pregnancy. • Cease upon pregnancy detection. • Increased quantities of consumption increase risk.

Tasmanian Department of Health and Human Services	Ideal	≤ 2 per day ≤ 7 per week	• No safe level is established. • Adhere to the NHMRC guidelines.
Victorian Department of Health	Ideal	≤ 2 per day ≤ 7 per week	• Adhere to the NHMRC guidelines. • Opinions on the safe level of consumption vary.
Western Australian Drug and Alcohol Office	Ideal	≤ 2 per day ≤ 7 per week	• Adhere to the NHMRC guidelines.
Western Australian Department of Health	Not stated	Not stated	• The consumption of alcohol while pregnant is hazardous or harmful. • This may increase the risk of growth restriction and low birth weight and premature birth.
		Medical and Nursing Organisations	
Royal Australian College of General Practitioners	Ideal	Restrict	• Assess pregnancy and planning to become pregnant women annually for levels of consumption. • Intervene for those women who drink high levels.
Royal Australian and New Zealand College of Obstetricians and Gynecologists	Not stated	Not stated	• Not stated.
Royal Australasian College of Physicians, Royal Australian and New Zealand College of Psychiatrists	Ideal	No level has been deemed safe	• Inform women of risks associated with consumption during pregnancy.
Australian Medical Association	Ideal	Not stated	• Guidelines were based on the NHMRC guidelines of 2001, stating to abstain from alcohol during pregnancy. • It was suggested that NHMRC need to review their guidelines again to recommend abstinence.

1.4 Prenatal alcohol exposure and offspring outcomes

Despite, numerous references in non-scientific literature, the earliest scientific manuscript discussing the teratogenic effects of alcohol was published in 1973 with Jones and Smith associating "maternal alcoholism and aberrant morphogenesis in the offspring" [48]. Alcohol rapidly disperses into all cell types, including the placenta and fetal tissues, contributing to a wide range of outcomes including miscarriage, preterm birth, growth restriction and subsequent low birth weight [49–53]. The most severe outcome associated with teratogenic levels of alcohol consumption in pregnancy is fetal alcohol syndrome (FAS). A recent meta-analysis performed by Popova *et al.* suggests a prevalence of FAS to be 14.6 per 10 000 [54]. FAS is associated with altered brain structure, craniofacial abnormalities, cardiac defects and prenatal growth restriction [48,55]. Once born, children with FAS display developmental delays, behavioural difficulties and have a greater susceptibility to conditions such as addiction and mental illnesses in later life [55–57]. However, the severity of FAS symptoms depends on variables such as dosage, the timing of alcohol exposure and genetics [47]. Since this first identification of FAS, the umbrella term, Fetal Alcohol Spectrum Disorder (FASD), has been adopted to include all diagnostic criterion of FAS, including partial Fetal Alcohol Syndrome, Alcohol-Related Birth Defects and Alcohol-Related Neurodevelopmental Disorder [58]. FASD is a complex condition to diagnose, even with the guidelines describing only two specific categories; FASD with three sentinel facial features and FASD with less than three sentinel facial features (diagnosis details can be seen in Table 1. 3) [58]. As such although epidemiological research has determined the prevalence of FASD as greater than overt FAS, with 1.1-5% of the population in the United States, and up to 19% in some regions of Australia impacted [59–61], this has been suggested to be an under-estimate.

Table 1. 3: The diagnostic criteria for Fetal Alcohol Spectrum Disorder, as of 2016 [58].

Diagnostic Criteria	FASD without three facial features	FASD with three facial features
Prenatal alcohol exposure	Confirmed/unknown	Confirmed
Neurodevelopmental • Altered brain structure • Impaired motor skills • Difficulty with language • Impaired Memory • Attention deficits • Poor school performance • Impulse control • Hyperactivity • Inappropriate social awareness	3 or more	3 or more
Sentinel Facial Features • Short palpebral fissure • Smooth philtrum • Thin upper lip	3 or more	Less than 3

Animal models have been utilised to interrogate the impact of prenatal alcohol exposure on offspring without the confounding impact of nutrition and social demographics on childhood outcomes. The earliest studies using animal models, pre-dating the classification of FAS and FASD were performed in the early 1890s by Charles Stockard and team. Evidence was provided that following soaking of zebrafish eggs in water containing 1-5% ethanol, embryos displayed deformities in the Fundulus in central nervous system [62]. Following this, Stockard *et al.*, repeatedly intoxicated guinea pigs via alcohol vapour inhalation, and demonstrated that offspring were "defective", and although some died, some also continued to live in "monstrous form" [63]. Investigations directly aimed at modelling FASD did not commence until the 1970s. Numerous studies have supported knowledge that PAE results in reduced brain weight [64,65], and reduced volume changes in the forebrain, cerebellum, brainstem [66], olfactory bulb and

hippocampus [66,67], corpus callosum [67,68] and amygdala [69] in several animal models. PAE has also been associated with alterations to pathways associated with the aetiology of mental illness, including reduced 5-HT-ir neurons in the dorsal raphe nucleus of the brainstem [70], reduced dopamine D2 receptor (D2R) mRNA and protein levels [71], and the HPA, which is the focus of this thesis and will be discussed in greater detail in Section 1.6 Hypothalamic-pituitary-adrenal axis.

Many systemic changes are observed in offspring following PAE, including a low birth weight [72–76] and metabolic abnormalities, such as altered insulin and glucose homeostasis [74,77,78], altered skeletal muscle utilisation [79], increased adiposity [76] and elevated gluconeogenesis [74]. Morphological changes have been seen in PAE offspring, with reduced nephron count [80], myocardial contractile function [81] and altered liver weight [82]. These changes collectively indicate that many physiological systems can be influenced by PAE, increasing the risk of NCDMI outcomes.

The diagnostic criteria for FASD are quite rigid, which, unfortunately, does not allow individuals with more subtle deficits to fall into the current guidelines [83,84]. This is despite clear evidence that PAE can lead to a range of long-term diseases outcomes related to developmental adaptations to alcohol exposure. Therefore, if PAE were to be considered in the context of the Developmental Origins of Health and Disease (DOHaD) hypothesis, FASD diagnosis may apply to a higher number of impacted individuals.

1.5 The Developmental Origins of Health and Disease (DOHaD) hypothesis

The DOHaD hypothesis, which associates the intrauterine environment and later life disease originated from what was formerly known as the "Barker Hypothesis". This hypothesis was established in the 1980s when David Barker and his team observed an inverse relationship between infant mortality and cardiovascular-related deaths within lower-income regions of the United Kingdom [85]. The subsequent investigation further established the link between low birth weight and the risk of hypertension and cardiovascular disease, which was suggested to be as a result of intrauterine nutrient deficiency [86]. Further evidence determined a link between *in utero* growth restriction (IUGR), glucose tolerance and insulin resistance, with a significant increase in the likelihood of developing type 2 diabetes mellitus and other metabolic conditions [87,88], as well as lung disease and mental illness [89].

Since this time, DOHaD has been defined more succinctly, that a sub-optimal *in utero* environment may influence fetal development and increase the risk of developing chronic diseases in later life. This may occur by directly altering fetal development or indirectly by altering maternal physiology and fetal exposure to key nutrients and hormones. These initial insults may induce changes in the developing fetus known as predictive adaptive responses [90–92]. These adaptations are often observed in key organs such as the kidney, heart and brain, and are essential for continued *in utero* development and survival [93,94]; however they may increase the risk of adverse offspring outcomes [92,95].

It is now understood that although low birth weight is commonly reported in DOHaD, it is not the deciding factor in offspring disease risk, with adverse outcomes demonstrated across a range of birth weights. Since this discovery, the relationship between perturbations such as alcohol consumption, maternal diet, placental insufficiency, hypoxia, maternal stress, glucocorticoid exposure and drug exposure can result in both fetal and offspring outcomes [96–100]. These outcomes include altered growth profiles and impaired function of key systems such as renal, metabolic, cardiovascular and endocrine systems, changes which may increase the risk of associated diseases. The range of disease outcomes in response to various maternal perturbations is too extensive to discuss in detail within this thesis and as such, Table 1. 4 displays a non-exhaustive collection of published review articles within the last five years.

Of particular focus for this thesis are programmed alterations to neuroendocrine systems such as the HPA and related psychiatric disorders [101–103]. However, before programmed disease outcomes related to the HPA are discussed in detail, it is first essential to outline the structure and function of this axis.

Table 1. 4: A non-exhaustive collection of published reviews of a range of maternal perturbations associated with adverse offspring outcomes, within the last five years.

Perturbation	Altered physiological system	Ref
Nutrition, Obesity, Gestational Diabetes Mellitus	• Behavioural • Cardiovascular and metabolic • Neuroendocrine and neuropsychiatric • Renal • Respiratory	104–129
Placental dysfunction and hypoxia	• Behavioural • Cardiovascular and metabolic • Neuropsychiatric • Pregnancy complications	121,130–137
Drug use (amphetamines, cannabis, cocaine, nicotine)	• Behavioural • Immune • Neuropsychiatric • Pregnancy complications • Physical abnormalities	121,138–161
Endocrine Disrupting Chemicals	• Cancer • Endocrine • Metabolic • Neuropsychiatric	162–172

	• Pregnancy complications • Respiratory	
Maternal stress, mental illness and glucocorticoid exposure	• Allergies • Behaviour • Birth weight • Central nervous system development • Metabolic and obesity • Neuropsychiatric	133,173–189
Overall review of the field	• Numerous	190,191

1.6 Hypothalamic-pituitary-adrenal axis

The HPA is a complex neuroendocrine signalling pathway connecting the hypothalamus, pituitary gland and adrenal gland (Figure 1. 1). This axis is necessary for the communication of both psychological (also known as processive), and physiological (also known as homeostatic) stress through the action of glucocorticoids. The activity of the HPA is driven by neurosecretory neurons located within the dorsomedial parvocellular division of the paraventricular nucleus (PVN) [192–194]. Upon stimulation of these neurons, secretagogues such as corticotropin-releasing hormone (CRH, historically known as corticotropin-releasing factor [CRF]) and arginine vasopressin (AVP) are released into the median eminence [195]. These hormones act on CRH and AVP receptors respectively, on the corticotrophs of the anterior pituitary gland, stimulating the synthesis of adrenocorticotropic hormone (ACTH) from the proopiomelanocortin (POMC) gene, for packaging into vesicles and release into systemic circulation [193,196]. Within the adrenal cortex, ACTH acts on the melanocortin receptor (MC2R), which stimulates the transport of low-density lipoprotein (LDL) into the cell, producing free cholesterol within the lysozymes [197–199]. The initial and rate-limiting step of the steroidogenesis pathway is the transfer of cholesterol from the outer mitochondrial membrane to the inner membrane by steroidogenic acute regulatory protein (StAR) [197,200], whereby (CYP11A1, P450scc) converts cholesterol to pregnenolone [199,201].

Pregnenolone is a key precursor for a range of steroid hormones including androgens, mineralocorticoids and glucocorticoids, all of which are produced by the adrenal gland. Various members of the Cytochrome P450 family regulate which steroid is produced in which tissue. Cytochrome p450 family 21, subfamily a, member 1 (CYP21A1, P450c21a) is expressed in both the zona glomerulosa and zona fasciculata of the adrenal gland where it is essential for the production of glucocorticoids. These are further converted to aldosterone within the zona glomerulosa by the actions of CYP11B2 (P450C18, aldosterone synthase). In humans, much of the pregnenolone involved in the production of glucocorticoids is firstly catalysed into 17 alpha-hydroxy pregnenolone by CYP17A1 prior to conversion into cortisol. Rodents (rats and mice), however, do not express CYP17A1 within the adrenal gland, so they instead produce corticosterone as their predominant glucocorticoid hormones. This pathway can be seen in Figure 1. 2. Unless species-specific, the term glucocorticoids will be used throughout this thesis.

Regardless of species, the secretion of glucocorticoids undergoes a daily rhythm, as discussed in Section 1.6.1 HPA regulation, with the highest plasma concentration at wakening [202,203]. This secretion allows glucocorticoids to occupy their receptors optimising the functional tone of many systems [204]. Glucocorticoids are lipophilic and exert their effects by cell membrane diffusion, binding to their receptors, the mineralocorticoid receptors (MR, *Nr3c2*), and the glucocorticoid receptors (GR, *Nr3c1*) [205,206]. Within systemic tissues, the MR is predominantly involved in regulating fluid and sodium homeostasis and is primarily the target for aldosterone [207].

The GR acts via the binding of glucocorticoids, resulting in changes to chaperone proteins, such as heat shock proteins (HSP) 70 and 90 occur, to facilitate translocation to the nucleus and interaction with glucocorticoid response elements within the promoter region of genes [199,208–210]. This cascade of events will ultimately impact transcription factor binding, influencing gene transcription. In addition to this traditional transcriptional pathway, glucocorticoids and their receptors can regulate cellular biology through additional mechanisms such as trans-repression and protein-protein interactions [211].The GR acts via the binding of glucocorticoids, resulting in changes to chaperone proteins, such as heat shock proteins (HSP) 70 and 90 occur, to facilitate translocation to the nucleus and interaction with glucocorticoid response elements within the promoter region of genes [199,208–210]. This cascade of events will ultimately impact transcription factor binding, influencing gene transcription. In addition to this traditional transcriptional pathway, glucocorticoids and their receptors can regulate cellular biology through additional mechanisms such as trans-repression and protein-protein interactions [211].

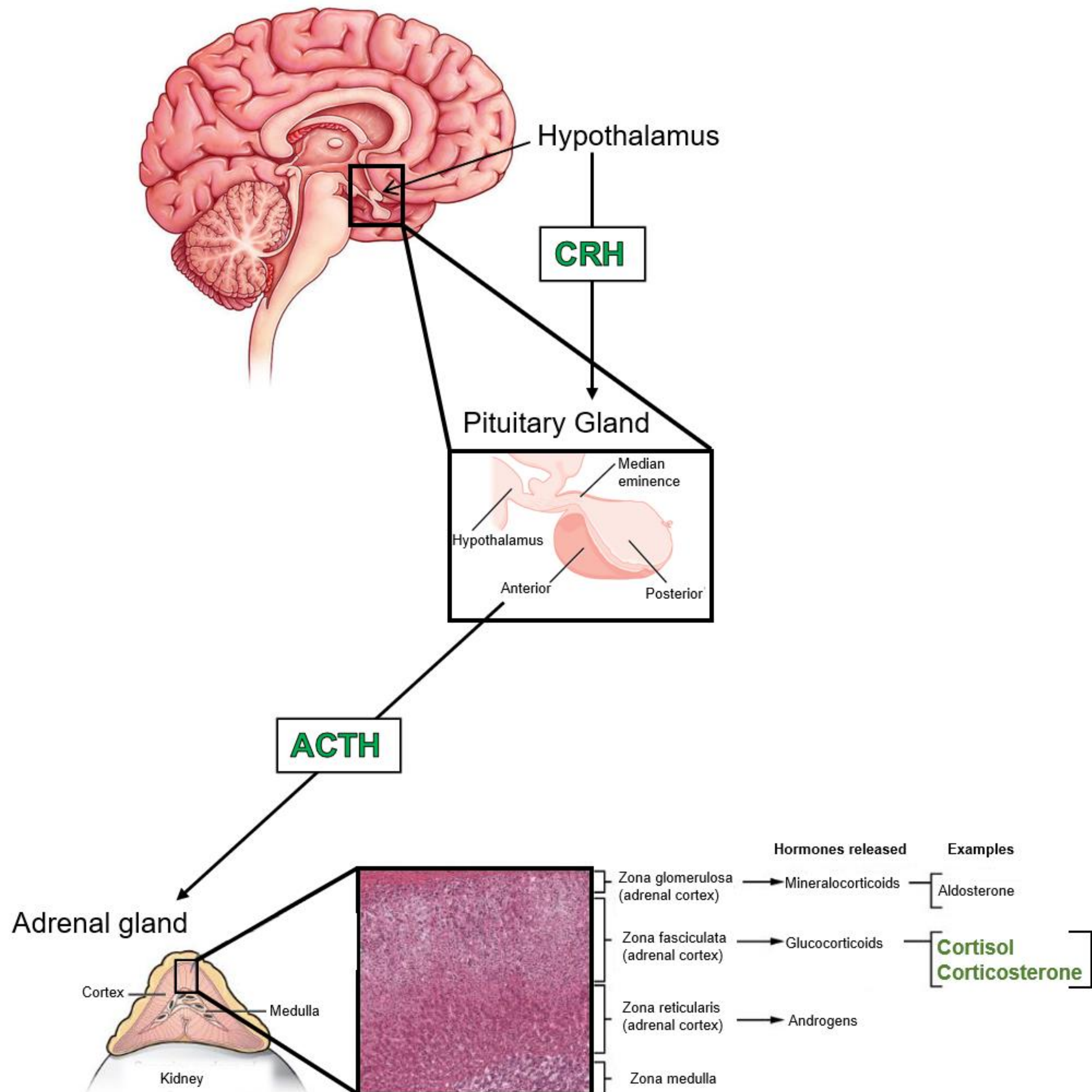

Figure 1. 1: A simplified schematic of the hypothalamic-pituitary-adrenal axis. Stress is detected by the hypothalamus, which results in a release of corticotropin-releasing hormone (CRH). This signals the release of adrenocorticotrophic hormone (ACTH) from the anterior pituitary gland. ACTH acts on the adrenal gland and results in the synthesis and release of glucocorticoids, cortisol (human) and corticosterone (rodent).

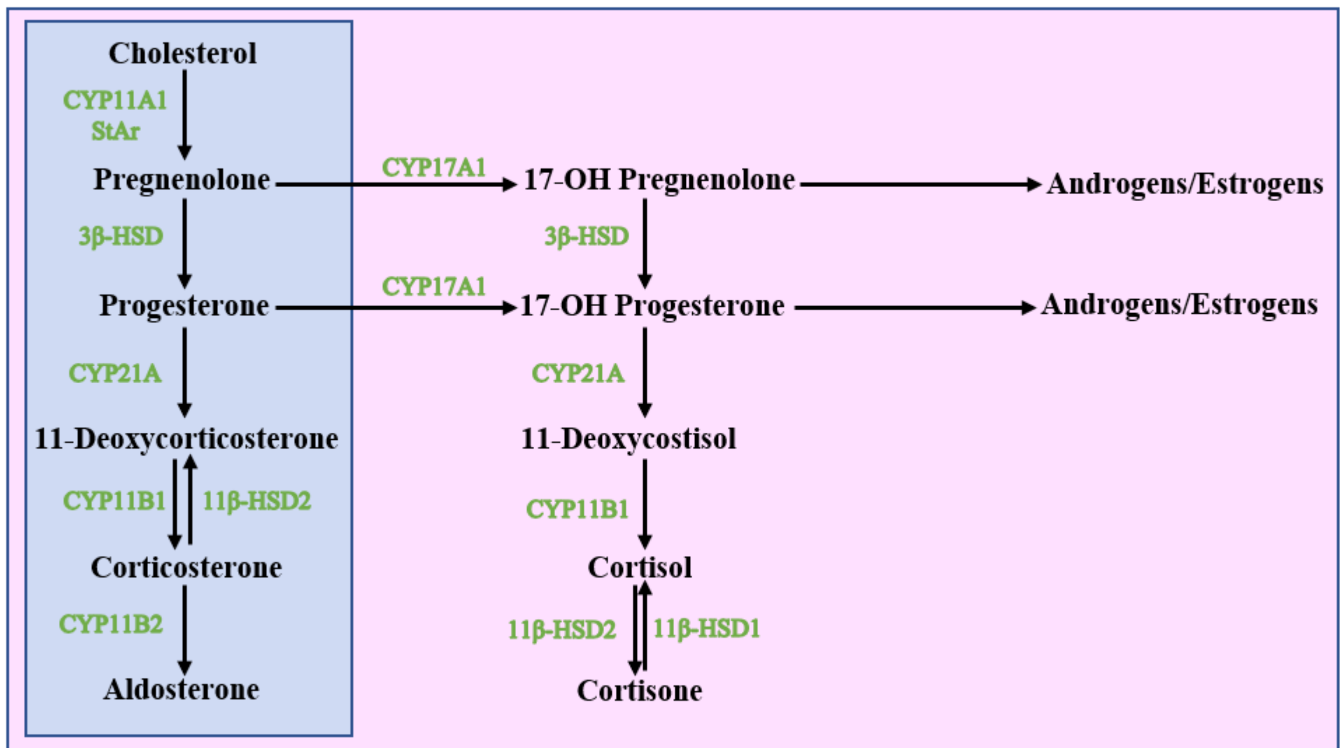

Figure 1. 2: The adrenal steroidogenic pathway of the zona glomerulosa and zona fasciculata, producing mineralocorticoids and glucocorticoids. Blue represents rodent, and pink represents the human pathways involved in glucocorticoid production. A key difference between humans and rodents is that rodents do not express CYP17A1 in the zona fasciculata of the adrenal gland. As such, corticosterone is the predominant glucocorticoid in rodents while cortisol is the predominant glucocorticoid in humans. *Abbreviations:* CYP11A1: cytochrome p450, family 11, subfamily a, polypeptide 1, STAR: steroidogenic acute regulatory protein, 3B-HSD: 3β-hydroxysteroid dehydrogenase; CYP21A1: cytochrome p450, family 21, subfamily a, polypeptide 1, CYP11B1: cytochrome p450, family 11, subfamily b, polypeptide 1, CYP11B2: cytochrome p450, family 11, subfamily b, polypeptide 2, HSD-11B1: 11-beta-hydroxysteroid dehydrogenase 1, HSD-11b2: 11-beta-hydroxysteroid dehydrogenase 2, CYP17A1: cytochrome p450, family 17, subfamily a, polypeptide1. Image adapted from Arlt *et al.* [199].

1.6.1 HPA regulation

Regulation of the HPA is also under the control of numerous systemic and local factors with both negative and positive feedback pathways. The secretion of glucocorticoids is under tight circadian control with external light, regulated by the master circadian clock known as the suprachiasmatic nucleus (SCN) of the ventral hypothalamus [213,214]. The SCN receives information from the external environment via light signals reaching the retina, stimulating the retino-hypothalamic tract and autonomic systems and subsequently, the entire organism. The core circadian feedback loop occurs with the stimulation of CLOCK in the morning, which binds to BMAL1 to drive the transcription of several genes including *Period* (*Per1* and *2*) and Cryptochrome (*Cry1* and *2*). Throughout the day, these genes act via negative feedback to repress CLOCK/BMAL1 mediated transcription until the next morning [215]. Intrinsic regulators of the SCN can also maintain a level of circadian rhythm indefinitely without a light source; however, this can be disrupted by several external reasons. Humans, non-human primates and rodents demonstrate robust circadian rhythmicity of glucocorticoid secretion, with glucocorticoids increasing before waking, waning through the day and falling in preparation for sleep cycles [215,216].

Regulation of the HPA occurs via the SCN, with studies demonstrating that lesions within this region result in reduced secretion of glucocorticoids and expression of adrenal clock genes [217,218]. The SCN stimulates the rhythmic release of CRH via projections to the PVN, stimulating ACTH production and subsequent activation of the adrenal gland [219], regulating the diurnal rise in glucocorticoids [216,220,221]. The SCN also activates the autonomic nervous systems via multiple projections to the adrenal gland [222], which is likely to be the primary regulator for maintenance of adrenal circadian rhythm. This is supported by studies demonstrating that removal of the pituitary gland does not alter the rhythmicity of the adrenal gland [223]. The adrenal gland itself acts as a peripheral circadian clock, whereby rhythmic expression of several genes impacts adrenal sensitivity to ACTH, as well as adrenal steroid metabolism (reviewed in Oster *et al.* [224]). Research suggests that this is not under the regulation of the steroidogenic pathway, as neither *Star* nor *Cyp11a1 et al.* circadian transcription [224].

The circadian rhythm is essential in regulating the sexual dimorphism of the HPA. Studies have demonstrated that female rats have a higher peak in the circadian release of corticosterone,

resulting in higher plasma and adrenal concentrations than male rats [225,226]. It is suggested that this is due to circadian regulation of the hypothalamic-pituitary-gonadal axis, whereby estradiol has a significant role in the release of glucocorticoids, as discussed below, and testosterone has an essential feedback role in reducing HPA functionsaw A recently identified pathway occurs via the activation of CXCR7, a β-arrestin-biased G-protein coupled receptor, selectively expressed in the adrenal cortex of females only, and has a significant role in glucocorticoid amplitude (see Ikeda *et al.* [228]). Disruption to the regulation of the HPA has an extensive impact on normal physiology and increases the risk of disease outcomes including obesity, insulin resistance, hyperglycaemia, dyslipidaemia and hypertension (as reviewed by Nader [229]), sleep patterns, behaviours and mental health [215].

In addition to circadian regulation, glucocorticoids concentrations are maintained by systemic mechanisms. Corticosteroid binding globulin (CBG) is produced by the liver and binds up to 80% of endogenous glucocorticoids to regulate the biological availability of these hormones for transfusion through membrane and binding to steroid receptors [212,213]. However, CBG itself is down-regulated by glucocorticoids forming a negative feedback loop, consequently increasing glucocorticoid bioavailability [212,214]. Additional proteins such as albumin within the blood are also involved in mediating glucocorticoid bioavailability. Both systemic and local concentrations of glucocorticoids are also regulated by two different 11-beta-hydroxysteroid dehydrogenases (HSD11b) enzymes, which are expressed in key tissues throughout the body and placenta. These act to interconvert active corticosterone to its inactive precursor, 11β-deoxyhydro-corticosterone and reducing bioactive concentrations [215–217]. Systemically, HSD11b2 converts active glucocorticoids into their inactive metabolites and is highly expressed in tissues with high MR expression patterns, preventing glucocorticoid-induced regulation of the MR [218]. Withing the brain, however HSD11b is not expressed, and therefore does not mediate the binding of glucocorticoids to the MR, which is in high abundance here. Therefore MR is more easily stimulated by endogenous glucocorticoids than GR, and is therefore bound by ligand even when circulating hormone levels are low [219–221]. Regulation of the HPA axis is also mediated by the balance between expression of GR and MR, particularly within the limbic system. Discussed in the review published by Joels *et al.*, both GR and MRs are essential for stress responsiveness, cognition, behaviour and negative feedback to the HPA [210,222,223].

Within the brain, glucocorticoids act in multiple areas including cortical regions with both direct and indirect actions mediating positive and negative feedback pathways. This regulation can

occur via direct pathways, such as neuronal connections into the PVN CRH neurons from the nucleus of the solitary tract and circumventricular organs of the brainstem [202], as well as humoral inputs from other endocrine glands [224]. The regulation also occurs via indirect pathways including the limbic forebrain circuits, such as the hippocampus [225,226].

These structures act indirectly, via synapses on intermediate neurons of the bed nucleus of stria terminalis, hypothalamic nuclei and brainstem nuclei, which provide descending information to CRH neurons to regulate HPA function [202,222,224,227]. Numerous studies have demonstrated that damage to the hippocampus is associated with elevated ACTH and corticosterone, both in basal concentrations and following immobilisation stress [228–230], as well as increased in PVN CRH mRNA levels [231,232]. Both human and rodent studies have shown that electrical stimulation to the hippocampus results in long term suppression of ACTH and corticosterone response to photonic stimulation stress [233,234]. Glucocorticoid receptor antagonism in the hippocampus, via injections of RU38486, resulted in a reduction of corticosterone and ACTH [235].

1.6.2 Physiological actions of the HPA

Glucocorticoid hormones stimulate approximately 20% of genes within the human genome and participate in a vast array of physiological actions [205]. Reactivity to stress responses is an essential role of the HPA. However, many physiological actions required for survival are also regulated by glucocorticoids. This includes mobilisation of energy stores via gluconeogenesis, amino acids and fat breakdown, as well as immune activity, fluid homeostasis, pro-inflammation, bone homeostasis, mammary gland function, memory and emotional regulation [210,236]. For many years, dysregulation of the HPA has been associated with psychiatric illness. Furthermore, 50% of depressed patients present with an impaired ability to suppress cortisol concentrations following dexamethasone administration [237,238]. Furthermore, studies have shown reduced expression of glucocorticoid receptors and CRH receptors in the hippocampus of suicide victims [239]. Interestingly, while depressive patients show a greater concentration of glucocorticoids, patients who suffer Post Traumatic Stress Disorder following combat, earthquake or sexual abuse show low to normal 24-hour free urinary cortisol [240–244]. Collectively, these studies indicate impaired HPA function and responsivity with several psychiatric illnesses.

Human studies to investigate the underlying molecular mechanisms that may explain why some individuals are more prone to poor mental health are limited. Much of the current

knowledge is based on imaging techniques such as Magnetic Resonance Imaging and Positron Emission Tomography scans. For this reason, animal models are vital in advancing the field of knowledge. Although it is difficult to translate findings from an animal model to human mental illness, many behavioural tests have been used. Mental illness-like outcomes in the rodent have traditionally been referred to as emotionality, which encompasses depressive-like and anxiety-like and social behaviours. Depressive-like and social interactions are the primary focus of behavioural investigation in this thesis, utilising the forced swim test, and an open arena social interaction test is used to investigate behaviour that may be associated with mental-health like phenotypes, as is further discussed in Chapter Three.

Stress and psychiatric illnesses are often associated with increased cardiovascular function and increased risk of cardiovascular disease [245–249]. Stress responses are generally associated with increased cardiovascular activity, with studies demonstrating that psychological stress results in arrhythmia and fatal cardiac events. In one such study, it was seen that following the Northridge earthquake, sudden deaths from cardiac causes increase by 4.6 per day in the week before to 24 per day on the day of the earthquake [250], with similar results being shown following the 1995 Hanshin-Awaji earthquake in Japan [251]. Furthermore, other studies that individuals with high cardiac reactivity to stress also demonstrate hyperactivity of the HPA and sympathetic pathways [252].

The main neurological pathways activated by stress are stimulation the HPA and sympathetic nervous system (SNS) and withdrawal of parasympathetic activity [253]. These systems interact to generate adaptive responses to both physiological and psychological threat, known as the ‘fight-or-flight” response. Neurosensory signals are processed in the PVN of the hypothalamus, where CRH neurons directly innervate the locus coeruleus (LC) in the brainstem [252,254,255], stimulating the release of noradrenaline (NA). This is a bidirectional pathway, with the SNS further stimulating the release of CRH and further activation of the HPA [252]. Stress-induced activation of the LC-NA pathway further stimulates peripheral catecholamine release, activation of α - and β-adrenergic receptors and the intermediolateral nucleus of the spinal cord. This subsequently increases blood pressure and heart rate [253,256,257] and highlights the intrinsic link between the stress response and the cardiovascular system. This interaction will be investigated in Chapter Four of this thesis.

1.7 Programming of the HPA

Dysfunction of the HPA is implicated in many systemic and psychological disorders, in a complex and bi-directional manner, acting as both a risk factor and a consequence of disease. As such, much literature suggests that the HPA as a central pathway impacted by *in utero* perturbations.

Programming of the HPA is observed in studies across several maternal perturbations, including altered nutrition, intrauterine insufficiency, exposure to endogenous and synthetic glucocorticoids and drug and alcohol use. This programming is suggested as advantageous for offspring, regulating nutrient availability, or preparing for awareness necessary for survival in what is predicted to be an adverse *ex-utero* environment [258,259]. However, the conditions in the external environment are often not equivalent to that during *in utero*, resulting in a hyperactive or increased basal tone of the HPA in offspring. This triggers a cascade of inappropriate physiological responses collectively contributing to the progression of disease state.

The most extensively researched maternal perturbation related to the offspring HPA is maternal stress. These studies range from the administration of exogenous glucocorticoids through to physiological and psychological disorders that increase the concentration of endogenous glucocorticoids. Human studies associate maternal psychosocial stressors, including anxiety and elevated cortisol, with altered, albeit varied, offspring HPA outcomes. Davis *et al.* and Gutteling *et al.* demonstrated that infants of mothers with these conditions present with elevated cortisol in response to stressors, such as their first day of school and heel-stick blood draw [260,261]. Another study determined that infants of mothers who experience high anxiety during pregnancy had elevated cortisol at five weeks of age; however, blunted cortisol at two months and one year [262].

Similarly, elevated maternal amniotic fluid cortisol concentrations resulted in increased basal cortisol, but a blunted response to stress in offspring [263]. This was also observed in two studies of adolescents of mothers who suffered from depression and anxiety during pregnancy [264,265]. Maternal treatment with synthetic glucocorticoids has also been associated with a suite of HPA changes in both infants and young children, such as increased basal cortisol concentrations, exaggerated stress responses and altered cortisol upon awakening [266–269]. Numerous studies also reveal impaired offspring behaviour following maternal stress (reviewed by Van Den Bergh

et al. [187]), including attention deficit hyperactive disorder (ADHD) symptoms [270–272], elevated anxiety [272] and cognitive development [273].

In rodents, maternal social defeat stress programs elevated offspring stress response, with increased corticosterone and ACTH in both male and female offspring [274]. Likewise, maternal exposure to predator odours resulted in elevated female offspring ACTH reactivity, as well as changes to glucocorticoid signalling genes [275]. Maternal forced swimming during pregnancy revealed that although male offspring had reduced basal corticosterone, there was an elevated and prolonged stress response [276] and in a guinea pig model of maternal repeated exposure to strobe light (10Hz), offspring cortisol was reduced in both plasma and faeces [277]. Studies have also determined that offspring exposed to maternal stress display significantly altered neurological morphology, and suffer from a range of emotional deficits such as anxiety-like behaviour, as well as learning deficits and altered attention [278–284].

Exogenous treatment with synthetic glucocorticoids such as dexamethasone and betamethasone have similarly revealed several changes in the HPA function in offspring. Rodent models have demonstrated that offspring of dams treated with synthetic glucocorticoids have elevated basal corticosterone levels [285], diminished adrenal structure [286] and altered expression of the *Mc2r* [287] in the adrenal gland. HPA hyperactivity in these offspring is observed, with increased urinary corticosterone following overnight isolation stress and plasma corticosterone following anaesthesia [287].

Sheep models have demonstrated that offspring exposed to maternal treatment with betamethasone have increased basal and stress cortisol levels, at one year of age, which had normalised at two years, suggesting transient but relevant changes in HPA activity [288,289]. Other studies have revealed that a single maternal dexamethasone treatment in guinea pigs results in elevated corticosterone in male offspring, but decreased responsiveness in female offspring [290], whereas multiple course treatment of synthetic glucocorticoids throughout pregnancy results in the opposite outcome [291,292]. In contrast to the above studies, when betamethasone was administered to ewes, only during early gestation, female but not male offspring had decreased cortisol concentrations [293]. This again highlights that events that occur in early pregnancy may induce offspring deficits that are different from those that occur following exposure later in pregnancy.

Alterations within the placenta are associated with HPA programming outcomes in offspring. As HSD11b2 regulates fetal exposure to glucocorticoids, alterations in this enzyme can have significant implications for offspring development. As such, numerous studies demonstrate that HSD11b2 either fails to increase its activity or has reduced expression following prenatal stress [294–296]. Placental HSD11b2 activity is also observed to be diminished in human growth-restricted pregnancies, with subsequent elevated fetal cortisol concentrations [297]. Similarly, animal models of protein restriction suggest that reduced HSD11b2 activity is likely to contribute to the adverse outcomes in offspring [298]. Other placental alterations that occur in response to a maternal insult may similarly contribute to programmed deficits in HPA activity. Genetically manipulated reductions in placental expression of O-linked N-acetylglucosamine transferase is associated with disrupted hypothalamic gene expression profiles and elevated corticosterone concentrations following restraint in offspring [299].

Numerous studies have demonstrated that protein or calorie restriction during pregnancy can program deficits in the HPA in offspring. In a sheep model, Bloomfield *et al.* restricted food consumption to 15% of the recommended intake for ten days during late pregnancy (day 105 to 146) and revealed that female offspring showed increased HPA response to a CRH stress challenge [300]. Conversely, Hawkins *et al.* performed a more mild restriction, 85% of daily requirement during the first 70 days of gestation and showed that the fetus had significantly reduced gene expression of C*rh* in the PVN, deceased *Nr3c1* in the anterior pituitary and reduced cortisol and ACTH concentration [301,302]. Rodent models of undernutrition have demonstrated that prenatal nutrition restriction of 50% protein calories results in offspring adrenal atrophy, decreased gene expression of *Nr3c1* and *Nr3c2* in the hippocampus and C*rh* in the PVN, as well as altered corticosterone and ACTH responses to ether inhalation stress but a delayed corticosterone return to baseline compared to control offspring [303].

Maternal overnutrition and obesity have also been associated with significant offspring HPA outcomes. A human study performed in Kingston, Jamaica, demonstrated that children of women with truncal obesity had elevated cortisol secretion at eight years of age with subsequent high blood pressure [304]. Sheep studies have also revealed that a prenatal diet of 150% recommended daily calorie intake programs offspring to have elevated basal cortisol and ACTH concentrations [305]. Maternal hypoxia is also associated with altered offspring HPA function, as determined by Fan *et al.* observing that 10.8% oxygen throughout gestation in a rat model was associated with increased offspring plasma ACTH and corticosterone

concentration, elevated relative gene expression of C*rh* and C*rh-r1* in the PVN, as well as elevated expression of C*rh-r1* and C*rh-r2* in the anterior pituitary gland [306]. Additionally, this study performed a restraint test on offspring to reveal an even greater elevation in these parameters, demonstrating that stress-responsiveness is also impaired following prenatal hypoxia. Similarly, human studies of maternal cigarette smoking, which can also result in hypoxia, showed that offspring at two months of age had elevated cortisol concentrations and attenuated stress responses [307]. Rodent models of nicotine exposure are associated with altered HPA outcomes, including elevated ACTH and corticosterone concentration during the early post-natal period. Hyperactivity of the stress response is also observed with excessive concentrations of these hormones [96,308]. Cannabis smoking is associated with greater startle responses to stress [309], disrupted sleep patterns [98,310] and some other HPA axes related disorders such as hyperactivity and delinquent behaviour [98,311].

