Faculty of Health Sciences
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Stress exposure from in-utero to adolescence, the HPA-axis response to stress, and symptoms of depression and anxiety in adulthood

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This thesis is presented for the degree of Doctor of Philosophy
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Declaration

To the best of my knowledge and belief this thesis contains no material previously published by any other person except where due acknowledgment has been made.

This thesis contains no material which has been accepted for the award of any other degree or diploma in any university.

Human Ethics

The research presented and reported in this thesis was conducted in accordance with the National Health and Medical Research Council Statement on Ethical Conduct in Human Research (2007) – updated in March 2014. Ethics approval for this research was obtained from the University of Western Australia (UWA) (ref: RA/4/1/5686) and from Curtin University Human Research Ethics Committee (ref: HRE2019-0118).

Carly McLaughlin (nee Herbison)

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Abstract

Currently, the lifetime prevalence of mental health problems in many westernised countries is approaching 50% and the World Health Organisation estimates depression as the second leading contributor to the global burden of disease. Stress exposure and adversity across the life course has consistently been linked to the development of depression and anxiety. Life stress events, whether in early or later life, have also been associated with modifications in the Hypothalamic, Pituitary, Adrenal (HPA)-axis response to a stressor, via hormones such as cortisol. These same hormones are often dysregulated in depression and anxiety disorders. Whilst there are a plethora of studies examining some aspect of these relationships, there is limited quality evidence directly linking common early life stress exposure, differences in HPA-axis regulation, and later depression and anxiety over the life course in the same individuals. The literature has focused on more severe trauma at limited time points, often involving retrospective studies of clinical populations. Furthermore, studies testing stress-responses have often been restricted in sample size, potentially due to test expense and time-consuming nature, limiting investigations into sex differences.

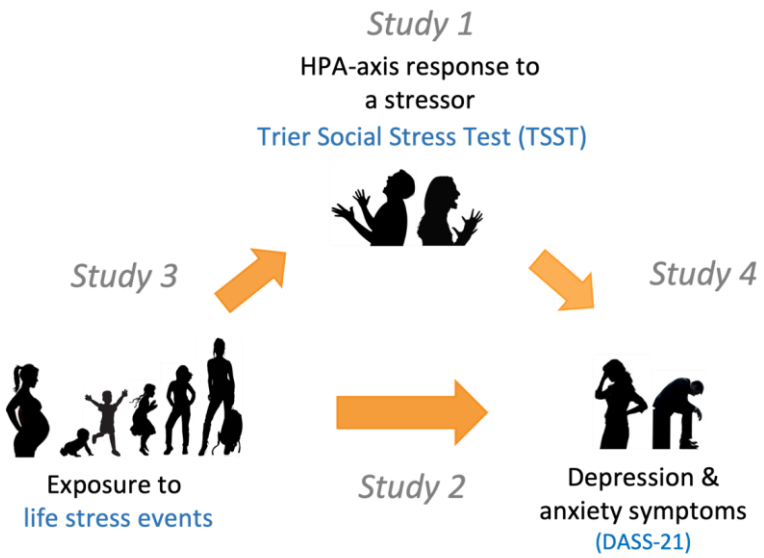
This doctoral thesis investigates these links with a prospective longitudinal study design, utilising data from the large, well characterised Raine Study cohort. This data includes repeated measures of life stress events from in utero to age 17 years, response to a gold standard psychosocial stressor test at age 18, and anxiety and depression symptoms at age 20, in over 700 participants. This thesis fills gaps in the literature as it investigates more common life stress events over multiple timepoints in a community sample and symptomatology instead of clinical disorders, rendering it more relevant to the general population. In addition, this study examines a very large sample for a psychosocial stressor test, allowing for novel characterisation of the stress response and consideration of sex differences. Finally, this is investigated at a time when the HPA-axis has reached a set point after adolescence and the prevalence of depression and anxiety escalates. These findings are important as developmental stress exposure is potentially modifiable and may help inform the timing of intervention and support strategies.

Therefore, the aims of this doctoral thesis are to:

1. Characterise the Trier Social Stress Test (TSST) data (individual cortisol and adrenocorticotrophic hormone (ACTH) responses to an acute psychological stress) at age 18 years in the Raine Study cohort and examine the relationships with sex, body mass index (BMI), smoking, oral contraceptive use and menstrual cycle.
2. Examine the relationship between life stress events from in-utero to age 17 and symptoms of depression and anxiety at age 20 in male and female Raine Study participants.
    i) Define postnatal trajectories of life stress exposure from birth to age 17 in Raine Study participants, incorporating the timing and number of life stress events.
    ii) Establish the impact of prenatal stress and postnatal trajectory of stress exposure on depression and anxiety symptoms at age 20 in Raine Study male and female participants.
3. Examine the relationship between prenatal/postnatal stress exposure and the HPA-axis response to the TSST at age 18 in Raine Study males and females.
4. Examine the relationship between the HPA-axis response to the TSST at age 18 and depression/anxiety symptoms at age 20 in Raine Study males and females.

A diagrammatic overview of this PhD project is illustrated in the following figure.

Graphical representation of this doctoral thesis integrating four distinct studies



Each aim is addressed in a separate study and these four studies have been published.

Study 1

Title: Characterisation and novel analyses of acute stress response patterns in a population-based cohort of young adults: influence of gender, smoking and BMI

Aims: This study aimed to examine the age 18 years Trier Social Stress Test data in the Raine Study cohort, using standard traditional measures and novel responder-category approaches, and examine their relationships with sex, BMI, smoking, oral contraceptive use and menstrual cycle.

Methods: Analyses were conducted on the acute stress response of 798 18-year-old participants from a community-based cohort using the TSST. Plasma adrenocorticotrophic hormone (ACTH), plasma cortisol and salivary cortisol levels were quantified. Participants were categorised as Reactive-Responder (RR), Anticipatory-Responder (AR) or Non-Responder (NR), based on serial measures of cortisol over 105 mins.

Results: RR, AR and NR patterns comprised 56.6%, 26.2% and 17.2% of the cohort respectively. Smokers were more likely to be NR than (RR or AR) (adjusted, $p<0.05$). Overweight and obese subjects were less likely to be NR than the other patterns (adjusted, $p<0.05$). Males were more likely to be RR than NR (adjusted, $p=0.05$). In addition, a novel area under the curve measure (AUC_R) is presented for use when the TSST baseline concentration is higher than later time points.

Conclusions: These results show that in a young adult cohort, stress-response patterns, in addition to standard traditional approaches, vary with gender, smoking and BMI. The distribution of these patterns has the potential to vary with adult health and disease and may represent a biomarker for future investigation.

Study 2

Title: The impact of life stress on adult depression and anxiety is dependent on gender and timing of exposure

Aims: The second study aimed to define postnatal trajectories of life stress exposure from birth to age 17 years and examine the relationship of prenatal and postnatal trajectory of stress with symptoms of depression, anxiety and stress at age 20, in males and females.

Methods: Exposure to life stress events was examined in the Raine Study during pregnancy and at ages 1, 2, 3, 5, 8, 10, 14 and 17 years. At age 20, participants completed the Depression Anxiety Stress Scale. Prenatal stress and trajectories of stress events from age 1-17 were analysed in linear regression analyses.

Results: Five post-natal stress trajectories were identified. In females, medium to high chronic stress exposure or increasing exposure during puberty/adolescence predicted depression and anxiety symptoms, while low or reduced stress exposure over the life course did not, after adjustment for relevant confounders. High stress early in pregnancy contributed to male depression and anxiety symptoms independent of postnatal stress trajectory. In females, postnatal stress trajectory was more important than prenatal stress in predicting depression/anxiety symptoms.

Conclusions: The findings suggest that interventions focused on reducing and managing stress events around conception/pregnancy and adolescence, particularly in people experiencing recent or chronic stress exposure, may have beneficial outcomes on rates of depression and anxiety in adults.

Study 3

Title: Prenatal and childhood stress exposure and the sex-specific response to psychosocial stress in adulthood

Aims: The third study aimed to investigate whether common early life stressors, experienced prenatally or throughout childhood and adolescence, play a role in the dysregulation of the HPA-axis in early adulthood.

Methods: Exposures to common life stress events were examined prenatally and as longitudinal trajectories of stress exposure from birth to age 17 years in males and females from the Raine Study. At age 18 years, 986 participants were assessed for their salivary cortisol response to a psychosocial stressor - the Trier Social Stress Test (TSST) which was used as the outcome measure in regression models.

Results: In males, there was an association between high prenatal stress exposure at 18 weeks gestation and a heightened TSST response. Evidence was found for sex-specific associations with increasing stress exposure during adolescence (the ascending trajectory), whereby males were more likely to be non-responders to the TSST and females were more likely to be responders.