1.8 Prenatal alcohol and HPA programming

Interestingly, many of the outcomes observed in human studies of drug use are often also seen in offspring of PAE, with some studies indicating that it is difficult to elucidate exactly which outcome is an effect of which perturbation. Indeed, many of the neurological changes associated with FAS and FASD, are linked with the HPA, in an essential regulatory role. Autopsies of FASD individuals have demonstrated central nervous system disorganisation, gross microcephaly and migration errors (as reviewed by Clarren [312]), as well as deformities in limbic regions such as hippocampus [313,314]. Ramsay *et al.* demonstrated that cortisol was decreased in FASD children at two months of age [307], a result similarly observed in a study by Ouellet-Morin *et al.* with offspring at 19 months of age having reduced basal cortisol, but a hyperactive response following the stress of an unfamiliar environment [315]. Conversely, other studies have demonstrated that cortisol concentrations in PAE infants are higher under basal conditions, with a further increase following stress [316,317].

Animal models have been utilised to understand the impact of PAE with the common consensus that PAE results in altered offspring HPA function and hyperactivity to stressors. Comprehensive research carried out by Weinberg and colleagues has investigated HPA outcomes following prenatal exposure to moderate to high dose alcohol in rats throughout gestation. These studies demonstrated that basal corticosterone and ACTH was elevated in

offspring, with HPA hyperactivity in response to stressors [318–321]. These animals also displayed a range of anxiety and depressive-like signs. A separate laboratory, using the same dose of PAE exposure, administered to offspring during the weaning period, which is commonly compared to the third trimester in humans [322], demonstrated these same offspring outcomes. Anxiety-like phenotypes in offspring have also been observed in other models treating dams with 6.5% ethanol (liquid diet, E0-E21) throughout pregnancy, and gavage administration of 25% volume per volume ([v/v], gavage, E7-E21) [323,324].

Rat models have also revealed changes in central regulatory gene expression profiles, such as in the limbic system [325,326]. Interestingly, studies also indicate sexual dimorphism in response to stressors, depending on duration and type [319], which may be related to alternate but interlinking pathways, such as within the aforementioned limbic system. Other studies using alcohol vapour from embryonic day 7 to 18 demonstrated elevated CRH and ACTH concentrations in offspring following stressors [327]. Another model of maternal alcohol exposure (6.7% v/v liquid diet) from embryonic day 7 to 21 resulted in several offspring changes including elevated basal corticosterone, decreased POMC expression in the anterior pituitary gland and increased pituitary gland weight. Following stressors, these offspring demonstrated increased corticosterone, ACTH and CRH concentrations [71,328]. These outcomes were also seen in another study using maternal gavage of 4g/k.day from embryonic day 11 to 21 [99]. These studies are summarised in Table 1. 5.

While these studies investigated the impact of alcohol exposure either late in pregnancy or throughout gestation on offspring HPA outcomes, less is known about the impact of alcohol when exposure occurs around conception. Given that this is likely to be the most common exposure in humans, it is essential to understand how this may contribute to diseases in offspring.

Table 1. 5: A non-exhaustive list of prenatal alcohol effects on the programming of the HPA and related behaviours.

Species	Dose	Mode	Timing	Outcomes		Ref
				Behaviour	HPA	
Human	Maternal reporting of alcohol during pregnancy	Liquid	Over gestation		↑ basal cortisol levels ↑ stressed cortisol levels ↓ HPA return to baseline after stressors	307,316,317307,316,317
Mice	25% v/v, gavage, 4 hours apart	Gavage	E7–E21	Anxiety-like in males (Light dark)	↑ CORT (males) (FS) ↑ ACTH (females) (FS)	323323
Rat	175 ± 7 mg/dl blood ethanol levels	Vapour	2nd-week gestation		↑ ACTH secretion (FS) ↔ CORT secretion (FS) ↑ CRH mRNA (hypothalamus)	329329
	36% ethanol derived calories 145-155 mg/dl	Liquid	E0–E21	Depressive-like in male (FST) Anxiety-like in male and female (OFT and EPM)	↑ basal CORT (males) ↑ CORT (FS) ↑ ACTH (FS) ↓ HPA return to baseline (FS) ↓ adrenal weight ∆ pituitary sensitivity to CRH and ACTH ↑ HPA activity to dexamethasone ↑ CORT (FS) ∆ feedback mechanisms ↓ CRH mRNA (hypothalamus) (FS)	319,330–335319,330–335
	BAL 273 ± 11mg %	Vapour	E7–E18		↑ CRH following (FS) ↑ ACTH (FS) (females)	327327
	5% v/v – 129.2 mg/dl		E8–E21		↑ CORT (FS)	336336
	4g/kg.d. 87 – 58mM	Gavage	E11–E21		↓ basal CORT ↓ basal ACTH	99

					↑ CORT (FS) ↑ ACTH (FS)	
	6.7% v/v	Liquid	E7 to E21		↑ CORT (FS) ↓ POMC expression Epigenetic changes to POMC ↑ CORT following stressors ↑ ACTH following stressors ↑ CRH following stressors ↑ pituitary weight	71,328,33771,328,337
	6.5% (~14.01g/kg/d)	Liquid	(E0-22/23)	↑ Activity in male and females		324324
	Dams: 4.3g/kg/d 36%EDC Pups: 4g/kg 12%v/v	Liquid	E1-PN10	Depressive-like in male and female (FST) Anxiety-like: male and females (EPM)		322322

Abbreviations: ACTH: adrenocorticotropic hormone; CORT: cortisol (human, sheep), corticosterone (rodent [rat and mice]); CRH: corticotropin-releasing hormone; E: embryonic day; EDC: ethanol derived calories; EPM: Elevated plus maze; FS: the following stress; FST: Forced swim test; HPA: hypothalamic-pituitary-adrenal axis; OFT: open field test; POMC: proopiomelanocortin; Δ: change in.

1.9 Limitations of animal models.

Animal models have provided the opportunity to investigate the structural and molecular changes that occur as a consequence of prenatal alcohol exposure. However, these models are not without limitations when comparing the clinical outcomes. It is important to note that the many animal studies performed have resulted in outcomes that differ depending on factors such as timing of exposure, ethanol dose and duration. While timing and dose are often discussed in-depth, the method of alcohol administration is rarely discussed. Studies administer alcohol in a variety of ways including addition to drinking water, a liquid diet, gavage, vapour or injection. Some modes of administration, in particular oral gavage, have been demonstrated to induce a maternal stress response and cause a significant impact on corticosterone concentration in rats [329]. As such, this may be inducing additional prenatal perturbations of maternal stress, which is associated with several adverse offspring outcomes. For this reason, this thesis will administer alcohol in a liquid diet to reduce stress and mirror human consumption as closely as possible.

The metabolism of alcohol between human and rodents differs, with humans metabolizing alcohol at an approximate rate of 100mg per kilogram per hour and rodents metabolizing at a rate three times greater [62]. It is important to consider this when comparing to those studies performed in humans. However, there is a significant amount of research which indicate the similarities between human and rodent response to alcohol, particularly endocrinology and behavioural outcomes (as discussed in Crabbe et al., [338] and Tabakoff et al., [339]). However, information from animal studies needs to be interpreted with caution as the dose and timing of the exposure, social interaction, housing, genetics and a wide range of factors have significant implication in results obtained from animal studies. Regardless, these models have proven valuable for determining neurochemical and molecular pathways that may be common between species following alcohol exposure.

1.10 Periconceptional alcohol and programming outcomes.

The timing and duration of the maternal perturbation dictate the extent and severity of disease outcomes in offspring. While later in pregnancy, specific organs and systems may be particularly vulnerable to a perturbation, during the periconceptional period these systems are yet to begin development. This period is characterised by numerous cellular events occurring

over a short period, leading to the formation of distinct embryonic and placental cell structures. These processes include meiotic division, maturation and oocyte release from the ovary, conception, and development of the blastocyst leading to implantation [340]. As such, perturbations occurring during the periconceptional period may influence any of these processes and lead to permanent alterations to the entire embryo.

While very few studies have investigated the impact of alcohol exposure around conception on offspring outcomes, numerous studies published in recent years have investigated the impact of other insults around conception on offspring disease. A classic study utilised survivors of the Dutch Winter Famine, where during World War II, a large population in the Netherlands was restricted to a diet of only 400-800 calories per day, from 26 November 1944 to 5 May 1945 [341,342]. This population provided an invaluable tool for research into developmental programming during specific critical windows. It was observed that offspring conceived during these harsh conditions showed a propensity to develop cardiovascular and metabolic disease [343–345], and had an increased risk of mental illness [346,347].

Animal models of periconceptional undernutrition have also observed some systemic alterations in offspring including increased fat mass [348,349], impaired muscle development [350], increased blood pressure [351–353], altered renal structure and function [354,355] and have significant metabolic outcomes such as impaired glucose and insulin homeostasis [352,356]. Likewise, deficiencies in key vitamins such as folate, choline, methionine and B12 during the periconceptional period have been associated with increased body weight, fat mass, insulin resistance, glucose intolerance and elevated blood pressure in offspring [357].

As mentioned above, the impact of periconceptional ethanol exposure (PC:EtOH) is poorly researched, with human studies often compounded by other factors including poor diet, drugs and domestic abuse [358]. However, it has been shown that women who drink 4 or more standard drinks per week within the first trimester of pregnancy had a greater risk of miscarriage, with the strongest association occurring when alcohol consumption occurred prior to ten weeks of gestation [359]. Babies born of mothers who drank up to five standard drinks within the first trimester are observed to have increased rates of cleft lip, cleft pallet [360,361], conotruncal heart deficits [362] and gastrointestinal abnormalities including omphalocele and gastroschisis [363].

However, beyond these studies, very little research has been performed regarding the long-term outcomes of offspring following this common pattern of human drinking in pregnancy.

Animal models have started to investigate specific aspects of offspring physiology following alcohol exposure around conception. Lo *et al.* utilised Rhesus Macaques to investigate the impact of 1.5g/kg per day of self-administered ethanol (4%) during the periconceptional period (preconception to E60 of a gestational time of 168 days) and demonstrated reduced fetal weight, reduced growth and development of the fetal brain and reduced placental blood oxygenation [364]. It must be noted, however, that the length of time of alcohol exposure in this Macaque model includes a significant proportion of pregnancy and would not be representative of women ceasing alcohol consumption immediately at the recognition of pregnancy. Sheep models of intravenous injection of alcohol from embryonic day 4 to 41 results in reduced fetal brain weight. Coll *et al.* demonstrated that maternal exposure to 10% ethanol from 17 days before mating to E10 in mice, decreased embryonic differentiation, inhibited the rate of embryonic development and increased neural tube deficits [365], however, this model again exposed the embryo for a large proportion of pregnancy. Recently, Asimes *et al.* showed that alcohol consumption during five days prior to conception only, altered offspring body weight, with decreased play behaviour and altered hypothalamic-pituitary-gonadal axis function with decreased testosterone and changes to luteinising hormone and gonadotropin-releasing hormone [366]. Within the Moritz laboratory, the rat model used administers 12% v/v ethanol from 4 days before conception to embryonic day 4. This is representative of one oestrous cycle before conception and up until the time of implantation. This model had been shown to induce fetal growth restriction associated with glycogen accumulation in the placenta [367]. When these rats aged, they developed metabolic dysfunction including glucose intolerance and insulin insensitivity [367]. Male offspring had an increased preference of a high-fat diet with decreased dopamine receptor type 1 expression in the ventral tegmental area of the mesolimbic system [368], suggesting that PC:EtOH may also impact neurological pathways. In studies conducted in parallel to those in this thesis, female offspring exposed to PC:EtOH displayed increased anxiety-like behaviour, and spatial memory deficits associated with changes in hippocampal expression of memory-related genes and epigenetic regulatory pathways [369]. Interestingly, many of these offspring outcomes may relate to the HPA. Furthermore, as offspring HPA activity is highly dependent on maternal physiology, it is important to consider just how alcohol

exposure around conception may indirectly program offspring disease by affecting maternal physiology.

1.11 Impact of periconceptional exposures on maternal physiology

The periconceptional timeframe is marked by extensive maternal physiological, metabolic and endocrine changes [340,370], necessary to initiate the cascade of events required for the maintenance of a healthy pregnancy. If insults occur in early pregnancy that can impact how these processes are initiated, then subsequent stages of development can be perturbed leading to a cascade of events that collectively harm the developing embryo. These physiological and endocrine adaptations start even before conception when the previous menstrual cycle is initiated.

1.11.1 Physiological changes in a healthy pregnancy

The increase in estrogen production that occurs as the selected follicle begins to expand plays a major role in regulating when ovulation occurs. Similarly, the hypothalamus and pituitary must respond to this increase in estrogen and produce sufficient luteinizing hormone to stimulate ovulation. Following ovulation, the corpus luteum secretes progesterone to maintain the endometrial lining. This progesterone will decline if conception does not occur. However, if the ovulated oocyte is fertilised, the early embryo secretes hormones such as Human Chorionic Gonadotropin to maintain the corpus luteum and allowing continued production of progesterone and prevention of menstruation (reviewed in Kumar *et al.* [371]). As pregnancy advances, increased production of estrogen and progesterone from the corpus luteum and later by the developing placenta progressively alter maternal hemodynamic and cardiovascular function. Plasma volume increases by 45% and cardiac output increases from 15% within the first trimester to 50% at parturition [372,373]. Additionally, blood pressure decreases, and heart rate increases to ensure appropriate uterine blood flow, placental perfusion and nutrient delivery to the fetus. These changes, however, collectively place an increased strain on kidney function, whereby activation of the renin-angiotensin-aldosterone system (RAAS) occurs, catalysed by the increased concentration of estrogen, inducing a two to three-fold increase in circulating aldosterone concentrations. The elevation in aldosterone will subsequently alter sodium handling, filtration, plasma osmolality and increase erythropoietin production to elevate red

blood cell count, which may have implications for both maternal and fetal cardiovascular function and nutrient provision via the placenta [374,375].

Importantly, many of these processes interact with the HPA. Cortisol concentrations are known to decline during the follicular phase of the menstrual cycle, important for follicular maturation leading up to ovulation and fertilisation. Following conception, cortisol concentrations begin to increase by 2% each day until the 4th week of gestation [376]. These changes in cortisol are attributed to the increased production of estrogen, which stimulates CBG levels and reduces the rate of cortisol clearance [375]. The early rise in cortisol is vital for the allocation of energy to specific tissues ensuring that pregnancy is sustained. However, excessive increases in cortisol during this period may impair rates of conception, implantation, regulation of anti-rejection pathways and maintenance of a successful pregnancy, acting itself as a maternal perturbation. Studies utilising patients undergoing *in vitro* fertilisation have additionally shown reduced fecundability and a 90% increased risk of miscarriage when cortisol levels are perturbed [376,377]. The vast changes associated with the establishment of pregnancy strongly highlights the importance of the HPA around the timing of fertilisation.

Although there are slight fluctuations in cortisol concentration around the fourth week of gestation, cortisol continues to increase by 9% per day up to 42 days post-conception [376]. From this time onwards, cortisol concentrations remain elevated. This sustained increase is essential for the regulation of cardiovascular changes discussed above and adaptations of maternal glucose control and insulin resistance, as well as the final developmental wave of fetal tissues and the onset of parturition [378–380].

1.12 Perturbations that impact maternal physiology

Food restriction, undernutrition, overnutrition and drug exposure have all been observed to result in alterations to the maternal HPA including changes to both plasma glucocorticoid concentrations, ACTH and adrenal pathways [303,351,381–384]. However, many of these studies investigated perturbations that occurred later in pregnancy. Studies which have investigated the impact of periconceptional insults on the maternal HPA have predominantly involved maternal undernutrition. In sheep fed a restricted nutrient diet until 30 days after conception, maternal cortisol was decreased during the undernutrition period [385]. Similarly, sheep fed a diet

that contained 70% of recommended calories until day 7 of gestation, had lower levels of cortisol prior to conception but normal cortisol concentrations at day 55 of pregnancy [382]. In a mouse model of hypoxia, Cuffe *et al.* demonstrated that dams had significantly elevated corticosterone concentrations following a short exposure of 12% oxygen from embryonic day 14.5 to 18.5 in a hypobaric chamber [386]. These studies and the strong association between maternal glucocorticoid exposure and offspring disease highlight that periconceptional exposure to adverse conditions may program offspring disease outcomes by disrupting maternal HPA function.

Limited studies have investigated the effect of alcohol consumption during pregnancy on maternal HPA outcomes. However Weinberg *et al.* determined that alcohol stimulates maternal adrenal activity, with elevated adrenal weights and elevated basal and stress-induced corticosterone concentration [387]. Research performed by Wilcoxon and Schwartz has demonstrated that adrenalectomy of the dam following PAE (5% wt/vol, 35% ethanol derived calories) prevented many of the outcomes caused by maternal alcohol consumption, including reduced placental weight and increased fetal corticosterone at embryonic day 21, as well as hypertrophy of the left ventricle of the heart in adult female offspring [388,389]. These studies indicate the essential role of the maternal HPA in both pregnancy and offspring outcomes and resulted in the question: does PC:EtOH program similar outcomes in offspring through alterations to maternal HPA physiology, thus inflicting a “maternal stress” paradigm.

1.13 Rationale

This chapter has reviewed the evidence that alcohol consumption during pregnancy is associated with some adverse offspring outcomes; the most severe being FAS and FASD, but includes a spectrum of other outcomes including hyperactivity and mental illness, with the suggestion that this is related to dysfunctional neuroendocrine signalling [57]. Animal models used to explore these underpinning mechanisms have shown PAE is associated with offspring HPA hyperactivity, reduced corticosterone normalisation after stressors and changes within the central regulatory pathways [318]. This dysfunction may have significant impacts on offspring outcomes, as the HPA and associated pathways are critical in the homeostatic maintenance of most physiological systems within the body, as well as underlying cognitive and emotional tone.

Furthermore, as similar outcomes have been demonstrated following prenatal exposure to glucocorticoids, the potential role of PC:EtOH on the maternal HPA is also of interest [318].

As dysfunction in the HPA may contribute to the worldwide burden of NCDMI, understanding how prenatal alcohol may influence this axis, is essential. Although the current recommendations are that "for women who are pregnant, or planning a pregnancy, not drinking is the safest option" [41], it has recently been established that that up to 64% of women admit to drinking throughout pregnancy [46]. However, although up to half of these women will cease consumption upon pregnancy detection, little is known of the maternal impacts of ethanol (EtOH) exposure, or if the periconceptional period is a sensitive time to this maternal perturbation. For this reason, the research within this PhD aimed to determine the impact of PC:EtOH on offspring outcomes, including behaviour and function and regulation of the HPA. To complement these studies in offspring, the examination of maternal HPA activity following PC:EtOH was conducted throughout pregnancy.

1.14 Aims

This thesis aims to determine if PC:EtOH exposure results in

1. Altered offspring behaviour and HPA responses to both physiological and psychological stressors, in both adult and aged offspring, associated with changes in genes regulating adrenal steroidogenesis.
2. Altered basal corticosterone concentration in both adult and aged offspring.
3. Altered maternal corticosterone throughout gestation, associated with changes to adrenal steroidogenic pathways.
4. Altered maternal plasma aldosterone and renal physiology parameters.
5. Altered placental glucocorticoid signalling gene expression.

1.15 Hypotheses

It is hypothesised that PC:EtOH will stimulate the maternal HPA throughout gestation, programming a mental illness-like phenotype and HPA dysfunction in offspring.

Specifically, this will be divided into three experimental chapters with the respective hypotheses:

1. *Chapter Three:* It is hypothesised that PC:EtOH will result in a depressive-like and altered social phenotype in offspring, with HPA hyperactivity in response to a dexamethasone suppression/corticotrophin-releasing hormone stimulation test and restraint test, with alterations in underlying adrenal gland steroidogenesis gene expression.
2. *Chapter Four:* It is hypothesised that basal corticosterone concentration will be altered in adult and aged offspring, with HPA hyperactivity following restraint in aged offspring. This will be associated with altered cardiovascular stress response, as well as altered adrenal gland steroidogenesis gene expression.
3. *Chapter Five:* It is hypothesised that PC:EtOH exposure will increase maternal plasma corticosterone concentration throughout gestation, with associated changes in adrenal steroidogenic gene expression. This will be associated with alterations in plasma aldosterone and renal physiology. It is also hypothesised that PC:EtOH will alter gene expression of key glucocorticoid signalling genes in the placenta at the end of gestation.

Chapter Two

General Methods

"Research is formalised curiosity.
It is poking and prying with a purpose"
- Zora Neale Hurston –

This general methods chapter will include a more detailed description of techniques used in multiple chapters but will leave out details relevant only to specific experimental protocols such as sample size and statistical analyses. Behavioural testing, stress responsiveness and metabolic cage analysis will be discussed in relevant chapters.

2.1 Ethics

All animal experimentation performed throughout this thesis were approved by The University of Queensland's Anatomical Bioscience Animal Ethics Committee (SBS/022/12/NHMRC, SBMS/467/14/NHMRC and SBMS/085/17/NHMRC). The code of practice for animal care and use for scientific purposes was followed. Power calculations were performed prior to the commencement of this work to determine the minimal sample size required to achieve meaningful statistical significance.

2.2 PC:EtOH treatment

Sprague Dawley rats (*Rattus norvegicus*) at 12 weeks of age, were ordered from the animal resource centre (ARC, Perth Western Australia), and housed within a 12:12 hour light-dark cycle at 22°C and 60% humidity at the Institute for Bioengineering and Nanotechnology animal facility at the University of Queensland. This cycle commenced at 1200h with lights off, to allow for optimal lighting, breeding and experimental conditions for the rats during standard work hours. Rats were provided standard laboratory chow (4% fat, 13.6% protein, 64.3% carbohydrates; 15.5 MJ/kg, SF-08-020 Specialty Feeds, Glenforrest, Western Australia, Australia) and water *ad libitum* for a minimum of one week prior to experimental protocols. Once female rats reached 230 grams of body weight, they were placed on a liquid control or ethanol diet established using the Lieber-DeCarli nutritionally complete diet [390] and optimised prior to the commencement of this research study [102] for 21 hours, with diet consumption and body weight monitored. The composition of these diets is outlined in Table 2. 1.

Rats were tested for oestrous daily using the EC40 oestrous cycle monitor (Fine Science Tools, Foster City CA, USA), with a vaginal impedance reading of 4.5x $10^3\Omega$ or greater indicating oestrous. Upon this reading, rats were allocated to control or ethanol liquid diets, with this day being denoted as embryonic day (E)-4. These diets were made fresh daily and were placed on

the cage at the commencement of the dark period (1200h). The diet bottle was shaken at 1700h daily to prevent blockages from forming. At 0900h on the following day, the diet bottle was removed, and water was provided *ad libitum* for three hours before replacement with a freshly made liquid diet. This continued for four days or until an oestrous reading of 4.5x $10^3\Omega$ was recorded again. If a rat did not reach this reading by six days, they were removed from the protocol. At this point, female rats were placed in a wire-based floor cage and a male rat introduced to the cage. Successful mating was detected by the presence of seminal plugs either at 1700h, with the following day denoted as E1. If seminal plugs were not present at 1700h, mating continued until 900h, with successful mating resulting in this day being denoted as E1. Dams were returned to their home cages, and diet treatment continued until 4 days post-mating (E5). Dams were weighed daily prior to and throughout pregnancy. Dams were mated for two consecutive days and were removed from the protocol if unsuccessful.

Table 2. 1: The composition of control and ethanol liquid treatment diets.

Ingredient	Control	Ethanol	Supplier
Sustagen hospital formula (g)	19.50	22.00	Mead Johnson Nutritionals, Auckland, New Zealand
Corn flour (g)	15.70	15.00	Coles Supermarket Australia Pty Ltd
Low-fat milk (mL)	58.33	50.00	Dairy Farmers Australia, LD&D Milk Pty Ltd
Ethanol (12.5%, mL)	0.00	10.00	General Laboratory
Selenium (g)	0.03	0.60	Selemite B; Blackmores, New South Wales, Australia
Sunflower oil (mL)	0.83	2.00	Crisco
Copper (II) sulphate (50mM)	0.01	0.01	Sigma Aldrich Inc, Missouri, United States of America
Ferric Citrate (199mM)	0.01	0.01	Sigma Aldrich Inc, Missouri, United States of America
Manganese sulphate (303mM)	0.01	0.01	Sigma Aldrich Inc, Missouri, United States of America

2.3 Experimental cohorts

Six separate cohorts of rats were set up for experiments within this research project to ensure appropriate handling and to eliminate the impact of experimental stress on subsequent

experiments. Due to the labour intensiveness of the protocols, additional students and staff were involved in the treatment of these animals. Of the six cohorts, the first was established for the generation of offspring. Offspring were then divided into three groups with some animals undergoing experiments at 3 months of age, a separate group undergoing experiments at 5 months of age and a final group undergoing experiments at 12-14 months of age.

The subsequent cohorts were established for maternal investigation. One was set up to measure plasma corticosterone at E-2 and E2, before rats were culled, others for tissue collection at E5, E15 and E20. The final cohort was subject to experimental protocols on E16 and E18. The various cohorts of rats used throughout this project are described in detail in Figure 2. 1.

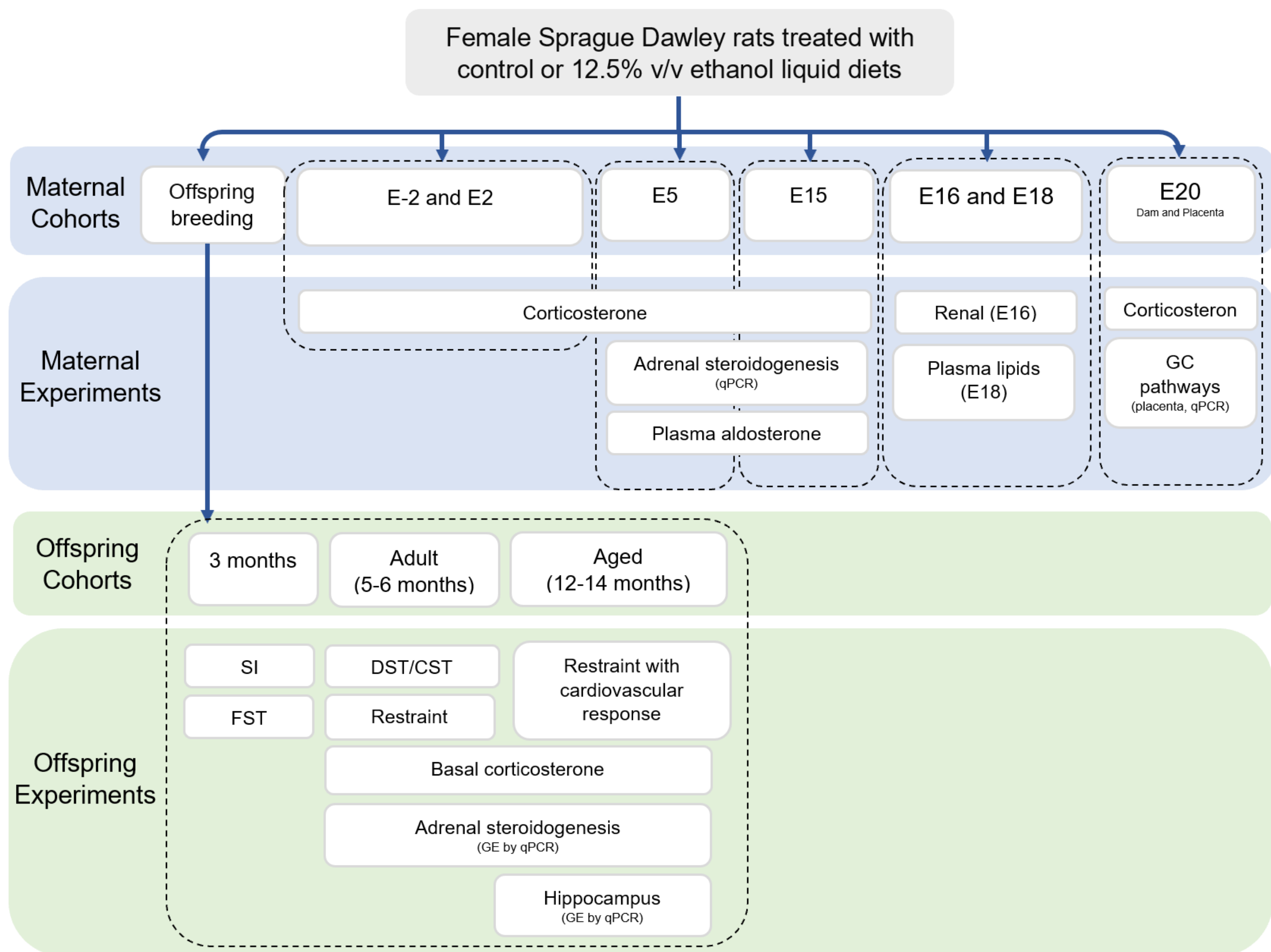

Figure 2. 1: The different cohorts of rats used throughout this thesis for various experimental protocols (within the dotted lines are details about each of the 6 cohorts of animals used). Dams were treated with 12.5% ethanol during the periconceptional period (PC:EtOH). Separate cohorts of dams were established to measure outcomes at embryonic day (E) 2. E2, E5, E15, E16/E18 and E20) for analysis of plasma corticosterone, adrenal steroidogenesis, plasma aldosterone, metabolic and plasma lipid analysis and glucocorticoid (GC) pathways within the placenta. Offspring cohorts were established from another cohort of dams (3, 5-6 and 14-16 months of age). These offspring underwent behavioural testing including the forced swim test (FST) and social interaction test (SI), stress tests including the dexamethasone suppression test (DST) and corticotropin-releasing hormone stimulation test (CST) as well as restraint testing. Basal corticosterone, which refers to corticosterone concentration within animals who were not exposed to stressors, was also measured, as well as gene expression (GE) in the hippocampus.

2.4 Animal handling

As this thesis aimed to investigate the HPA, special care was taken to ensure animals were unstressed and in an enriched environment wherever possible. All dams were housed individually, except when housed with male rats for mating. Dams were acclimatised to artificial lighting for a minimum of two weeks prior to mating. Bodyweight was recorded daily by the team of researchers, rapidly and with utmost care to ensure the lowest possible stress levels. On the day of birth (denoted as post-natal (PN) day 0), neither dams nor offspring were interrupted. However, all were weighed daily from PN1 to PN30. Rats were weaned into sex-specific cages at PN28, with no more than two male and three female rats per cage. After PN30, offspring were handled and weighed weekly, until two weeks before behavioural testing at 3 months of age. At this time, rats were placed into individual cages and handled daily for a minimum of 10 minutes.

Following behavioural testing, rats remained individually housed but returned to handling and weighing weekly until one week prior to the stress reactivity tests, whereby animals were handled again for 10 minutes per day. Aged animals were handled and weighed weekly until experimentation at 14-16 months of age.

2.5 Sampling and tissue collection in maternal cohorts

Prior to the commencement of this PhD, a tail tip blood sample was collected from dams at E-2 and E2. At 1230h, dams from control and PC:EtOH treatment groups were briefly restrained within a fabric sock. A scalpel was used to slice a 3-millimetre section of the tail from the tip, followed by a collection of blood (0.3mL) into two tubes. Each tube was coated with heparin and ethylenediaminetetraacetic acid (EDTA). Blood was centrifuged at 3500rpm for 5 minutes and plasma collected and stored at -80°C for subsequent hormonal analysis.

A separate subset of dams was euthanised at E5 using a guillotine. Maternal trunk blood was collected and stored with heparin or EDTA. Samples were centrifuged at 3500rpm for 5 minutes, and plasma stored at -80°C for analysis of corticosterone and aldosterone. Maternal adrenal glands were collected and snap-frozen in liquid nitrogen and stored at -80° Celsius for subsequent molecular analysis.

Another cohort of rats was generated and handled daily until E15. Dams were transported to a separate facility (approximately 5 minutes in duration). Here, rats were anaesthetised rapidly using an intraperitoneal injection (IP) of 50:50 ketamine and xylazine (0.1ml/100g of body weight, Lyppard Australia Ltd, Queensland, Australia). Once anaesthetised, a midline incision was made along the abdomen and fetuses externalized for tissue collection. Dams were euthanised using cardiac puncture for the rapid collection of blood from which plasma was collected as described above. Maternal adrenal glands were collected and snap-frozen in liquid nitrogen and stored at -80°C for molecular analysis.

The final cohort for blood and tissue collection was generated, and blood was collected at E20 via tail-tip, as described above, from conscious, restrained dams at 0830h. This blood sample was collected prior to transportation, to eliminate the compounding impacts of external stress on corticosterone values. Following the tail bleed, dams were transported to a separate laboratory for tissue collection. Anesthetisation occurred as above, and fetal tissues were collected prior to maternal cardiac puncture and blood collection. Maternal adrenal glands were snap-frozen in liquid nitrogen and stored at -80°C for molecular analysis. All blood samples were centrifuged at 3500rpm for 5 minutes, and plasma was stored at -80°C for subsequent analysis of corticosterone and aldosterone.

It is important to note that all blood collection, excluding collections at E-2 and E2, were performed at 0900h. This is nine hours following lights on (1200h) and was chosen for consistency across animals, as well as for logistical purposes. It would be expected that corticosterone would be close to peak concentration [391]. Blood was collected from dams at E-2 and E2 at 1230h, when blood was simultaneously collected for measurement of blood alcohol concentration [102]. At this time, corticosterone concentrations would at a trough [391]. For these reasons, direct comparisons between corticosterone concentrations at these different time points have not been discussed within this thesis.

2.6 Offspring cohorts

2.6.1 Adult cohort

Three age groups of offspring were established using one male and one female per litter. Offspring were handled and weighed as outlined in Section 2.4 Animal handling. All rats had ad libitum access to standard laboratory chow and water. All female rats were tested in diestrus. One of these groups underwent behavioural testing at 3 months of age. Behavioural testing occurred during the dark phase of the light cycle (1400h – 1600h), when

both plasma corticosterone and activity are at its peak [391,392]. At 5-6 months of age, animals underwent a dexamethasone suppression (DST) and corticotropin-hormone stimulation test (CST). Following a minimum of two days of recovery, these animals underwent a 30-minute restraint test. These tests were performed during the light phase of the light cycle, whereby plasma corticosterone would be at its trough [391]. Tissues were collected from rats at 5 months of age.

In addition to the above ages, the impact of PC:EtOH on aged offspring was also of interest. Although key parameters were measured in both ages, due to the range of experiments and collaborative nature of offspring cohorts, a different range of tests was performed as the focus of experiments were altered to measure as many outcomes as possible. Therefore, the final group was aged to 14-16 months and underwent telemetry surgery for cardiovascular measurements during a 15-minute restraint test. These animals recovered for two days before being culled for tissue collection. Cohorts and experiments are illustrated in Figure 2. 1.

2.6.2 Tissue collection

For all tissue collection, rats were transported to a separate facility for cull between 0900h and 1000h by an IP injection of pentobarbitone sodium (Lethobarb [325.73g/L]; 0.1 ml/kg body weight). Adrenal and pituitary glands were collected and weighed from offspring at all ages. Hypothalamus and hippocampus were dissected from aged (12 months) offspring using coordinates obtained from Paxinos and Franklin [393]. Hippocampal tissue was collected from Bregma −2.28 to −3.64. Likewise, the hypothalamus was collected with coordinates Bregma -3.24 to -4.44. Tissues were snap-frozen in liquid nitrogen and stored at -80°Cfor molecular analysis. Pituitary glands from aged rats were fixed in 4% paraformaldehyde for histological analysis using methodologies similar to those performed previously [286]. Pituitary glands were processed to paraffin and sectioned before staining with hematoxylin and eosin and sent to an expert veterinary pathologist for blinded assessment [286].

2.6.3 Hormone analysis

The corticosterone analysis was performed using an in-house radioimmunoassay as described in Spiers et al [394]. Plasma corticosterone was extracted from 5µL of plasma using dichloromethane (Sigma Aldrich, Castle Hill, NSW, Australia) and reconstituted into assay buffer (porcine gelatin with phosphate-buffered saline). Standards of known concentration

range (39pg/µl to 10000pg/µl) and samples were incubated for 12 hours at 4°C with corticosterone antibody (anti-corticosterone antibody, AB1297, Merck-Millipore) and a tritiated [1,2,6,7-^{3}H] corticosterone tracer. The unbound steroid was removed using centrifugation (1000 x g for 10 minutes at 4°C) with activated dextran charcoal. Radiation readings were collected using a scintillation spectrometer (Tri-Carb 3100 TR, Perkin Elmer). The assay sensitivity ranges from 39ng/ml up to 5000ng/ml based on 5µl of plasma per sample, and all samples were run in triplicate. The intra-assay coefficient of variation and inter-assay coefficient of variation across assays performed were 6.8% and 7.3% respectively. This assay has been shown to have minimal cross-reactivity (<1%) for progesterone, testosterone, cortisol or 11 deoxycorticosterone.

Plasma aldosterone was measured using an enzyme-linked immunosorbent assay (ELISA) (Alpha Diagnostic International Inc, Texas, US) as per the manufacturer's details. Briefly, 50µl of plasma stored in EDTA and standards were combined with 100µl avidin conjugate into a 96 well plate and incubated for 60 minutes. Samples were washed with provided wash buffer, and 150µl of TMB substrate (provided) was added. 50µl of stop solution was used following a 10-minute incubation on a shaker. Absorbency was measured at 450nm using and absorbency scanner (TECAN, Life Sciences, Switzerland). Concentration was determined using a logarithmic line of the best fit curve of standards. The assay sensitivity ranges from 15pg/mL up to 2000pg/ml based on 50µl of plasma per sample. This assay has been shown to have minimal cross-reactivity (1.1%) for 11 deoxycorticosterone, and negligible cross-reactivity with androsterone, cortisone, 11-deoxycortisol, 21-deoxycortisol, dihydrotestosterone, estradiol, estriol, estrone, and testosterone. All samples were assayed on a single plate, in duplicate with an intra-assay coefficient of variation of 3.8%.

2.7 Molecular analysis

2.7.1 RNA extraction

Total mRNA was extracted from adrenal glands, hypothalamus and hippocampus using the RNeasy mini kit column extraction (QIAGEN, Doncaster, Australia) as per the manufacturer's details. Adrenal glands, whole hypothalamus and hippocampal samples were used for extraction. Samples were homogenised using beta-mercaptoethanol and appropriate lysis buffer, followed by required procedural washes. Samples underwent a DNase digestion (10µL) with a 25-minute incubation period. Final washes were performed and samples were eluted in RNase free water. RNA yield was assessed using the Nanodrop

1000 spectrophotometer (Thermo Fisher Scientific, Scoresby Vic, Australia), with the absorbance of A260: A280nm wavelengths. Pure RNA samples were accepted with an optical density reading between 1.8 and 2.1. These samples were used for complementary DNA (cDNA) synthesis.

2.7.2 Reverse transcription for cDNA synthesis

cDNA (10µL) was synthesised using the iScript cDNA synthesis kit (Bio-rad, Gladesville, New South Wales, Australia) as per manufacturers details. 1µg of RNA was combined with 2µL of iScript reverse transcription master-mix and nuclease-free water for a total reaction volume of 10µL. cDNA was synthesised using a thermocycler (StepOne Plus Real-Time PCR System, Applied Biosystems) with the protocol as seen in Table 2. 2. cDNA was diluted to 1:10 in RNase free water for gene expression analysis.

The iScript reverse transcription MasterMix contains an optimal mix of oligodT and random primers to ensure unbiased representation of the 5' and 3' regions of target genes. Based on this information, 18S is an appropriate housekeeper as cDNA was synthesised using random primers.

Table 2. 2: The reaction set-up for cDNA synthesis using the StepOne Plus Real-Time PCR System.

Priming	5 minutes at 25ºC
Reverse Transcription (RT)	30 minutes at 42ºC
RT inactivation	5 minutes at 25ºC

2.7.3 Relative gene expression analysis

Quantitative PCR was performed to investigate gene expression levels. Assays were performed using the RT2 Profiler PCR Array (QIAGEN, Doncaster, Victoria, Australia) for maternal samples, and TaqMan Assay-on-demand primers (Thermo Fisher Scientific, Scoresby, Victoria, Australia) for offspring studies (details to be discussed in relevant chapters), with details of primers in Table 2. 3. Samples were run in duplicate. 96 well plates were utilised and analysed with the Quantstudio6 Flex Real-Time PCR systems (Life Technologies, Carlsbad, CA). Relative gene expression was measured using the comparative cycle of threshold fluorescence ($\Delta\Delta C_T$) method. The cycle threshold (C_T) value of the housekeeper was subtracted from that of the gene, to give the change in C_T (Δ_T).

Samples were normalised to the mean of the control group, which was designated control (control male for the offspring studies) and was labelled $\Delta\Delta C_T$. Relative expression was determined by calculating $2^{-\Delta\Delta CT}$. Gene expression was normalised to the geometric mean of endogenous controls, 18S ribosomal RNA (*Rn18s*), peptidylprolyl isomerase B (*Ppib*) and glucuronidase (*Gusb)* or beta-actin (*Actb*).

2.8 Statistical details

Statistical details will be discussed in each chapter relevant for the data included. All data are presented as the mean ± standard error of the mean and statistical significance was accepted when p-values were significantly less than 0.05. Statistical abbreviations are Ptrt representing P-value for treatment, Psex representing P-value for sex and Pint representing P-value for a treatment x sex interaction. Post hoc analysis significance is denoted as p. P values of * < 0.05, ** < 0.01, *** < 0.001 and **** < 0.0001.

Table 2. 3: The genes investigated within this research study using Taqman AOD probes.

Gene	Gene Name	Assay on demand
HSD11b2	11-beta hydroxysteroid dehydrogenase 2	Rn00492539_m1
Crh	Corticotrophin releasing hormone	Rn01462137_m1
Crh-r1	Corticotrophin releasing hormone receptor 1	Rn00578611_m1
Cyp11a1	Cytochrome P450, Family 11, Subfamily A, Polypeptide 1	Rn00568733_m1
Cyp11b1	Cytochrome P450, Family 11, Subfamily B, Polypeptide 1	Rn00568733_m1
Cyp21a1	Cytochrome P450, Family 21, Subfamily A, Polypeptide 1	Rn00588996_g1
Hsp90a1	Heat shock protein 90	Rn00822023_g1
Mc2r	Melanocortin receptor 2	Rn02082290_s1
Nr3c1	Nuclear receptor subfamily 3, group C, member 1	Rn00561369_m1
Nr3c2	Nuclear receptor subfamily 3, group C, member 2	Rn00565562_m1
Star	Steroidogenic acute regulatory protein	Rn00580695_m1

Chapter Three

Periconceptional ethanol exposure alters behaviour and hypothalamic-pituitary-adrenal axis activity in young adult offspring

"foolish, drunken, or haire-brain women
most often bring forth children
like unto themselves, morose and languid."
- Burton –

3.1 Abstract

Alcohol consumption throughout pregnancy has been associated with mental illness, hyperactivity and social difficulties in offspring. Human and animal studies have demonstrated that this may be due to programmed disruption of the hypothalamic-pituitary-adrenal (HPA) activity and responsiveness. However, it is unknown if the HPA is affected following alcohol exposure during the periconceptional period and if so, whether this results in similar outcomes. This study hypothesised that periconceptional ethanol (PC:EtOH) exposure would alter offspring behaviour, resulting in depressive-like and altered social phenotypes. Additionally, that HPA reactivity and molecular pathways regulating steroidogenesis within the adrenal gland would be altered.

Female Sprague-Dawley rats were treated with PC:EtOH (12.5% v/v EtOH liquid diet) or a control diet from 4 days before conception, until embryonic day 4. At 3 months of age, rats underwent behavioural testing, including the forced swim test (FST) and social interaction test, followed by HPA reactivity tests (combined dexamethasone suppression test [DST] and corticotropin-releasing hormone test [CST], restraint stress) at 5 months of age. Tissues including adrenal glands, liver and pituitary glands were also collected at this age in a separate cohort.

PC:EtOH exposure resulted in a significant increase in immobility ($p < 0.05$) in both male and female offspring in the FST. PC:EtOH also increased the duration of affiliative behaviour ($p < 0.05$) within the social interaction test in female offspring. There was no impact of PC:EtOH on non-affiliative or rearing behaviour in either male or female offspring although males displayed more non-affiliative behaviour than females. When administered dexamethasone, plasma corticosterone suppression was less in both male and female offspring exposed to PC:EtOH ($p < 0.05$). Female but not male offspring exhibited an exaggerated plasma corticosterone response to the corticotropin-releasing hormone (CRH) ($p < 0.05$). There was no impact of PC:EtOH on plasma corticosterone concentration in response to restraint. Adrenal mRNA expression of genes regulating steroidogenesis (melanocortin receptor 2 [*Mc2r*]*,* steroidogenic acute regulatory protein [*Star*]*,* cytochrome p450, family 11, subfamily a, polypeptide 1 [*Cyp11a1*], 3β- hydroxysteroid dehydrogenase [*Hsd3ab*]*,* : cytochrome p450, family 21, subfamily a, polypeptide 1 [*Cyp21a1*], cytochrome p450, family 11, subfamily b, polypeptide 1 [*Cyp11b1*], cytochrome p450, family 11, subfamily b, polypeptide 2 [*Cyp11b2*], 11-beta-hydroxysteroid dehydrogenase 2 [*Hsd11b2*],

glucocorticoid receptor [*Nr3c1*] and heat shock protein 90a1 [*Hsp90a1*]) were not altered by PC:EtOH in the unstressed cohort.

This study supports the hypothesis that PC:EtOH exposure programs sex-specific alterations in HPA responsiveness, which occurred without underlying changes in adrenal gene expression. This HPA hyperresponsiveness may underlie the depressive-like outcomes and altered social behaviours observed in this study following PC:EtOH. However, outcomes suggest that HPA pathways independent of the adrenal gland may be influenced by PC:EtOH, such as within central regulatory pathways, warranting greater investigation.

3.2 Introduction

Prenatal alcohol is associated with adverse cognitive functioning in children and adults [395,396]. These deficits are often observed as language and learning difficulties [395,396], inappropriate social interaction with peers [397,398] and dysfunctional behaviours such as hyperactivity [396,399–402]. However, secondary to these outcomes, mental illness is highly prevalent, with studies revealing that over 90% of persons diagnosed with fetal alcohol spectrum disorder (FASD) report depression, anxiety and mood disorders [395,396]. These disorders, as well as many physiological conditions, are often associated with impaired function of the HPA, in a complex and bi-directional manner, acting as both a risk factor and a consequence of disease. Higher diurnal cortisol concentration and cortisol awakening responses are predictive of poor mental health in children and the onset of depressive and anxiety disorders in adolescence and adulthood [403–409]. Furthermore, patients with depression have increased adrenocorticotropin hormone (ACTH) and cortisol pulses, reflected in total cortisol levels and elevated corticotropin hormone (CRH) in cerebrospinal fluid [410,411].

Within the clinic, the dexamethasone suppression (DST) and CRH stimulating tests (CST) are used to interrogate disorders of the HPA including depression and Cushing's disease [412–415]. Patients suffering from depression resist dexamethasone suppression of cortisol but have elevated responses to the combined dexamethasone suppression and corticotropin-releasing hormone stimulation test (DST/CST). These outcomes correlate with a four-fold increased likelihood for the reoccurrence of depressive symptoms in depressed patients, compared to those who have a normal suppression response, potentially as a consequence of a hyperactive neural response to stressful events [237,412,416–418]. Mouse models of glucocorticoid receptor knock out in the forebrain (FBGRKO) display increased anxiety and depressive-like behaviour, with increased basal and stress-induced corticosterone secretion [419–421]. Importantly, these depressive-like outcomes in FBGRKO mice are reversed with treatment of the anti-depressant imipramine [419].

The HPA is highly susceptible to programming by prenatal alcohol exposure (PAE). Studies demonstrate elevated basal and stress-induced cortisol concentrations in infants following PAE [307,315–317]. Animal models have indicated a range of adverse outcomes following prenatal alcohol exposure, including depressive and anxiety-like phenotype [318,331,422], altered HPA function, including hyper-responsiveness to stressors, negative feedback pathways and gene expression in offspring [321,330,335,423–425]. To date, research has focused

on understanding how alcohol intake throughout the entirety of pregnancy, or specifically during organogenesis and brain development, impairs long-term outcomes. It is yet to be determined if alcohol exposure during the periconceptional period can program altered HPA activity in response to stressors, depressive-like and social behaviour in offspring. Of significant interest, sexual dimorphism is evident in mental illness with three-fold greater prevalence in females than males [426]. Although the factors mediating this increased incidence are not well understood, sexually dimorphic programming of the HPA is likely to contribute. For these reasons, this study hypothesised that PC:EtOH would result in altered depressive-like behaviour and altered social interactions, with impairments in underlying HPA activity in response to physiological and psychological stressors, with different outcomes in male and female offspring.

3.3 Methods

3.3.1 Ethics

All experiments were performed with approval by The University of Queensland's Anatomical Bioscience Animal Ethics Committee (SBS/022/12/NHMRC and SBMS/467/14/NHMRC).

3.3.2 PC:EtOH treatment

Sprague-Dawley rats were housed individually in a 12-hour light-dark cycle (lights on 00:00 h to 12:00 h, light off 12:00 h to 24:00 h) and treated as discussed in General Methods Section 2.2 PC:EtOH treatment. Briefly, Sprague Dawley dams were treated with 12.5% v/v ethanol in a liquid diet (PC:EtOH, sample size = 30 dams), or an isocaloric control liquid diet (Control, sample size = 30 dams) from four days before until four days after mating. Water was provided *ad libitum* for the remaining three hours. Dams were paired with a male rat overnight, and mating was confirmed by the presence of a seminal plug. This day was denoted as embryonic day 1 (E1). On E5, dams were returned to standard laboratory chow and water *ad libitum.* Dams were handled daily and allowed to litter down naturally at E21-22, with the day of birth was designated as postnatal day (PN) 0. Offspring were weighed daily from PN1 to PN30, weaned at PN28 and housed in groups of three female or two males per cage. All offspring were weighed weekly throughout the testing protocol. Two cohorts of rats were used for these studies, with one undergoing behavioural testing, and the other being euthanised for tissue collection. Often, each test was performed once, on

one animal of each sex from each litter. If more than one male or female rat was used from one litter, results were averaged.

3.3.3 Behavioural tests

All rats were housed individually and handled daily for 14 days prior to the commencement of behavioural testing. Testing occurred between two and four hours after lights off (14:00h to 16:00h) within a separate room of the animal facility. All experiments were performed when females were in diestrus. Two days prior to behavioural testing, rats were relocated to the behavioural room within 30 minutes of lights off. Rats were habituated to the room for one hour (12:00 – 13:00h) in the dark and one hour (13:00 – 14:00h) in red light (approximately 20 lux). Rats were returned under dark conditions to the main housing room. Behavioural tests were video recorded using a generic webcam located above the arena. Videos were blinded before manually scored for the appropriate behaviours. All areas were cleaned with 70% ethanol prior to testing of the next rat.

3.3.3.1 Social interaction

The social interaction (SI) test was adapted from Burne *et al.* [427]. All offspring were habituated to the 60cm square arena for 15 minutes on day one. On day two, rats of the same sex, and within 20% body weight were placed into the arena for 15 minutes and movement was electronically recorded. Affiliative, non-affiliative and rearing behaviour was measured manually by an assessor blinded to prenatal treatment. Affiliative behaviour is defined as self-grooming, circling, following and social rest. Non-affiliative behaviour is classified as aggressive behaviour including biting and dragging, as well as rough and tumble play, wrestling and pinning.

3.3.3.2 Forced swim test

The protocol for the forced swim test (FST) was adapted from Porsolt *et al.* [428]. On day one, rats were placed in a 60cm tub filled with water at 25°C for 15 minutes. On day two, water was refreshed, and swimming was digitally recorded. Immobility was defined as anytime the rat was stationary, excluding leg movements which ensured its head was above water and was assessed blinded to prenatal treatment.

3.3.4 HPA reactivity tests

Following behavioural testing, offspring remained in individual housing and were weighed weekly until 5 months of age. At this age, rats were handled daily for seven days. HPA reactivity tests, as outlined below, were performed. These tests were used to investigate how PC:EtOH impacts offspring sensitivity to stressors.

3.3.4.1 Combined dexamethasone suppression and corticotropin-releasing hormone stimulation test

The DST/CST was adapted from work published by Osborn *et al.* [429]. Testing was performed within the home cage to reduce any stress effects. The protocol for this experiment is illustrated in Figure 3. 1A. Six hours after lights on (0600h), 150µL of blood was collected via tail snip into ethylenediaminetetraacetic acid (EDTA) coated capillary tubes. Immediately following an IP injection of dexamethasone (15µg/kg for male and 30µg/kg for female), rats were returned to their home cage for 90 minutes. 150µL of blood was collected, as well as at 120, 150 and 210 minutes after injection. Following the blood collection at 210 minutes, CRH (2µg/100g in 50µl saline) was administered by IV tail vein injection. Blood was collected 20, 40 and 60 minutes following CRH injection. No more than 150µl of whole blood was collected at any each time point. All blood samples were centrifuged at 3500rpm for 5 minutes, and plasma separated within 30 minutes of collection for analysis of plasma corticosterone concentrations. Plasma samples were frozen rapidly and stored at -80°C until analysis.

At the commencement of this experiment, blood glucose readings were taken. At 90 minutes, following dexamethasone injection, another blood glucose reading was recorded. In the rare instance where blood glucose concentration had not increased at 90 minutes after dexamethasone injection, the rats were removed from the protocol. Rats were also removed from protocol if IV injection of CRH was unsuccessful. Sample sizes, including those removed from the protocol, are shown in Table 3.1.

Table 3. 1: The sample sizes for rats who underwent the dexamethasone suppression test (DST) and corticotropin-releasing hormone stimulation test (CST). Rats were removed from both DST and CST protocols if blood glucose had not increased at 90 minutes after dexamethasone injection or if IV injection was unsuccessful. *two rats were used from one litter, and therefore the result was averaged.

		Total	DST		CST	
			Removed	Total	Removed	Total
Male	**Control**	12	3	9	1	8
	PC:EtOH	8	1	7	0	7
Female	**Control**	10	1	8*	2	6*
	PC:EtOH	7	1	5*	0	5*

3.3.4.2 30-minute restraint test

Following a minimum of three days of recovery from the DST/CST rats underwent a 30-minute restraint test. The protocol for this experiment is illustrated in Figure 3.1. A. After approximately 7 hours of light (0700h), 150μL of blood was collected from rats via tail snip into EDTA tubes. The restraint test occurred within the home cage using clear Perspex dividers, holding the rats firmly in place, with a limited ability to rear. One divider ran along the rat's body, holding the animal against the wall, and the other on its rear. The rat had a limited ability to rear, and food and water were withheld. At the end of the 30 minutes, the restraint device was removed, and food and water were returned. At 30, 60 and 90 minutes post-initiation of restraint, 150 μL of blood was collected. All blood samples were centrifuged at 3500rpm for 5 minutes, and plasma separated within 30 minutes of collection. Samples were frozen rapidly on dry ice and stored at -80°C for subsequent plasma corticosterone concentration measurements.

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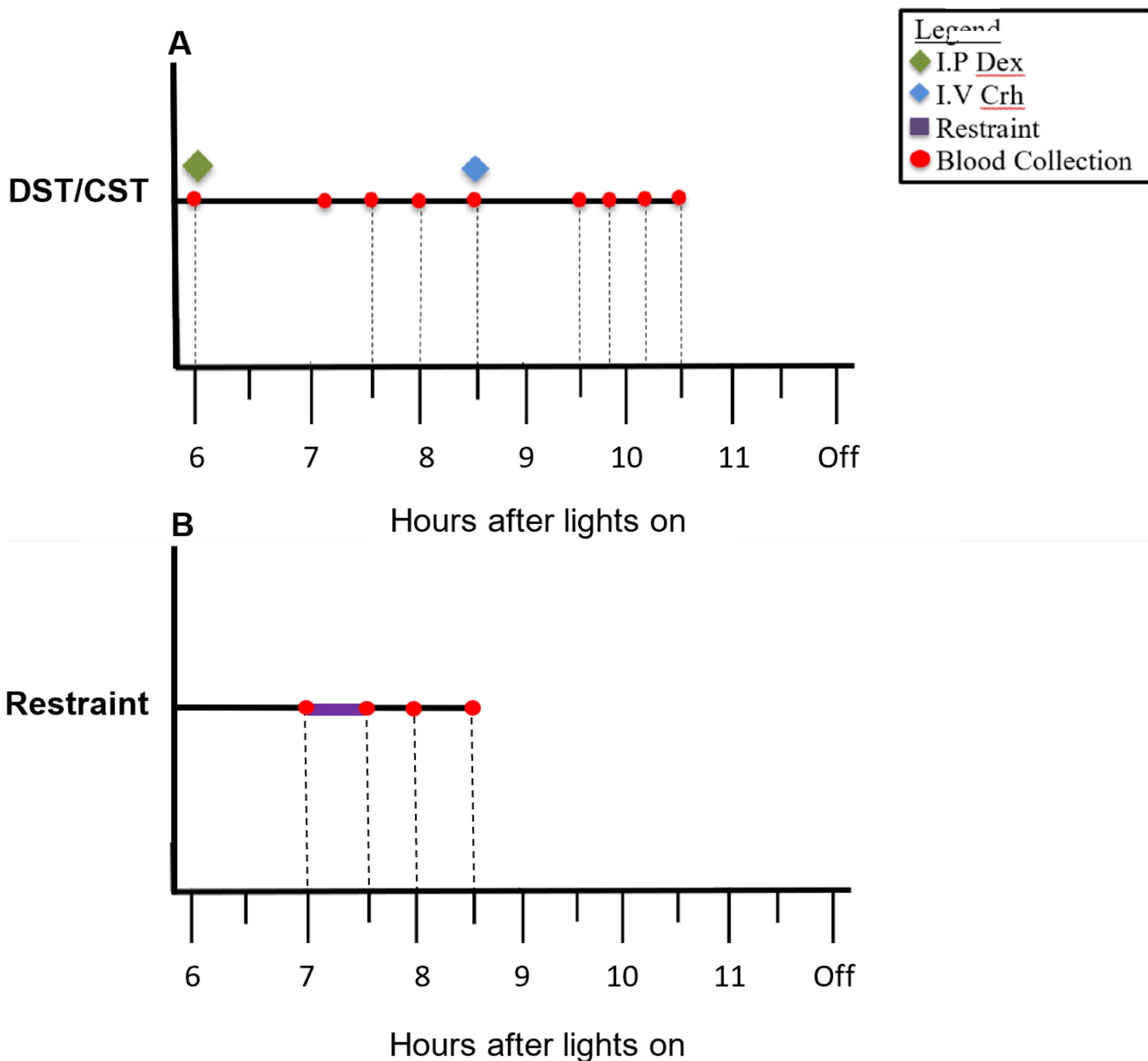

Figure 3. 1: The protocols for DST/CST (A) and restraint test (B), performed at least three days apart, on offspring at 5 months of age who were prenatally exposed to control or PC:EtOH treatment. Colours represent different events within protocols relative to the number of hours after lights on. *Abbreviations*: CRH; corticotropin-releasing hormone, CST; corticotropin-releasing hormone stimulation test, Dex; dexamethasone, DST; dexamethasone suppression test, IP; intraperitoneal injection, IV; intravenous.

3.3.5 Post-mortem and tissue collection

Tissues were collected from rats which did not undergo behavioural testing, at five months of age. Rats were euthanised with sodium pentobarbital (1mL/kg) and the brain, liver, pituitary and adrenal glands collected, weighed and snapped frozen in liquid nitrogen and stored at -80°C for subsequent molecular analysis.

3.3.6 Adrenal steroidogenesis relative gene expression

RNA was isolated from the left adrenal glands of rats in the unstressed cohort, to ensure baseline gene expression was being analysed. RNA was extracted using the RNeasy mini kit (QIAGEN, Doncaster, Australia) and cDNA synthesised using the iScript cDNA synthesis kit (Bio-rad, Gladesville, New South Wales, Australia) as described in Section 2.7.2 Reverse transcription for cDNA synthesis. Quantitative PCR was utilised to investigate the expression of melanocortin receptor (*Mc2r*, Rn02082290_s1), steroidogenic acute protein (*StAr*, Rn00580695_m1), 3β-hydroxysteroid dehydrogenase (*Hsd3a*, Rn01789220_m1), cytochrome p450 family 21 subfamily a polypeptide 1 (*Cyp21a1*, Rn00588996_g1), 11-beta-dehydrogenase isozyme 2 (*Hsd11b2*, Rn00492539_m1), cytochrome P450 family 11 subfamily B polypeptide 1 (*Cyp11b1,* Rn02607234_g1), cytochrome P450 family 11 subfamily polypeptide 2 (*Cyp11b2*, Rn01767818_g1), nuclear receptor subfamily 3 group C member 1 (*Nr3c1*, Rn00561369_m1) and heat shock protein 90 alpha 1 (*Hsp90a1*, Rn00822023_g1). Gene expression was normalised to the geomean of housekeepers *Rn18s* and *Rpl19*. Relative gene expression was measured using the comparative cycle of threshold fluorescence (ΔΔCT) method. The cycle threshold (CT) value of the housekeeper was subtracted from that of the gene, to give the change in CT (ΔCT). Samples were normalised to the mean of the male control group and were labelled ΔΔCT. Relative expression was determined by calculating $2^{-\Delta\Delta CT}$.

3.3.7 Hormonal analysis

An in-house radioimmunoassay was used to analyse plasma corticosterone concentration as previously described in Section 2.6.3 Hormone analysis.

3.3.8 Statistics

All data were presented as the mean ± standard error of the mean and analysed using GraphPad Prism 8 for Windows (GraphPad Software, Inc., San Diego, CA). All repeated measures data were analysed using a repeated measure analysis of variance (ANOVA). All

other data were analysed by two-way ANOVA or a students t-test. Relevant posthoc analysis was performed as necessary. Outcomes of behavioural testing were compared using treatment (trt) and time as factors. Weights at post-mortem were compared using treatment and sex as factors. For analysis of DST/CST and restraint test, Area Under the Curve (AUC) of plasma corticosterone concentration was calculated and analysed separately for sex using a student's t-test. This was deemed necessary as basal corticosterone concentration are significantly different between sexes (females having a two-fold higher basal concentration). Gene expression data was assessed by two –way ANOVA, using treatment and sex as factors. Statistical abbreviations are Ptrt representing P value for treatment, Psex representing P value for sex and Pint representing P value for a treatment x sex interaction. Post hoc analysis significance is denoted as p. P values of * < 0.05, ** < 0.01, *** < 0.001 and **** < 0.0001.

3.4 Results

3.4.1 Body and relative organ weights

PC:EtOH did not significantly alter the bodyweight of male or female offspring at 5 months of age (Table 3. 2), however female offspring were significantly smaller than male offspring ($P_{sex} < 0.0001$, Table 3. 2). At post-mortem, PC:EtOH significantly reduced relative liver weight ($P_{trt} < 0.01$, Table 3. 2), with a post-hoc analysis revealing that this was significant in male offspring ($p < 0.05$, Table 3. 2). Although female offspring had significantly smaller total adrenal, spleen, brain and pituitary weights than male offspring ($P < 0.01$, 0.001, 0.001 and 0.001, respectively), there was no difference in any other relative organ weights in male or female offspring following PC:EtOH.

Table 3. 2: The effect of PC:EtOH on body (gBW) and relative organ weights (mg/gBW) in offspring, at 5 months of age.

Organ	Male		Female		ANOVA		
	Control	PC:EtOH	Control	PC:EtOH	Trt	Sex	Int
Sample Size	8	9	8	8			
Body Weight (g)	632 ± 16	602 ± 14	339 ± 8	340 ± 8	NS	P<.0001	NS
Liver (mg/gBW)	36.96 ± 1.97	32.29 ± 1.09[#]	33.63 ± 1.33	31.92 ± 0.89	P<.01	NS	NS
Spleen (mg/gBW)	1.44 ± 0.08	1.42 ± 0.03	1.61 ± 0.08	1.74 ± 0.12	NS	P<.01	NS
Total Adrenal (mg/gBW)[a]	0.08 ± 0.01^	0.08 ± 0.01^	0.19 ± 0.02	0.20 ± 0.03	NS	P<.0001	NS
Brain (mg/gBW)	3.55 ± 0.09	3.55 ± 0.12	5.79 ± 0.22	5.90 ± 0.15	NS	P<.0001	NS
Pituitary (mg/gBW)[b]	24.98 ± 1.46	25.50 ± 0.85	54.6 ± 2.84	55.9 ± 3.67	NS	P<.0001	NS

Trt: treatment, Int: treatment by sex interaction, NS: non-significant, BW: body weight.

[a]Average of two adrenal glands.

[b]Total pituitary weight, including anterior gland, posterior gland and median eminence.

Bodyweight and relative adrenal weight data presented as mean ± standard error of the mean. Analysed by two-way ANOVA comparing sex and treatment. A Bonferroni posthoc test was performed for post-hoc analysis; #p<0.05.

3.4.2 Depressive-like and social phenotypes at 3 months of age

3.4.2.1 Forced swim test

Immobility in the forced swim test increased over the 5 minutes in both male and female offspring ($P_{time} < 0.001$, Figure 3. 2). In male offspring, PC:EtOH significantly increased immobility ($P_{trt} < 0.05$). The post-hoc analysis demonstrated that immobility was significantly greater at the fourth minute ($P < 0.0001$, Figure 3. 2A). In female offspring, immobility was consistently greater in those exposed to PC:EtOH ($P_{trt} < 0.05$) compared to the control counterparts (Figure 3. 2B).

3.4.2.2 Social interaction

Affiliative, non-affiliative and rearing behaviours were measured by observing social interaction with a novel rat. PC:EtOH resulted in increased affiliative behaviours during the 15-minute social interaction test in female exposed offspring ($P_{trt} < 0.05$, Figure 3. 3B). This behaviour was significantly greater in the 5 to 10 minute period as determined by a Bonferroni post-hoc analysis ($p < 0.01$) when compared to control offspring. PC:EtOH did not significantly affect affiliative behaviour in male offspring. There was no difference in the number of times rearing (Figure 3. 3E and F) in male or female offspring over the 15 minutes time course of the social interaction, regardless of periconceptional treatment. There was also no difference in non-affiliative behaviours (Figure 3. 3E and F) in either male or female offspring of each treatment, with many rats not displaying these behaviours at any time.

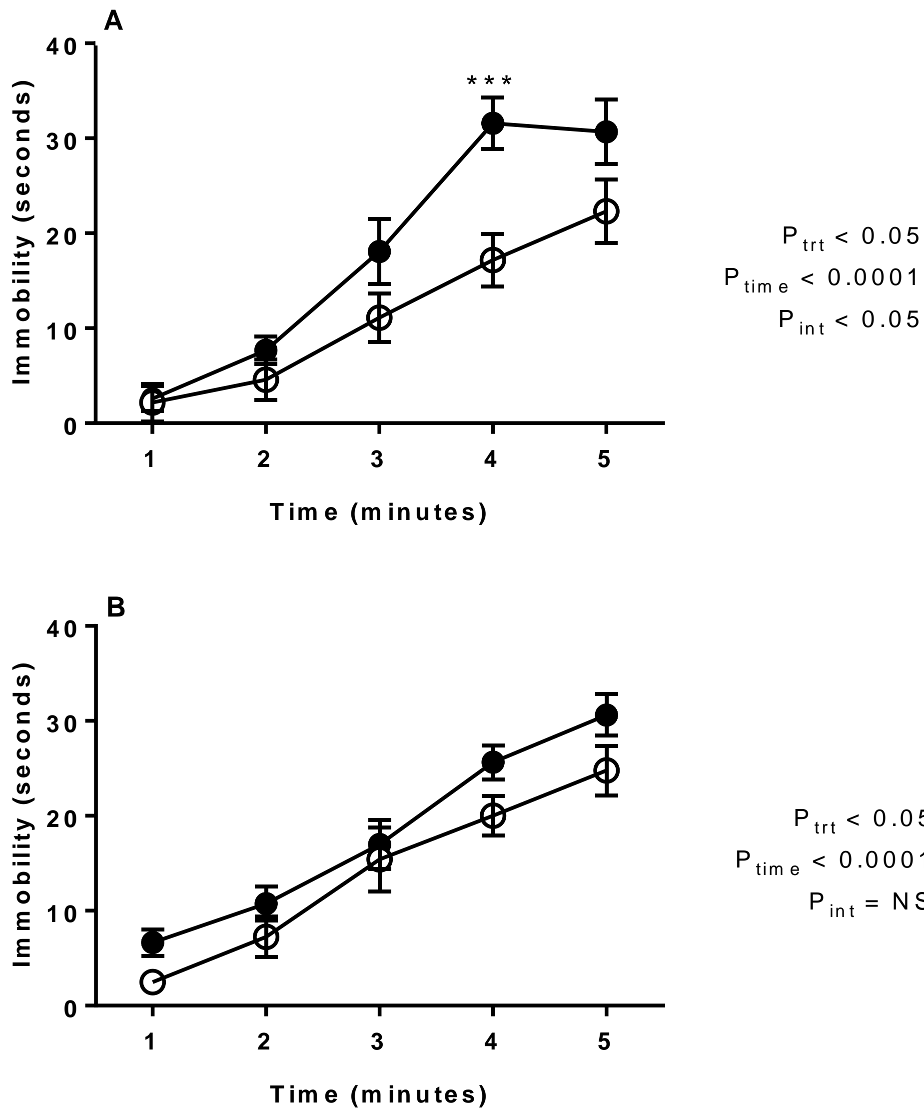

Figure 3. 2: Immobility (seconds) in the forced swim test (FST) over 5 minutes in male (A) and female (B) 3 month old offspring treated with control (white circles) or PC:EtOH (black circles) diet. Data presented as the mean ± standard error of the mean, analysed by a repeated measured two-way analysis of variance and a Bonferroni post hoc analysis, *** p < 0.0001. One male and female was utilised from each litter, or a litter average was taken. Sample size = 12 per sex, per group.

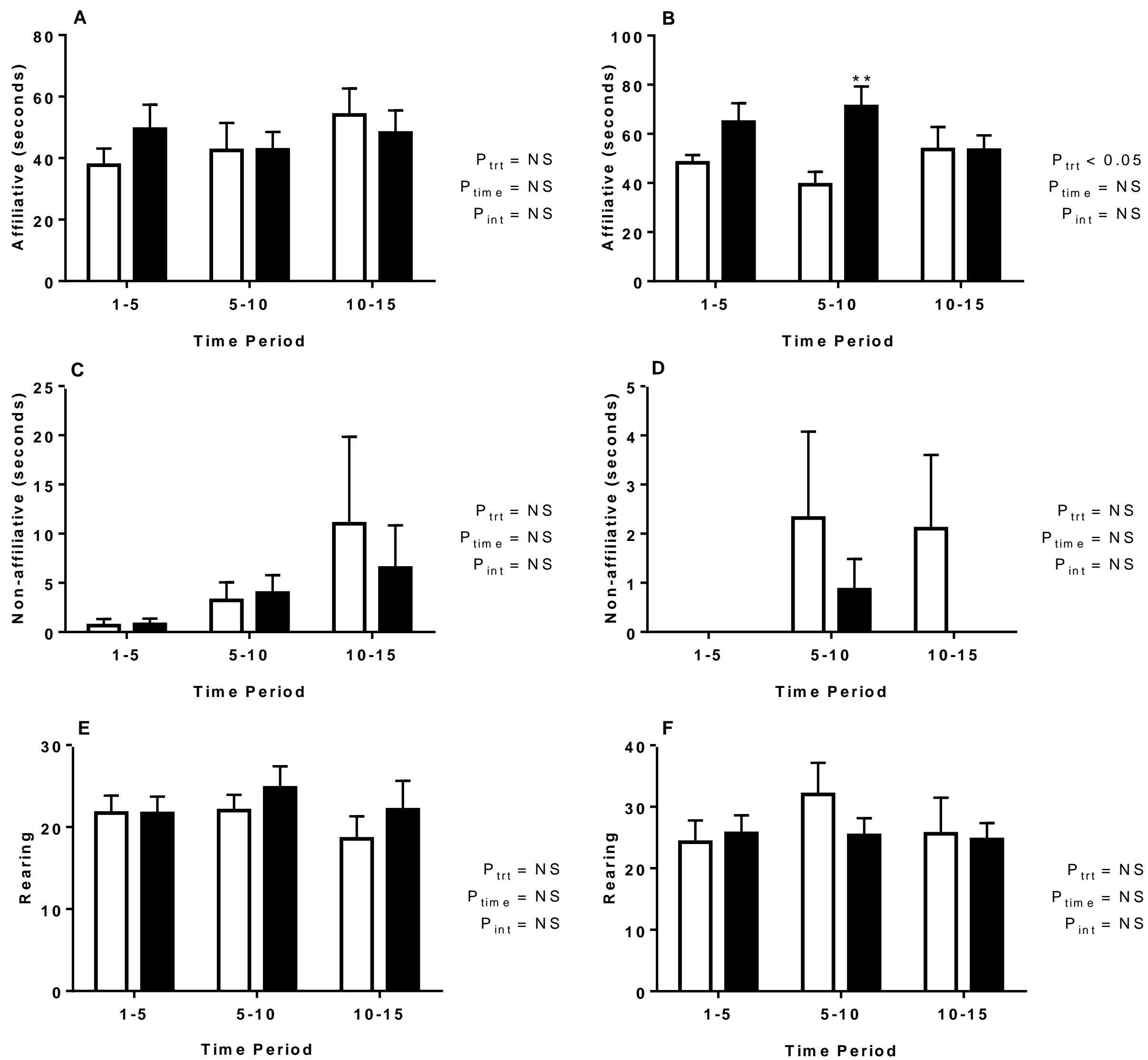

Figure 3. 3: Affiliative (A and B), non-affiliative (C and D) and rearing (E and F) behaviour in male (A, C and E) and female (B, D and F) offspring at three months of age exposed to control (white bars) or PC:EtOH (black) bars diet. Data presented as the mean ± standard error of the mean, analysed by a two-way repeated-measures Analysis of Variance, with a Bonferroni post hoc test. ***P < 0.01. One male and female was utilised from each litter, or a litter average was taken. Samples sizes: male control = 9, male PC:EtOH = 14; female control = 9, female PC:EtOH = 12.

3.4.3 HPA reactivity at 5 months of age

3.4.3.1 DST/CST

Dexamethasone administration reduced plasma corticosterone concentrations in both male and female offspring as expected ($P_{time} < 0.001$, Figure 3. 4 A and B). However rats of both sexes exposed to PC:EtOH had an impaired suppression of plasma corticosterone concentration following the dexamethasone injection compared to control offspring ($P_{trt} < 0.05$, Figure 3. 4A and B). In PC:EtOH females, plasma corticosterone concentrations were significantly greater at time point 2 compared to the control offspring ($P < 0.05$, Figure 3. 4B), indicating a delayed response to dexamethasone. In male PC:EtOH offspring, AUC analysis of plasma corticosterone during the DST revealed increased concentration overall, when compared to control offspring supporting a lack of suppression ($p < 0.01$, C). In female PC:EtOH offspring, the plasma corticosterone AUC was not significantly different ($p = 0.09$, Figure 3. 4D), despite the average being 55% higher than control offspring. This was likely due to the variability in response at time point 2.

Following the CRH administration, the plasma corticosterone concentration increased as expected in both male ($P_{time} < 0.01$, Figure 3. 4A) and female offspring ($P_{time} < 0.05$, Figure 3. 4B). There was no significant impact of PC:EtOH exposure on plasma corticosterone concentrations (Figure 3. 4A) or the AUC (Figure 3. 4E) following CRH administration in male offspring. However, in PC:EtOH exposed female offspring, there was a significantly greater plasma corticosterone response to CST, as indicated by AUC analysis, compared to control ($P < 0.01$, Figure 3. 4F).