Conclusion: These results point to sex differences in how stress exposure in-utero and increasing stress exposure during adolescence may affect regulation of the HPA-axis later in life. However, overall common life stress events experienced in-utero, during childhood and adolescence, show limited impact on the HPA-axis stress response in early adulthood.

Study 4

Title: The anticipatory response to stress and symptoms of depression and anxiety in early adulthood

Aims: The fourth and final study aimed to examine the relationship between the psychosocial response to the TSST at age 18 and depression/anxiety symptoms at age 20 in males and females.

Methods: At age 18 years, 748 males and females from the Raine Study were assessed for their salivary cortisol response to a psychosocial stressor using the Trier Social Stress Test (TSST). Participants later completed the Depression Anxiety Stress Scale (DASS-21) at age 20, which was used as the outcome measure in regression models.

Results: Differences were found in DASS-21 across TSST responder categories in females but not males. Female reactive-responders (RR) and non-responders (NR) had increased symptoms of depression and anxiety compared to anticipatory-responders (AR). AR were associated with the lowest symptomology in females. In addition, we found evidence for a small negative association between salivary cortisol (C_{BL}, C_{MIN}, AUC_G) and anxiety symptoms at age 20 in females only.

Conclusions: This study sheds new light on potentially adaptive and maladaptive physiological responses to psychosocial stress in terms of depression and anxiety symptoms. The pattern of response to a psychosocial stressor may contribute to individual vulnerability for stress-related diseases in a sex-specific manner.

Overall thesis conclusions

This doctoral thesis significantly contributes to understanding of the interrelationships between early life stress exposure, the HPA-axis (cortisol) response to a stressor in adulthood, and symptoms of depression and anxiety. This work (1) characterized a novel method of examining the acute response to a stressor using responder-categories that were associated with known modifiers of the stress response; sex; BMI and smoking. (2) showed that high stress exposure at specific timepoints prenatally and postnatally increases risk for the development of depression/anxiety in adulthood. (3) indicated that exposure to stress early in pregnancy or around adolescence was related to cortisol response to a stressor later in life and (4) showed the cortisol response to an acute stressor was associated with differing risk for depression/anxiety. Finally, the sex-specific differences in each of these pathways indicates the need to consider these relationships separately in males and females. Together these findings, with the relatively small associations found in studies 3 and 4, also suggest pathways other than the HPA-axis may be involved mechanistically in linking early life stress to later life mental health. Furthermore, this work has translational implications including driving pre-emptive strategies to manage and reduce controllable stress events around pregnancy and early life and guiding the delivery of mental health support for adolescents at risk due to recent or prolonged stress exposure.

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List of abbreviations

ACE	adverse childhood experiences
ACTH	adrenocorticotrophin hormone
ANS	autonomic nervous system
AR	anticipatory responder
AUC	area under the curve
BMI	body mass index
CAR	cortisol awakening response
CBG	corticosteroid binding protein
CI	confidence interval
CRF	corticotrophin releasing factor
DASS	depression anxiety stress scale
DOHaD	developmental origins of health and disease
DSM	Diagnostic and Statistical Manual of mental disorders
ELS	early life stress
HPA	hypothalamic-pituitary-adrenal
MDD	major depressive disorder
NR	non responder
OC	oral contraceptive
PTSD	post-traumatic stress disorder
RR	reactive responders
TSST	Trier Social Stress Test

List of publications arising from this thesis

Chapter 3

Herbison, C. E., Henley, D., Marsh, J., Atkinson, H., Newnham, J. P., Matthews, S. G., Lye, S. J., & Pennell, C. E. (2016). Characterization and novel analyses of acute stress response patterns in a population-based cohort of young adults: influence of gender, smoking, and BMI. *Stress*, 19(2), 139-150. doi: http://doi.org/10.3109/10253890.2016.1146672

Chapter 4

Herbison, C. E., Allen, K., Robinson, M., Newnham, J., & Pennell, C. (2017). The impact of life stress on adult depression and anxiety is dependent on gender and timing of exposure. *Dev Psychopathol, 29*(4), 1443-1454. doi: http://doi.org/10.1017/S0954579417000372

Chapter 5

McLaughlin, C., Schutze, R., Henley, D., Pennell, C., Straker, L., & Smith, A. (2021). Prenatal and childhood stress exposure and the sex specific response to psychosocial stress in adulthood. *Psychoneuroendocrinology, 125*, 105109. doi: http://doi.org/10.1016/j.psyneuen.2020.105109

Chapter 6

McLaughlin, C., Schutze, R., Pennell, C., Henley, D., Robinson, M., Straker, L., & Smith, A. (2022). The anticipatory response to stress and symptoms of depression and anxiety in early adulthood. *Psychoneuroendocrinology, 136*, 105605. doi: http://10.1016/j.psyneuen.2021.105605

Statement of contributors

Candidate

The candidate, Carly McLaughlin, was responsible for all aspects of the research presented in this thesis, including study design, data analysis and interpretation, original manuscript writing and subsequent editing for the publications entitled:

Herbison, C. E., Henley, D., Marsh, J., Atkinson, H., Newnham, J. P., Matthews, S. G., Lye, S. J., & Pennell, C. E. (2016). Characterization and novel analyses of acute stress response patterns in a population-based cohort of young adults: influence of gender, smoking, and BMI. *Stress*, 19(2), 139-150. doi:10.3109/10253890.2016.1146672

Herbison, C. E., Allen, K., Robinson, M., Newnham, J., & Pennell, C. (2017). The impact of life stress on adult depression and anxiety is dependent on gender and timing of exposure. *Dev Psychopathol, 29*(4), 1443-1454. doi:10.1017/S0954579417000372

McLaughlin, C., Schutze, R., Henley, D., Pennell, C., Straker, L., & Smith, A. (2021). Prenatal and childhood stress exposure and the sex specific response to psychosocial stress in adulthood. *Psychoneuroendocrinology, 125*, 105109. doi:10.1016/j.psyneuen.2020.105109

McLaughlin, C., Schutze, R., Pennell, C., Henley, D., Robinson, M., Straker, L., & Smith, A. (2022). The anticipatory response to stress and symptoms of depression and anxiety in early adulthood. *Psychoneuroendocrinology, 136*, 105605. doi:10.1016/j.psyneuen.2021.105605

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I, as Co-author, endorse that this level of contribution by the candidate indicated above is appropriate.

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Conferences, co-authored publications and prizes

Oral presentations

2013 Rising Stars Symposium, Women's and Infants Research Foundation, Perth, 9th October. "Factors affecting the adolescent psychological response to stress"

2013 Raine Study Annual Scientific Meeting, Perth, 2nd August, "Characterisation of novel psychosocial stress response profiles in a large adolescent cohort – The "Challenge Me" study"

2013 Endocrine and Reproductive Biology Society of WA, Perth, 14th August, "Novel stress response patterns in adolescence and their relationship with gender, oral contraceptive use, smoking and BMI"

2014 Perinatal Society of Australia and New Zealand (PSANZ), Perth, 6-9th April. "Trajectories of stress events from early life to adolescence predict depression and anxiety in young adults"

2014 DOHaD Society of ANZ, Perth, 9-10th April, "Trajectories of stress events from early life to adolescence predict depression and anxiety in young adults"

2015 International Society for Psychoneuroendocrinology (ISPNE), Edinburgh, 8-11th Sept, "Trajectories of stress events from early life to adolescence predict depression, anxiety and stress in young adults"

2016 DOHaD Society of ANZ, Adelaide, June 23-24th, "Gender-specific impact of prenatal stress versus postnatal stress exposure on depression and anxiety"

2016 Raine Study Annual Scientific meeting, Perth, 30th Sept, "Stress exposure during development and adult depression and anxiety"

2019 Raine Annual Scientific Meeting, Perth, 1st Nov, "Prenatal, childhood and adolescent stress exposure and the response to psychosocial stress at age 18"

Co-authored publications

Le-Ha, C., **Herbison, C. E.**, Beilin, L. J., Burrows, S., Henley, D. E., Lye, S. J., Matthews, S. G., Pennell, C. E., & Mori, T. A. (2016). Hypothalamic-pituitary-adrenal axis activity under resting conditions and cardiovascular risk factors in adolescents. *Psychoneuroendocrinology, 66*, 118-124. doi: http://doi.org/10.1016/j.psyneuen.2016.01.002

Martin, W. N., Wang, C. A., Lye, S. J., Matthews, S. G., Reynolds, R. M., **McLaughlin, C. E.**, Smith, R., & Pennell, C. E. (2021). A Life Course Approach to the Relationship Between Fetal Growth and Hypothalamic-Pituitary-Adrenal Axis Function. *J Clin Endocrinol Metab, 106*(9), 2646-2659. doi: http://doi.org/10.1210/clinem/dgab341

Tearne, J. E., Allen, K. L., **Herbison, C. E.**, Lawrence, D., Whitehouse, A. J., Sawyer, M. G., & Robinson, M. (2015). The association between prenatal environment and children's mental health trajectories from 2 to 14 years. *Eur Child Adolesc Psychiatry, 24*(9), 1015-1024. doi: http://doi.org/10.1007/s00787-014-0651-7

Zhu, K., Henley, D., Pennell, C., **Herbison, C. E.**, Mountain, J., Lye, S., & Walsh, J. P. (2016). Associations between hypothalamic-pituitary-adrenal axis function and peak bone mass at 20 years of age in a birth cohort. *Bone, 85*, 37-44. doi: http://doi.org/10.1016/j.bone.2016.01.016

Prizes/Awards

2014 DOHaD Society of ANZ Prize : Best Student Poster

2015 International Society for Psychoneuroendocrinology Prize for Best Abstract by an early career researcher.

2015 University of Western Australia Convocation Travel Award

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There is a certain irony about experiencing stress events whilst studying them, having children whilst examining the effects of prenatal and early life stress, and presenting talks with effects similar to the psychosocial stressor in Study 1.