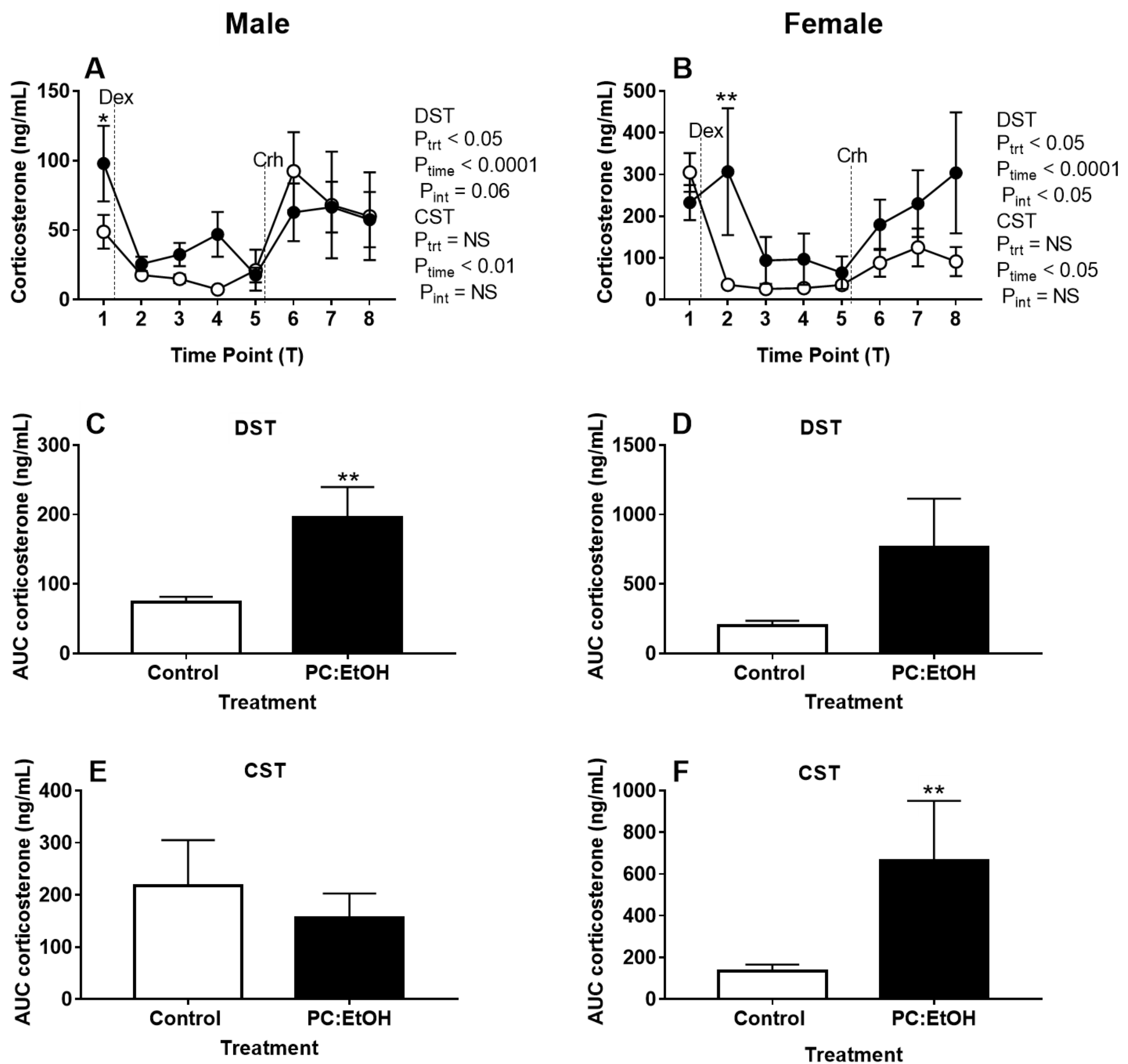

Figure 3. 4: Plasma corticosterone concentration in response to a combined dexamethasone suppression and corticotropin-releasing hormone stimulation test (DST/CST) (A and B) and the area under the curve (AUC) of plasma corticosterone concentration during the DST (C and D) and CST (E and F). Experiments were performed with male (A, C and E) and female (C, D and F) offspring exposed to control (white circles/bars) or PC:EtOH (black circles/bars) diet. Data presented as the mean ± standard error of the mean. A and B are analysed by a two-way repeated-measures analysis of variance, with a Bonferroni post hoc test. *$P < 0.05$, ** $p < 0.01$. C – F is analysed with Mann-Whitney non-parametric t-test, **$P < 0.01$. One male and female was utilised from each litter, or a litter average was taken. Sample sizes for DST: male control = 9, male PC:EtOH = 7, female control = 8, female PC:EtOH = 5. CST: male control = 8, male PC:EtOH = 7, female control = 6, female PC:EtOH = 5.

3.4.3.2 Restraint

Both control and PC:EtOH male and female offspring responded to the restraint test with increased plasma corticosterone (Ptime < 0.0001 and < 0.01 respectively, Figure 3. 5A and B). However, the response was similar in both groups as shown by the comparable plasma corticosterone as analysed by AUC (Figure 3. 5C and D).

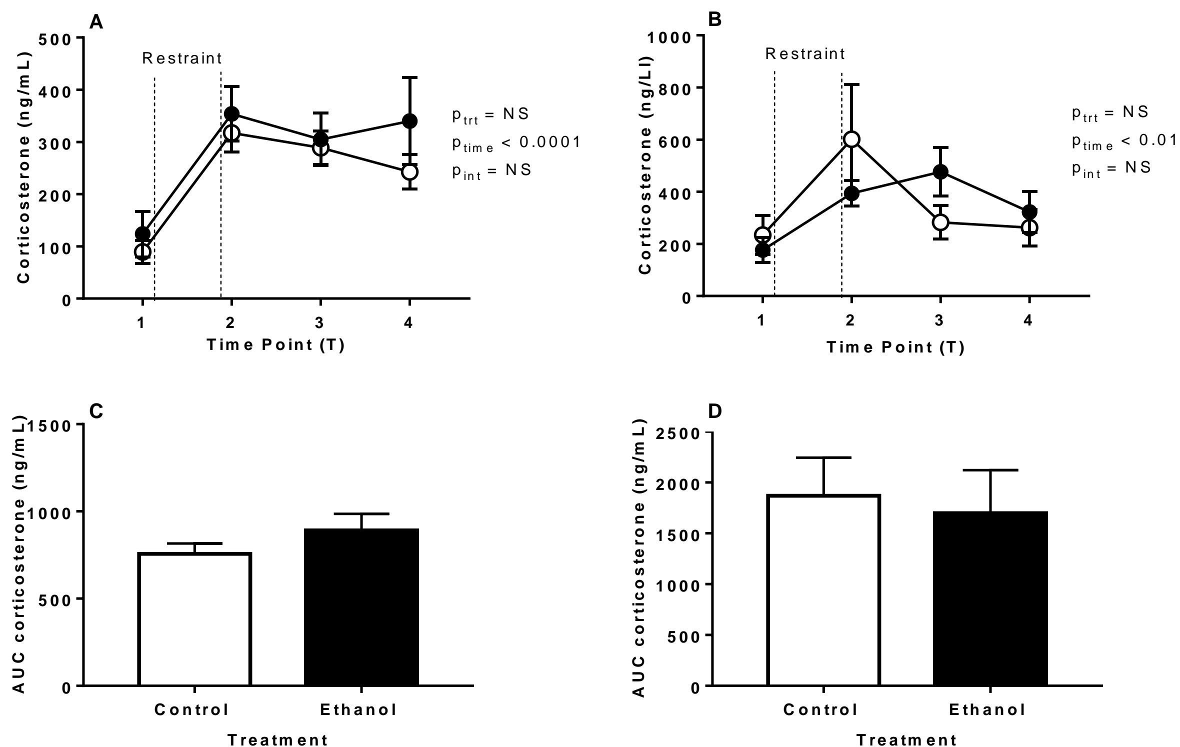

Figure 3. 5: Plasma corticosterone concentration in response to restraint (A and B) and the area under the curve (AUC) of plasma corticosterone concentration (C and D) in male (A and C) and female (B, D) offspring at 5 months of age exposed to control (white circles/bars) or PC:EtOH (black circles/bars) diet. Data presented as the mean ± standard error of the mean, analysed by a two-way repeated-measures analysis of variance (A and B) and Mann-Whitney non-parametric t-test (C and D). One male and female was utilised from each litter, or a litter average was taken. Sample sizes: male control = 9, male PC:EtOH = 7,female control = 8, female PC:EtOH = 5.

3.4.4 Adrenal gland gene expression

PC:EtOH did not significantly alter relative gene expression of *Mc2r*, *Star*, *Cyp11a1*, *Hsd3b1*, *Cyp21a1*, *Cyp11b1*, *Cyp11b2* and *Hsd11b2* in male or female offspring (Figure 3. 6). Sexual dimorphism was observed, with female offspring having significantly lower gene expression of *Mc2r* ($Psex < 0.01$), *Star* ($Psex < 0.01$), *Hsd3ba* ($Psex < 0.01$), *Cyp21a1* ($Psex < 0.05$), *Cyp11b1* ($Psex < 0.01$), *Cyp11b2* ($Psex < 0.01$), and *Hsd11b2* ($Psex < 0.01$) (Figure 3. 6) compared to males. Relative gene expression of glucocorticoid signalling genes *Nr3c1* and *Hsp9a1*, were not different in offspring exposed to PC:EtOH (Figure 3. 7).

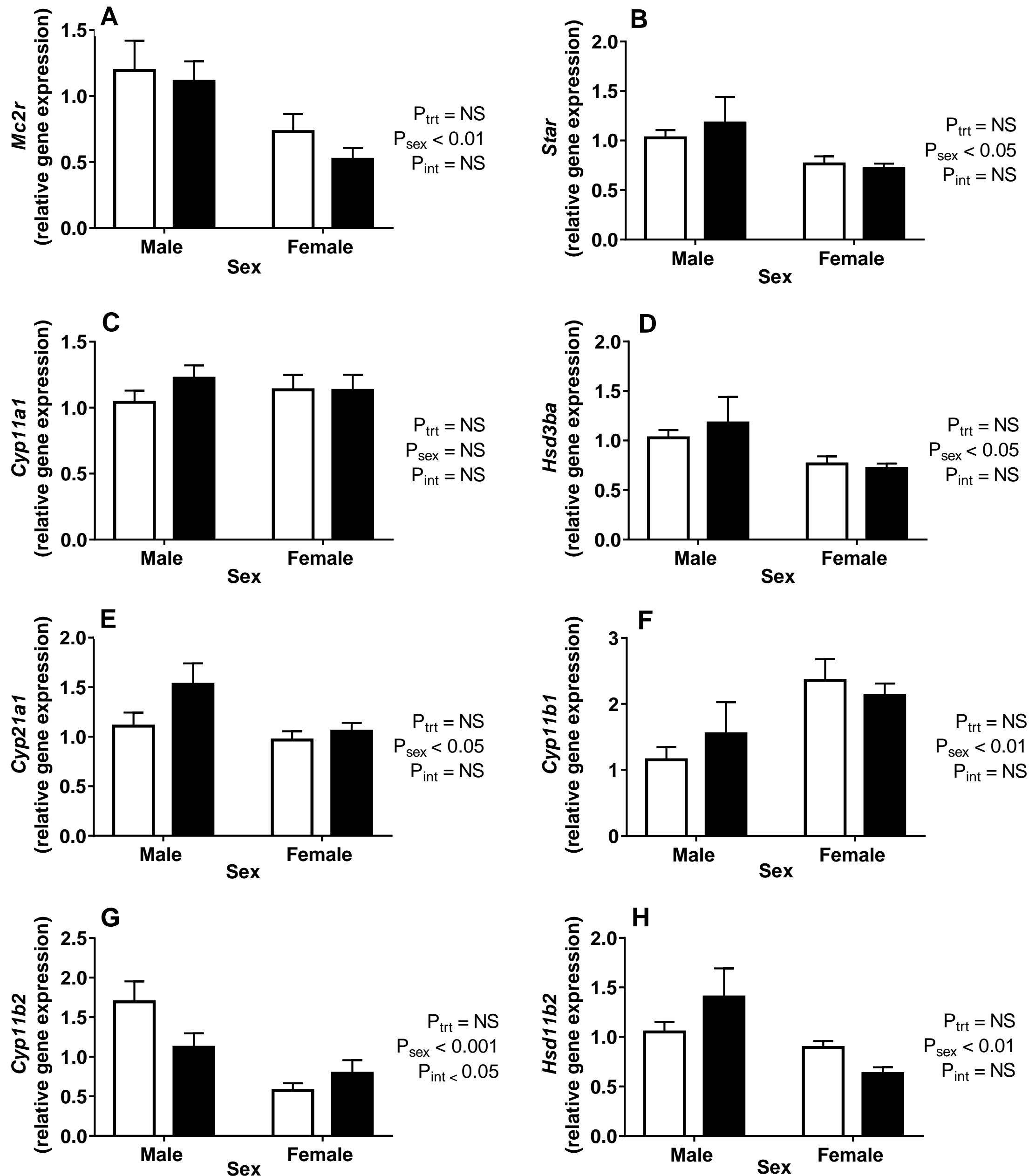

Figure 3. 6: Relative gene expression of melanocortin receptor (*Mc2r*, A), steroidogenic acute protein (*Star*, B), cytochrome P450 family 11 subfamily A member 1 (*Cyp11a*, C), 3β-hydroxysteroid dehydrogenase (*Hsd3a*, D), cytochrome P450 family 21 subfamily A polypeptide 1 (*Cyp21a1*, E), cytochrome P450 family 11 subfamily B polypeptide 1 (*Cyp11b1*, F), cytochrome P450 family 11 subfamily polypeptide 2 (*Cyp11b2*, G) and 11-beta-dehydrogenase isozyme 2 (*Hsd11b2*, H) in the adrenal gland of 5 month old offspring exposed to control (white bars) or PC:EtOH (black bars) diet. Data presented as the mean ± standard error of the mean and analysed by two-way analysis of variance. One male and female was utilised from each litter, sample size = 10 per sex per group.

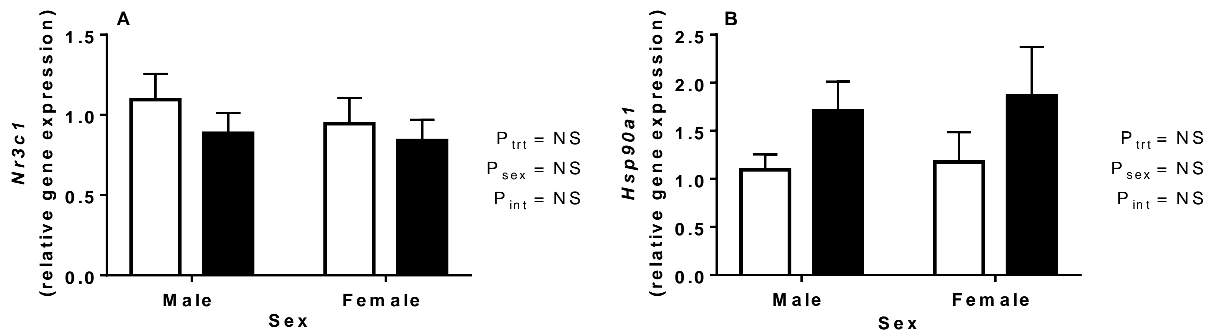

Figure 3. 7: Relative gene expression of nuclear receptor subfamily 3 group C member 1 (*Nr3c1*, A) and heat shock protein 90 alpha 1 (*Hsp90a1*, B) in the adrenal gland of 5 month old offspring exposed to control (white bars) or PC:EtOH (black bars) diet. Data represented as the mean ± standard error of the mean and analysed by two-way analysis of variance. One male and female was utilized from each litter, sample size = 10 per sex per group.

3.5 Discussion

The results of the current study demonstrated that PC:EtOH exposure has a significant impact on behaviour and HPA activity in young adult rat offspring. Both male and female offspring exhibited increased immobility in the FST suggestive of a depressive phenotype, while female offspring displayed increased social interactions with a novel rat. Both male and female offspring exposed to PC:EtOH had reduced sensitivity to dexamethasone treatment, while female offspring had a delayed response to dexamethasone and an exaggerated plasma corticosterone response to the CRH challenge, as determined by an AUC analysis. Interestingly, although plasma corticosterone concentration increased as expected during a 30-minute restraint test, there was no impact of PC:EtOH in either sex nor was there any impact of PC:EtOH on the adrenal expression of *Mc2r, StAr*, *Hsd3ba, Cyp21a1*, *Cyp11b1*, *Cyp11b2* and *Hsd11b2.* Overall, these results suggest that PC:EtOH exposure programs sex-specific alterations in HPA responsiveness, which occurred without underlying changes in adrenal gene expression. This HPA hyperresponsiveness may underlie the depressive-like outcomes and altered social behaviours observed in this study following PC:EtOH. However, outcomes suggest that HPA pathways independent of the adrenal gland may be influenced by PC:EtOH, such as within central regulation from the hippocampus or hypothalamus, warranting further investigation.

3.5.1 PC:EtOH results in a depressive-like phenotype

This study is the first to indicate that alcohol exposure, even prior to implantation and well before brain development begins, increases offspring immobility during the FST in both males and females. Immobility in this test is considered to be representative of a depressive-like phenotype, also known as “behavioural despair” in rodents [428,430–434], a claim supported by studies demonstrating a reversal of the behaviour with the treatment of antidepressants [428,435–440]. The depressive-like phenotype observed within PC:EtOH offspring is consistent with studies of alcohol exposure at other developmental stages. Alcohol exposure from E8 to birth or from E1 to postnatal day 10 resulted in increased immobility in male and female offspring [322,422,441]. However, Hellemans *et al.* administered alcohol to rats throughout the entire of gestation, and exposed offspring to ten days of chronic mild stress, following by the FST. Female offspring displayed increased immobility, whereas males had less immobility than the control counterparts [330].This decreased immobility in male offspring (female were not investigated) was also observed within a study performed by Bilitzke *et al.* who administered alcohol from E7 to 20 [442]. Interestingly, all of these studies administered a

similar dose of alcohol, of around 35-36% ethanol derived calories, and results seem inconsistent regardless of if alcohol was administered throughout the entire of pregnancy, or if administration occurred from mid-gestation. However, it appears that the stress experienced by the rat may be an essential factor in the behavioural response in the FST, and indicates that studies need to closely regulate and report handling, housing and other stressors that may be experienced during protocols. Within this thesis, although rats were handled with the utmost care, this is an important variable to consider. Additionally, as the HPA is critical in the detection and interpretation of stressors, this further supports the critical role the HPA has in behavioural outcomes, and the susceptibility this axis has to the programming effects of PAE.

While the FST is routinely used to indicate depressive-like behaviour, Nelson *et al.* has suggested that immobility may be interpreted as a sign of elevated anxiety [443]. Indeed, within our laboratory, PC:EtOH has been shown also to program anxiety-like behaviours as determined by Elevated Plus Maze tests and Hole-Board tests, within the same cohort of rats utilised in this thesis. Importantly, these recently published results display anxiety-like behaviour in adult female offspring at both 6 and 18 months of age, while male offspring only showed anxiety-like behaviour at 18 months of age [369]. Therefore, we suggest that young female offspring display symptoms of both depressive and anxiety-like phenotypes, with males potentially having a later onset of disease. These results reflect findings from clinical investigations in which 70-90% of adults exposed to fetal alcohol are diagnosed with psychiatric conditions including depression and anxiety [444,445]. Furthermore, 29% of the population suffers from comorbid depression and anxiety [446,447] and the prevalence is three times more common in women than men [35]. A study by O'Connor *et al.* demonstrated that 19% of PAE children reported depressive symptoms, compared to 0% of non-exposed controls [448]. Another small clinical study from this group established that of 23 children prenatally exposed to alcohol, 87% were diagnosed with psychiatric illness, and 61% of these were a mood disorder such as major depressive disorder [449]. Therefore, the results from the current study support the clinical presentation of psychiatric conditions in the general population and highlight that exposure even prior to implantation can have long term mental illness sequelae.

3.5.1.1 Sex differences in the depressive-like phenotype

The sexual dimorphism observed in behavioural outcomes associated with PAE may occur as a consequence of various treatment dosages, timing and stress protocols. It is interesting

that sexual dimorphism was not observed during the FST following PC:EtOH, however, male offspring do reach peak immobility sooner than females, as seen during the fourth minute of the test. These results may suggest altered neurological interpretation and signalling between the sexes, such as within the hypothalamic-pituitary-gonadal axis (HPG) and associated hormones. 17β-estradiol for example, can have significant effects on mood by interacting with neurotransmitter pathways associated with depression [450]. Interestingly, it has been determined by Dorey *et al.* (unpublished data) that 17β-estradiol is elevated in female offspring following exposure to PC:EtOH, supporting this hypothesis. Although the HPG is not investigated within this thesis, it is a critical progression of these studies to elucidate if this underlying pathway may contribute to the observed phenotype.

While these studies did not focus on gender as a major factor, others have begun to elucidate sex differences in the long-term outcomes of PAE. Sayel *et al.* investigated sex-specific mental illness in children following low-level alcohol exposure during the first 18 weeks of pregnancy, demonstrating that female offspring only had greater levels of mental health deficits [451].

3.5.2 PC:EtOH results in altered social interaction

In addition to the impact of depression and anxiety on quality of life for PAE offspring, altered social behaviours are also commonly reported, making it challenging for these individuals to integrate well with other members of society. Indeed, PAE individuals are often described as inappropriately friendly and hyperactive [452–455]. Results obtained from the current study suggest that PC:EtOH can induce similar outcomes in rats, with greater affiliative behaviour during the social interaction test being displayed in female offspring. We suggest that this is indicative of inappropriately friendly behaviour. In support of these findings, PAE exposure (35% calories derived from ethanol, from E6 to E20) in a rat model, has been shown to increase social interaction in females rats more than male rats [456]. Similarly, a study by Hamilton *et al.* demonstrated increased social interaction in female but not male offspring following treatment with 5% v/v ethanol throughout gestation [457]. The increased social interaction in the current study may also indicate hyperactivity. This is particularly true in children with hyperactivity being the most prevalent behavioural symptom following PAE [402,458]. Likewise, we have determined that female PC:EtOH offspring have increased locomotor activity (Wing et al, *unpublished data*).

In contrast, some studies have shown that PAE impairs social interaction, but results in hyperactivity particularly in males. Mooney *et al.* demonstrated that after treatment with 20%

v/v alcohol on E7 only, male offspring had greater social interaction [459]. Similarly, Hellemans *et al.* revealed that a high dose of alcohol from E7 to E21 resulted in hyperactivity in both sexes but reduced social interaction in males [330]. In clinical studies, sex differences within outcomes are rarely discussed, however of those that do, varied results are often reported. Some studies demonstrate that PAE exposed female individuals suffer greater social difficulties and hyperactivity than males [399,460], with others demonstrating converse results [461]. In human studies, increased internalising problems were observed in both male and female offspring when heavy exposure only occurred in the first and last trimesters of pregnancy, with 10% of individuals displaying altered social behaviour at eight years. Overall, these results likely suggest that both sexes are impacted by PAE, but with altered severity of the phenotype depending on the timing and dose of alcohol exposure.

One caveat to the current study is that only a limited number of behavioural tests were performed, and the behavioural tests used in the current study are open to interpretation,. Therefore, the results of the current chapter must be treated with caution. For instance, immobility in the FST has been suggested to be an advantageous learnt behaviour performed in an attempt to reduce energy expenditure during a stressor rather than an indicator of despair (as reviewed in Molendijk & de Kloet. [462]). This article based its conclusion on data from a range of publications which investigated the involvement of the sympathetic nervous system, the HPA and neurotransmitter pathways in behavioural outcomes. It was suggested that increased immobility is an enhanced ability for acquisition, consolidation and retention of danger, resulting in increased immobility for survival. Similarly, alternative interpretations of the social interaction test suggest that this may also be an advantageous behaviour, rather than an anxious and/or hyperactive state. Indeed, some rat studies have demonstrated that social interaction increases in a state of lower cortisol [463] and decreases in the case of reported stress [464,465], where elevated cortisol would be expected. It would be of benefit to perform a greater range of behavioural testing related to these phenotypes to support the conclusion that PC:EtOH induces depressive-like and social interaction alterations.

3.5.3 PC:EtOH programs altered HPA activity: as a cause of altered offspring behaviour?

The phenotypes presented in this chapter may occur as a consequence of altered HPA activity. Patients with Cushing's disease and those treated with glucocorticoids for a variety of reasons often suffer depressive symptoms [466–470]. Additionally, HPA dysregulation is

shown in both human and animal models of altered social interaction [471,472], with a strong association between mental illness and stressful life experiences [473]. Furthermore, the DST/CST is used in the diagnosis of depressive disorders, with depressed patients failing to respond to cortisol suppression following dexamethasone treatment, and demonstrate increased cortisol concentration following CRH treatment [237,413,474–478]. In the current study, both male and female PC:EtOH offspring had significantly reduced plasma corticosterone suppression in the DST test, and female offspring had an increased plasma corticosterone response to CST. In another study, Osborn *et al.* demonstrated that male and female offspring exposed to PAE (35% ethanol derived calories, E0 to E21) both had greater plasma corticosterone concentrations following the DST and an increased response to CST [332,429]. Similarly, Coe *et al.* demonstrated that prenatal stress exposure, in a Rhesus Monkey model, resulted in reduced suppression during the DST, with other HPA related changes including altered behaviour and elevated basal cortisol levels[479]. It is known that dexamethasone binds with high affinity to the glucocorticoid receptor, predominantly in the anterior pituitary gland, to suppress ACTH and inhibit feedback pathways, ultimately inhibiting glucocorticoid production [413,480]. CRH stimulates these pathways [192,481] and is often administered to investigate the secretory function of pituitary corticotropes [482]. The results from the current study suggest that PC:EtOH exposed offspring would have elevated ACTH levels due to a loss of sensitivity to dexamethasone. It would be expected that this would similarly be elevated following the CRH challenge. This hypothesis is supported by the aforementioned studies performed by Osborn *et al.* which revealed similar corticosterone results as in this study, with elevated ACTH response [332,429]. Previous studies investigating the effect of prenatal alcohol exposure on the regulation of the ACTH precursor, proopiomelanocortin, have shown increased mRNA levels in male rats [483] and epigenetic dysregulation [484]. Therefore, it would be of value to measure ACTH concentrations in this study, however, due to time restraints and small sample volume ACTH analysis was not possible.

In addition to the effects on pituitary and hypothalamic suppression, dexamethasone suppression of the HPA can occur through inputs from neural tissues including various regions of the limbic system [485]. While the role of PC:EtOH on limbic regulation of the HPA is unclear, prenatal alcohol is well documented to induce multiple deficits in limbic structures in humans [486,487]. The limbic system is also critical in behavioural outcomes, suggesting that the effects of PC:EtOH as seen in this study, may also be as a result of programming within this central system. Indeed, models utilising rats, mice and rhesis monkeys have

demonstrated that prenatal alcohol exposure results in altered limbic system signalling and structure [468,488–490]. The fact that PC:EtOH had no impact on adrenal regulators of steroidogenesis further supports the hypothesis that the effect of PC:EtOH may be occurring centrally, such as within the higher levels of the HPA, or in the limbic system, which is investigated in Chapter Four of this thesis.

Despite the differences in the DST, PC:EtOH had no impact on the reactivity of the HPA to the ‘psychological’ stressor, physical restraint. Many animal studies investigating PAE throughout pregnancy have demonstrated that although basal corticosterone is often normal, HPA activity in response to prolonged or acute stress is elevated in offspring [424,425,491,492]. Infants exposed to alcohol had increased cortisol responsivity to a “still face” protocol, and at two and six months of age, children had greater cortisol responses to vaccination stress [307,316]. However, Jacobson *et al.* revealed elevated basal cortisol levels in PAE infants, but no change in cortisol to vaccination stress [317]. Interestingly, animal studies have shown that lesions in the hippocampus, glucocorticoid receptor deletion and glucocorticoid receptor inactivation all diminish HPA feedback efficacy to psychological stress, but not physiological stressors [235,493]. Stress can be classified into psychological (also known as processive), defined as those that are perceived as a danger to the organism, or physiological (also known as homeostatic), defined as those that pose a threat to the organism’s survival. Psychological stressors are detected by the limbic system, before stimulating the HPA. Physiological stressors on the other hand, are of immediate attention and act on the HPA directly [494]. It may be hypothesised, therefore, that the DST/CST and restraint may be centrally processed and regulated differently, posing an explanation for the unexpected discrepancy in results observed in this chapter. An alternative explanation may relate to the threshold of sensitivity of the HPA with the positive and negative feedback pathways being more strongly affected than responsiveness to stress. Again, this may be as a result of PC:EtOH induced alterations within alternate regions of the HPA; the hypothalamus and pituitary gland, or the hippocampus, which is highly susceptible to the programming effects of prenatal alcohol exposure [69,487,495,496].

3.5.4 Conclusion

In summary, this is the first study to establish that PC:EtOH has a significant impact on depressive-like and social interaction phenotypes, with underlying dysfunction relating to suppression but not stimulation of the HPA. A lack of altered adrenal steroidogenic gene expression suggests that PC:EtOH exposure may result in underlying changes within other

levels of the HPA or within the limbic system, primarily the hippocampus. These changes may be mechanistic in the development of altered behavioural phenotypes observed and leads to the necessity of investigating these pathways. As many individuals inadvertently consume alcohol prior to pregnancy recognition, this study has significant implications relating to the high prevalence of mental illness in society. Furthermore, HPA dysfunction and dysregulation is associated with many diseases, including metabolic and cardiovascular [446,497,498], lending to the necessity to further characterise the effects and mechanism of PC:EtOH exposure in offspring.

Chapter Four

Periconceptional ethanol exposure alters the stress axis in adult female but not male rat offspring.

"Parents' Drinking Weakens Children's Vitality"

- World's and National Woman's Christian Temperance Union, 1914 -

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This chapter is presented adhering to guidelines requested by the publishing journal.

4.1 Abstract

Ethanol consumption during pregnancy is associated with altered offspring hypothalamic-pituitary-adrenal axis (HPA) regulation, however little is known about the outcomes of alcohol consumption confined to the periconceptional period. This study investigated periconceptional ethanol (PC:EtOH) exposure on corticosterone concentrations, response to restraint stress and gene expression of adrenal, hypothalamic and hippocampal glucocorticoid-related pathways in rat offspring. Female Sprague-Dawley rats were treated with PC:EtOH (12.5% v/v EtOH liquid diet) or a control diet from 4 days before conception, until embryonic day 4. At 6 (adult) and 12-14 (aged) months of age, basal corticosterone concentrations were measured, while in a separate cohort of aged rats, blood pressure, heart rate and plasma corticosterone concentrations were measured during a 30-minute restraint stress. Adrenal gland, hypothalamic and hippocampal tissue from aged rats were subjected to transcriptomic analysis. PC:EtOH exposure reduced basal plasma corticosterone concentrations in adult and aged female but not male offspring ($p<0.05$). The corticosterone and pressor response was significantly reduced in aged PC:EtOH female offspring following restraint ($p < 0.05$). Adrenal steroidogenesis genes (melanocortin receptor 2 [*Mc2r*], cytochrome p450, family 11, subfamily a, polypeptide 1 [*Cyp11a1*], cytochrome p450, family 21, subfamily a, polypeptide 1 [*Cyp21a1*], 11-beta-hydroxysteroid dehydrogenase 2 [*Hsd11b2*] and glucocorticoid receptor [*Nr3c1*]) and hypothalamic genes (Corticotropin-releasing hormone [*Crh*], Corticotropin-releasing hormone receptor 1 [*Crh-r1*], glucocorticoid receptor [*Nr3c1*] and heat shock protein 90a1 [*Hsp90a1*]) were not affected by PC:EtOH. In aged female offspring exposed to PC:EtOH, adrenal mRNA expression of *Hsp90a1* was significantly elevated, and within the hippocampus, relative gene expression of *Nr3c1* and *Hsp901a1* were increased ($p < 0.05$). This study supports the hypothesis that prenatal alcohol exposure programs sex-specific alterations in the HPA and provides the first evidence that the periconceptional period is a critical window for programming of this axis.

4.2 Introduction

Alcohol consumption is a worldwide concern, contributing to the top 5 risk factors for disease, disability and death [499]. Regardless, up to 50% of women drink at some point during pregnancy [46], despite warnings that consumption may increase the risk of fetal alcohol spectrum disorders (FASD) and increased offspring risk of neurological dysfunction including changes in learning, behaviour, reward pathways and mental illness [453,500].

Alcohol exposure during pregnancy has been associated with alterations in the hypothalamic-pituitary-adrenal axis (HPA) of offspring in both human and rodent studies. Alcohol intake throughout pregnancy increased basal and post-stress cortisol concentrations in children [501]. Similarly, increased cortisol reactivity and elevated heart rate (HR) have all been demonstrated in children following prenatal alcohol exposure [307,316,502], suggesting that prenatal alcohol exposure may alter endocrine pathways and related physiological stress responses [503,504]. Rat models have established similar outcomes with alcohol exposure from mid-gestation until embryonic day 20 (E20), increasing offspring basal plasma corticosterone concentrations by 90% and altering the expression of adrenal steroidogenic enzymes [505]. Prenatal alcohol exposure throughout pregnancy also resulted in offspring with elevated plasma corticosterone concentrations [330,334]. Other studies have demonstrated prenatal alcohol consumption does not alter basal corticosterone concentrations but greater plasma corticosterone concentrations following a challenge, including a delayed return to baseline in the alcohol-exposed group [323,332,335,429,506]. This may be due in part to alterations in the hypothalamus or pituitary gland as prenatal alcohol exposure alters basal hypothalamic mRNA expression of the glucocorticoid receptor (*Nr3c1*, GR) and corticotropin-releasing hormone (CRH) [321,507]. Long-term regulation of HPA function may also be driven by alterations to the hippocampus, which plays a major role in regulating the HPA response to stress. Previous studies support this, as prenatal alcohol exposure also alters HPA negative feedback pathways in the hippocampus via dysregulation of the glucocorticoid receptor [496,508].

Recent statistics suggest that most women cease or decrease alcohol consumption once they are aware of their pregnancy. However, 20% admit to episodes of binge drinking before pregnancy detection [46]. Currently, there are few studies which investigate the long-term outcomes of alcohol consumption around the time of conception on the HPA. One clinical study found that alcohol consumption before pregnancy recognition increased infant cortisol concentrations and heart rate in a sex-specific manner, but alcohol consumption after

pregnancy recognition had no effect [316]. Our study, therefore, aimed to investigate the effects of PC:EtOH exposure on the function and regulation of the HPA in rat offspring. We have demonstrated PC:EtOH exposure results in long-term neurological and metabolic disorders in offspring [367,368,509] and is currently investigating a potential role of the HPA in this dysfunction. We hypothesised that PC:EtOH exposure would disrupt the regulation of corticosterone production in offspring under both basal and stressed conditions due to dysregulated adrenal steroidogenesis or negative feedback through the hippocampus.

4.3 Methods

All rat experiments and procedures were approved by The University of Queensland Anatomical Bioscience Animal Ethics Committee and performed according to the Guidelines from the National Health and Medical Research Council of Australia.

4.3.1 PC:EtOH treatment

Rats were obtained from the animal resource centre (ARC, Perth Western Australia). Sprague-Dawley rats were housed individually in a 12-hour light-dark cycle (lights on 00:00h to 12:00h, off 12:00h to 24:00h) and treated as previously described [367]. Briefly, rats were tested for stage of oestrous using the EC40 oestrous cycle monitor (Fine Science Tools, Foster City CA, USA). Upon a reading of 4.0 x 103 Ω or higher, indicating oestrous, rats were randomly allocated to either a liquid diet containing alcohol (PC:EtOH [12.5% v/v ethanol, n =19 dams]) or an isocaloric diet containing no alcohol (control). This day was denoted as embryonic day (E) -4. After 4 days of being on the diet, female rats were paired with a male rat overnight and the day of pairing was designated E0. Mating was confirmed the following day by the presence of seminal plugs, and this day was denoted as E1. The liquid diet continued until embryonic day 4 (E4) and was offered for 21 hours each day. Water was provided *ad libitum* for the remaining 3 hours. On E5, rats were placed back on a standard chow diet and delivered naturally on approximately day 22. The day of birth was designated as postnatal (PN) day 0. Offspring were weaned at day 28. Littermates of the same sex were housed together after weaning, with two male or two to three female offspring per cage. No animals were housed alone. Rats were handled weekly. At six months of age (adult), a tail vein blood sample was collected from conscious, restrained rats. At 1030h, rats from a subset of control and PC:EtOH groups (1 rat/sex/litter) were briefly restrained within a fabric sock for a 3mm tail tip slice and collection of 0.3mL of blood. Rats were aged to 12-14 (aged) months before being assigned to two groups: rats from Group 1 were sacrificed for blood and tissue collection (1-2 rats/sex/litter) while Group 2 underwent radiotelemetry surgery for measurement of blood pressure during a restraint challenge (see below).

4.3.2 Tissue collection (Group 1 at 12 months)

Rats were transported to a facility for blinded tissue collection with all tissue samples being collected between 0900h and 1000h. Rats were euthanised by an intraperitoneal injection of pentobarbitone sodium (Lethobarb; 0.1 ml/kg body weight). Blood was collected via cardiac puncture for analysis of corticosterone. In aged rats, adrenal and pituitary glands

were weighed. Hypothalamus and hippocampus were collected. The coordinates for brain regions were determined using Paxinos and Watson [510]. Hippocampal tissue was collected from Bregma −2.28 to −3.64. Likewise, the hypothalamus was collected with coordinates Bregma -3.24 to -4.44. Adrenal glands, hypothalamic and hippocampal tissues were snap-frozen and stored at -80°C for qPCR analysis. While tissues were being collected, anomalies were noted, and a number of pituitary glands were recorded as abnormal and enlarged. These were fixed in 4% paraformaldehyde for histological analysis using methodologies similar to those performed previously [286]. Pituitary glands were processed to paraffin and sectioned before staining with hematoxylin and eosin and sent to an expert veterinary pathologist for blinded assessment [286].

4.3.3 Measurement of the cardiovascular stress response (Group 2)

Due to the intrinsic link between the HPA and cardiovascular function, a subset of aged rats at 14 months of age underwent surgery for implantation of radiotelemetry monitors to allow for measurements of cardiovascular responses during a restraint challenge. These rats were transported to a separate facility with a 12-hour light-dark cycle (0600h to 1800h) and allowed to adjust for two weeks prior to telemetry surgery. Anaesthesia was induced using 5% isoflurane in oxygen and maintained using 2-3% isoflurane. Upon confirmation of reflex abolishment, a 2cm incision was made in the left hind limb, and the cannula of the telemetry probe (PA-C40, DSI, St Paul, USA) inserted into the femoral artery and advanced into the descending aorta. The radiotelemeter transmitter was then placed subcutaneously. Each rat was housed individually in a cage located on a PhysioTel-Receiver (Model # RPC-1, DSI, St Paul, USA). After a recovery period of 10 days, the radio transmitters were turned on, and measurements were assessed for 14 days before restraint test. Following this, cardiac parameters of systolic and diastolic blood pressure and HR were measured before and from 2 to 15 minutes of a 15-minute restraint challenge to measure the change in cardiovascular parameters from baseline, adapted from the protocol published previously by O'Sullivan *et al.* [511]. Results were analysed by calculating the change from baseline (delta, Δ) for each parameter. The restraint stress was performed 3 to 5 hours after lights on, and once per rat, within its home cage. Rats were restrained by inserting custom-made Perspex dividers [462mm (length) x 125mm (height) and 325 mm (width) x 125 mm (height)] into the cage. These dividers press against one side and rump, to confine the rat within the corner of their home cage, limiting its ability to rear, turn or move forward and backwards. Rats had adequate air holes, however food and water were removed for the 30-minute duration of testing. The restraint stress was performed with rats in their home cage with animals

restricted from nose to rump, on both sides and with a limited ability to rear, using a custom-made transparent device. A tail vein blood sample was collected (0.3mL) immediately upon removal from restraint for plasma corticosterone concentration analysis as described below.

4.3.4 Corticosterone radioimmunoassay

An in-house radioimmunoassay was used to analyse plasma corticosterone concentration in both adult and aged rats, as previously described [386,394]. The assay sensitivity ranges from 39ng/ml up to 5000ng/ml based on 5ul of plasma per sample. The intra-assay coefficient of variation and inter- assay coefficient of variation for assays performed for this manuscript were 6.8% and 7.3% respectively. This assay has been shown to have minimal cross-reactivity (<1%) for progesterone, testosterone, cortisol or 11 deoxycorticosterone.

4.3.5 Quantitative PCR

RNA was extracted from adrenal glands (n=8/group/sex), hypothalamic (n=8/group/sex) and hippocampal (n=5-7/group/sex) samples using the commercially available RNeasy Mini-kit (Qiagen, Doncaster, VIC) and cDNA was synthesised using iscript (Bio-Rad, Gladesville, NSW, Australia). Commercially available assay-on-demand primer and probe sets (Life Technologies) were used to analyse gene expression of key steroidogenic genes within the adrenal gland including *Mc2r* (rn02082290_s1), *Cyp11a1* (rn00568733_m1), *Cyp21a1* (rn00588996_g1), *Hsd11b2* (rn00492539_m1), *Nr3c1* (rn00561369_m1) and *Hsp90a1* (rn00822023_g1). Hypothalamic gene expression of *Crh* (rn01462137_m1), *Crh-r1* (rn00578611_m1), *Nr3c1* and *Hsp90a1* (rn00822023_g1) and hippocampal gene expression of *Nr3c1* and *Hsp90a1* was assessed. A panel of housekeeper genes was measured, with the geomean of *Rn18S* and *Actb* being used for aged adrenal gland and hypothalamus, and *Rn18S* for hippocampal gene expression. PC:EtOH did not significantly alter housekeeper gene expression.

4.3.6 Statistical analysis

All data are presented as mean ± standard error of the mean (SEM). All data were analysed using a two-way Analysis of Variance (ANOVA). Weights, plasma corticosterone concentrations and gene expression analyses compared treatment (trt) and sex as factors with an LSD Fisher post hoc test. Two-way ANOVA was used to analyse radiotelemetry parameters with an LSD Fisher post hoc test. Statistical analysis was performed using GraphPad Prism 7 for Windows (GraphPad Software, Inc., San Diego, CA). Pituitary gland

weight was tested for normality using a D'Agostino-Pearson test, and a Fishers Exact Test was used to determine the association between tumours and treatment.

4.4 Results

4.4.1 Body weights and relative organ weights of offspring

PC:EtOH did not alter body weight of aged rats (Table 4. 1). However, females were significantly lighter than male offspring ($F_{(sex)\ (1,67)} = 179.2$, $P_{sex} < 0.0001$). In aged rats at post-mortem, total and relative adrenal weights and pituitary weights were not significantly different between treatment groups (Table 4. 1).

4.4.2 Basal corticosterone concentrations in adult and aged offspring

Plasma corticosterone concentrations were higher in adult females compared to male offspring ($F_{(sex)\ (1,22)} = 6.86$, Figure 4. 1A, $P_{sex} < 0.05$). A sex by treatment interaction ($F_{(trt*sex)\ (1,22)} = 4.03$, $P_{int} < 0.05$) was further examined by post hoc analysis, demonstrating that plasma corticosterone concentrations were 25% lower in adult female but not male PC:EtOH exposed offspring ($T(21) = 2.19$, $P < 0.05$). Within aged rats, PC:EtOH offspring had lower plasma corticosterone concentrations ($F_{(trt)\ (1,26)} = 9.80$, $P < 0.05$, Figure 4. 1B) with a post hoc analysis demonstrating that this was due to a 34% reduction in female offspring exposed to alcohol ($T(26) = 3.2$, $P < 0.05$). Of interest, plasma corticosterone concentrations were similar in male and female offspring at this age

.

Table 4. 1: The impact of periconceptional alcohol on body and organ weight in aged male and female offspring.

	Male		Female		ANOVA		
	Control	**PC:EtOH**	**Control**	**PC:EtOH**	**Trt**	**Sex**	**Int**
Body (g)	761 ± 26 (n = 19)	739 ± 26 (n = 18)	434 ± 18 (n = 19)	445 ± 19 (n = 16)	NS	<0.0001	NS
Average adrenal (mg)[a]	40.3 ± 3.7 (n = 19)	39.9 ± 3.0 (n = 17)	46.4 ± 3.6 (n = 17)	39.4 ± 1.9 (n = 15)	NS	NS	NS
Relative adrenal (mg/BWg)	0.05 ± 0.01 (n = 19)	0.06 ± 0.01 (n = 17)	0.11 ± 0.01 (n = 17)	0.10 ± 0.02 (n = 15)	NS	<0.0001	NS
Pituitary (mg)[b]	43.4 ± 12.2 (n = 8)	85.4 ± 36.8 (n = 9)	52.5 ± 7.8 (n = 9)	150.9 ± 66.3 (n = 10)	NS	NS	NS
Relative pituitary (mg/BWg)	0.05 (0.13, 0.01) (n = 8)	0.05 (0.58, 0.01) (n = 9)	0.11 (1.7, 0.03) (n = 9)	0.16 (1.49, 0.03) (n = 10)	-	-	-

Trt: treatment; Int: interaction between treatment and sex.

Bodyweight and relative adrenal weight data presented as mean ± standard error of the mean. Analysed by two-way ANOVA comparing sex and treatment, with a Bonferroni post hoc test. Relative pituitary weight data are presented as median (upper, lower limit). [a]average of two adrenal glands.

[b]total pituitary weight, including anterior gland, posterior gland and the median eminence.

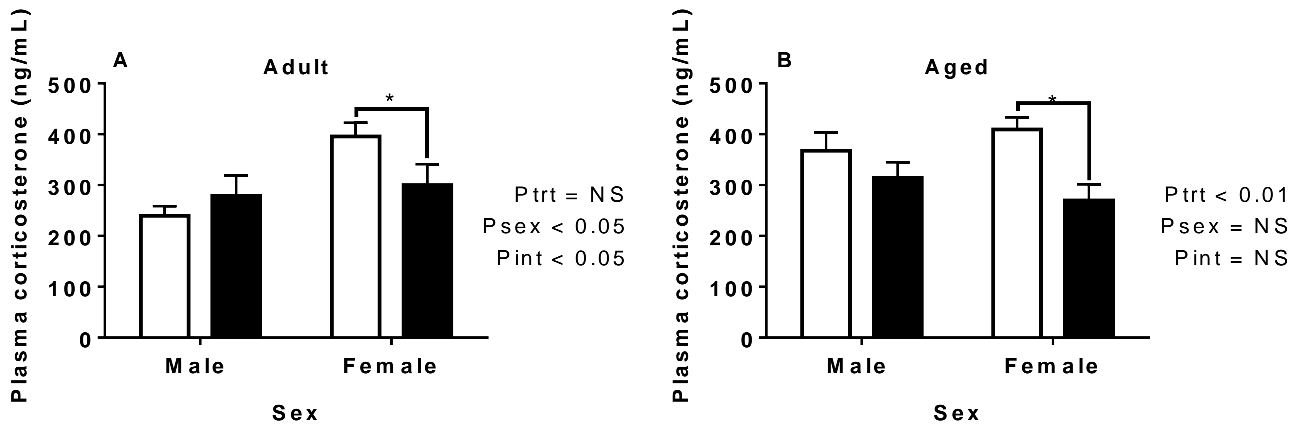


Figure 4. 1: The basal plasma corticosterone concentration (ng/mL) in adult (A) and aged (B) offspring exposed to control (white) or periconceptional ethanol (PC:EtOH, black) diets. Samples were collected prior to experimental paradigms and within 4 hours of light. Sample size at 6 months: Control = 6 PC:EtOH = 7 per sex. Sample size at aged: Control = 8, PC:EtOH = 7 per sex). Data represented as mean ± standard error of the mean, analysed by a two analysis of variance, with a Bonferroni post-hoc analysis, *p <0.05.

4.4.3 Adrenal gland gene expression in aged offspring

Given that female PC:EtOH offspring had reduced plasma corticosterone concentrations, the gene expression of key factors in the adrenal gland, the hypothalamus and hippocampus in the aged cohort were investigated to establish if this may be as a result of an adrenal or central deficit. PC:EtOH did not affect the expression of *Mc2r, Cyp11a1, Cyp21a1, Hsd11b2* or *Nr3c1* in adult or aged offspring (Figure 4. 2). However, analysis of *Hsp90a1* gene expression revealed a sex by treatment interaction ($F_{(trt*sex)\ (1,25)} = 6.12$, $P < 0.05$), which when further examined by post hoc analysis, demonstrated significantly higher in aged female offspring exposed to PC:EtOH when compared to control ($T(25) = 2.81$, $P < 0.05$).

4.4.4 Hypothalamus and hippocampus gene expression in aged offspring

PC:EtOH did not significantly alter *Crh*, *Crh-r1*, *Nr3c1* or *Hsp90a1* within the hypothalamus of aged offspring (Figure 4. 3A-D, respectively), neither was there any difference between males and females. In contrast, hippocampal expression of both *Nr3c1* and *Hsp90a1* were altered by

sex and treatment (Figure 4. 3E-F, respectively). PC:EtOH significantly increased hippocampal expression of *Nr3c1* ($F_{(trt)\ (1,20)}$ = 13.32, P < 0.05) which was also more highly expressed in females compared to males ($F_{(sex)\ (1,20)}$ = 8.99, P < 0.05). A sex by treatment interaction ($F_{(trt*sex)\ (1,20)}$ = 6.61, P <0.05) and post hoc analysis (T(20) = 4.07, P < 0.01) demonstrated a 4.3-fold increase in hippocampal gene expression of *Nr3c1* of PC:EtOH female but not male offspring. Similarly, PC:EtOH increased mRNA expression of *Hsp90a1* within the hippocampus ($F_{(trt)\ (1,20)}$ = 5.65, P < 0.05). Hippocampal *Hsp90a1* mRNA were higher in females compared to males ($F_{(sex)\ (1,20)}$ = 6.91, P < 0.05), with a sex by treatment interaction ($F_{(trt*sex)\ (1,20)}$ = 4.47, P < 0.05) and post hoc analysis (T(20) = 2.94, P < 0.05) demonstrating a 2 fold increase in *Hsp90a1* expression in female PC:EtOH offspring only (Figure 4. 3F).

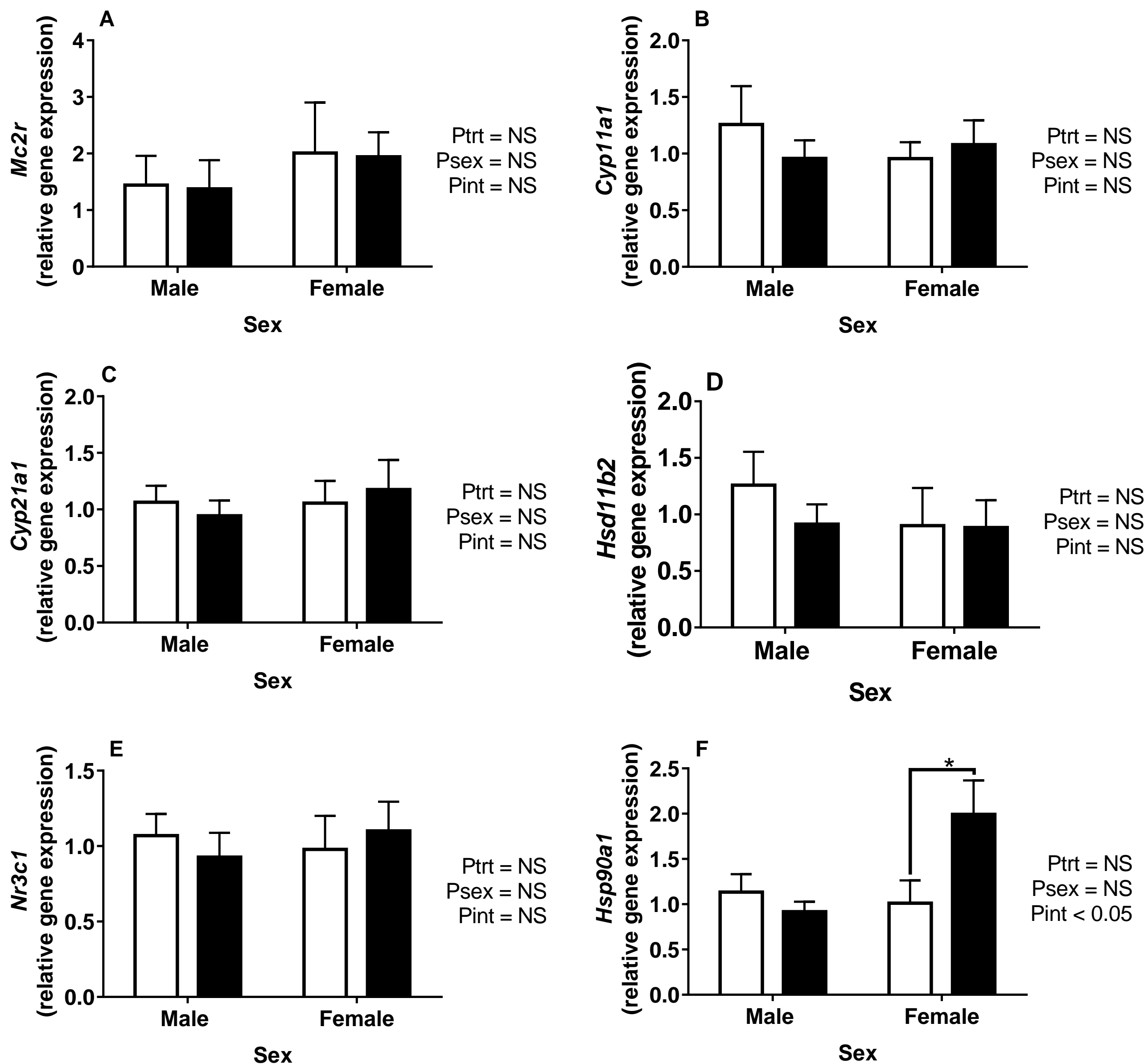


Figure 4. 2: The relative gene expression of melanocortin receptor 2 (*Mc2r,* A), cytochrome P450 family 11 subfamily A member 1 (*Cyp11a1,* B), cytochrome P450 family 21 subfamily A polypeptide 1 (*Cyp21a1,* C), 11-beta-dehydrogenase isozyme 2 (*Hsd11b2,* D), nuclear receptor subfamily 3 group C member 1 (*Nr3c1,* E) and heat shock protein 90 alpha 1 (*Hsp90a1,* F) in the adrenal gland of aged offspring (sample size = 5-8 litters per group) exposed to control (white) or periconceptional ethanol (PC:EtOH, black) diets. Data represented as mean ± standard error of the mean and analysed by two-way analysis of variance with a Bonferroni post hoc test.

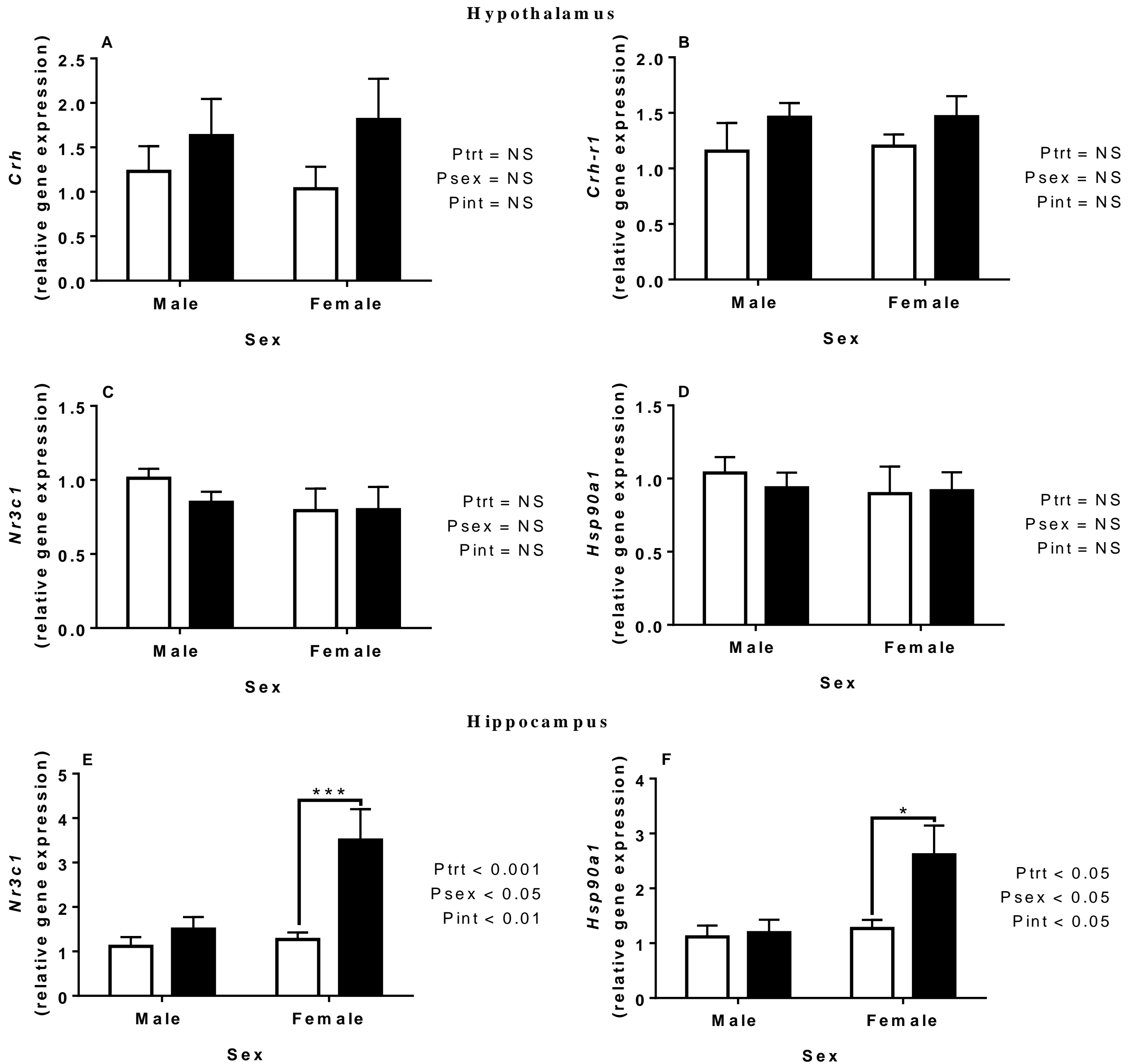


Figure 4. 3: The relative gene expression of corticotropin-releasing hormone (*Crh,* A), corticotropin-releasing hormone receptor 1 (*Crh-r1,* B) in the hypothalamus, nuclear receptor subfamily 3 group C member 1 (*Nr3c1,* C, E) and heat shock protein 90 alpha 1 (*Hsp90a1,* D, F) in the hypothalamus and hippocampus respectively, of aged offspring exposed to control (white) or periconceptional ethanol (PC:EtOH, black) diets. Data represented as mean ± standard error of the mean and analysed by two-way analysis of variance with a Bonferroni post hoc test. Sample size =7-8 for hypothalamus and 6-7 for hippocampus).

4.4.5 Effects of restraint stress on cardiovascular parameters in aged offspring

As expected, heart rate, blood pressure (systolic, diastolic and mean arterial) all increased during the restraint protocol. As the cardiovascular response to restraint stress was the primary focus of this protocol, all cardiovascular parameters are reported as the change from baseline (delta, Δ). After a large initial increase in heart rate and blood pressure, the delta heart rate (ΔHR), delta systolic blood pressure (ΔSBP) and delta diastolic blood pressure (ΔDBP) decreased towards the initial reading of respective parameters in both male and female rats, regardless of treatment over the 15 minutes of restraint (Figure 4. 4, Ptime < 0.001. [Male: $F_{(time)\ (13,\ 130)}$ = 21.59, 5.93 and 8.24 for ΔHR, ΔSBP and ΔDBP respectively]. [Female: $F_{(time)\ (13,\ 156)}$ = 17.50, 10.98, 5.18 for ΔHR, ΔSBP and ΔDBP respectively]). The ΔSBP was lower in female PC:EtOH offspring ($F_{(trt)\ (1,\ 12)}$ = 5.17, P < 0.05) with post hoc analysis revealing that this was significant at all time-points after the first two minutes except the 7 and 9 minute mark (3, 4 and 5 minutes [T(168) = 2.12, 2.53, 2.61, p < 0.01], 6, 8 to 10 [T(168) = 2.12, 2.26, 2.07, 1.99, P < 0.05] and 13 to 15 minutes [T(168) = 2.15, 2.40, 2.53, p < 0.05]). In contrast, the ΔSBP was not affected by treatment in male offspring. Both ΔDBP ($F_{(trt)\ (1,\ 12)}$ = 4.04, P=0.06) and ΔMAP ($F_{(trt)\ (1,\ 12)}$ = 4.25, P=0.06) trended towards being lower in female PC:EtOH offspring, however, these parameters were not altered in male offspring. ΔHR was not changed by PC:EtOH in either male or female offspring.

Although plasma corticosterone concentrations following restraint were not affected overall by PC:EtOH, a sex by treatment interaction ($F_{(trt*sex)\ (1,\ 29)}$ = 5.32, P<0.05) was observed and post hoc analysis demonstrated that plasma corticosterone concentrations were lower in female PC:EtOH offspring compared to control female offspring (T(29) = 2.23, p<0.05, Figure 4. 4E). PC:EtOH had no effect on plasma corticosterone concentration following restraint stress in male offspring. Plasma corticosterone concentration following restraint was greater in female compared to male offspring ($F_{(sex)\ (1,\ 29)}$ = 66.46, P < 0.001, Figure 4. 4E).

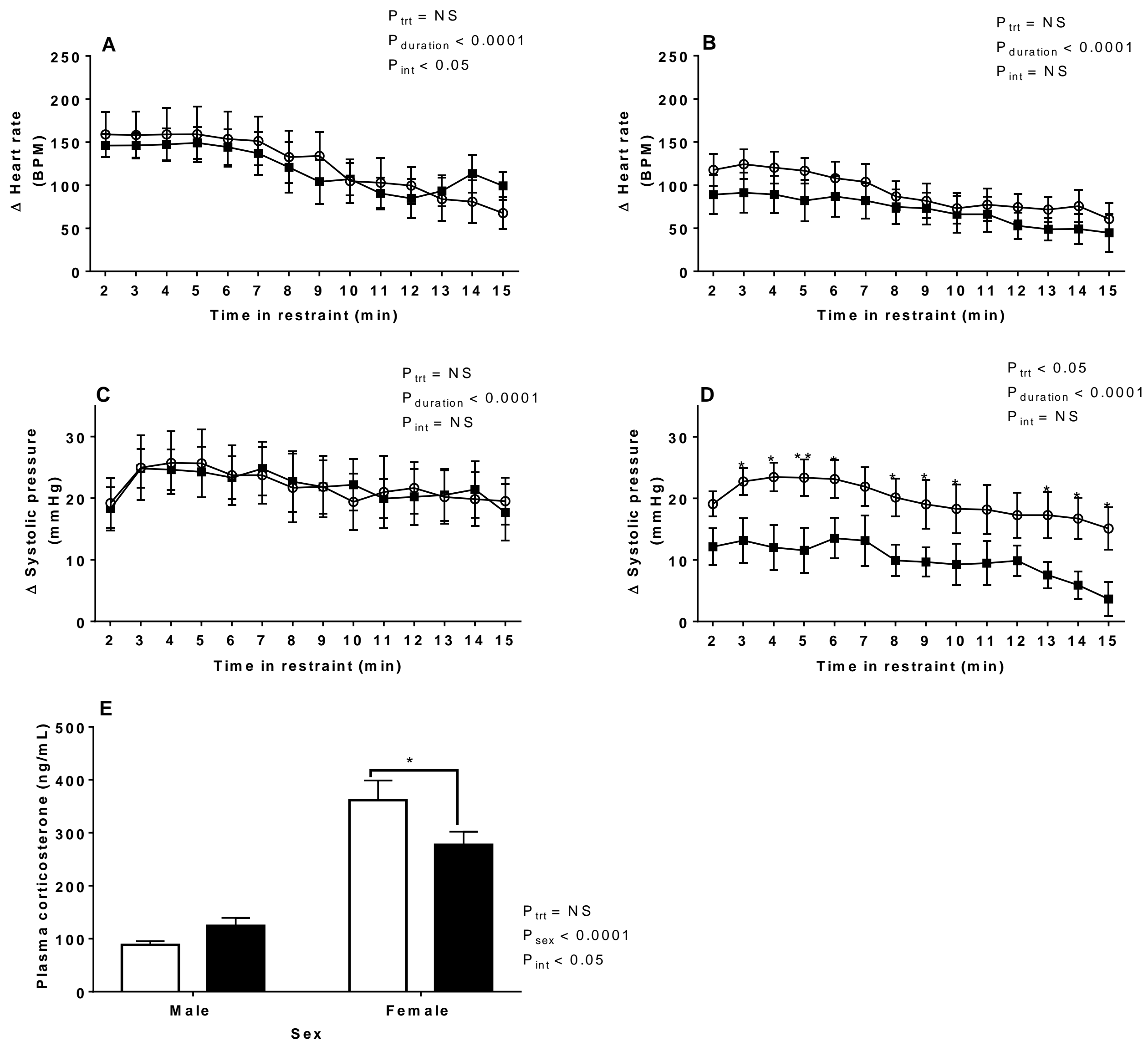


Figure 4. 4: The delta (Δ) heart rate (A, B) and Δ systolic blood pressure (C, D) during the 2 to 15 minute duration of the restraint test, and plasma corticosterone concentration (ng/mL) at the end of the restraint test (E), in male (left) and female (right) offspring exposed to control (white) or periconceptional ethanol (PC:EtOH, black) diets. Sample size = 7-8 per group. The data was represented as mean ± standard error of the mean analysed by a two-way analysis of variance with a Fishers least significant difference test, *p<0.05, **p<0.01.

4.4.6 Pituitary weights and abnormalities in aged offspring

Interestingly, at tissue collection a number of pituitaries from the PC:EtOH group were identified as being enlarged and sent for histopathological assessment. Mean pituitary weight was not significantly different between groups. However, pituitary weight was normally distributed in control rats but highly variable in PC:EtOH exposed rats for both males ($P_{normality} < 0.05$) and females ($P_{normality} < 0.05$). It was noted that of the ten male PC:EtOH offspring, two were recorded as enlarged and abnormal compared to one of the eight control rats (Figure 4. 5). Within the female cohort, five of the ten PC:EtOH rats were abnormal, compared to zero within the control group (Figure 4. 5). A Fisher's exact test demonstrated that there was an increased number of enlarged pituitaries in female PC:EtOH offspring compared to control offspring ($p < 0.05$). The expert pathological analysis identified mitotic figures in representative sections from the enlarged pituitaries of both male (Figure 4. 5C) and female offspring exposed to PC:EtOH (Figure 4. 5D).

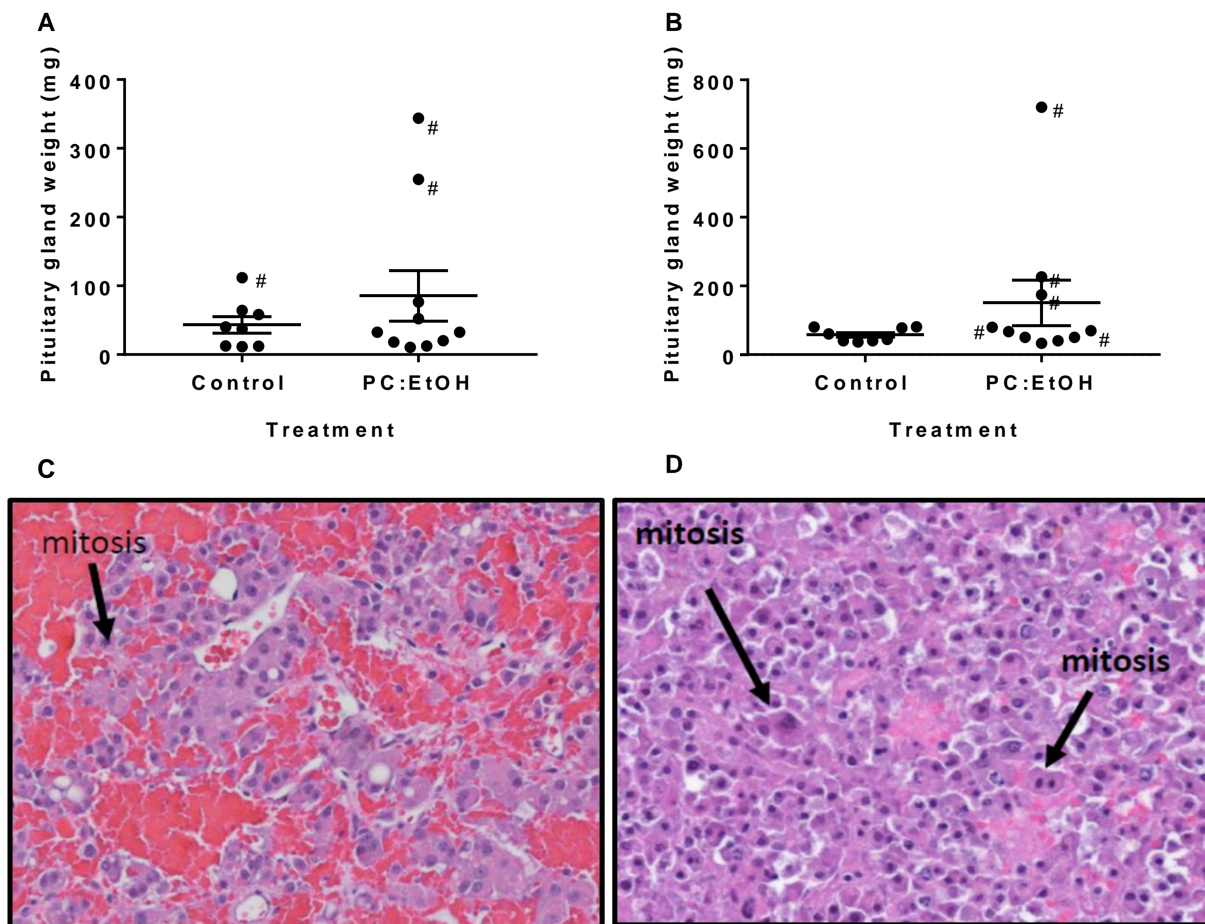


Figure 4. 5: Pituitary gland weights (mg) and pathology from control or periconceptional ethanol exposure (PC:EtOH) treated male (A) (nControl = 8, nPC:EtOH = 10) and female (B) (nControl = 9, nPC:EtOH = 10) offspring. # indicates rats with observed pituitary abnormalities determined during post-mortem. (C) and (D) show pathology results in an abnormal gland, as determined by a hematoxylin and eosin stain, in male and female offspring, respectively.

4.5 Discussion

This study demonstrated that PC:EtOH resulted in lower plasma corticosterone concentrations in adult female offspring, and concentrations remained lower in aged female offspring under both resting and stressed conditions. Interestingly, this was sex-specific with no differences observed in male offspring exposed to PC:EtOH. These lower corticosterone concentrations in females occurred despite no effect of PC:EtOH exposure on the gene expression of steroidogenesis regulators in the adrenal gland and hypothalamus. Further analysis demonstrated that this dysregulation of corticosterone production might be due to altered expression of *Nr3c1* and *Hsp90a1*, genes involved in negative feedback pathways within the hippocampus. Previous studies have demonstrated that exposure to alcohol throughout the entire pregnancy period programs HPA dysfunction in offspring [512–515]. These results highlight that alcohol exposure during this early stage of gestation, prior to implantation and before initiation of brain development, may have long-term impacts on central control of HPA function which may impact physiological outcomes.

To date, no studies have investigated HPA outcomes following alcohol exposure limited to the time before implantation and organogenesis. Within this study, the finding that plasma corticosterone concentrations were lower in adult and aged female offspring exposed to alcohol around conception contrasts with those following prenatal ethanol exposure throughout pregnancy. These studies indicated that even though the adrenal weight was unchanged in offspring, there are significant elevations in basal corticosterone concentrations in both male and female offspring [334,491]. Animal studies of chronic and binge alcohol consumption during pregnancy have also revealed elevated basal corticosterone levels, albeit in male offspring only [323]. Conversely, other studies have demonstrated no alterations in basal plasma corticosterone levels [491,516]. The reasons for these differences have not been determined but may be due to the dose of alcohol, strain and species of animal or age at which the measurement was made. In humans, heavy drinking around the time of conception and throughout pregnancy has been associated with increased basal cortisol levels in 13-month old children [501]. Similarly, children diagnosed with FASD have increased cortisol concentrations [316,517] although there is evidence this may be dependent upon the time of day, with FASD diagnosed children displaying higher cortisol levels than healthy children in the afternoon and at bedtime [517]. Although the current study is the first to report reduced corticosterone concentrations in offspring following PC:EtOH, a recent publication investigated high dose ethanol administration (via gavage) during adolescence but prior to pregnancy, on offspring HPA outcomes. Male offspring from dams exposed to

alcohol before pregnancy had reduced basal corticosterone concentrations. This study suggests that these alterations may be due to intergenerational inheritance of epigenetic markers [366].

Within this study, basal plasma corticosterone concentrations reported were slightly elevated when compared to values reported in rats by other groups. While basal values in our study were approximately 230ng/mL in males and 400ng/mL in females at 6 months, others report basal concentrations of approximately 114ng/mL and 292ng/mL respectively [429]. The values obtained from the study by Osborn *et al.* were from blood collected within 3 hours of the light cycle, while in our study blood samples are collected 9-10 hours after the commencement of the light cycle, as our animals were housed in an alternative cycle. Work by Waddell and colleagues demonstrate that basal corticosterone should peak when light is withdrawn [391] supporting the values from the current study being basal for the time of day they were collected.

In addition to alterations to basal corticosterone concentrations following various prenatal alcohol exposure paradigms, alcohol exposure throughout pregnancy has been shown to dysregulate HPA activity following a stress challenge. Rats exposed to stressors such as immune challenges, repeated restraint and foot shock show elevated corticosterone, and ACTH levels and a reduced return to baseline [335,424,425,518] Other studies of prenatal alcohol exposure throughout pregnancy have shown HPA hyperactivity following challenges such as the dexamethasone suppression test [332,429]. Additional parameters measured as a response to stress, such as perturbed heart rate or changes in blood pressure from baseline further support alterations to the stress pathways within PC:EtOH offspring. In the current study, the restraint induced corticosterone concentrations and ΔSBP were significantly lower in female offspring exposed to PC:EtOH. Results collected within this thesis (Section 3.3.4.1 Combined dexamethasone suppression and corticotropin-releasing hormone stimulation test revealed that PC:EtOH programmed a hyperactive HPA in both male and female offspring, however, given the link between the HPA and the cardiovascular system, these results were counter-intuitive. Furthermore, clinical studies have demonstrated that high-frequency alcohol consumption before pregnancy recognition increases children's cortisol and cardiovascular response to stress when compared to children of women with low-frequency alcohol consumption [316]. It may be hypothesised that HPA activated pathways to the LC-NA, withdrawal of the parasympathetic regulation of cardiovascular activity and other neural cardiovascular centres may be perturbed by PC:EtOH, an avenue of research worth pursuing.

Interestingly, while prenatal alcohol consumption increased infant cortisol response to stress to a greater extent in males compared to females, female offspring had a greater increase in heart rate in response to stress. This suggests that the underlying mechanisms and outcomes of prenatal alcohol exposure is sex-specific and, in part, corroborates the current findings that PC:EtOH did not affect restraint induced changes in male offspring. Earlier studies using this animal model have demonstrated sex-specific placental adaptations which may have contributed to these female-specific disease outcomes, and support the hypothesis that sex-specific adaptations due to perturbations in early pregnancy may protect male offspring but increase disease risk in female offspring [102,519]. Furthermore, sex-specific outcomes within our study may be related to PC:EtOH programming of sex steroids, as the hypothalamic-pituitary-gonadal axis, has significant cross-communication with the HPA. Changes in hormones, such as estrogen, which has a role in the production of the glucocorticoid regulatory protein, corticosteroid-binding globulin [520] may have significant implications in the observed sexually dimorphic outcomes.