To Rob (McLaughlin) – my lifebuoy through waves and alternating tides. The wind in my sails. There was a marriage proposal 2 weeks after I enrolled in a PhD and a wedding 6 months later. We have been on this journey together. This is a man of wisdom, who

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Through this journey I did commit the scientific *faux pas* of publishing under 2 different names as halfway through after two children and a change of supervisory team and university, I found I identified more as a McLaughlin than a Herbison.

To my parents, Linda and Ian, who forged my metal, gave me endless support and in part made me who I am. I am grateful for the privilege of even being able to do such a thing as this.

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“It is easier to build strong children than to repair broken men.”

Frederick Douglass (1819–1895)

Chapter 1 Introduction

1.1 Background

This thesis builds on the current understanding of links between early life exposure to common stressors, adult physiological responses to a stressor, and adult symptoms of depression and anxiety.

Depression and anxiety disorders and their symptoms are common, often comorbid and recurrent across the lifespan (Kessler et al., 1996). They occur at double the rate in women than in men and appear to be increasing (WHO, 2017). For the individual, they result in reduced quality of life, inability to function effectively and reduced capacity for employment. These disorders also show high co-morbidity with other chronic conditions (Clarke & Currie, 2009), substance use (Davis et al., 2008) and suicide (Hawton et al., 2013) and reduced life expectancy (Laursen et al., 2016). This has resulted in a substantial burden of depression and anxiety-related disorders worldwide, with the World Health Organisation assessing depression as the leading cause of disability in the world and the second leading contributor to the global burden of disease (Rehm & Shield, 2019; WHO, 2017).

A substantial portion of the population will suffer from symptoms of depression and anxiety but not reach clinical thresholds. Whilst subclinical depression and anxiety are less severe, they are also more prevalent and can be associated with similar costs to the individual and society in terms of poorer quality of life and vast economic costs (Ebert et al., 2018). Moreover, individuals with subclinical symptoms are at greater risk of developing diagnosed depression and anxiety disorders later. Much of the research conducted that examines links between stress exposure and/or stress response and depression and anxiety has considered depression and anxiety as disorders. However, the validity of categorising symptoms that exist on a continuum via a threshold for diagnoses has been questioned (Solis et al., 2021; Strong, 2019), and analyses of symptoms provide additional important information (Markon et al., 2011).

The association between life stressors and depression/anxiety disorders has been described as one of the most robust findings in psychology (LeMoult, 2020). However, there has been a focus on more severe stressors (trauma) and diagnosed disorders in clinical populations, and it remains to be seen whether there is a relationship between

common early life stress events and symptoms of depression and anxiety in adulthood which would be more relevant to the general population. The timing at which the stress exposure occurs may be an important factor in determining later life mental health, as the brain regions regulating the stress response grow and mature at different times over development (Lupien et al., 2009). However, there are gaps in the human literature as retrospective studies tend to collapse childhood exposure into one measure 'before the age of 18', thereby negating issues of timing (Felitti et al., 1998; Green et al., 2010; Ouellet-Morin et al., 2015). Furthermore, whilst prenatal stress exposures have been linked to poor mental health in children (Talge et al., 2007), they have never been systematically examined alongside childhood exposures in the same individuals with adult depression/anxiety. Life stress events rarely occur in isolation and are highly correlated across the lifespan (Cole et al., 2006), therefore analyses taking repeated measures of stress exposure from the same individuals are vital to understanding the impact of stressor timing on later life depression and anxiety. Finally, few studies have systematically considered the potential for differences between males and females concerning the association between early life stress and symptoms of depression/anxiety in adulthood.

Exposure to stressors triggers a physiological stress response, most notably activation of the Hypothalamic Pituitary Adrenal (HPA)-axis. Upon detection of a threat, neural pathways trigger a sequential cascade of hormone release, ultimately producing cortisol which targets many different cell types via the bloodstream to cope with the anticipated increased demands on the body. The pattern and amount of cortisol release vary across individuals. An exaggerated stress response is represented by a greater than average increase in cortisol after a stressor, and an inappropriately low response to stress is represented by a lower than average or undetectable increase in cortisol after a stressor. Both these types of responses have been associated with negative health outcomes, including depression. The gold-standard laboratory procedure for measuring HPA-axis/cortisol reactivity to a stressor is called the Trier Social Stress Test (TSST) and uses a combination of social evaluation, public speaking and mental arithmetic to induce a cortisol response (Allen et al., 2017; Kirschbaum, Pirke, et al., 1993). TSST studies are extremely valuable for determining the real-time physiological response to a life stressor. However, they are also time-consuming and expensive; therefore studies that use this test tend to be limited in size to under 150 participants. There are discrepancies in the

literature over the size and direction of differences in TSST response attributable to BMI, oral contraceptive use and menstrual cycle, and the overall response pattern is rarely assessed as a separate measure. Furthermore, studies have tended to focus on only one source (generally saliva), one sex, one demographic or clinical samples, reducing the generalisability of findings to the general population.

There is mounting evidence that exposure to early life stress is associated with dysregulated HPA-axis reactivity in adulthood (Bunea et al., 2017). Stressors that occur while the HPA-axis is developing and maturing, may alter its regulation such that adult reactivity to stressors is sensitised or recalibrated (potentially increasing vulnerability to poor adult mental health). However, there are conflicting results concerning the direction of this effect, with some studies showing increased cortisol reactivity with exposure to early life adversity (Harkness et al., 2011; Ouellet-Morin et al., 2019) and others showing no effect (Fogelman & Canli, 2018) or blunted cortisol reactivity (Bunea et al., 2017). The timing of the stressor may, in part explain these diverging effects, although longitudinal early life stress exposure measures in the same individuals are rare. In general, there has been a focus on more severe trauma and clinical populations, and more common life stress exposures in the general population have been neglected. In addition, sex differences in the relationship between early life stressors and adult HPA-axis reactivity have been largely overlooked.

Dysregulation of HPA-axis reactivity has been associated with depression and anxiety (Herbert, 2012; Zorn et al., 2017) and is assumed more generally to play an important role in the relationship between early life stress exposure and psychopathology (Heim et al., 2008). Whilst cortisol reactivity has been associated with depression and anxiety disorders, research examining cortisol reactivity with depression and anxiety symptoms in males and females within the general population is limited. Large samples with a comprehensive investigation of novel and traditional TSST parameters are also lacking.

1.2 Aims of the research

Given the limitations outlined above in these fields of research, this doctoral thesis aimed to:

1. Characterise the Trier Social Stress Test (TSST) data (individual cortisol and adrenocorticotrophic hormone (ACTH) responses to an acute psychological stress) at age 18 years in the Raine Study cohort and examine the relationships with sex, body mass index (BMI), smoking, oral contraceptive use and menstrual cycle.
2. Examine the relationship between life stress events from in-utero to age 17 and symptoms of depression and anxiety at age 20 in male and female Raine Study participants.
    iii) Define postnatal trajectories of life stress exposure from birth to age 17 in Raine Study participants, incorporating the timing and number of life stress events.
    iv) Establish the impact of prenatal stress and postnatal trajectory of stress exposure on depression and anxiety symptoms at age 20 in Raine Study male and female participants.
3. Examine the relationship between prenatal/postnatal stress exposure and the HPA-axis response to the TSST at age 18 in Raine Study males and females.
4. Examine the relationship between the HPA-axis response to the TSST at age 18 and depression/anxiety symptoms at age 20 in Raine Study males and females.