To investigate the mechanism underlying the programmed decrease in corticosterone concentrations in adult females, the mRNA expression of key markers of adrenal steroidogenesis and hypothalamic function were investigated. There were no major changes in the adrenal or hypothalamus apart from the fact PC:EtOH increased adrenal *Hsp90a1* gene expression in female offspring. This may suggest an increase in negative feedback at the level of the adrenal, and although overall gene expression of the steroidogenic pathway was not changed, protein or activity levels were not investigated in this study and may be altered due to PC:EtOH. These results contrast with previous studies which demonstrated significant changes in adrenal steroidogenic enzymes *Star* and *3β-hsd*, as well as alterations in mRNA expression of *Crh* within the hypothalamus of both weanlings and adult offspring following prenatal ethanol exposure [321,329,507]. Interestingly, while the traditional regulators of HPA function were not affected, hippocampal expression of *Nr3c1* and *Hsp90a1* were increased in female offspring exposed to PC:EtOH. The expression of *Nr3c1* and *Hsp90a1* within the hippocampus are essential for the interpretation of sensory information, as well as mediating glucocorticoid negative feedback within the HPA [521]. This suggests that alterations in hippocampal *Nr3c1* and *Hsp90a1* expression may influence glucocorticoid production and responses to stress. The reduction in corticosterone is hypothesized to be, in part, driving the increased expression of hippocampal *Nr3c1*. Although limited studies have been performed investigating the outcomes of these changes, adrenalectomised rats (i.e. removal of endogenous corticosterone production) prenatally exposed to alcohol had

increased hippocampal GR and MR expression, suggesting that prenatal alcohol consumption may reduce the sensitivity of the hippocampus to glucocorticoids [321]. These findings provide support for the contention that PC:EtOH may program increased expression of GR and its chaperone protein HSP90, with an inability to normalise HPA output due to decreased sensitivity. It is important to note here, however, that many other limbic structures, such as the amygdala and the prefrontal cortex also play a necessary role in the regulation of the HPA [321], and it cannot be discounted that PC:EtOH may also have a programming role within these structures.

An interesting observational aspect of this study was the increased occurrence of pituitary abnormalities, particularly in female PC:EtOH offspring. Alcohol exposure throughout pregnancy in rats on resulted in an increased incidence of tumorigenesis in offspring including in the pituitary gland [328]. While immunohistochemistry was not performed in the current study due to limitations in the fixation methodology, further studies should focus on identifying the cell types associated with the enlarged pituitary glands. Prenatal alcohol exposure can also increase pituitary weight in offspring and perturb critical hormonal pathways [71], suggesting that further investigation of these abnormalities is warranted, as functional alterations within the pituitary gland may contribute to altered hormonal status following PC:EtOH exposure.

This study has demonstrated the adverse impacts of PC:EtOH exposure on offspring HPA but further studies are required to fully understand the mechanisms involved. Considering that this exposure is before organogenesis, it is likely that mechanisms are indirect. The periconceptional period encompasses many drastic changes, including reprogramming of methylation profiles [522] and maternal undernutrition in women during the periconceptional period results in alterations DNA methylation at metastable epialleles [523] including the *Igf2* gene [345]. This has been similarly observed in animal models with periconceptional undernutrition results in altered DNA methylation in pig embryos [524], and epigenetic changes to adrenal gene expression [525], hypothalamic proopiomelanocortin and glucocorticoid receptor genes in the sheep [526].

Another possible mechanism may be alterations in the maternal physiology. Maternal alcohol consumption stimulates the HPA, including increase cortisol concentrations, reduced ACTH and HPA responsiveness [527–530]. Alcohol exposure throughout the entirety of pregnancy has demonstrated increased corticosterone concentration, adrenal weight and HPA activity in rat dams [387,531–533].

4.5.1 Conclusion

This study is the first to show that PC:EtOH exposure results in a persistent reduction in plasma corticosterone concentrations and reduced pressor responsiveness to stress in female but not male offspring. This reduction in corticosterone was likely driven by an increase in negative feedback through the hippocampus as indicated by an increase in *Nr3c1* and *Hsp90a1* expression rather than alterations to adrenal steroidogenesis or hypothalamus gene expression. Furthermore, morphological alterations in offspring pituitary glands of aged rats suggest more significant alterations in the HPA which warrant further investigation. This study demonstrates that the effects of alcohol exposure around the periconceptional period are significantly different from those induced by chronic alcohol consumption.

Chapter Five

Periconceptional ethanol exposure alters maternal glucocorticoid concentration throughout gestation in a rat.

"The angel of the Lord appeared to her and said,
'You are barren and childless, but you are going to
become pregnant and give birth to a son. Now see to it
that you drink no wine or other fermented drink
and that you do not eat anything unclean'"
- Judges 13: 3-4 –

5.1 Abstract

A disrupted hypothalamic-pituitary-axis (HPA) during pregnancy is associated with a range of disease outcomes in offspring. Many of these are similar to those observed following periconceptional alcohol exposure, as demonstrated within this thesis. However, very few studies to date have investigated the impacts of alcohol consumption during pregnancy on the maternal HPA function, particularly when alcohol exposure occurs only around the time of conception. Therefore, this chapter aimed to establish the impact of periconceptional ethanol (PC:EtOH) exposure on maternal corticosterone, aldosterone concentrations and adrenal pathways necessary for hormone production. Homeostatic systems regulated by the HPA, including metabolic and renal function, were also investigated. Importantly, as these processes are dynamic throughout pregnancy, this study investigated both the direct and delayed consequences of alcohol exposure. Additionally, as the placenta regulates fetal exposure to disruptions to maternal HPA status, expression of placental glucocorticoid signalling pathways were investigated in late pregnancy.

Female Sprague Dawley dams were treated with PC:EtOH (12.5% v/v EtOH liquid diet) or a control diet from 4 days before conception, until embryonic day (E) 4. Two days prior to mating (E-2), a plasma sample was collected. During early gestation (E2 and E5), mid-gestation (E15) and at the end of pregnancy (E18 and E20), further plasma samples were collected, for analysis of corticosterone. A plasma sample was collected at E18 for measurement of creatinine and plasma lipids. At E16, a separate subset of dams underwent 24-hour metabolic cage testing to assess renal function, and urine was analysed for electrolyte excretion (sodium, potassium, chloride and creatinine), followed by plasma collection at E18 for assessment of creatinine and plasma lipids. Adrenal glands were collected at E5, E15 and E20 for transcriptomic analysis of the steroidogenic pathway.

PC:EtOH consumption significantly increased plasma corticosterone in the non-pregnant dam at E-2. These changes were not seen following conception at E2. However, a significant decrease was observed at E5. Although plasma corticosterone was not different between treatment groups at E15, there was an increase during late gestation, at E20, in the PC:EtOH group. Interestingly, only minor changes in the expression of genes which regulate adrenal steroidogenesis were observed in PC:EtOH dams, with a significant increase in cytochrome p450, family 11, subfamily b, polypeptide 2 (*Cyp11b2*), angiotensin receptor, type 2a (*Agtr1a*) and heat shock protein 90a1 (*Hsp90a1*) at E5 and a significant increase in gene expression of cytochrome p450, family 21, subfamily a,

polypeptide 1 (*Cyp21a1*) and cytochrome p450, family 11, subfamily b, polypeptide 1 (*Cyp11b1*) at E15. PC:EtOH exposure did not alter renal function (urinary flow and excretion of electrolytes) at E16 or plasma high-density lipoprotein (HDL), low-density lipoprotein (LDL) and triglycerides at E18. PC:EtOH elevated placental expression of corticotropin-releasing hormone (*Crh-r1*) in male and female and glucocorticoid receptor (*Nr3c1*) in female only.

In summary, these results demonstrate that PC:EtOH exposure alters circulating concentrations of corticosterone across gestation and that the long-term effects in of this exposure in offspring, may in part be related to these maternal hormonal alterations. The variability in plasma corticosterone throughout gestation, potentially as an effort to maintain homeostasis throughout pregnancy, indicates the burden that PC:EtOH places on the maternal system. While it is difficult to separate the impact of direct alcohol exposure and altered maternal physiology on offspring outcomes, this study highlights the complex interaction of these two perturbations.

5.2. Introduction

This thesis has determined that periconceptional ethanol exposure (PC:EtOH) results in several changes to offspring behaviour, including increased depressive-like and altered social phenotypes, hypothalamic-pituitary-adrenal axis (HPA) hyperactivity, and changes within central feedback pathways. However, the underlying mechanism causing these changes is yet to be elucidated. Alcohol has the potential to act directly as a teratogen on the developing fetus; however, within this model, PC:EtOH exposure occurs prior to implantation and fetal development. For this reason, it is hypothesised that PC:EtOH may be programming offspring outcomes via alterations in the maternal HPA and associated physiology.

Pregnancy is characterised by a series of remarkable physiological alterations that places the maternal system at an increased vulnerability to additional challenges, such as alcohol consumption. This includes changes to cardiovascular, renal, metabolic and hormonal physiology, which are discussed in detail in Section 1.11.1 Physiological changes in a healthy pregnancy. During early pregnancy, changes in estrogen, progesterone and glucocorticoids are essential for conception, implantation, regulation of anti-rejection pathways and maintenance of the early embryo [379]. These findings are evident in patients undergoing *in vitro* fertilisation who have reduced fecundability and a 90% increased risk of miscarriage when glucocorticoid levels are either greater or lower than healthy pregnancy glucocorticoid ranges [376,377]. A sustained increase in the concentration of this hormone throughout the remainder of pregnancy is essential for pregnancy-specific alterations to maternal physiology including glucose control, insulin resistance, lipoproteins, triglycerides and the renin-angiotensin-aldosterone system (RAAS) [372,373,534,535]. The RAAS is essential for the regulation of maternal vasculature and has a role in fetal nutrient delivery, as well as maternal thirst and renal function including electrolyte handling, plasma osmolality and vasopressin regulation for fluid homeostasis, as well as several other systemic and nervous system pathways [536,537]. These factors are regulated by mineralocorticoid signalling by both glucocorticoids and aldosterone, both of which are produced by the adrenal steroidogenic pathway. Finally, at the end of pregnancy, a surge in glucocorticoid concentration to three times the level of that during non-pregnancy is essential for the final wave of fetal organ developmental, and in some species, regulates the onset of parturition [378–380,538,539].

Alcohol-induced changes to the maternal endocrine system may have significant implications. Animal studies have shown that alcohol exposure throughout gestation stimulates the HPA of the

dam. These studies demonstrate increased adrenal gland weight during late gestation, as well as elevated glucocorticoid levels and increased HPA activity in response to stress, without a change in corticosterone binding globulin (CBG) concentration during mid and late gestation [387,388,531]. In studies which treated dams with ethanol, maternal adrenalectomy ameliorated the alcohol-induced low birth weight [540] and reversed the immobility observed in the forced swim test (FST) [441] in both male and female offspring. Sexually dimorphic outcomes observed in offspring exposed to prenatal alcohol exposure (PAE) were ameliorated following maternal adrenalectomy, with gene expression changes in the anterior pituitary gland of male offspring being similar to control values [483]. Adrenalectomy in PAE animals restored placental expression of 11β-hydroxysteroid dehydrogenase-2 and placental weight in females to the level of female control [389]. These results demonstrate the necessity of glucocorticoids in the programming effects of alcohol consumption during pregnancy.

Furthermore, several studies have associated prenatal stress exposure, due to either endogenous maternal glucocorticoids, or exogenous administration of glucocorticoids, with significant impacts on offspring outcomes. Human studies have associated maternal stress with adverse pregnancy-related conditions such as preterm birth and *in utero* growth restriction [49–53,541,542]. Relevant to this thesis, human studies also show an association between maternal stress, both during early pregnancy and throughout gestation, with altered infant behaviour, depression, schizophrenia and attention deficit hyperactivity disorder [270,271,543–546]. In animal models, prenatal stress and exposure to glucocorticoids are associated with altered offspring mental illness-like phenotypes, HPA activity and physiological abnormalities [278,286,295,547–550].

Therefore, it has been hypothesised that a mechanism of PC:EtOH exposure resulting in offspring mental illness-like phenotypes and HPA hyperactivity may be due to the maternal HPA during pregnancy. This chapter aimed to determine if PC:EtOH would result in altered plasma corticosterone and aldosterone concentrations and expression of critical adrenal regulators of steroidogenesis. Importantly, as the HPA is essential throughout the entirety of pregnancy, these outcomes will be investigated early in pregnancy, during mid-pregnancy and in late pregnancy where possible.

5.3 Methods

5.3.1. Ethics

All animal experiments and procedures were approved by The University of Queensland Anatomical Bioscience Animal Ethics Committee (AEC approval number SBS/022/12/NHMRC) and performed according to the Guidelines from the National Health and Medical Research Council of Australia.

5.3.2 PC:EtOH treatment

The details of animal treatments are outlined in Chapter Two. Briefly, Sprague Dawley dams were treated *ad libitum* with a 12.5% v/v EtOH (PC:EtOH) or control liquid diet from 4 days before conception, to 4 days after. Diets were made fresh daily and were provided *ad libitum* from 1200h to 0900h. Water was provided *ad libitum* for the remaining hours. Numerous cohorts of animals were used throughout this study to determine the effects of PC:EtOH on maternal parameters at multiple stages of pregnancy. The first cohort of rats had plasma collected from a tail tip at embryonic day (E) -2 and E2. As blood collection may have impaired breeding outcomes, these rats were then culled and not used for the remainder of this study. The second cohort of rats was treated as above but not subjected to tail bleeding and culled at E5. The third cohort of rats was treated as the E5 cohort, but on the fifth morning after mating, were returned to a standard laboratory chow and water *ad libitum* and culled on E15. Separate cohorts of rats were treated as per the E15 cohort but were placed in metabolic cages at E16 or culled at E20 for tissue collection. A separate subset of rats were treated as above and used to provide offspring for previous chapters of this thesis, however, were not utilised for data collected in this thesis. These rats were used for the analysis of weight gain over pregnancy. All dams were handled and weighed daily until end-point experiments.

5.3.3 Plasma and tissue collection

5.3.3.1 E-2 and E2 cohort

Following two days of treatment, the tails of female rats were warmed and had topical analgesia applied before a 1mm section of the tail tip was removed and 200µL blood collected. This occurs between 900h and 1030h. These rats were then mated two days later, and a second tail tip blood collection was performed at E2. As blood collection may have impaired pregnancy establishment, these animals were not used for subsequent analysis. The blood was centrifuged at 3500 rpm and the plasma collected and frozen at -20°C for subsequent corticosterone and aldosterone analysis.

5.3.3.2 E5 cohort

The second cohort of dams were transferred to a separate facility and culled via guillotine at E5. Maternal trunk blood (approximately 6mL) was collected in both heparin-coated or ethylenediaminetetraacetic acid (EDTA)-coated tubes, centrifuged at 3500 rpm and the plasma collected and frozen at -20°C for subsequent hormonal analysis. Tissues including liver, adrenal glands and pituitary glands were dissected, weighed and either snap-frozen in liquid nitrogen for molecular analysis or fixed in 4% paraformaldehyde for histological analysis.

5.3.3.3 E15 and E20 cohorts

For the E15 cohorts, dams were transported to a separate facility for tissue and blood collection. For the E20 cohorts, tail tip blood (200µL) was collected from E20 dams immediately prior to being transported to a separate facility for tissue collection. Dams were anaesthetised using 50:50 ketamine:xylazine (0.1ml/100g body weight, Lyppard Australia Ltd, QLD, AUS). Placental regions were separated and stored at -80ºC for subsequent molecular analysis. Once fetal tissues were collected, dams were culled by cardiac puncture. Cardiac blood (approximately 3mL) was collected in heparin-coated or EDTA-coated tubes from the E15 cohort. All blood from both cohorts were centrifuged at 3500 rpm and plasma frozen at -20°C for subsequent hormonal analysis. Tissues were collected as described above.

5.3.4 Plasma corticosterone and aldosterone analysis

Plasma corticosterone was measured via an in-house radio-immunoassay as described in Section 2.6.3 Hormone analysis. All samples were analysed in triplicate. This measured plasma corticosterone in plasma from dams at E-2, E2, E5, E15 and E20 with all samples from each time point being analysed within a single assay.

Plasma aldosterone was measured using an enzyme-linked immunosorbent assay (ELISA) (Alpha Diagnostic International Inc, Texas, US) as per the manufacturer's details. Briefly, 50µl of plasma stored in EDTA and standards were combined with 100µl avidin conjugate into a 96 well plate and incubated for 60 minutes. Samples were washed with provided wash buffer, and 150µl of TMB substrate (provided) was added. 50µl of stop solution was used following 10 to 15-minute incubation on a shaker. Absorbency was measured at 450nm using and absorbency scanner (TECAN, Life Sciences, Switzerland). Concentration was calculated using a logarithmic line of the best-fit curve of the standards. The assay sensitivity ranges from 15pg/mL up to 2000pg/ml based on 50µl of

plasma per sample. This assay has been shown to have minimal cross-reactivity (1.1%) for 11 deoxycorticosterone, and negligible cross-reactivity with androsterone, cortisone, 11-deoxycortisol, 21-deoxycortisol, dihydrotestosterone, estradiol, estriol, estrone, and testosterone. All samples were assayed on a single plate in duplicate with an intra-assay coefficient of variation of 3.8%.

5.3.5 Renal function

Renal function was assessed at E16 in a separate cohort of dams that underwent metabolic cage testing within the homeroom facility. These dams were acclimatised to the metabolic cages for two hours on E14 and one hour on E15. Food and water were provided *ad libitum* during this time. On E16, dams were placed within the metabolic cages for 24 hours for quantification of food and water consumption, as well as urine output. Urinary electrolytes (Sodium [Na^{2+}], Chloride [Cl^{-}] and potassium [K^{+}]) were assessed used the Cobas Integra 400 Plus Chemistry Analyzer System (software version 3.5, Block Scientific, NY, USA) by ion-selective electrode potentiometry with assay detection. The Cobas Integra 400 Plus Chemistry Analyzer System was also used to measure creatinine by an enzymatic colourimetric reaction.

5.3.6 Plasma lipid measurements

At E18, tail tip plasma was collected from dams (approximately 300μL), via a tail slice into ethylenediaminetetraacetic acid (EDTA) coated tubes. Samples were centrifuged at 3500 rpm and plasma frozen at -20°C. Using the Cobas Integra 400 Plus Chemistry Analyzer System (software version 3.5, Block Scientific, NY, USA), plasma low-density lipoprotein (LDL), high-density lipoprotein (HDL) and triglycerides were measured using enzymatic colourimetric reactions.

5.3.7 Gene expression of maternal adrenal glands and placental labyrinth

RNA was isolated from the left maternal adrenal gland at E5, E15 and E20 and placental labyrinth at E20, using the RNeasy mini kit column protocol (QIAGEN, Doncaster, Australia) and iScript cDNA synthesis kit (Bio-rad, Gladesville, New South Wales, Australia). Relative gene expression of key steroidogenic genes was investigated in adrenal glands from E5, E15 and E20. *Mc2r*, *Star*, *Cyp21a1*, *Cyp11b1*, *Cyp11b2* and *Agtr1a* were measured using the RT2 Profiler PCR Array (QIAGEN) and normalised to the geomean of housekeeper genes *Ppib*, *Gusb* and *Rn18S*. *Nr3c1* and *Hsp90a1* in adrenal glands and labyrinth gene expression of *Crh*, *Crh-r1*, *Nr3c1*, *Cyp17a1* was measured using TaqMan assay on demand primers (Applied Biosystems, Foster City, CA) and normalised to the expression of *Rn18S*. Expression was analysed by comparing to the average of

control dams and by using the $\Delta\Delta C_T$ method as described in Chapter Two for adrenal glands, and compare to the average of male control for the placental labyrinth.

5.3.8 Statistics

All graphs and statistical tests were performed using GraphPad Prism 7 software (GraphPad, Inc., San Diego, CA). As samples were collected from different cohorts of rats, no analysis was made between animals of different gestational ages except for the change in body weight over gestation, which was collected from animals from the E20. Statistical significance was accepted when p-values were significantly less than 0.05 (*$p < 0.05$, **$p < 0.01$, ***$p < 0.001$). All data presented as the mean ± standard error of the mean. Student t-tests were used to compare control and PC:EtOH groups for tissue weights, gene expression, hormone analysis, urinary and plasma parameters. When an unequal variance was determined, Mann-Whitney non-parametric tests were used. Placental gene expression was analysed using a two-way measured of analysis of variance (ANOVA), with a Bonferroni posthoc analysis. Bodyweight gain over gestation was measured using an ANOVA.

5.4 Results

5.4.1 Body and relative organ weights throughout pregnancy

The body weight and change in body weight of dams significantly increased over gestation (Ptime < 0.001, Figure 5. 1A and B) however, there was no significant difference due to treatment. At post-mortem, maternal weight body weight at E5, E15 and E20 were not significantly different in either control or PC:EtOH dams (Table 5. 1). Furthermore, maternal organ weights were not significantly different following treatment with PC:EtOH at any time points (Table 5. 1).

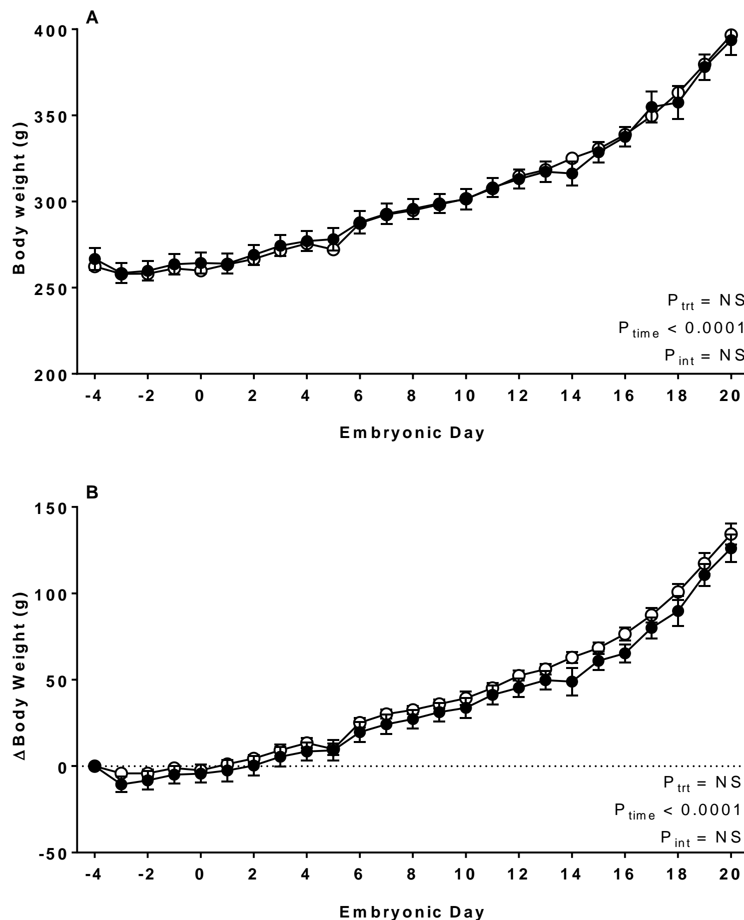


Figure 5. 1: Bodyweight (grams [g], A) and change (Δ) in body weight (as gram body weight change from the day of mating [g/gBW], B) of dams from embryonic day -4 to 20, exposed to control (white circles) or PC:EtOH (black circles) diet. Data presented as the mean ± standard error of the mean and analysed by two-way repeated-measures Analysis of Variance, with a Bonferroni posthoc analysis. Sample size: control = 15, PC:EtOH = 16.

Table 5. 1: Maternal body weight, relative total adrenal and relative pituitary weight following exposure to control or PC:EtOH diet at embryonic day (E) 5, 15 and 20.

Organ	E5			E15			E20		
	Control	PC:EtOH	Sig	Control	PC:EtOH	Sig	Control	PC:EtOH	Sig
Bodyweight (g)	282 ± 6 (n = 8)	269 ± 7 (n = 11)	NS	333 ± 12 (n = 9)	349 ± 8 (n = 9)	NS	376 ± 6 (n = 10)	364 ± 10 (n = 11)	NS
Relative adrenal (mg/gBW)[a]	0.21 ± 0.02 (n = 8)	0.22 ± 0.02 (n = 10)	NS	0.21 ± 0.02 (n = 9)	0.22 ± 0.02 (n = 9)	NS	0.20 ± 0.01 (n = 14)	0.22 ± 0.01 (n = 15)	NS
Relative pituitary (mg/gBW)[b]	0.06 ± 0.01 (n = 5)	0.05 ± 0.01 (n = 9)	NS	0.04 ± 0.01 (n = 8)	0.04 ± 0.01 (n = 6)	NS	0.04 ± 0.01 (n = 6)	0.04 ± 0.01 (n = 5)	NS

Data presented as the mean ± standard error of the mean, analysed by t-test. Sample size (n) is listed in the table. Sig: Significance, BW: body weight, g: grams, mg: milligrams, mg/gBW: milligrams per gram body weight.

[a]Average of two adrenal glands.

[b]Total pituitary weight, including anterior gland, posterior gland and the median eminence.

*5.4.2 **Early gestation**: Hormonal status and adrenal steroidogenesis*

At E-2, plasma corticosterone was significantly increased by 45% in dams treated with PC:EtOH compared to animals treated with a control diet ($P < 0.05$, Figure 5. 2A). There was no effect of PC:EtOH on plasma corticosterone at E2 (Figure 5. 2B). However, following the rats return to chow at E5, there was an observed reduction in plasma corticosterone by 62% ($P < 0.05$, Figure 5. 2C).

Given that dams treated with PC:EtOH showed altered plasma corticosterone concentrations at E5, relative expression of adrenal gland steroidogenesis genes was analysed. Interestingly, PC:EtOH did not alter expression of *Mc2r, StAr, Cyp21a1, Cyp11b1*, *Hsd11b2* or *Nr3c1* at E5 (Figure 5. 3). Interestingly, *Cyp11b2, Agtr1a* and *Hsp90a1* (Figure 5. 3, $P_{trt} < 0.05$) expression were significantly higher in these dams.

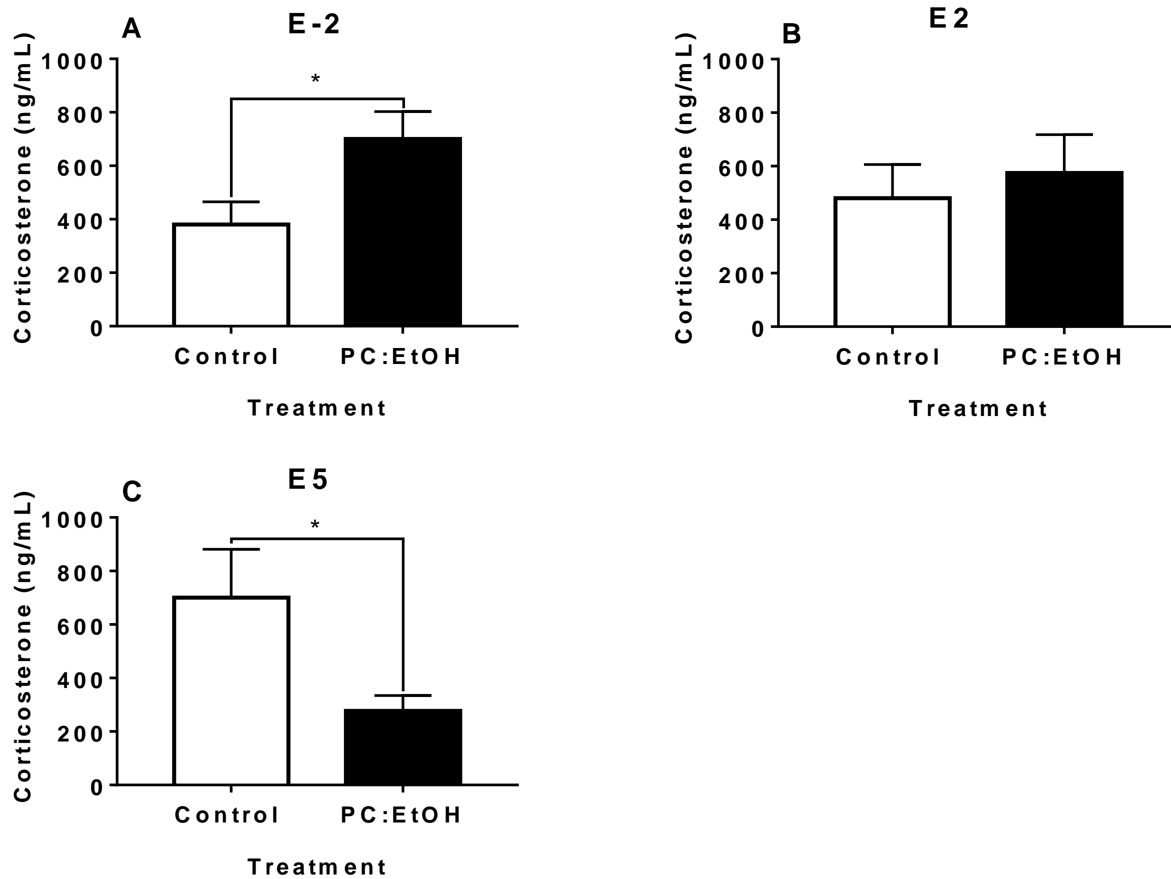


Figure 5. 2: Plasma corticosterone concentrations (ng/mL) at embryonic day (E) -2 (A), E2 (B) and E5 (C) in dams following exposure to control (white bars) or PC:EtOH (black bars) diet. Data presented as the mean ± standard error of the mean and analysed by Student's t-test or Mann-Whitney test, as required. *$p < 0.05$. Sample size: E-2; control = 7, PC:EtOH = 8. E2; control = 4, PC:EtOH = 6. E5; control = 8, PC:EtOH = 10**.**

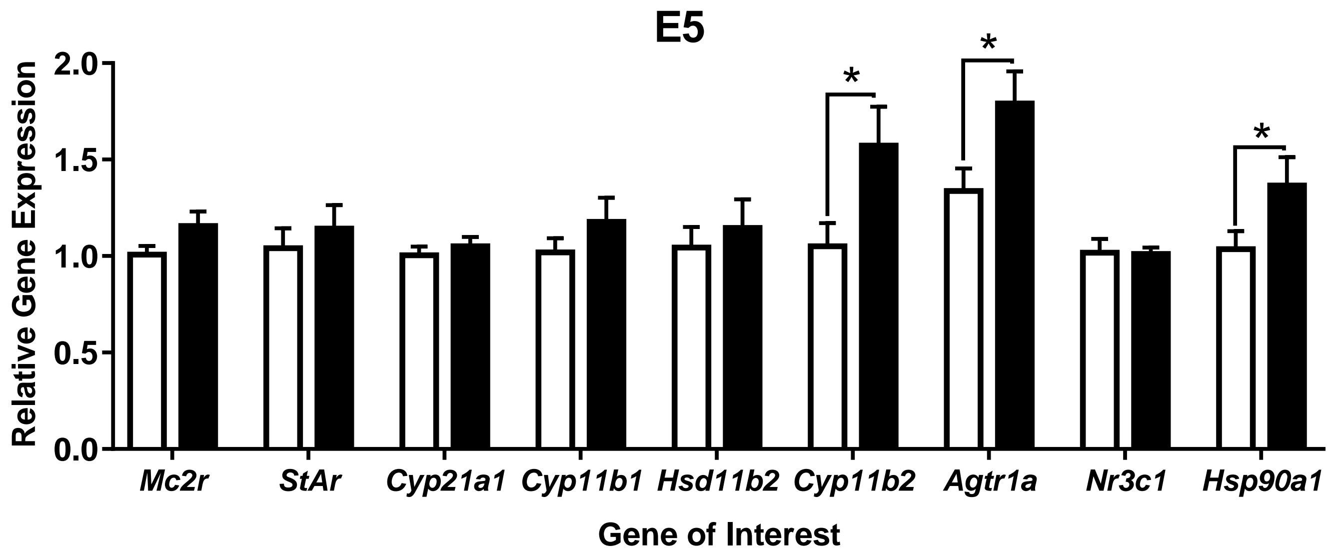


Figure 5. 3: Relative adrenal gland gene expression of melanocortin receptor 2 (*Mc2r*), steroidogenic acute protein (*Star*), cytochrome P450 family 21 subfamily A polypeptide 1 (*Cyp21a1*), cytochrome P450 family 11 subfamily B polypeptide 1 (*Cyp11b1*), 11-beta-dehydrogenase isozyme 2 (*Hsd11b2*), cytochrome P450 family 11 subfamily polypeptide 2 (*Cyp11b2*), angiotensin 2, receptor, type 1a (*Agtr1a*), nuclear receptor subfamily 3 group C member 1 (*Nr3c1*) and heat shock protein 90 alpha 1 (*Hsp90a1*) in dams at embryonic day (E) 5 exposed to control (white bars) or PC:EtOH (black bars) treatments. Data presented as the mean ± standard error of the mean and analysed by Student's t-test or Mann-Whitney non-parametric test, as required. *P<0.05. Sample size = 8 per treatment.

5.4.3 Mid to late gestation: Hormonal status and adrenal steroidogenesis

PC:EtOH did not result in changes to plasma corticosterone in dams at E15 (Figure 5. 4). However, PC:EtOH induced a 30% increase in plasma corticosterone concentration in dams at E20 ($P < 0.05$, Figure 5. 4B). However, there was no significant difference in relative expression of steroidogenic genes, *Mc2r*, *Star*, *Hsd11b2*, *Cyp11b2*, *Agtr1a* or glucocorticoid signalling genes, *Nr3c1* or *Hsp90a1* in dams at E15, however there was a significant increase in expression of *Cyp21a1* ($P < 0.05$) and *Cyp11b1* ($P < 0.001$) at this age (Figure 5. 5). PC:EtOH did not change gene expression of key steroidogenic genes *Mc2r*, *Star*, *Cyp21a1*, *Cyp11b1*, *Hsd11b2*, *Cyp11b2*, *Agtr1a*, *Nr3c1* or *Hsp90a1* at E20 (Figure 5. 6).

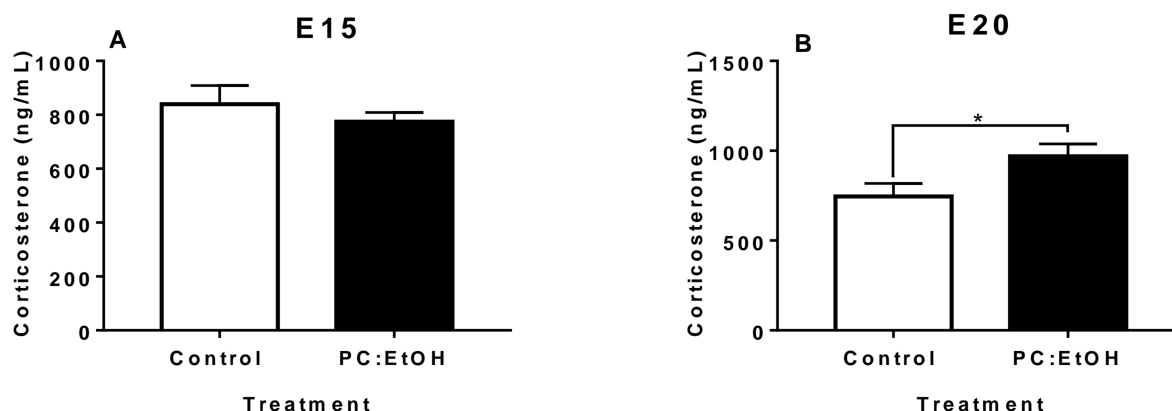


Figure 5. 4: Plasma corticosterone concentration (ng/mL) in dams at embryonic day (E)15 (A) and E20 (B), exposed to control (white bars) or PC:EtOH (black bars) diet. Data presented as the mean ± standard error of the mean and analysed by students t-test. Sample size: E15; control = 9, PC:EtOH = 8. E20; control = 8, PC:EtOH = 9.

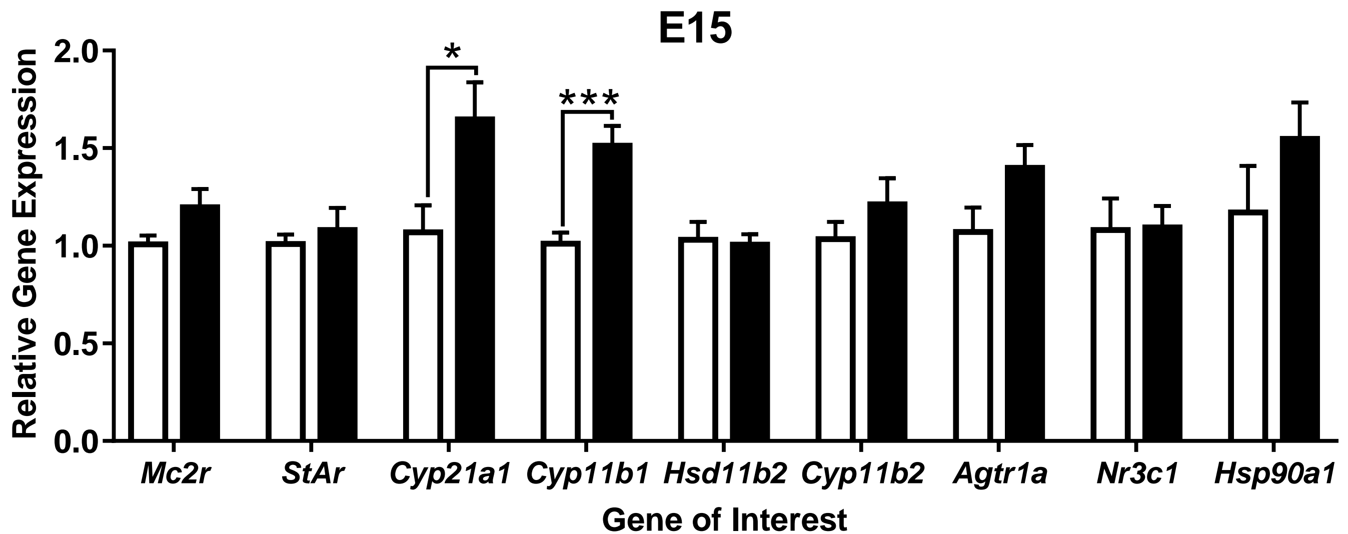


Figure 5. 5: Relative adrenal gland gene expression of melanocortin receptor 2 (*Mc2r*), steroidogenic acute protein (*Star*), cytochrome P450 family 21 subfamily A polypeptide 1 (*Cyp21a1*), cytochrome P450 family 11 subfamily B polypeptide 1 (*Cyp11b1*), and 11-beta-dehydrogenase isozyme 2 (*Hsd11b2*), cytochrome P450 family 11 subfamily polypeptide 2 (*Cyp11b2*), angiotensin 2, receptor, type 1a (*Agtr1a*), nuclear receptor subfamily 3 group C member 1 (*Nr3c1*) and heat shock protein 90 alpha 1 (*Hsp90a1*) in dams at embryonic day (E) 15, exposed to control (white bars) or PC:EtOH (black bars) diet. Data presented as the mean ± standard error of the mean and analysed by Student's t-test or Mann-Whitney non-parametric test, as required. Sample size = 8 per treatment.