A prospective longitudinal cohort study, the Raine Study, was used to address the thesis aims. Long-term prospective cohort studies have several design advantages for examining the relationship between early life stress exposure, the HPA-axis and adult depression and anxiety. Capture of repeated measures from the same individuals over time allows exploration of the natural course of exposure to risk factors and the development of disease (Caruana et al., 2015). Recall bias is minimised as data is collected prospectively over a series of follow-ups (Sedgwick, 2013; Setia, 2016). Cohort studies also offer better generalisability to the general population as they include a broad range of participants, in contrast to clinical populations.

The Raine Study is an excellent example of a large prospective cohort study with longitudinal information collected over two decades from early gestation. Established in 1989-1991 and with 2868 live births, the Western Australian Pregnancy Cohort (Raine) Study aimed “to develop a large cohort of Western Australian children studied from 18 weeks gestation to ascertain the relative contributions of familial risk factors, fetal growth, placental development and environmental insults to outcome in infancy and to

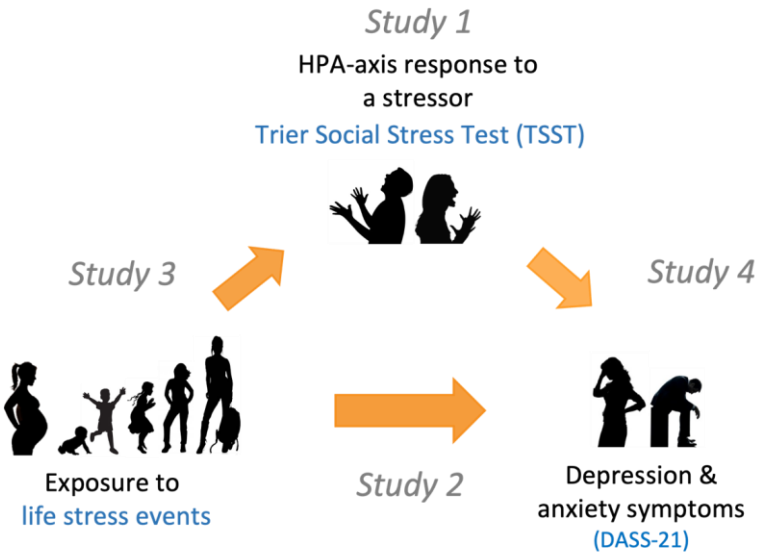
the precursors of adult morbidity"(Straker et al., 2017). The study started with a randomised control trial investigating the effect of ultrasounds during pregnancy on birth outcomes (Newnham et al., 1993). However, additional funding was obtained to examine the 'origins of disease in the fetus, the child and young adult' (Straker et al., 2017), and this concept has evolved into a life-course framework. One of the main strengths of the Raine Study is the longitudinal nature of the stress exposure data with regular cohort follow-ups at ages 1, 2, 3, 5, 8, 10, 14, 17, and 20+ years, in addition to the rare stress event data captured at two timepoints in-utero (18 and 34 weeks gestation). Furthermore, another major strength of the Raine study is the HPA-axis reactivity data collection at age 18 using the gold-standard Trier Social Stress Test with both blood and saliva collection. This represents (to our knowledge) the largest TSST conducted in a young adult population (n = 1137), allowing investigation of sex differences and measurement over the three levels of plasma ACTH, plasma cortisol and salivary cortisol. Lastly, Raine Study participant characteristics were compared to those of similarly aged youth in the general population at the 8-year, 14 and 17-year and 20-year follow-ups (using corresponding census data from 2001, 2006 and 2011 surveys) and the Raine Study sample were found to be widely representative of the general population, over a wide range of variables (Straker et al., 2017).

Stress exposure, HPA axis reactivity analysis and depression/anxiety have not been collectively examined over the life course in the same population. This thesis will overcome some of the limitations of previous research to better understand the stress-related risk factors and mechanisms behind the development of depression and anxiety problems. Such knowledge could inform research towards early detection and the targeted delivery of interventions for these problems, resulting in both personal and economic savings for society. A detailed understanding of the timing of early life stress events may help determine critical windows of development during which exposure to stress has a greater impact on HPA-axis function and future mental health. Consequently, this knowledge may aid in developing specific, early life targeted interventions directed towards high-risk groups to reduce stress exposures, provide education, resources and support to reduce the likelihood and burden of depression and anxiety symptoms in future.

1.3 Structure of the thesis

An overview of the studies within this thesis is depicted visually in Figure 1.1.

Figure 1.1
Graphical representation of this doctoral thesis integrating four distinct studies



This thesis has seven chapters. This chapter provides an overview of the topic, gaps in the literature and the overall rationale behind the research conducted as part of this thesis.

Chapter 2 presents a more detailed literature review on the problems of depression, anxiety and stress exposure and existing evidence on the associations between early life stress exposure, HPA-axis reactivity (the stress response) and adult symptoms of depression and anxiety.

Chapter 3 reports the findings of Study 1, examining factors contributing to variation in the response to stressors measured by the TSST, using traditional methods and novel analyses of stress-response patterns. This study has been published in *Stress*.

Chapter 4 contains the outcomes of Study 2, examining the association between prenatal stress exposures, the postnatal trajectory of stress exposure over 17 years and symptoms of depression and anxiety in adult males and females. This study has been published in *Development and Psychopathology.*

Chapter 5 presents the findings of Study 3, examining the association between prenatal stress exposures, the postnatal trajectory of stress exposure over 17 years and the reactivity of the HPA-axis at age 18 using the TSST. This study has been published in *Psychoneuroendocrinology.*

Chapter 6 reports the outcomes of Study 4, examining the association between the reactivity of the HPA-axis at age 18 using the TSST and symptoms of depression and anxiety in adult males and females. This study has also been published in *Psychoneuroendocrinology.*

Chapter 7 presents a discussion of the main findings of this thesis, integrating the results and situating them within the existing literature. This section highlights the overall significance of this thesis and contribution to the field and considers limitations, implications, translation and future directions.

Supplementary data for each study are presented at the end of each relevant chapter.

Chapter 2 Literature review

This thesis investigates the associations between common early life stress exposures, the adult cortisol response to a stressor and adult symptoms of depression and anxiety.

This review of the relevant literature begins with an overview of depression and anxiety conditions, how they are defined, their prevalence, problems, measurement and origins in Section 2.1. Section 2.2 defines stress and the HPA-axis and describes the physiology of the stress response, and then Section 2.3 reviews what is known about the relationship between exposure to stressors and the development of conditions or symptoms of depression and anxiety, noting gaps in the literature. This section is relevant to Study 2 (Chapter 4) in this thesis, where this association is examined. In Section 2.4, the different theoretical frameworks relevant to a deeper understanding of early life stress exposure and adult mental health are summarised. Section 2.5 examines the measurement of the HPA-axis, the Trier Social Stress Test and gaps in the literature. This section is relevant to Study 1 (Chapter 3) in this thesis, where the TSST is examined in a large young-adult sample. Section 2.6 reviews the relationship between early life stress exposure and the adult cortisol response to a stressor, noting gaps in the literature. This section is relevant to Study 3 (Chapter 5) in this thesis, where this association is examined. Section 2.7 summarises what is known about the relationship between cortisol stress response and depression and anxiety problems, highlighting gaps in the literature. This is relevant to Study 4 (Chapter 6) in this thesis, where this association is examined. Section 2.8 reviews a combination of evidence suggesting the involvement of the HPA-axis in the relationship between early life stress exposure and depression/anxiety symptoms but also points to other pathways that may be involved. Finally, Section 2.9 provides the overall summary and Section 2.10 outlines Studies 1-4.

2.1 Depression and Anxiety

2.1.1 Definitions and classifications

Depressive symptoms are numerous but can include pervasive feelings of sadness, emptiness, or irritability, accompanied by somatic, behavioural and cognitive changes. Clinically, depressive symptoms exist on a continuum and are relatively common in the

general population. Particular clusters of symptoms with functional impacts form psychiatric diagnoses according to classification systems such as DSM (Diagnostic and Statistical Manual of Mental Disorders, DSM5), APA (2013) and ICD (International Classification of Diseases, ICD-10)(WHO, 2004). These diagnoses include disruptive mood dysregulation disorder, major depressive disorder (including major depressive episode), persistent depressive disorder (dysthymia), premenstrual dysphoric disorder, substance/medication-induced depressive disorder, depressive disorder due to another medical condition, other specified depressive disorder, and unspecified depressive disorder (APA, 2013). The term 'depression' is often used non-specifically in the literature and can refer to depressive symptoms or a depressive disorder. The disorder most often implied here is major depressive disorder (MDD), otherwise known as unipolar depression or clinical depression. For the purposes of this thesis, depression as a clinical disorder will be referred to as conditions or disorders of depression, whilst depression symptoms will be specified as such. Depression problems will refer to both depression symptoms and disorders.

Anxiety symptoms include an excessive fear and anticipation of a potential future threat, often along with physical arousal symptoms such as increased tension, heart rate, blood pressure and sometimes sweating, trembling, dizziness or shortness of breath. As per depressive symptoms above, anxiety symptoms exist on a spectrum and are frequently found within the general population. Clustering symptoms over time with functional impact form various psychiatric diagnoses according to the DSM5 or ICD-10. These diagnoses include separation anxiety disorder, selective mutism, phobias, social anxiety disorder, panic disorder, generalised anxiety disorder and substance-induced anxiety disorder (APA, 2013). For the purposes of this thesis, anxiety as a disorder will be referred to as conditions or disorders of anxiety, whilst anxiety symptoms will be specified as such. Anxiety problems will refer to both anxiety symptoms and disorders.

2.1.2 Prevalence and concerns with depression and anxiety problems

Depression and anxiety disorders are common, and currently, the lifetime prevalence of depressive and anxiety disorders in many westernised countries is around 20% and 30%, respectively (Kessler et al., 2005), although some studies put estimates closer to 50% (Andrews et al., 2005). Australian statistics put depressive and anxiety

disorders at 5.9% and 7% of the population, respectively with both being more common in females compared to males (WHO, 2017).

These conditions are often comorbid, chronic and recurrent across the life course, and there is evidence that their prevalence is increasing. From 2005 to 2015, depression increased by 18.4% and anxiety disorders by 14.9% globally (WHO, 2017). The World Health Organisation assesses clinical depression as the leading cause of disability in the world (WHO, 2017) and the second leading contributor to the global burden of disease (Murray et al., 1996).

Globally, depressive disorders account for 43.1 million disability-adjusted life years (DALYs) (95%CIs 30.5–58.9), and anxiety disorders account for 27.1 million DALYs (95%CIs 19.2–36.1 million)(GBD-Collaborators, 2018). In the US, the estimated economic burden of depression was just over $83 billion in 2000 (Greenberg & Birnbaum, 2005). In Australia, the total annual productivity losses of depression and anxiety-related disorders were estimated to be AU$11.8 billion, together with annual income tax losses of AU$1.2 billion and welfare payments of AU$12.9 billion in 2007 (Lee et al., 2017). All these estimates probably underestimate the true economic burden of the disease because the available data and analysis techniques do not capture all of the subtle costs of these conditions (Greenberg & Birnbaum, 2005). Furthermore, subthreshold depressive symptoms (beneath the threshold for diagnosis), although rarely taken into account, are also highly prevalent, related to increased mortality, poorer quality of life, increased healthcare service utilisation and vast economic costs (Ebert et al., 2018).

Symptoms of depression and anxiety commonly occur together, and comorbidity estimates range from 25-75% (approx. 50%) (Choi et al., 2020; Gorman, 1996; Kessler et al., 1996). Comorbid depression and anxiety are associated with increased severity of illness (Hirschfeld, 2001), reduced response to treatment, longer recovery time (Kessler et al., 1998), increased recurrence rates (Brown et al., 1996) and higher risk for suicide (Lepine et al., 1993).

Depression and anxiety show high comorbidity and multimorbidity with other chronic illnesses such as heart disease, stroke, cancer, diabetes (Clarke & Currie, 2009), back pain and arthritis (Harris et al., 2018). As such, depression and anxiety are risk factors for chronic physical health conditions. People with a chronic physical health

condition combined with depression/anxiety have worse health outcomes than those with the physical health condition alone (Harris et al., 2018).

From the perspective of the individual, their family and community, depression and anxiety often represent a significant burden in terms of reduced quality of life, inability to function and parent effectively, need for extra care and treatment, lost income, and lack of participation in society.

2.1.3 Categorical diagnoses versus a continuum of symptoms

A diagnosis of a particular disorder is a way of categorising clinical symptoms, and this categorical system is useful for capturing prevalence rates, guiding treatment recommendations and identifying patient groups for clinical research. However, the validity of categorising collections of symptoms that exist on a continuum via a threshold for diagnosis has been questioned (Johnstone & Boyle, 2018; Strong, 2019). In contrast to the categorical method, depression and anxiety severity can be seen as continuous variables, and symptoms assigned to a single disorder may occur in other disorders at varying levels of severity. Genetic and environmental predisposition studies also raise concerns over using separate categorical diagnoses as they may not adequately capture the shared symptoms and risk factors across disorders with high comorbidities (Johnstone & Boyle, 2018).

Essentially, the boundaries between many disorder categories are more fluid and more porous over the life course than originally thought (APA, 2013). There have been calls to introduce dimensional approaches to mental disorders (examining a continuum rather than a categorical diagnosis), and the clustering of 'internalising' factors now represents an empirically supported framework, with the internalising grouping representing disorders of anxiety, depressive, and somatic symptoms (APA, 2013).

Research from the Netherlands Study of Depression and Anxiety measuring the clinical trajectories of depression and anxiety disorders over nine years in adulthood found that categorical diagnoses alone failed to capture the perseverance, recurrence and comorbidity of affective symptoms observed using a dimensional approach (Solis et al., 2021). Diagnostic recovery does not mean symptomatic recovery, and categorical diagnoses lead to overestimation of recovery, underestimation of chronic illness and persistence of symptoms (Solis et al., 2021). Meta-analyses have suggested a 15%

increase in reliability and 37% increase in validity using dimensional measures over categorical measures of psychopathology (Markon et al., 2011). Dimensional measures offer richer and more detailed information, and examining symptoms is important because their expression below diagnostic thresholds is extremely common. As such, it may be more helpful to examine risk factors for symptomatology as a continuum rather than categorical diagnoses set by a threshold.

2.1.4 Measurement of depression and anxiety symptoms

Several self-report instruments exist that capture depressive and/or anxious symptomatology, including the Beck Depression Inventory (BDI)(Beck et al., 1996), the Beck Anxiety Inventory (BAI)(Beck et al., 1988), the Hospital Anxiety and Depression Scale (HADS) (Zigmond & Snaith, 1983) and the Depression Anxiety Stress Scale (DASS)(Lovibond & Lovibond, 1995). Whilst they all measure aspects of psychological distress, the DASS measures the overlapping and interrelated dimensions of depression, anxiety and stress.

The DASS-42 (42 items) and DASS-21 (21-item shortened version) are based on a dimensional rather than a categorical concept of psychological disorders. The DASS was developed using a non-clinical Australian population (Crawford & Henry, 2003; Lovibond & Lovibond, 1995), although it has been found to have good internal consistency with both clinical and non-clinical populations. Each negative emotional symptom is rated on a 4-point Likert scale of frequency or duration ranging from 0 "did not apply to them at all" to 3, "apply to them very much or most of the time", referring to the past week. The DASS consists of three subscales; the first two measure items more specific to depression or anxiety; the third stress subscale measures non-specific arousal and forms a coherent measure in its own right. This stress subscale contains items that do not map specifically to the depression or anxiety constructs but relate to both, capturing negative affect and emotional distress. Investigation has shown that doubling the DASS-21 scores is equivalent to deriving scores from the original DASS-42, and results from clinical populations suggest the DASS-21 has a cleaner factor structure relative to the longer DASS-42 (Henry & Crawford, 2005). Although not originally developed as a unidimensional instrument, the total DASS score has been validated as a summary measure of general psychological distress (Osman et al., 2012). It is therefore useful to examine alongside the more specific continuous subscales as necessary. Support for the

derivation and use of the DASS-21 total scale score also acknowledges the substantial comorbidity between depression, anxiety and stress (Osman et al., 2012).

2.1.5 Origins and risk factors of depression and anxiety problems

A large proportion of depression and anxiety problems have their origins in childhood. It is estimated that up to 50% of these disorders originate before age 14, with 75% before age 24 (Kessler et al., 2005). It is therefore important to investigate risk factors throughout these early years. Some risk factors include poverty (Ridley et al., 2020), female sex, experience of trauma/abuse/life stress events, and family history of depression/anxiety disorders. Heritability studies estimate the concordance rate of major depression in monozygotic twins at 31% (for males) to 44% (for females). In contrast, the concordance rates in dizygotic twins ranged from 11% to 16% (Kendler et al., 2006; Sullivan et al., 2000), suggesting a substantial genetic component for these problems. The experience of additional risk factors superimposed on an underlying background of genetic predisposition contributes to the expression of depression and anxiety.

2.1.6 Sex differences in depression and anxiety problems

Sex and gender differences are recognised in the DSM as contributing to the expression of depression and anxiety (APA, 2013). Sex refers to anatomy at birth, biology and chromosomes in the determination of male and female and gender refers to the socially constructed roles, behaviours, expressions and identities of men and women.