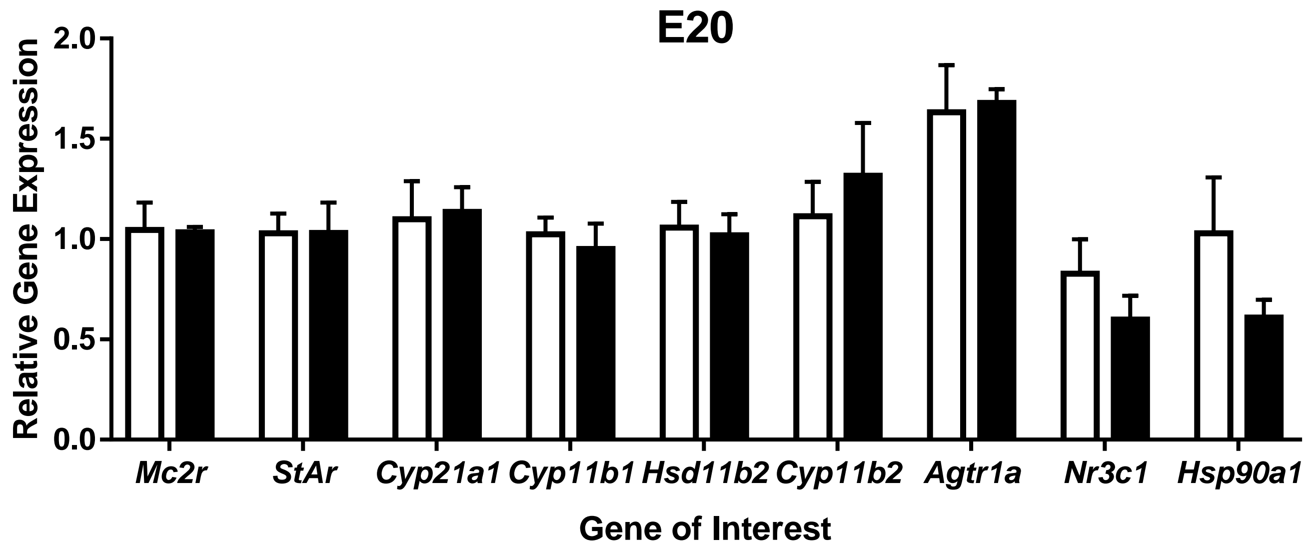


Figure 5. 6: Relative adrenal gland gene expression of melanocortin receptor 2 (*Mc2r*), steroidogenic acute protein (*Star*), cytochrome P450 family 21 subfamily A polypeptide 1 (*Cyp21a1*), cytochrome P450 family 11 subfamily B polypeptide 1 (*Cyp11b1*), and 11-beta-dehydrogenase isozyme 2 (*Hsd11b2*), cytochrome P450 family 11 subfamily polypeptide 2 (*Cyp11b2*), angiotensin 2, receptor, type 1a (*Agtr1a*), nuclear receptor subfamily 3 group C member 1 (*Nr3c1*) and heat shock protein 90 alpha 1 (*Hsp90a1*) in dams at embryonic day (E) 20, exposed to control (white bars) or PC:EtOH (black bars) diet. Data presented as the mean ± standard error of the mean and analysed by Student's t-test or Mann-Whitney non-parametric test, as required. Sample size = 8 per treatment.

5.4.4 Maternal aldosterone concentration

As there were significant changes observed in gene expression of *Cyp11b2* and *Agtr1a* at E5, plasma aldosterone concentrations were investigated. However, PC:EtOH exposure did not result in any significant differences in aldosterone concentration at either E5 or E15 (Figure 5. 7A and B, respectively).

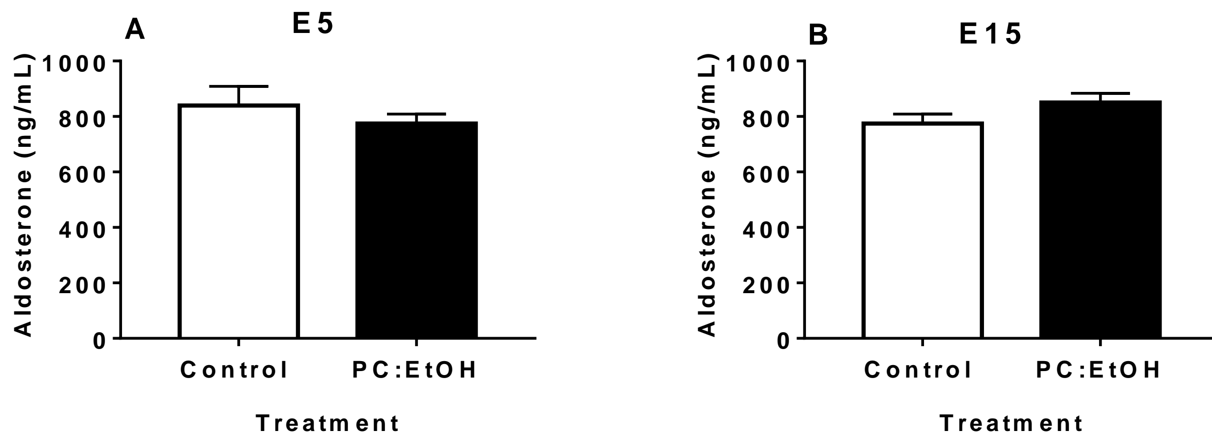


Figure 5. 7: Plasma aldosterone concentration (ng/mL) in dams at embryonic day (E) 5 (A) and E15 (B), exposed to control (white bars) or PC:EtOH (black bars) diet. Data presented as the mean ± standard error of the mean and analysed by students t-test. Sample size: E5; control = 9, PC:EtOH = 8. E15; control = 8, PC:EtOH = 9

5.4.5 Maternal renal and metabolic parameters

As HPA function within the dam is essential for regulating renal and metabolic pathways, these parameters were assessed at E16 and E18 respectively.

Food and water consumption, as well as urinary flow over the 24 hours of metabolic cage testing, was not significantly altered following PC:EtOH exposure (Table 5. 2). Urinary excretion of electrolytes and creatinine was not significantly altered between control and PC:EtOH dams over the 24 hours (Table 5. 2). HDL, LDL or triglycerides in the plasma of dams at E18 were not affected by PC:EtOH exposure (Table 5. 2).

Table 5. 2: Maternal urinalysis following metabolic cage testing at embryonic day 16 and plasma metabolic parameters at embryonic day 18.

	Parameter	Control	PC:EtOH	Sig
	Body Weight (g)	322 ± 7	311 ± 5	NS
	Food consumption (g)	25.9 ± 4.5	34.42 ± 10	NS
	Water consumption (mL)	34.7 ± 3.6	34.1 ± 2.5	NS
E16 Urinary analysis	Urinary excretion (mL/gBW)	0.04 ± 0.01	0.03 ± 0.01	NS
	Urinary Sodium (mmol/L)	65.7 ± 7.3	88.7 ± 13.2	NS
	Urinary Chloride (mmol/L)	105.1 ± 7.6	140.3 ± 14.8	NS
	Urinary Potassium (mmol/L)	209.1 ± 14.2	255.7 ± 17.4	NS
	HDL (mmol/L)	1.2 ± 0.3	1.0 ± 0.3	NS
E18 Plasma metabolic parameters	LDL (mmol/L)	0.5 ± 0.1	0.8 ± 0.3	NS
	Triglycerides (mmol/L)	4.7 ± 0.3	1.5 ± 0.5	NS

BW: body weight. g: grams. HDL: high-density lipoprotein, LDL: low-density lipoprotein. E: embryonic day. Sample size = 7 per treatment group. Data presented as mean ± standard error of the mean. Analysed by students t-test or Mann-Whitney non-parametric test. Sig: significance, NS: non-significant.

5.4.6 Placenta: Labyrinth gene expression

Given that maternal corticosterone was significantly elevated at E20, we investigated the expression of genes in the placenta that are known to be altered by or contribute to fetal exposure to glucocorticoid exposure. PC:EtOH significantly increased the relative gene expression of *Crh-r1* in both male and female labyrinth (Figure 5. 8A, Ptrt < 0.01), however only increased expression of *Nr3c1* in the female labyrinth, but not male was observed (Figure 5. 8B, p< .05). There was no significant difference in *Nr3c2* (Figure 5. 8C) following PC:EtOH in either male or female placenta.

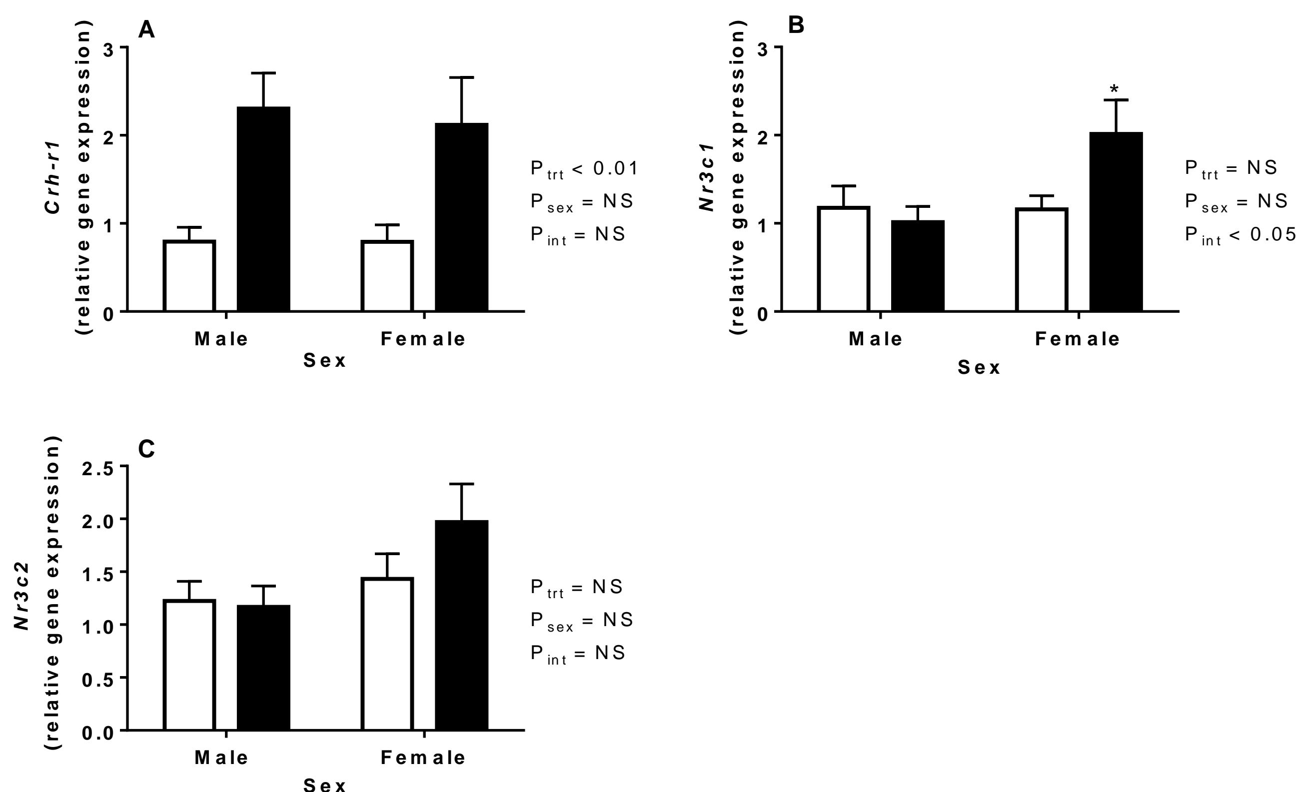


Figure 5. 8: Relative expression of glucocorticoid signaling genes, corticotropin-releasing hormone, receptor 1 (*Crh-r1,* A), nuclear receptor subfamily 3 group C member 1 (*Nr3c1,* B), and nuclear receptor subfamily 3 group C member 2 (*Nr3c2*, C) in labyrinth of male and female placenta at embryonic day (E) 20 following control (white bars) or PC:EtOH (black bars) treatment. Data are presented as the mean ± SEM and analysed by a two-way analysis of variance, with a Bonferroni posthoc test, *P<0.05. Sample size: Control; male = 6, female = 8. PC:EtOH; male = 8, female = 9.

5.5 Discussion

There has been minimal research performed to determine the impacts of ethanol consumption on maternal physiology across pregnancy. As such, this study investigated how alcohol intake around the time of conception influences maternal glucocorticoid concentrations over gestation using a highly clinically relevant animal model of alcohol consumption. Rats exposed to PC:EtOH had significantly greater plasma corticosterone concentrations during ethanol treatment prior to conception (E-2). However, this was not observed following conception (E2) despite this time point being within the PC:EtOH period. During early gestation (E5), PC:EtOH significantly reduced plasma corticosterone, although there was no significant difference in expression of adrenal steroidogenesis genes related to glucocorticoid signalling, except *Hsp90a1*. Interestingly, there were elevations in the relative gene expression of adrenal markers of RAAS, *Cyp11b2* and *Agtr1a* at E5, without a significant increase in plasma aldosterone at E5 or E15. In mid-gestation, at E15, PC:EtOH exposure resulted in significant elevations in *Cyp21a1* and *Cyp11b1* without changes in plasma corticosterone. At E16, renal function was not a by altered PC:EtOH and similarly at E18 plasma lipid measures were not different. Interestingly, at the end of gestation (E20), plasma corticosterone was elevated despite no changes to the expression of steroidogenic genes at this age. At E20, placental expression of glucocorticoid-responsive genes were elevated in the placenta suggests that the elevated corticosterone in addition to early exposure to PC:EtOH at this age may be impacting placental function. Overall, these dynamic alterations to maternal corticosterone following PC:EtOH is highly likely to be contributing to the observed programming of HPA function and regulation in offspring.

5.5.1 Early gestation

It is well accepted that alcohol consumption stimulates the HPA in both non-pregnant humans and animals [527,551]. It was therefore not surprising that this study demonstrated a significant increase in corticosterone at E-2 in dams treated with PC:EtOH. Interestingly, there was no increase in corticosterone following conception at E2, but a decrease in corticosterone at E5. To our knowledge, this is the first time that these dynamic changes in plasma corticosterone concentration have been observed at such an early time point. These results may suggest significant implications for pregnancy establishment and progression when alcohol is consumed at this time. Disrupted glucocorticoid status around the time of conception is known

to result in reduced fertility, altered ovarian and uterine receptivity and maintenance of a successful pregnancy. *In vitro* fertilisation patients with either increased or decreased cortisol, have reduced fecundability and a 90% increased risk of miscarriage [376,377]. It is important to note that despite changes in corticosterone at E-2, there was no significant difference in mating success within this study.

Contrary to the hypothesis of increased plasma corticosterone concentration throughout gestation following PC:EtOH, the corticosterone concentrations in PC:EtOH dams at E2 were similar to control, with a decrease in concentration at E5. One suggestion for these outcomes is an attempt to return to an optimal physiological baseline regardless of alcohol exposure, independent of the hormonal changes during early gestation. A study carried out by Wand *et al.* demonstrated a downregulation within the HPA of animals with repeat ethanol exposure, due to a reduction in POMC synthesis, and therefore decreased ACTH production and downstream corticosterone production [552]. Furthermore, it has been suggested that this habituation may also impair the ability for the HPA to respond to stressors. These claims also support the decrease in plasma corticosterone observed at E5, whereby this habituation may be overcompensatory for the PC:EtOH treatment.

Interestingly, a study of periconceptional undernutrition in a sheep model demonstrated reduced maternal cortisol during mid-gestation (day 42 to 80) [553]. Similarly, a study by Bloomfield *et al.* utilised a sheep model of undernutrition for 60 days before mating, and revealed maternal HPA suppression, with significantly reduced cortisol during treatment [385], suggestive of overcompensatory regulatory pathways. Limited literature is available for the impacts of decreased glucocorticoid levels during early pregnancy on long-term outcomes. However, it has been demonstrated that patients with undiagnosed Addison's Disease, characterised by underproduction of cortisol, show reduced fertility and increased risk of spontaneous abortion [554–556]. Women who suffer from Post-Traumatic Stress Disorder, where reduced plasma cortisol is observed, also demonstrate an increased risk of early complications such as ectopic pregnancies and miscarriage [557]. It is important to note that despite the changes in corticosterone presented in this chapter, there was no difference in the number of resorption sites within the uterus during early gestation, suggesting no early spontaneous abortions or fetal losses, however, increased resorptions/non-viable fetuses was observed later in pregnancy [102,558].

There are some pathways that may be impacted following PC:EtOH in dams, contributing to the altered plasma corticosterone concentration. These adaptations may include changes to function and signalling in the hypothalamus, pituitary, adrenal and associated hormones, or within the limbic system of the dams, all which are highly dynamic during the periconceptional period [559–561]. Hormonal changes including prolactin-like hormones which are secreted into the maternal system to mediate a range of biological processes including modulation of enzymes involved in the metabolism of hormones such as estrogen and progesterone [562] may be contributing to these results, with many having interactions with glucocorticoid pathways. Although these hormones were not significantly changed at a later stage in gestation (E5 and E7 respectively) within this model [558], several elements in downstream pathways were altered within the uterus. This may suggest that other maternal elements related to these hormonal pathways may be altered. As genes associated with the production of glucocorticoids (*Mc2r, StAr*, C*yp21a1, Cyp11b1*, and *Hsd11b2*) in the adrenal gland were not significantly different at E5, the reduction in plasma corticosterone may be a result of indirect changes. Corticosteroid Binding Globulin is regulated by estrogen, and upregulation of this would reduce free plasma corticosterone concentration resulting in the reduction observed at E5.

Interestingly, at E5, there was a significant increase in gene expression of *Cyp11b2* and *Agtr1a*. This suggested that PC:EtOH may be inducing alterations to the RAAS, crucial in regulating the production and release of plasma aldosterone. Furthermore, Gardebjer *et al.* demonstrated that dams treated with the PC:EtOH diet had greater water consumption only during the treatment period [102]. Interestingly though, there was no significant change in plasma aldosterone concentration at E5. Regardless of these findings, as it has been indicated in the literature that alcohol stimulates the RAAS [563], it would be advantageous to interrogate the impact of PC:EtOH on this system earlier in pregnancy and even before conception.

5.5.2 Mid and late gestation

This study hypothesised that PC:EtOH would program a sustained elevation in plasma corticosterone concentration. Plasma corticosterone was not elevated by PC:EtOH at E15, however, at E20 an increase was observed. These results suggest that there may have been critical adaptations to the maternal HPA to normalise corticosterone concentrations by E15 but may be contributing to the elevated corticosterone at E20. Indeed, the increased gene expression of *Cyp21a1* and *Cyp11b2* in PC:EtOH dams at E15 is likely to have a role in the

observed increased concentrations several days later at E20. The overcompensation of plasma corticosterone at E20 is similarly seen in a study by Bloomfield *et al.* which demonstrated that, upon return to normal nutritional status following periconceptional undernutrition, maternal HPA activity returned to a level higher than baseline for several weeks, albeit determined by measurement of ACTH [385]. These results collectively suggest that insults during the periconceptional period may result in a 'carry-over' adaptation whereby HPA activity is elevated. This may have adverse impacts on a range of maternal parameters as well as impacts on fetal development. Indeed, within our laboratory, studies have demonstrated increased resorptions at both E15 [558], and at E20 in separate cohorts, as well as *in utero* growth restriction at E20 [102], it may be implied that this PC:EtOH induced elevation in maternal corticosterone has significant implications on fetal viability and programming outcomes in those fetuses that survived pregnancy.

Glucocorticoids during late pregnancy are essential for regulating maternal metabolism, ensuring the appropriate fetal nutrient provision, as well as preparing the fetus for birth, including final maturation of the lungs, thermoregulatory systems and stimulation of parturition [564–567]. However, considering elevated glucocorticoid concentrations at E20, it was interesting to observe that at E16 renal parameters were unaffected by PC:EtOH, and at E18 metabolic parameters were not significantly altered. Although this would need to be assessed at E20 when corticosterone changes were observed, this suggests that appropriate renal and metabolic homeostasis is maintained in dams exposed to PC:EtOH. In another preliminary study within our laboratory, there was no evidence of elevated maternal glucose or insulin, with dams responding appropriately to glucose tolerance tests.

5.5.3 Placental labyrinth

A key link between the elevated maternal glucocorticoids at E20 with impaired fetal development and offspring outcomes may be due to placental changes observed at this time-point. Numerous studies within our laboratory have investigated the link between PC:EtOH and the placenta with evidence demonstrating a number of changes including in placental structure, morphology and gene expression, associated with *in utero* growth restriction [102]. Furthermore, PC:EtOH exposure resulted in elevated gene expression of *Hsd11b2* in both male and female placenta, suggesting perturbed glucocorticoid signalling pathways [102]. As the placenta regulates fetal exposure to elevations in maternal glucocorticoid concentrations and is

susceptible to the effects of glucocorticoids itself [568], this study aimed to further characterise changes in these pathways following this exposure. Increases in gene expression of *Crh-r1* in both male and female offspring, with only a significant increase in *Nr3c1* in female offspring, suggest that the placenta is adapting to the elevations in maternal corticosterone. These sex-specific responses to glucocorticoids may be contributing to disease phenotypes that are more overt in female offspring. While we would like to propose that these sex-specific placental adaptations may be occurring as a result of the increased maternal corticosterone concentrations at E20, additional studies are required to determine the relative contribution of this secondary insult and PC:EtOH exposure to the observed phenotype in offspring.

5.5.4 Limitations

While this study was designed with the intention of investigating maternal outcomes, by its very nature, this created limitations that suggest additional studies are required. As a large number of pregnancies were required to generate all the sample requires for this chapter, as well as other studies within the laboratory, maternal plasma samples were collected from several cohorts over the space of three years. Plasma samples, while collected at the same time of day, were not all from 'unstressed' rats, with dams at E5 and E15 having been transported prior to collection. These experimental conditions may have implications for the results collected. Ongoing studies using rats that have corticosterone concentrations measured in the same individuals at multiple time points of pregnancy are being carried out to confirm results observed in the current study. Additionally, this study is one of the first of its kind, particularly in regards to investigating the impact of alcohol during the periconceptional period on the maternal HPA over pregnancy. For this reason, a limitation in this study is the lack of direct comparison to other studies, but rather comparing and contrasting to other perturbations. Furthermore, the perturbed trajectory of maternal corticosterone concentration due to PC:EtOH may have significant implications on fetal adrenal gland function and development. Studies have determined that when maternal adrenalectomy has been performed, the fetal adrenal gland was activated to partly return the concentration of glucocorticoids to normal [569], potentially damaging their normal development, as well as influencing the maternal physiological system. Additionally, studies removing the fetal adrenal gland during pregnancy resulted in reduced maternal ACTH concentration and reduced corticosteroid concentration, suggesting that the impact of PC:EtOH on maternal and fetal physiology may be bidirectional.

5.5.5 Overall significance

The perturbations in HPA function demonstrated in this model as a result of PC:EtOH may have significant implications for both early and late pregnancy outcomes. The rates of infertility in Australia and worldwide ranging from 3 to 16 % of all women [570,571]. Given that PC:EtOH increases glucocorticoid production around conception, low rates of fertility may be partially due to high rates of alcohol consumption among women of reproductive age. HPA dysfunction in pregnancy increases the risk of pregnancy complications such as pre-eclampsia, hypertension and gestational diabetes. These subsequently increase the risk of post-partum cardiovascular disease and diabetes mellitus [572–577], highlighting that alcohol consumption should be avoided to minimise the risk of these complications.

Furthermore, long term maternal adaptations to alcohol exposure around conception may impair later HPA function having a significant implication on maternal health outcomes. Previous epidemiological studies following women whose pregnancies coincided with environmental disasters have indicated that elevated maternal glucocorticoids increase the risk of long-term adverse maternal outcomes, including cardiovascular conditions [578–580] and higher incidences of mental illness [542,581–583]. As the HPA is critical for mental health, and given that suicide is the leading cause of death in a pregnant woman [568,584], this again supports the importance of avoiding alcohol around conception.

Overall, alcohol consumption during pregnancy is preventable, and thus it is essential to increase knowledge of the impacts of alcohol consumption around the time of conception, and how this may contribute to the vast array of adverse outcomes that occur commonly in pregnancy.

5.5.6 Conclusions

To our knowledge, this is the first study that has investigated the impacts of PC:EtOH on maternal physiology, and contributes to a sparse field of knowledge. This research determined that PC:EtOH results in a dysregulation of the HPA contributing to both elevated and suppressed corticosterone concentrations in a gestational age-dependent manner. These findings suggest an essential role of the HPA in contributing to the programmed disease outcomes demonstrated in other chapters of this thesis.

Chapter Six

General Discussion

"The entire universe rested at my mothers' feet"
- Rupi Kaur-

6.1 Thesis summary

The primary focus of this thesis was to determine the impact of PC:EtOH exposure on the HPA of both mother and offspring in a rat model. This research is critical as up to 50% of pregnancies are unplanned [44,45], and 30% of women consume alcohol during the periconceptional period [44,46]. These statistics highlight that many pregnancies are exposed to alcohol during this early period and yet the full impact on both the mother and offspring is poorly understood. There were three predominant motives for investigating the impact of PC:EtOH on maternal and offspring HPA. Firstly, alcohol exposure throughout the entirety of pregnancy, or at later stages in gestation, is well accepted to result in altered offspring behaviour and HPA activity. Secondly, the HPA is essential for the homeostatic regulation of many systems within the body, and therefore, perturbations within this system may underlie many of the adverse outcomes observed in this model. Thirdly, the large array of data highlighting that the HPA is affected in many models of a perturbed *in utero* environment, suggest that elevated maternal glucocorticoids may be a common mechanism of developmental programming.

This chapter will discuss the findings of this thesis, which commenced with investigating the impacts of PC:EtOH on offspring behavioural outcomes, and HPA activity responses to physiological and psychological stressors (Chapter Three). This was followed by investigations to determine if HPA function is perturbed in unstressed offspring and how regulation of the HPA may be altered following PC:EtOH. This thesis then aimed to investigate if PC:EtOH is programming these offspring changes via alterations in maternal HPA physiology. These findings will be discussed within the context of the literature and other results demonstrated in this model. Finally, possible mechanisms (

Figure 6. 2), limitations and future directions will be discussed.

6.2 Chapter summaries

6.2.1 Chapter Three: Periconceptional ethanol exposure alters behaviours and HPA activity in young adult offspring

Chapter Three aimed to determine if PC:EtOH programs mental-illness like phenotypes and determine if PC:EtOH programmed HPA hyperactivity following both a physiological (DST/CST) and psychological (restraint) stressor. Finally, the adrenal steroidogenic gene expression was assessed.

Results of this chapter demonstrated that:

1. Both male and female offspring exposed to PC:EtOH displayed altered behaviour, indicative of a depressive-like phenotype, whereas only female offspring showed increased social interaction.
2. PC:EtOH exposed offspring demonstrated a hyperactive HPA response to the DST/CST but not the restraint stress, with sexual dimorphism observed in the results.
3. PC:EtOH did not program any changes in gene expression of key regulators of the adrenal steroidogenic pathway in either male or female offspring.

6.2.2 Chapter Four: Periconceptional ethanol exposure alters the stress axis in adult female but not male rat offspring

Next, Chapter Four investigated basal plasma corticosterone concentrations and aimed to determine if the expression of adrenal steroidogenic gene expression is altered at an older age in offspring exposed to PC:EtOH. This study also investigated plasma corticosterone and cardiovascular responses following a restraint stressor in aged rats. Finally, this chapter investigated hippocampal gene expression to determine if PC:EtOH altered pathways typically associated with HPA activity.

Results of this chapter demonstrated that:

1. PC:EtOH decreased corticosterone concentrations in both aged and adult female (but not male) offspring and altered the cardiovascular response to the restraint stress.

2. PC:EtOH did not impact adrenal steroidogenesis gene expression in aged male or female offspring.
3. PC:EtOH did not alter gene expression in the hypothalamus, but resulted in elevated expression of regulatory genes in the hippocampus (*Nr3c1* and *Hsp90a1)* of female PC:EtOH offspring only.

6.2.3 Summary of offspring outcomes in Chapter Three and Four

Overall, these results suggest that PC:EtOH may program mental illness-like phenotypes in offspring exposure, with underlying HPA hyperactivity. However, female offspring exposed to PC:EtOH may be more susceptible to adverse outcomes, with adult female offspring demonstrating reduced plasma corticosterone concentration. Aged female offspring also showed a significantly reduced plasma corticosterone but increased pressor responsiveness to restraint. These outcomes may be due to altered regulatory mechanisms within the hippocampus.

6.2.4 Chapter Five: Periconceptional ethanol exposure alters maternal and placenta glucocorticoid pathways throughout gestation in a rat

As altered phenotypes were observed within offspring following PC:EtOH exposure, the focus of this thesis turned to determine possible mechanisms. The maternal HPA, related pathways and glucocorticoid signalling in the placenta are essential in the development of the fetal HPA, offspring HPA function and behaviour. As such, Chapter Five investigated the impact of PC:EtOH consumption on maternal corticosterone, aldosterone and adrenal steroidogenesis pathways at significant stages in gestation. This was followed by an investigation of key glucocorticoid-related gene expression in the placenta.

Results of this chapter demonstrated that:

1. PC:EtOH resulted in alterations in maternal plasma corticosterone concentrations, including a decrease at E5 and an increase at E20 compared to animals on a control diet.
2. PC:EtOH resulted in increased gene expression of steroidogenic genes (*Cyp21a1* and *Cyp11b1*) at E15.
3. PC:EtOH programmed altered gene expression in genes regulating the RAAS system (*Cyp11b2* and *Agtr1a*) within the adrenal gland at E5. However, there were no changes in plasma aldosterone at E5 or E15, renal excretion of sodium, potassium

or chloride at E16, or plasma concentrations of low- and high-density lipoprotein or triglycerides at E18.

4. PC:EtOH resulted in increased gene expression of *Crh-r1* in placentas of both male and female fetuses and increased glucocorticoid receptor gene (*Nr3c1*) in female fetuses.

Overall, these results suggest that the HPA is altered in the mother, both during treatment (direct effects) and at the end of gestation (indirect effects), two weeks after the end of treatment. Changes were also observed late in gestation in the placenta throughout gestation, following PC:EtOH. As the appropriate function and regulation of the HPA is essential for the establishment and progression of pregnancy and fetal development, these changes are likely to be contributing to the adverse outcomes observed within the offspring. Although the research conducted during this PhD was investigated in the above order, the maternal HPA was altered during pregnancy and is likely central to the offspring outcomes. For this reason, this chapter will firstly discuss direct and indirect impacts of PC:EtOH on maternal and gestational outcomes, followed by a discussion of outcomes observed in adult and aged offspring.

6.3 The impact of PC:EtOH on maternal physiology

6.3.1 Maternal outcomes

Prenatal alcohol exposure and other perturbations related to the DOHaD hypothesis highlight, the effect of the maternal environment, can have on offspring health and disease outcomes. However, often the research focuses entirely on the offspring, and less research has been performed to comprehensively determine the impact of the perturbation itself on the maternal physiology. This is particularly true for insults and maternal behaviours around the time of conception, such as alcohol consumption, poor diet and smoking. Often it is assumed that once these are ceased or improved, pregnancy will progress healthily. However, this may not be the case as the maternal response to the perturbation may induce inappropriate pregnancy-related physiological changes likely to further contribute to poor fetal development and programming outcomes. Therefore, understanding the impact of the perturbation on maternal pathways would provide essential insight into mechanisms underlying offspring outcomes. Much literature has determined that the HPA is one such maternal system that may underlie outcomes following several perturbations.

6.3.1.1 Direct effects of PC:EtOH exposure

Within this model, PC:EtOH exposure occurred from 4 days prior to 4 days following conception, being removed on the morning of embryonic day 5. This is comparable to one menstrual cycle prior to pregnancy and the first six days of human pregnancy with a schematic shown in Figure 6. 1.

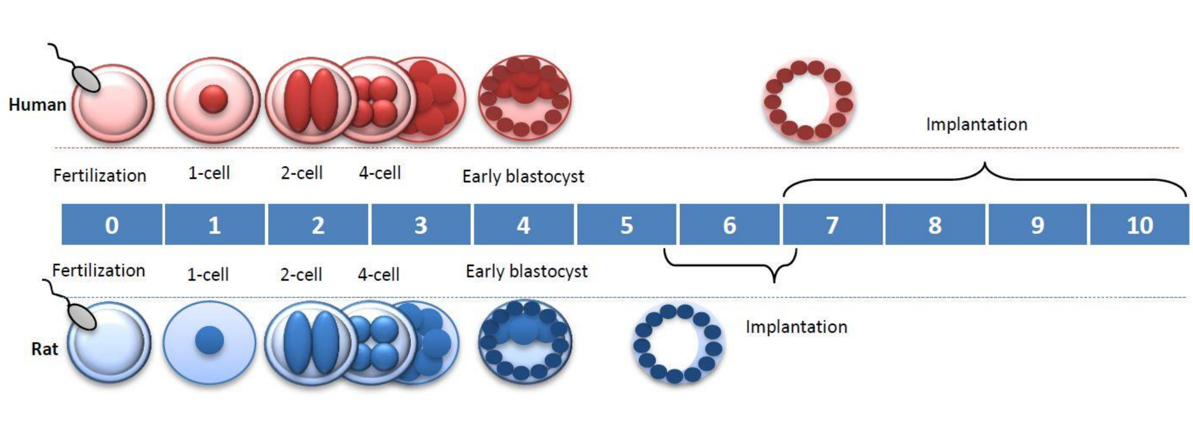


Figure 6. 1: Timing of peri-implantation development in the human and rat. Note that the peri-implantation period in humans and rodents (rat and mice) is comparable, allowing this model to be used to investigate the direct effects of periconceptional alcohol exposure. Image care of Gardebjer [585].

During this window of exposure, the 'direct' effects of alcohol exposure were investigated. As alcohol is well known to stimulate the HPA, the increased maternal corticosterone concentrations following two days of treatment (E-2) was predicted. This may have had significant impacts on pregnancy establishment and development. Studies of *in vitro* fertilisation demonstrated mothers had perturbed cortisol concentrations resulting in reduced fecundity and a 90% increased risk of miscarriage [376,377].

Interestingly, corticosterone concentration was comparable to control levels at E2, which may be indicative of maternal adaptations to normalise hormone levels. Contrary to the initial hypothesis that PC:EtOH treatment would increase plasma corticosterone throughout gestation, levels were significantly reduced at E5. This may suggest that these adaptations are maintained following alcohol exposure and would be of interest to investigate further in the days following cessation. It is important to note that, as discussed in Section 6.8 Limitations and future directions, this study had limitations, with plasma samples collected

from two separate cohorts. Future studies will investigate the ontology of corticosterone following PC:EtOH to further support this result.

Our previous work has demonstrated maternal hyperglycemia and increased water consumption during this period [102,367], which may be consequential of PC:EtOH induced changes to plasma corticosterone. Additionally, a number of other changes at this time have been determined within the Moritz laboratory (Table 6. 1). This includes reduced estrogen receptor expression, and changes to both the progesterone receptor and downstream gene pathways in the uterus, as well as physiological changes within the dam such as elevated triglyceride concentrations. These results indicate that this time is highly susceptible to the effects of PC:EtOH. As E5 corresponds with the time of implantation (Figure 6. 1), this suggests that the establishment of pregnancy may be impacted. Indeed, Kalisch-Smith *et al.* have determined that PC:EtOH impairs blastocyst outgrowth, reduces trophoblast differentiation, and alters embryo-uterine communication at E5 to E6 [558].

To our knowledge, reduced glucocorticoid concentrations during early gestation have not been observed in other prenatal ethanol exposure models. However, low glucocorticoid concentrations in early pregnancy have been observed in models of undernutrition [351,382,385,553,586], which also demonstrate altered development of the HPA and hyperactivity of the axis within the fetus [351,382,586] (discussed in Section 6.3.1.2 Indirect effects of PC:EtOH exposure). It is important to note that in the current PC:EtOH model, both control and treatment dams were receiving adequate and equivalent nutrition, including total calories and amounts of macro and micronutrients. However, the similar results across various perturbations indicate the converging mechanisms involving the maternal HPA This suggests that key role this system has in programming offspring HPA dysfunction.

Table 6. 1: The direct impacts of PC:EtOH on maternal parameters. Results are unpublished unless referenced.