Before adolescence, approximately equal numbers of boys and girls experience depression (Speier et al., 1995); however, after puberty, females are twice as likely to experience depression than males (Cyranowski et al., 2000). These observations occur across countries and cultures and suggest the involvement of sex hormones in the aetiology of these conditions. The hormonal transitions of menarche, pregnancy/birth and menopause represent windows of vulnerability for female depression, further solidifying a biological/hormonal contribution. However, the sex differences in the prevalence of anxiety problems occur earlier, prior to puberty, around age 9-10 years (Altemus et al., 2014; Van Oort et al., 2009). Accordingly, it is also recognised that societal expectations shaped by gender norms/stereotypes play a role in these sex differences, with girls and boys treated differently from birth (Nolen-Hoeksema, 1990).

In traditional patriarchal structures, social constructions of femininity characterise women as being more emotional than men and valued as submissive, nurturing and self-sacrificing (Gorham, 2012). This has potentially contributed to systematic psychological differences in women's sense of self. "Females tend to have a lower sense of their own competence, to interpret events more negatively, to evaluate themselves more harshly, to set lower goals for themselves, and to rely more on external feedback in making judgements about themselves than do males" (Nolen-Hoeksema, 1990). Moreover, according to some critiques, the definition of psychopathology through systems such as the DSM has inherent gender bias that results in overdiagnosis of women (Ussher, 2013). Although this thesis focuses on the biological aspects of sex differences in mechanisms underlying the development of depression and anxiety, it is acknowledged that these will exist in a complex interplay with social and cultural factors.

2.1.7 Summary

To summarise, depression and anxiety disorders and symptomatology are relatively common, show higher prevalence in females than males and have their origins in early life with environmental stressors superimposed on various genetic predispositions. The clear sex differences arise around puberty and are thought to result from a combination of biological, social and cultural factors.

Depression and anxiety disorders are often comorbid, chronic/recurrent and are increasing worldwide and depression remains a leading contributor to disability and the global disease burden. Whilst diagnoses are common via ICD-10 or DSM5 criteria, there is evidence that dimensional measures (symptoms) offer richer, more reliable information below diagnostic thresholds. Therefore, it may be beneficial to examine risk factors for a continuum of symptoms rather than categorical diagnoses set by a threshold.

2.2 Stress - What is stress?

2.2.1 Definitions of stress

Hans Selye coined the term 'stress' in 1956 to describe the "sum of all non-specific changes (within an organism) caused by function or damage". He also defined it as "a non-specific response of the body to any demand for change". His definition refers to the response within an individual, not the stimulus, which he referred to as the stressor

(Selye, 1976). More recent definitions expanding on this include stress representing a challenge to an individual's homeostasis, which exceeds their ability to cope physically or psychologically and demands some adaption (Koolhaas et al., 2011). Many situations are recognised as likely to induce stress – life stress events ranging from severe exposures such as physical or sexual abuse, and death of a close relative through to more common exposures such as financial strain or relationship difficulties and the cumulative effect of these experiences in daily life. Allostasis is defined as the ability of the organism to achieve stability through change, reflecting the view that healthy functioning requires continual physiological adjustments. Allostatic load refers to the cost of chronic exposure to repeated challenges in terms of increased 'wear and tear' on the body (McEwen & Seeman, 1999).

For this thesis, the stressful stimulus will be referred to as the *stressor* or *stress exposure,* e.g. life stress events, and the internal physiological changes will be referred to as the *stress response.*

2.2.2 The physiology of the stress response and the HPA-axis

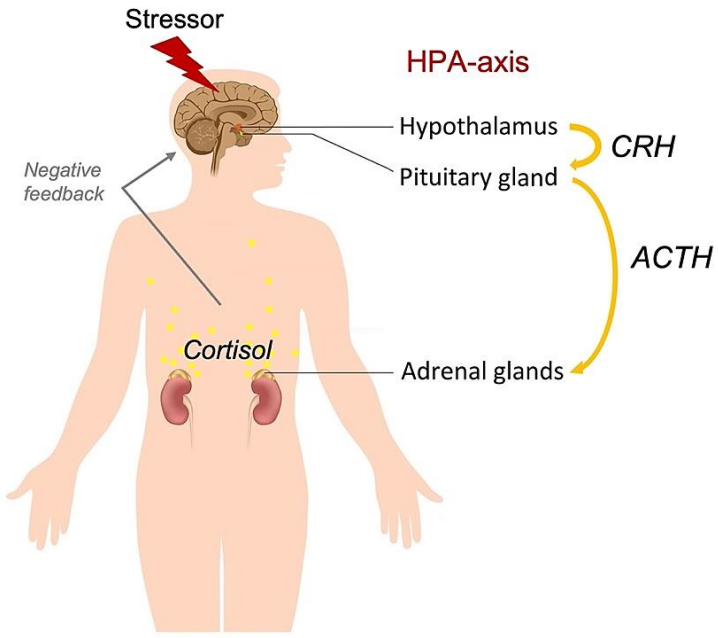
The stress response is a series of physiological changes, occurring in response to a stressor, that helps prepare an individual to cope with the anticipated increased demands on the body. This thesis will examine one part of the stress response, the HPA-axis response to a stressor. It refers specifically to the cascade of hormonal release via the Hypothalamus, Pituitary and Adrenal glands in response to a stressor (hence HPA), with cortisol as the end output. The mobilisation of this system is referred to as HPA-axis activation, HPA-axis reactivity or cortisol reactivity. A summary of the stress response and the part played by the HPA-axis is described below and is important to contextualise linking concepts in this thesis. More detail is presented later in Section 2.5 regarding measurement of the HPA-axis.

With a perceived stressor, the autonomic nervous system (ANS) will react immediately, with nerve-mediated activation of the sympathetic nervous system (SNS) and dampening of the parasympathetic nervous system (PNS). This fast response acts via the release of adrenalin and noradrenalin from the adrenal medulla, resulting in increased heart rate and blood pressure within a few minutes. However, the Hypothalamic-Pituitary-Adrenal (HPA)-axis is a second, slower-acting axis initiated simultaneously to

maintain stress arousal for a more prolonged period. See Figure 2.1. The activation of the HPA-axis begins when nerve impulses from the cerebral cortex trigger the release of corticotrophin-releasing factor (CRF) from the hypothalamus. CRF triggers the release of adrenocorticotrophin hormone (ACTH) from the anterior part of the pituitary gland. ACTH travels via the bloodstream to receptors on the adrenal glands. Binding to these receptors stimulates the secretion of glucocorticoids (primarily cortisol in humans) from the adrenal cortex. Most cortisol (95%) is immediately bound to corticosteroid-binding globulin (CBG) and albumin, thought to render it biologically inactive, whilst the remainder or 'free' portion is available to bind to target receptors and produce physiological effects on a wide variety of cell types (Kirschbaum & Hellhammer, 1989).

Saliva contains fewer binding proteins, so is perceived to more accurately represent the active cortisol compartment; that is, the unbound portion of cortisol that is free to bind to receptors and exert biological effects. Systemically, cortisol increases the availability of blood glucose for muscle action and dilates blood vessels to the skeletal and cardiac muscles for increased strength and capacity. It constricts other blood vessels, suppressing digestive and reproductive functions and increasing blood pressure. Cortisol also reduces the immune response and inflammation but increases alertness and short-term memory. Finally, cortisol levels are self-regulated via negative feedback pathways, acting on receptors in the hypothalamus, pituitary and other areas of the brain to reduce circulating levels of CRH, ACTH and cortisol (De Kloet & Derijk, 2004; Herman & Cullinan, 1997; Lopez et al., 1999). In contrast to adrenalin, cortisol can cross the blood-brain barrier and exert direct effects on the brain during development (M. Gunnar & K. Quevedo, 2007). Much attention has been spent on the detrimental outcomes of chronic hyperactivation of the HPA-axis resulting from long-term stress exposure (McEwen, 2008). However, hypoactivation of the HPA-axis is now also recognised as problematic when the response is not strong enough to meet the needs of the individual and mobilise resources to cope with a stressor (Fries et al., 2005; Phillips et al., 2013).

Figure 2.1
The HPA-axis and the cascade of hormone release following a stressor



2.3 The relationship of stress exposure with depression and anxiety problems

There is a longstanding consistent relationship between exposure to stress and the development of depression and anxiety disorders (Hammen, 2005). Increased exposure to and severity of stress events have been associated with poorer clinical outcomes, including more severe symptoms of distress (Briere et al., 2008), increased risk of developing a depressive disorder (Chapman et al., 2004), earlier onset (Kessler, 1997), recurrence (Francis et al., 2012; Gilman et al., 2013), longer time to remission (Fuller-Thomson et al., 2014) and reduced response to treatment (Heim et al., 2008). The following sections will further elucidate issues relating to the association between early life stressor exposure and depression/anxiety symptomatology, including the timing of measurements, study design, number and severity of stressors, sex differences and confounding factors.

2.3.1 Timing of stress exposure

When considering the timing of a stressor, data suggest that recent life stress exposure is important, with approximately 80% of depression in the community occurring after a major stressor (Mazure, 1998). Anxiety is associated with recent life stressors as

well (Francis et al., 2012). However, the experience of stressors throughout early life is also recognised to predispose individuals to depression and anxiety in adulthood (Hughes et al., 2017). Child development sees many changes in stress regulation, from brain functioning and maturation to hormonal fluctuations (Lupien et al., 2009). An accumulation of disadvantages and traumatic experiences, particularly during sensitive windows of brain plasticity in development, is thought to lead to re-calibrations of the individual's response to stress (Koss & Gunnar, 2018; Lupien et al., 2009).

2.3.1.1 Timing of stressors and brain development

There are three main areas of the brain involved in the regulation of the stress response; the hippocampus; the prefrontal cortex and the amygdala. The hippocampus attains maturation around the age of two years, the prefrontal cortex (PFC) matures between 8 and 14 years and finally, the amygdala continues to mature slowly until the late 20s (Giedd et al., 1996). Animal models have demonstrated that elevated stress hormone levels early in life lead to hippocampal damage (Sapolsky et al., 1986). Indeed, smaller hippocampal volumes in adults have been associated with early life adversity (Frodl et al., 2017; Frodl & O'Keane, 2013) and depression in adulthood (Arnone et al., 2012; Carrion et al., 2007). There is evidence for prefrontal cortex decreases in volume with chronic stress (van Harmelen et al., 2010) and in depression (Arnone et al., 2012). In contrast, findings are inconsistent with regard to the impact of early life trauma on amygdala volumes and amygdala reactivity may be more relevant (Zhu et al., 2019). The amygdala appears to be highly sensitised in patients with anxiety and post-traumatic stress disorder (PTSD) making the brain more responsive to stressors in general (Badura-Brack et al., 2018). Recent fMRI data suggests the timing of stressors may be relevant for the impact on the amygdala response. Maltreatment between ages 3 and 6 years of age was associated with a blunted amygdala response, whereas peer emotional abuse between ages 13-16 years was associated with augmented activation (Zhu et al., 2019). Therefore, the timing of stressor exposure is important to consider as it may impact brain development in such a way that stress regulation is altered and thus vulnerability to depression and anxiety problems in later life is increased.

2.3.1.2 *Timing of stressors in human studies of depression and anxiety*

Maternal exposure to stressors during pregnancy has been associated with behavioural/emotional problems in the child and the child's vulnerability to psychopathology (Talge et al., 2007; Van den Bergh et al., 2005; Weinstock, 2008), although there are no reported studies examining associations with adult symptomatology. Exposure to stressors and adversity during childhood and adolescence has been linked via meta-analyses with the development of depression and anxiety in adolescence (LeMoult et al., 2020) and adulthood (Gardner et al., 2019; Li et al., 2016; Mandelli et al., 2015). A meta-analysis of 96 large-scale studies using population samples found that any form of childhood maltreatment (including sexual/physical/emotional abuse, neglect and exposure to intimate partner violence) was associated with depressive disorders and symptomatology (OR 2.48, 2.14-2.87) and with anxiety disorders and symptomatology (OR 1.65, 1.35-2.02) later in life (Gardner et al., 2019). A meta-analysis examining early life stress also found 2.5 times the odds of early-onset depressive disorders (before age 18) with any form of childhood maltreatment mentioned above and also including poverty, illness, death of a family member and natural disaster (OR 2.5, 2.08-3.0) (LeMoult et al., 2020). These meta-analyses identified emotional abuse, neglect and death of a family member as the strongest contributors to later life depressive illness. The main limitations of studies in the human literature include retrospective study design, analyses involving one timepoint only and an inability to control for potential confounders. These are discussed further below.

2.3.1.3 *Repeated measurement of life stress events*

One issue in the literature regarding the measurement of life stress events is that most studies have considered only a single timepoint or a broad time window across the entirety of childhood/adolescence (0-18 years). Life stress events rarely occur in isolation, and experience of stressors over childhood and adolescence are highly correlated over time (Cole et al., 2006; Green et al., 2010). So, if an individual or family experiences high stress at one timepoint, they are more likely to have high-stress exposure at other times, and it can be hard to determine the particular relevance of one timepoint compared to others. Studies examining perinatal/early life stress exposure and adolescent/adult mental health have not controlled for the effects of stress exposure over childhood and adolescence (Van den Bergh et al., 2008; Yong Ping et al., 2020), so

it remains to be seen whether the effects of perinatal stress on adult depression and anxiety is independent of the effects of later life stress. The only longitudinal study that has partially addressed this reported that out of prenatal depression, postnatal depression, childhood trauma and family adversity, only childhood trauma was directly associated with offspring depression at age 24 when modelled together (Liu et al., 2022). Therefore, to address timing issues, analyses taking repeated measures of stress exposure from the same individuals over the life-course are vital to our understanding of the relative timing of stress exposure and the development of depression/anxiety symptoms.

2.3.2 Study design

Due to the long time period between early life stressors and adult mental health symptomatology, previous studies have generally retrospectively asked participants about their experience of stressors before the age of 15 or 18 years (i.e. at any time over their childhood), introducing the possibility of recall bias. Results from a large-scale cohort study using adult recall of childhood conditions estimated that over 20% of the association between childhood adversity/trauma and adult mental health is driven by differential recall bias (Sheikh et al., 2016). Other research has indicated that studies relying on retrospective recall provide different estimates for associations between childhood adversity and adult mental health symptoms compared to studies relying on prospectively collected data (Gardner et al., 2019; Newbury et al., 2018; Patten et al., 2015; Reuben et al., 2016). This may partially be due to negative mood or existing symptoms of depression/anxiety at the time of reporting resulting in negative recollection biases (Colman et al., 2016). In addition, healthy adults with positive dispositions are more likely to deny or forget adversity in childhood (Reuben et al., 2016), and adult participants, in general, may be unable to retrieve episodic memory from their early years (Usher & Neisser, 1993). Prospective measures of stressors over childhood as they occur are less subject to these recall biases, and prospective longitudinal studies are called for (Panagou & MacBeth, 2022).

2.3.3 Adjustment for confounders

There are confounders relevant to the relationship between early life stress and adult depression and anxiety, which are often not taken into account. Low

socioeconomic status (SES) is associated with higher exposure to stressors (Turner & Avison, 2003). Low SES in childhood is strongly associated with later life depression (Goodman et al., 2003). In particular, SES during early life appears more relevant to adult health and wellbeing than SES in adulthood (Surachman et al., 2019). SES proxy measures include maternal age in pregnancy and education and family income. Parental depression and anxiety problems are associated with both higher life stressors (Ge et al., 1994) and higher incidence of depression and anxiety (40%) in their offspring (Beardslee et al., 1998; Smith, 2004). Furthermore, lifestyle factors such as smoking, alcohol consumption, higher BMI, and less exercise are also linked to both early life adversity and adult depression (Brugiavini et al., 2022; Zhang et al., 2018). Analyses taking these relevant confounders into account are likely to yield more accurate estimates of the association between the timing of early life stressors and adult depression and anxiety symptoms.

2.3.4 Number and Severity of stressors

Adverse events in childhood are notably prevalent in the general population, with over 50% of US adults reporting at least one form of childhood adversity/stressor before the age of 18 and 25% reporting exposure to at least two forms of childhood adversity (Felitti et al., 1998; Green et al., 2010). These data were confirmed to be similar in Eastern Europe (Bellis et al., 2014), whilst 89% of Russians report being exposed to at least one form of childhood adversity (Meinck et al., 2016).

The unique Adverse Childhood Experiences (ACE) study of over 9000 adults in the US examined seven categories of stressors: psychological, physical or sexual abuse, violence against mother or living with household members who were substance abusers, mentally ill/suicidal or ever imprisoned. Researchers found a strong dose-response relationship between the number of adverse childhood experiences and depression, with those experiencing four or more ACEs at a 4.5-12 fold increased risk of depression and attempted suicide, respectively (Felitti et al., 1998). A 2019 meta-analysis of 96 studies found a significantly increased risk for depressive and anxiety disorders with more severe forms of child maltreatment, including sexual abuse, physical abuse, emotional abuse, neglect and exposure to intimate partner violence (Gardner et al., 2019). Parental loss in childhood due to death, separation or divorce is also associated with a two-fold increased risk for adult depression, and about 30% of depressed subjects have experienced parental loss before the age of 18 (Simbi et al., 2020). Finally, more common life stress

events such as daily hassles, financial or relationship problems have also been linked to the development of depression (Piechaczek et al., 2020; Tennant, 2002; Vinkers et al., 2014), and the cumulative effect of chronic stressors of this nature is recognised. Milder forms of life stressors are more prevalent and more relevant to the general population. However, research on the impact of these less severe forms of stress exposure has been relatively limited.