Parameter	E-2	E2	E5
Growth	↔	↔	↔
Corticosterone	↑		↓
Maternal hyperglycaemia	↑ (E-4 to 4)	↑ (E-4 to 4)	
Water consumption[102]	↑ (E-4 to E0)	↑ (E0 to E4)	
Aldosterone			↔
Estrogen/Progesterone			↔
Estrogen receptor mRNA in the uterus			↓
Estrogen receptor mRNA in the uterus			↓
Progesterone response genes			↓
Triglycerides			↑
Adrenal gene expression			Δ

Another potential direct effect of PC:EtOH on early development is through epigenetic modifications. Epigenetics is the process whereby DNA, histones and other regulatory factor are modified to alter the accessibility of genes, modulating expression and respective cellular function [587,588]. During very early development, the blastocyst and early embryo undergo global DNA demethylation, followed by a remethylation process, as well as X-chromosome inactivation in female embryos and telomere lengthening [589,590]. Studies of *in vitro* embryo cultures treated with ethanol have demonstrated altered DNA methylation profiles and growth retardation [591,592]. Following PC:EtOH treatment, preliminary data of Kalisch-Smith *et al.* [558] has confirmed altered methylation markers in the blastocyst at E5. These changes may have long-term impacts on both fetal development and offspring physiological function. This is supported by studies of PAE throughout pregnancy being associated with epigenetic modifications in several HPA pathways, including POMC and MeCP2, hippocampal DNA methylation profile and one-carbon metabolism [484,593–595]. These changes may ultimately inhibit appropriate development of the placenta and essential fetal physiological systems. It may be that epigenetic changes due to PC:EtOH occur.

6.3.1.2 Indirect effects of PC:EtOH exposure

This model of PC:EtOH exposure was designed to mimic one of the most common forms of alcohol exposure during pregnancy with treatment being removed on the morning of E5. However, it was hypothesised that PC:EtOH exposure will result in sustained alterations to maternal physiology throughout pregnancy, particularly corticosterone, that may indirectly impact fetal development and contribute to offspring outcomes.

This thesis determined maternal plasma corticosterone was elevated at E20, which may act as a perturbation, in its own right. In support of this claim, numerous studies have indicated maternal stress or glucocorticoid treatment during pregnancy, programs offspring physiology and behaviour [285–287] (as discussed in Section

Stress and psychiatric illnesses are often associated with increased cardiovascular function and increased risk of cardiovascular disease [245–249.] Stress responses are generally associated with increased cardiovascular activity, with studies demonstrating that psychological stress results in arrhythmia and fatal cardiac events. In one such study, it was seen that following the Northridge earthquake, sudden deaths from cardiac causes increase by 4.6 per day in the week before to 24 per day on the day of the earthquake [250,] with similar results being shown following the 1995 Hanshin-Awaji earthquake in Japan [251.] Furthermore, other studies that individuals with high cardiac reactivity to stress also demonstrate hyperactivity of the HPA and sympathetic pathways [252.]

The main neurological pathways activated by stress are stimulation the HPA and sympathetic nervous system (SNS) and withdrawal of parasympathetic activity [253.] These systems interact to generate adaptive responses to both physiological and psychological threat, known as the 'fight-or-flight" response. Neurosensory signals are processed in the PVN of the hypothalamus, where CRH neurons directly innervate the locus coeruleus (LC) in the brainstem [252,254,255,] stimulating the release of noradrenaline (NA). This is a bidirectional pathway, with the SNS further stimulating the release of CRH and further activation of the HPA [252.] Stress-induced activation of the LC-NA pathway further stimulates peripheral catecholamine release, activation of α - and β-adrenergic receptors and the intermediolateral nucleus of the spinal cord. This subsequently increases blood pressure and heart rate [253,256,257] and highlights the intrinsic link between the stress response and the cardiovascular system. This interaction will be investigated in Chapter Four of this thesis.

1.7 Programming of the HPA). Other studies of alcohol consumption throughout gestation (36% ethanol derived calories) in rats also demonstrate elevated maternal glucocorticoid concentration at the end of pregnancy [387]. Subsequent studies using this model demonstrated impaired behaviour and HPA responsiveness [318,332], results comparable to those observed within this thesis.

A study of maternal undernutrition, in a sheep model, during the periconceptional period firstly demonstrated that cortisol was reduced immediately following the return to optimal nutritional requirements, however with HPA hyperactivity as determined by increased plasma ACTH concentrations, at the end of pregnancy [385]. This study further demonstrated that fetal HPA development was perturbed, where treatment with an adrenal steroid synthesis inhibitor, metyrapone, resulted in increased ACTH, 11-deoxycortisol and mRNA concentration of *Pomc* in the anterior pituitary gland [385]. Additionally, glucocorticoid administration in late gestation both sheep and rat models have demonstrated elevated offspring glucocorticoid concentration and HPA hyperactivity to stressors, with associated cardiovascular and metabolic outcomes [288,596]. These similarities further cement the concept that the maternal HPA is highly susceptible to perturbation and may be a common pathway linking adverse maternal environment to long-term offspring disease outcomes.

Another mechanism whereby PC:EtOH may be impacting offspring development is by indirectly influencing placental function, potentially in response to the elevated maternal corticosterone concentration. The placenta is the interface between maternal and fetal systems and has a crucial role in fetal exposure to maternal glucocorticoid concentration. This thesis revealed elevated labyrinth mRNA expression of *Crh-r1* in placentas of both sexes and *Nr3c1* in female placentas at E20. These changes, in association with increased corticosterone concentration, would likely result in excess glucocorticoid exposure to the fetus, particularly in females. Although mechanisms may be occurring within the placenta to regulate fetal exposure to excessive maternal corticosterone, this is unlikely to be fully compensatory, therefore compromising fetal development and resulting in programmed physiological dysfunction in offspring. Other research within our laboratory has demonstrated decreases in cytoplasmic protein concentration of the pro-growth/anti-apoptotic related glucocorticoid receptor isoform GRα-D3 in the male and the isoform GRα-A, related to metabolism and inflammation, in females (Kent *et al. unpublished data).* Another significant finding in the placenta is elevated gene expression of *Hsd11b2* following PC:EtOH, at E20 [102]. As this enzyme is responsible for converting active glucocorticoid to its inactive precursor [597], it may be suggested that increased expression is a protective

mechanism to the excess glucocorticoid concentrations and signalling. Although the activity of this enzyme is yet to be examined, as it appears that overall glucocorticoid-related pathways are elevated, we speculate that this protection may not be enough and fetal exposure to glucocorticoids is in excess.

This theory is supported by a study utilising the treatment of pregnant rats with a placental *Hsd11b2* inhibitor, carbenoxolone, whereby fetal exposure to glucocorticoids would be elevated, demonstrated similar outcomes as seen within our laboratory. This included *in utero* growth restriction, cardiovascular alterations, and impaired glucose tolerance in offspring. Interestingly, the negative impact of the inhibition of this enzyme was prevented by maternal adrenalectomy. These outcomes, as well as central HPA regulatory changes, were similarly observed in rodent (rat and mouse) models of both synthetic and endogenous glucocorticoids in late pregnancy [295,549,598].

Furthermore, investigations of the outcomes following excess glucocorticoid exposure have demonstrated that children of mothers who experienced stress or were treated with glucocorticoids at the end of pregnancy had altered basal glucocorticoid concentrations, HPA response to stressors such as heel prick blood draw, public speaking and mental arithmetic tasks (Trier Social Stress Test for children) [260–262,266–269] and impaired behaviour, including, including ADHD symptoms [270–272], elevated anxiety [272] and cognitive development [273]. This was similarly observed in animal studies. Similarly, animal studies of maternal stress programs altered offspring stress response, including concentrations of corticosterone and ACTH [274276], changes to glucocorticoid signalling genes [275] and altered neurological morphology, and suffer from a range of emotional deficits such as anxiety-like behaviour, as well as learning deficits and altered attention [278–284]. Exogenous treatment with synthetic glucocorticoids such as dexamethasone and betamethasone revealed several changes in the HPA function in offspring. This includes dysregulated corticosterone levels and stress responsiveness [285,287] and altered adrenal structure and gene expression [286,287].

Elevated maternal corticosterone may also have significant impacts on maternal brain structure and maternal care, as reviewed by Francis and Meaney [599], and act as another indirect impact of PC:EtOH. Maternal brain changes have been particularly observed within the hippocampus, which has a significant role in maternal care of pups following birth [600,601]. Some studies have shown that PAE results in reduced nursing, reduced pup-retrieval [602,603] and increased self-directed and negative behaviours such as stepping on and dragging pups [604]. This can have significant implications on the physical and mental health of children [605–

[609], supported by numerous animal studies demonstrated similar adverse developmental outcomes [610–615].

Increasing circulating fetal glucocorticoid concentration are essential in late gestation for development of key organ systems as well as contributing to parturition [566,616–618]. The fetal adrenal gland is activated around E16 in the rat, and interestingly, the fetal loss was observed in culls at both E15 and E20 [558]. It is postulated that this is a consequence of the alterations to the maternal HPA and other changes associated between E15 and E20. Once the fetal adrenal gland is active, excess maternal corticosterone and impaired placental signalling may further impact its function. Other fetal systems such as the early HPA, other brain regions and physiological systems may also be perturbed and require greater investigation.

Overall, this thesis demonstrated both direct and indirect consequences of PC:EtOH on maternal corticosterone concentrations, with an initial increase and subsequent decrease during the time of treatment and a significant elevation at E20. This excess glucocorticoid concentration is likely to have a significant number of subsequent effects, such as within the placenta, maternal brain structure and subsequent care of offspring.

6.4 An altered HPA in PC:EtOH offspring may underlie physiological dysfunction

This model of PC:EtOH has been utilised within the Moritz laboratory since 2011, with a vast number of outcomes determined in the dam, embryo, placenta as well as in offspring (Table 6. 2). The HPA is essential for numerous homeostatic processes and behaviour, and changes in its function following PC:EtOH exposure is suggested to be a significant contributor to the broad range of physiological deficits. Of particular relevance to this study are behavioural alterations within offspring including hyperactive and anxiety-like phenotypes [369], as well as changes to central reward pathways and circadian rhythm regulation of corticosterone and glucose (Lucia *et al.*, *unpublished data* and Wing *et al.*, *unpublished data*). Systemic dysfunction is also observed with overall perturbation of metabolism and cardiorenal function [367,368]. However, with the complexity and interconnected function and regulation of the HPA, the impact of PC:EtOH is likely to be a multifaceted web of dysfunction.

6.5 Sexual dimorphism in PC:EtOH outcomes

A common observation throughout studies investigating PC:EtOH exposure is sexual dimorphism of offspring outcomes. Within this study, both male and female offspring exposed to PC:EtOH displayed a depressive-like phenotype, whereas altered social interaction was only seen in female offspring. Although both sexes exposed to PC:EtOH showed HPA hyperactivity during the DST/CST, females had a delayed response, as well as reduced basal plasma corticosterone concentration at both adult and aged. This finding is supported within the literature, with many models of alcohol exposure throughout pregnancy demonstrating sexual dimorphism in HPA hyperactivity. Studies using prolonged restraint and cold stress resulted in a greater response in PAE males with increased anhedonic behaviour and activity, whereas females show increased responses to ethanol, morphine or ether challenges and increased immobility in the forced swim test and social interaction [330,424,425,491,492,619]. Sexual dimorphism is also observed in human studies where alcohol exposure during the first trimester increased mental health issues in girls [451]. Similarly, May *et al.* revealed that although boys and girls diagnosed with FASD display similar outcomes following PAE, sex ratios indicated that females had more neurocognitive impairment than boys [620]. These results suggest that it is essential to consider males and females individually, in both research and within the FASD diagnostic criteria.

Table 6. 2: A summary of results in the adult and aged offspring following PC:EtOH exposure.

		Adult (3 -10 months)		Aged (10-19 months)		Ref
		Male	**Female**	**Male**	**Female**	
Behaviour	Depressive-like	↑	↑			Chapter 3
	Affiliative social	↔	↑			Chapter 3
	Rearing	↔	↔			Chapter 3
	Anxiety-like	↔	↑	↓	↔	369
	Learning and memory	↔	↓			369
	Overall activity	↑	↑	↔	↑	Unpublished
Metabolic	Bodyweight	↔	↔	↔	↔	509
	Total FM	↑	↑			509
	Glucose intolerance	↑	↑			367
	Insulin insensitivity	↑	↑			367
	Leptin	↑	↔			509
	Cholesterol	↑	↑			509
	Food preference:					
	- Alcohol			↔	↔	368 (aged only)
	- HFD	↔	↔	↑	↑	368 (aged only)
Circadian	Circadian glucose pattern	↔	Δ			Unpublished
	Circadian corticosterone	↔	Δ			Unpublished
HPG	Estrogen				↑	Unpublished

	Hippocampal GE				↑	Chapter 4
	Basal corticosterone	↔	↓	↔	↓	Chapter 4
	Hypothalamus GE			↔	↔	Chapter 4
HPA	Adrenal steroidogenesis GE	↔	↔	↔	↔	Chapter 3, 4
	DST/CST	↑	↑			Chapter 4
	Restraint – Cort response	↔	↔	↔	↓	Chapter 4
	Restraint – SBP response			↔	↑	Chapter 4
	Liver GR isoform	Δ	Δ			Unpublished

Data within this table is collated from this thesis, as well as unpublished and published as denoted. *Abbreviations:* Cort: corticosterone, CST: corticotropin-releasing hormone stimulating test, DST: dexamethasone suppression test, FM: fat mass, GE: gene expression, GR: glucocorticoid receptor. HFD: high-fat diet, SBP: systolic blood pressure.

6.6 Regulation of the HPA and sexual dimorphic outcomes following PC:EtOH

6.6.1 Hypothalamic-pituitary-gonadal axis

The HPG axis contributes to sexual dimorphism that is observed in physiological systems and behaviours [621], and both regulates and is regulated by the HPA. Within this thesis, female offspring were only tested during diestrus; however PC:EtOH alterations within HPG function and sex hormone production may have contributed to offspring phenotypes. Some evidence of PC:EtOH induced modifications within the system have been observed with Dorey *et al.* (*unpublished data*) with elevated plasma oestradiol observed in age female offspring. While testosterone and dihydrotestosterone are known to inhibit HPA [622,623], oestradiol both stimulates and inhibits HPA activity by alternative processes. These processes include inhibiting hypothalamic and pituitary pathways, which both express estrogen receptors and by regulating plasma glucocorticoid concentrations by stimulating CBG production within hepatocytes, necessary for the regulation of bioavailable glucocorticoids [623–628]. Investigation of both PAE and prenatal stress paradigms have demonstrated reductions in plasma CBG concentrations in offspring [334]. As such, PC:EtOH alteration in estrogen and CBG concentration may explain the decreased plasma corticosterone concentrations in females, with CBG concentration in male PC:EtOH offspring ensuring normalised plasma corticosterone concentration. This suggests the importance of measuring markers of HPG function, as well as CBG in offspring to determine if these alterations may be contributing to results observed within this thesis.

6.6.2 The impact of PC:EtOH on central pathways

6.6.2.1 Pituitary glands

Although adrenal steroidogenesis gene expression pathways were the focus for this thesis, evidence of pituitary abnormalities in PC:EtOH offspring highlights that HPA hyperactivity may be a consequence of changes within the more central parts of the axis. This is particularly relevant as the pathologies observed within the pituitary gland were of greater prevalence within female PC:EtOH offspring. Perturbations to the pituitary may impact multiple endocrine networks and link alterations seen in glucocorticoid responsivity to central regulation of sex hormones. Although hypothalamic gene expression was not significantly altered by PC:EtOH exposure within aged offspring, alternate pathways within this region may be impaired. This indicates that further investigation is required.

6.6.2.2 Limbic system

Another neurological pathway essential in the regulation of the HPA is the limbic system. Results of this thesis indicate that PC:EtOH may program altered limbic function, with elevated hippocampal expression of *Nr3c1* and *Hsp90a1* in female offspring. The hippocampus is the most well studied limbic region contributing to HPA regulation and is generally considered as inhibitory [421,629–631]. Abnormal hippocampal function is known to contribute to several cognitive and mental illness outcomes, including depression and stress dysregulation via direct connection with the hypothalamus and other HPA regulatory regions [632]. Furthermore, animal studies of reduced glucocorticoid receptor expression due to hippocampal lesions, genetic manipulation or pharmacological intervention, all show diminished HPA feedback efficacy to stressors [235,493], and it may be concluded that the opposite may be true. Therefore, the increased *Nr3c1* expression in the hippocampus of aged female offspring in the current model is in accordance with the hyperactivity following the DST/CST.

Numerous other regions of the limbic system including the medial prefrontal cortex, amygdala and the mesolimbic pathway have an essential role in the regulation of the HPA [421,629–631,633,634] The amygdala is known to stimulate the HPA [634], whereas the medial prefrontal cortex has both a stimulatory and inhibitory effect of the HPA depending on the type of stimulus being received [635]. Moderate PAE exposure throughout gestation is shown to reduced *Nr3c1* mRNA expression in the amygdala of male offspring only [636]. Similarly, although sex differences were not observed, previous studies from our team demonstrated that chronic low dose exposure to alcohol during pregnancy altered basolateral amygdala structure, associated with an anxiety-like phenotype and increased social interaction [69]. These studies support the susceptibility of these limbic regions to the effects of alcohol exposure pregnancy and that changes within these structures may contribute to the outcomes observed in PC:EtOH offspring.

Interestingly, PC:EtOH may alter other central regions and downstream physiological pathways that are related to HPA function and may contribute to observed outcomes. Recent data from our laboratory has associated PC:EtOH with significant modifications in the SCN (Lucia *et al. unpublished data*). This is related to altered circadian regulation of glucose and corticosterone concentration, whereby PC:EtOH exposure resulted in an increase in mesor, amplitude and total blood glucose concentration, as well as a 7-hour shift in plasma corticosterone rhythm and increase concentration in female offspring only (Wing

et al., *unpublished data)*. This may indicate a mechanism linking HPA function with the adverse metabolic outcomes observed following PC:EtOH. PAE is also associated with alterations to the production of neurotransmitters such as serotonin and noradrenaline, γ-aminobutyric acid, glutamate, adenosine and cannabinoids are implicated in offspring behaviour related to the HPA. These have yet to be investigated in our model, but PAE has been shown to alter serotonin and associated behaviours, similar to what is seen within this thesis and other studies within this PC:EtOH model. This includes increased depressive-like phenotypes, such as immobility in the FST, and increased passive behaviour in social interaction paradigms, which may be interpreted as elevated social interaction and anxiety-like behaviours in the EPM and open field test [637].

6.7 The contribution of age to programmed disease risk of PC:EtOH

A common finding in our PC:EtOH model is increased disease state in female offspring in aged cohorts, suggesting that age might exacerbate programmed disease risk in a sex-specific manner. Within this thesis, plasma corticosterone concentration was comparable between control and PC:EtOH offspring following restraint at 5 months of age, however at 12-14 months, female offspring demonstrated reduced plasma corticosterone response despite greater systolic blood pressure response to restraint. Additionally, it was demonstrated that females remained hyperactive at 18 months of age, whereas males no longer displayed that phenotype. Dorey *et al.* (*unpublished data*) also revealed that female offspring had altered cardiorenal function at 12 and 19 months of age respectively, which was not observed at 6 months or in male PC:EtOH offspring at any time. A number of other studies have demonstrated sex-specific age of onset of several illnesses, including OCD, bipolar, depression, as well as cardiovascular and metabolic diseases [638–641]. Several theories have been suggested to explain these differences, including puberty related sex hormone changes and HPG function, the timing difference in brain development, as well as varied response to stresses between men and women [642]. These may all be impacted by PC:EtOH exposure. Furthermore, women experience a rapid loss of ovarian sex hormones following menopause, including progesterone and 17 beta-estradiol, and although testosterone production decreases with age in men, this is a much more gradual process [643]. These hormonal changes with age have associated with an increased risk of disease in women more than men, including Alzheimer's, cardiovascular disease, metabolic disease, reduced stress resilience, and some studies suggesting an increased incidence of mental illness [643–645]. Given these findings, it would be of interest to investigate when behavioural

and HPA related outcomes begin to manifest in our model of PC:EtOH. This is important as the timing of disease onset may impact the optimal timing for intervention to minimise the impact of PC:EtOH on lifelong health.

6.8 Limitations and future directions

The results of this study highlight the necessity of gaining a greater understanding of the impact PC:EtOH has on maternal physiology, given that existing knowledge in this field is narrow. Some changes observed within this thesis and other studies of PC:EtOH indicate that maternal changes are not transient and restricted to the timing of exposure, but instead result in long-lasting alterations throughout the entirety of gestation. Furthermore, as altered maternal HPA function following PC:EtOH may be a significant contributor to the adverse offspring outcomes, further investigation should be a primary goal. Methods such as maternal adrenalectomy and exogenous corticosterone replacement may be used, as previous studies have indicated that maternal adrenalectomy is capable of ameliorating adverse offspring outcomes seen in a model of PAE [389]. However, as temporal changes in the maternal corticosterone observed in this model may have individual impacts on offspring outcomes, this method may make changes difficult to interpret.

Within this thesis, as animals were used for multiple studies, maternal corticosterone was measured at each time point in a different cohort, with various collection techniques. and related pathways across gestation, within the same cohort of minimally stress animals, using consistent techniques. This would allow for the establishment of a more accurate baseline of how corticosterone changes over gestation in rats, something that is mostly lacking in the literature. This would also provide information about key time points of maternal corticosterone variation following PC:EtOH and allow targeted investigation of other metabolic and cardiovascular parameters. This would aid in a more comprehensive understanding of the role of maternal physiological adaptations in PC:EtOH induced programmed disease. This targeted approach would also provide key investigation points for interrogating changes to fetal developing organ systems, as well as for intervention at the most appropriate times, or knowledge of when to avoid treatment with drugs such as synthetic glucocorticoids.

We hypothesise that PC:EtOH induced alteration in maternal HPA have as a potential contributor to offspring behaviour and HPA activity. In order to better understand the impact of PC:EtOH on mental illness, a greater suite of behavioural tests are required, ensuring a

more robust interpretation of the behavioural phenotype currently observed. Furthermore, a limitation of this study was that behavioural and HPA activity testing occurred between 3-5 months of age. Although all care was taken to ensure there was no age difference between control and PC:EtOH animals, this age spread needs to be considered when interpreting and comparing across the literature. It would be advantageous to measure hormone concentration at these ages to determine if there are changes within HPA output cross these three months. A more in-depth analysis of molecular pathways in the HPA and related regulatory regions would also allow a greater understanding of this axis and its contribution to mental illness and other diseases. To achieve this, genetic manipulation of key regulatory factors within specific limbic regions could be established in a mouse model of PC:EtOH. A caveat of this study was the lack of protein and enzymatic activity measurements, with the necessity to perform these analyses in the future. Further investigation of epigenetic modifications as well as regulation of the HPG and other endocrine pathways, as well as systemic regulation, would also prove beneficial in elucidating the extent of outcomes following PC:EtOH.

Another consideration of this model is that treatment occurs during both the pre-conception and post-conception periods, making it difficult to determine the most critical time of exposure. A recent study by Asimes *et al.* treated rats with alcohol from postnatal day 37 to 45, followed by mating at postnatal day 67, and determined several HPA related outcomes including behavioural, epigenetic and hormonal changes within offspring of these treated rats [646]. This, therefore, raises the question of how long before conception, women should cease alcohol consumption. Finally, as this model utilised an *ad libitum* liquid diet, the dose rats were exposed to was difficult to ascertain. Although, blood alcohol levels have been previously established in high drinking rats as 0.18 ± 0.04% (E-2) and 0.25 ± 0.04% (E2), we believe this would be different across dams and may explain some variability in observed results. Regardless of this, we also believe that although this presents a limitation, it is representative of women's drinking patterns across a population.

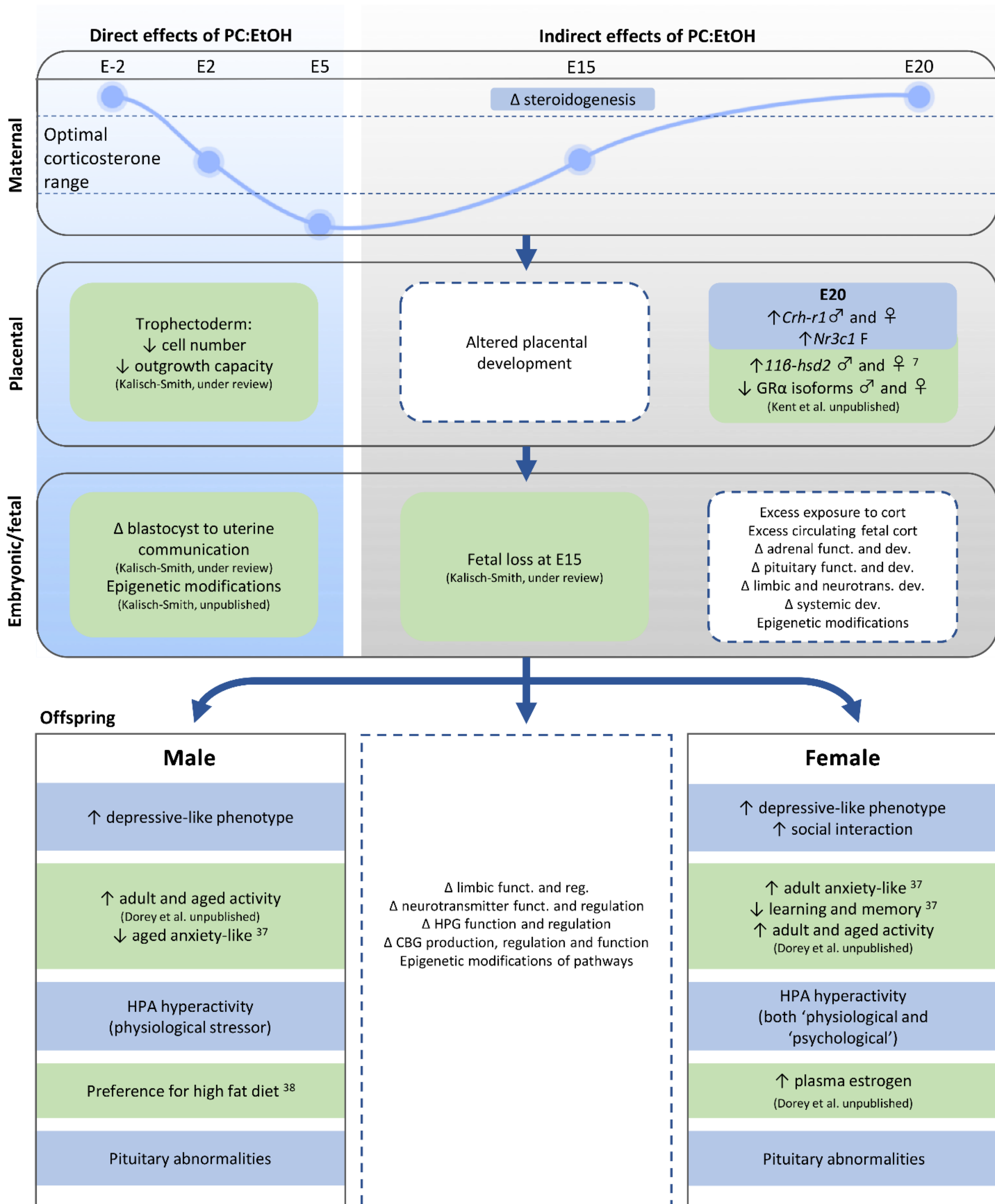
6.9 Summary

This thesis is one of the first studies to demonstrate that PC:EtOH has a significant impact on maternal physiology, even after cessation of consumption, and results in behavioural changes in offspring. Linking the maternal exposure and offspring outcomes is significant and sustained alterations to the HPA. A summary, including hypothesised mechanisms, is outlined in

Figure 6. 2.

PC:EtOH resulted in dynamic changes in plasma corticosterone concentration throughout gestation, with a significant elevation at the end of gestation. Dams also expressed altered gene expression in the adrenal glands at varying time points of gestation, and the placenta displayed altered glucocorticoid signalling pathways. These results are suggesting impaired protection from elevated maternal corticosterone concentrations association of PC:EtOH with behavioural alterations in offspring, associated with HPA dysfunction. Although not investigated, many changes would be hypothesised to be occurring within the fetus. This may include the development and function of the early HPA and related neurological pathways, as well as systemic organs which require an appropriate concentration of corticosterone to develop.

My work suggests these maternal and *in utero* changes are underlying the results observed, whereby behaviour and HPA activity was altered in a sexually dimorphic manner. Female offspring exposed to PC:EtOH demonstrated both increased depressive-like and altered social phenotypes, with a sustained reduction in basal corticosterone concentration. Female PC:EtOH offspring also demonstrated a delayed and hyperactive response to a systemic stressor and a disposition to an enhanced response to psychological stressors when aged. Furthermore, at the aged time point female offspring had elevated *Nr3c1* and *Hsp90a1* expression in the hippocampus, suggesting altered regulation of the HPA. Male PC:EtOH offspring on the other hand, while displaying a depressive-like phenotype and HPA hyperactivity to a systemic stressor, did not have a propensity for altered social interaction, altered basal corticosterone or elevated response to the psychological stressor. These results propose that male and female offspring may have differentially altered pathways or that female offspring are more susceptible following PC:EtOH exposure.



Direct effects of PC:EtOH
Indirect effects of PC:EtOH
E-2
E2
E5
E15
E20
Maternal
Δ steroidogenesis
Optimal corticosterone range
Placental
Trophectoderm:
↓ cell number
↓ outgrowth capacity
(Kalisch-Smith, under review)
Altered placental development
E20
↑Crh-r1 ♂ and ♀
↑Nr3c1 F
↑11β-hsd2 ♂ and ♀ 7
↓ GRα isoforms ♂ and ♀
(Kent et al. unpublished)
Embryonic/fetal
Δ blastocyst to uterine communication
(Kalisch-Smith, under review)
Epigenetic modifications
(Kalisch-Smith, unpublished)
Fetal loss at E15
(Kalisch-Smith, under review)
Excess exposure to cort
Excess circulating fetal cort
Δ adrenal funct. and dev.
Δ pituitary funct. and dev.
Δ limbic and neurotrans. dev.
Δ systemic dev.
Epigenetic modifications
Offspring
Male
↑ depressive-like phenotype
↑ adult and aged activity
(Dorey et al. unpublished)
↓ aged anxiety-like 37
HPA hyperactivity
(physiological stressor)
Preference for high fat diet 38
Pituitary abnormalities
Δ limbic funct. and reg.
Δ neurotransmitter funct. and regulation
Δ HPG function and regulation
Δ CBG production, regulation and function
Epigenetic modifications of pathways
Female
↑ depressive-like phenotype
↑ social interaction
↑ adult anxiety-like 37
↓ learning and memory 37
↑ adult and aged activity
(Dorey et al. unpublished)
HPA hyperactivity
(both ‘physiological and ‘psychological’)
↑ plasma estrogen
(Dorey et al. unpublished)
Pituitary abnormalities

Figure 6. 2: A schematic of overall PC:EtOH induced changes in mother, placental, fetus and offspring. PC:EtOH resulted in dynamic changes to maternal corticosterone concentration throughout gestation (discussed in Section 6.3). This is represented by the concentration curve (measured time points represented in blue circles) falling outside the optimal corticosterone range. A number of changes were observed within the developing blastocyst as a direct consequence of PC:EtOH, along with indirect effects on placenta across gestation (discussed in Section 6.3.1.2). This thesis determined placental gene expression changes to *Crh-r1* and the glucocorticoid receptor *Nr3c1* at E20, likely as a consequence of elevated maternal corticosterone at this time. Although not yet investigated, it is hypothesised that these changes result in excessive fetal glucocorticoid exposure, impacting some fetal developmental processes. Collectively, these PC:EtOH induced changes have resulted in some offspring behavioural, HPA and other alterations, which may also be related to change in a number of other central and systemic pathways related to the function and regulation of the HPA (discussed in Section 6.6). *Legend:* Blue: determined in this thesis; green: previous research with PC:EtOH model; dashed boxes: hypothesised outcomes. *Abbreviations:* Δ: change; cort: corticosterone; dev: development; funct: function; HF: high-fat.

6.10 Conclusion

The knowledge gained from this thesis demonstrates that alcohol consumption immediately prior to conception and at the very beginning of pregnancy can have a significant impact on the HPA of both the mother and her offspring. Considering the widespread physiological, psychological and lifestyle upheaval that occurs with pregnancy, several problems could ensue from PC:EtOH consumption. Additionally, as the HPA is a critical regulator of numerous physiological systems and is essential in endocrine, cardiorenal and metabolic homeostasis during pregnancy, alterations in the function of this system may result in multiple pregnancy complications. These complications include miscarriage, the development of gestational diabetes or preeclampsia. Furthermore, changes in maternal HPA function throughout gestation may have significant implications for long-term maternal disease, including obesity, diabetes mellitus type 2, and cardiovascular disease [572]. The vulnerability of the HPA to *in utero* perturbations is well established, however until now; few studies had investigated the axis' susceptibility to periconceptional perturbations. As up to 30% of women admit to drinking prior to pregnancy detection [44,46], the lack of knowledge regarding outcomes following and medical research to date should be a priority for research. Understanding the contributions that PC:EtOH has on pregnancy, and long-term maternal health would provide critical clinical information for monitoring mothers, babies and pregnancy outcomes.

Alcohol consumption is a common practice in many societies and knowing the impacts of its consumption before pregnancy detection on common non-communicable diseases and mental illness would have an enormous knowledge impact. This would provide an essential foundation for a preventative health impact and a greater understanding of the aetiology of these illnesses. This study also provides an explanation for sexual dimorphism often seen within the clinic, whereby women are three times likely to suffer depression and anxiety than men [35] and would provide an early marker for observation of patient throughout their lifespan. Furthermore, considering the impact of PC:EtOH in the context of DOHaD may extend the diagnostic criteria in place for FASD allowing for a greater number of individuals experiencing social, cognitive and stress-related difficulties to receive assistance and support to ensure high quality and healthy life.

As alcohol consumption is entirely preventable, these results highlight the critical importance of education towards the outcomes associated with consumption during this time, as well as the importance of preparing for pregnancy. While it is unrealistic to expect alcohol consumption around pregnancy to be eliminated, this model provides a basis for knowledge of the mechanisms underlying programming of mental illness, HPA function and downstream physiological systems in both mother and offspring.

7.0 References

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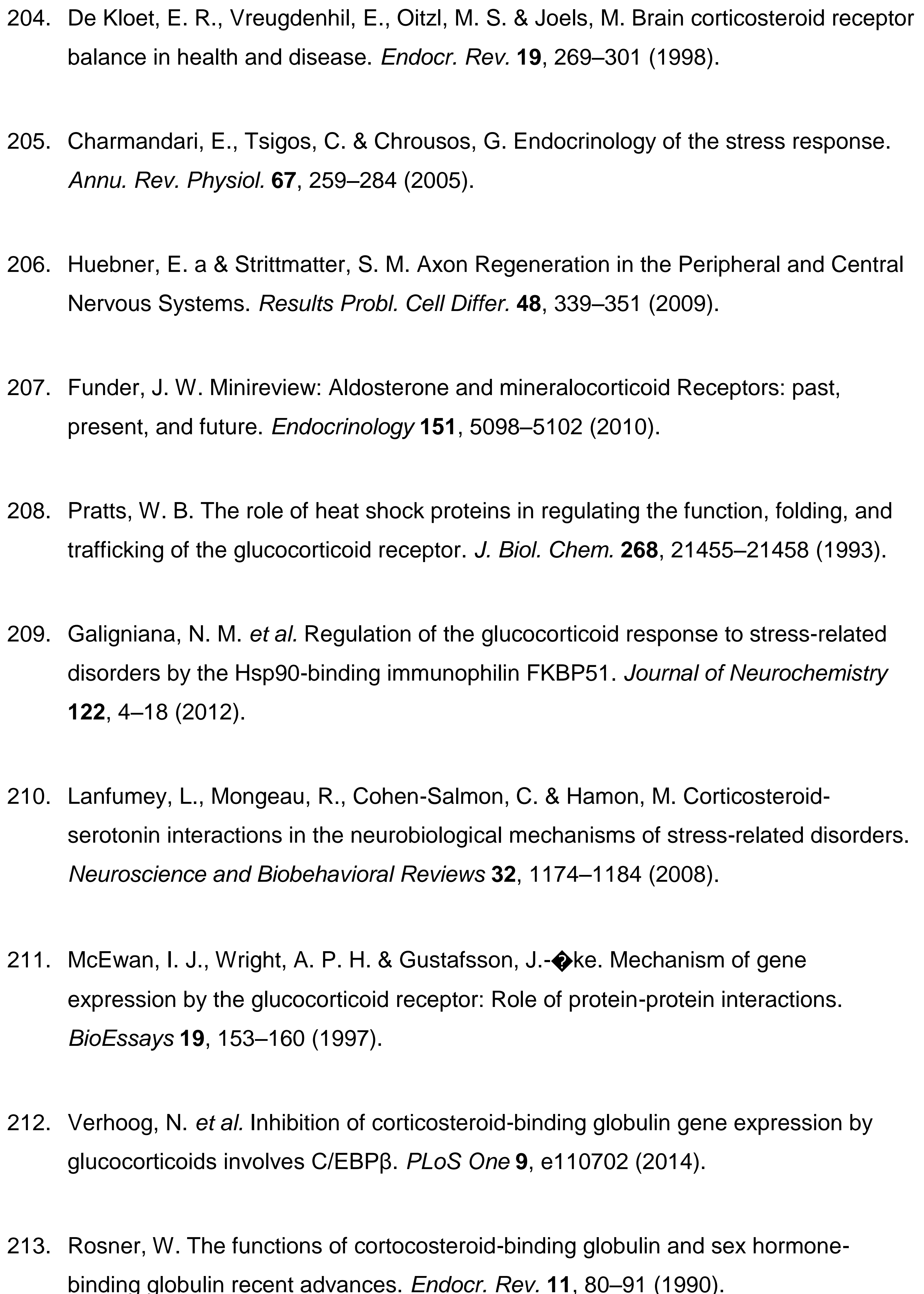

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