2.3.5 Sex differences

With sex differences in the prevalence of depressive and anxiety disorders, and their established link with stressors, research theories have proposed that males and females may differ in their exposure to early life stressors or their sensitivity to them. Although some studies have shown sex differences in the types of stressors experienced, for example, higher exposure to sexual abuse in childhood in girls and higher exposure to physical trauma in boys (Chapman et al., 2004; Tolin & Foa, 2006), overall there is little evidence to support differences in the total number of adverse childhood events in girls and boys (Young & Korszun, 2010). Yet, women are more likely to develop symptoms of depression and anxiety with exposure to life stress events (Maciejewski et al., 2001; Oldehinkel & Bouma, 2011), pointing to potential sex differences in the response to stressors (i.e. sensitivities to stress exposure in females). More detailed analyses into categories of life stressors and depressive illness in adults have found that women are more sensitive to interpersonal stressors involving others in their close network and emotional responsiveness (such as death/illness of a friend or relative) whilst men are more sensitive to work and job-related stressors (Kessler & McLeod, 1984; Maciejewski et al., 2001). Therefore, perhaps what is perceived as threatening differs in men and women (Dedovic et al., 2009). A meta-analysis of population-based studies showed that associations between childhood sexual/physical abuse with depression and anxiety were larger for women than for men, although sex interaction effects were not significant (Gallo et al., 2018). Therefore, there is some evidence that the impact of early life/childhood stressors on depression/anxiety outcomes is more pronounced in females. However, effects of sex are not always reported, and there has been no investigation into sex differences with less severe, more common life stressors and the impact of stressor timing. Recent reviews of the field have called for longitudinal studies and a focus on sex

differences and the impact of adversity timing with long-term outcomes (Gobinath et al., 2015; Koss & Gunnar, 2018; Raymond et al., 2018).

Furthermore, the brain regions that regulate the HPA-axis (the hippocampus, prefrontal cortex and the amygdala) have shown sex differences in their structure, morphology, function and developmental trajectories (Cahill, 2006; Korosi et al., 2012; Oldehinkel & Bouma, 2011). Fetal and pubertal exposure to sex hormones and the distribution of estrogen receptors throughout the brain result in sexually dimorphic brain organisation and pruning of synapses (Andersen & Teicher, 2008). Amygdala volume increases more in adolescent boys, and hippocampal volume increases faster in girls (Giedd et al., 1996). The hippocampus is larger in women than men (adjusted for total brain size), yet the amygdala is larger in men than women (Cahill, 2006). MRI studies show sex-specific patterns of brain responsiveness during a stressor (Mather et al., 2010) and macroscopic changes after experiencing a stressor (Shalev et al., 2020). Therefore, evidence suggests the impact of stressors may differ between males and females.

2.3.6 Summary

In summary, while increasing evidence indicates that specific windows of vulnerability to stress exposure are relevant to future mental health (Oldehinkel & Bouma, 2011; Romeo, 2010a), their relative importance remains in question. There is also evidence of sex-specific pathways from stress exposure to mental health symptoms; however, research examining relationships separately in males and females is lacking. Prospective longitudinal measures of stress exposure and repeated measures analyses in males and females, with adjustment for relevant confounders, are required to advance our understanding of how the timing of stress exposures is related to adult symptoms of depression and anxiety.

2.4 Theoretical perspectives relevant to early life stress exposure and adult mental health

Different theoretical perspectives have guided research as to whether and how early life stress exposure influences stress regulation and adult mental health outcomes. These perspectives are useful for considering the relationships explored in this thesis and will be briefly discussed below. With additional data, these models can

be further refined and extending knowledge in this area requires longitudinal life-course research approaches.

The **Developmental Origins of Health and Disease** (abbreviated DOHaD) perspective emphasises the role of perinatal and early life factors in contributing to human disease in adulthood (Barker et al., 2002). It incorporates the effects of stress during pregnancy and early life on adult mental health (O'Donnell & Meaney, 2017; Wadhwa et al., 2009) and recognises the potential for transmission across generations (Hoffman et al., 2017). Importantly, DOHaD emphasises how early life exposures can have a disproportionately large effect on later life health outcomes (Gluckman & Hanson, 2006).

With a focus on how 'sensing' the early environment enables adaptations to take place to allow optimal functioning, problems can occur when the early and later life environments are markedly different. This is the basis for the **mismatch hypothesis** and suggests individuals are more likely to suffer depression or anxiety when a mismatch occurs between stress exposures in-utero or in early life and stress exposures later in life (Gluckman & Hanson, 2006; Gluckman et al., 2005). For example, high-stress exposures during pregnancy may indicate the environment in which the baby will be born is hostile. This induces changes to stress regulation systems to ensure hyper-vigilance in order to increase chances of survival. However, if the child does not live in a high-stress environment, they may suffer higher rates of depression and anxiety problems.

Complementary to this perspective is the theory of **allostatic load,** which posits that modifications in the activity of various physiological systems over time in response to stressors, including the stress response, immune, metabolic and cardiovascular systems, although adaptive in the short term, are harmful in the long term. This perspective hypothesises that the cumulative effects of these changes, termed allostatic load, increase wear and tear in the body, decrease resilience to future stressors and increase the risk of psychopathology (McEwen, 1998). In other words, allostatic load is the price the body pays for adapting to exposures of early and later life stressors.

With exposure to cumulative or successive stressors, **sensitisation** to future stressors can occur with multiple 'hits' or stress exposures (Hammen et al., 2000; Harkness et al., 2006). However, not all stressors are necessarily harmful and the ability of the individual to adequately cope with mild stressors is important for health. In a form

of **stress inoculation**, exposure to mild/moderate stress during childhood may help stress regulatory systems to develop and function optimally when later challenges occur (Garmezy, 1991; Rutter, 1993). In this way, both the experience of too little or too much stress during early life may be detrimental. The **biological sensitivity to context theory** (Boyce & Ellis, 2005) suggests a U-shaped relationship between early life stress and HPA-axis reactivity such that high or low early life stress produces greater reactivity of the stress response system.

All these perspectives recognise that individuals vary in their susceptibility to developing depression and anxiety problems via genetic or epigenetic predisposition (Monroe & Simons, 1991) and this may be exacerbated or buffered by exposures in the environment during development. In a prominent study illustrating gene x environment interaction, only carriers of a specific polymorphism in the serotonin transporter gene that reduced its efficiency, who also had a history of stressful life events, showed an increased risk for the development of depression (Caspi et al., 2003).

When examining links between early life stress exposure, the stress response system, and adult mental health an important consideration is that stress responses can be viewed as adaptive as well as maladaptive, functional as well as dysfunctional. From an evolutionary point of view, adaptation to the environment ensures threat reduction and survival. The acute short-term stress response is important and highly beneficial when responding to threatening stimuli, avoiding attack, defending the pack, or escaping ostracism from the group. Current modern day stress exposures, whilst different and arguably more psychological, still harness the same stress regulatory responses developed in early humans (McEwen, 2007).

Whilst it is natural when examining associations with psychopathology such as depression and anxiety to describe exposure to stressors and stress responses as aberrant, abnormal, good or bad, in an evolutionary context, changes in stress responses are viewed as primarily adaptive to a certain situation because adaption facilitates survival and resilience in a changing environment. All adaptations have fitness costs as well as benefits and to be adaptive a trait only has to yield a positive overall contribution to fitness (Ellis & Del Giudice, 2019). Thus, adaptive stress responses may come with a cost of increased vulnerability to certain psychopathologies (McEwen, 2007).

In summary, these overlapping theoretical perspectives all encompass the idea that mental health vulnerabilities have their origins in early life, and that the timing of the stress exposure may be an important factor in determining reactivity of stress response systems and later life mental health. In general, many of these theoretical perspectives address only one aspect of developmental stressors, stress reactivity and health and hence may be an oversimplification of the spectrum of pathways observed in reality. Nonetheless, they are useful overlapping perspectives for understanding how early life stress exposure may influence the HPA-axis response to stress and development of symptoms of depression and anxiety. There remain unanswered questions to be addressed concerning the timing of the stress exposure and the existence of sex differences and this will require longitudinal lifespan approaches.

2.5 Measurement of the HPA-axis in the laboratory

The work in this thesis involved measuring the stress response via the HPA-axis and cortisol release. This section puts the choice of measurement in context, considers sources of variation and highlights gaps in the literature. HPA-axis function and regulation can be assessed by measuring cortisol levels in blood, saliva, urine or hair. Whilst urine and hair cortisol are better suited to measuring daily or long-term cortisol output, blood and salivary cortisol are more appropriate for measuring short-term cortisol changes over the day or responses to a stressor over 60-90 minutes.

Basal cortisol (whether measured from blood or saliva) represents unstimulated non-stressed HPA activity, although cortisol does show natural variation over the day (circadian rhythm). The Cortisol Awakening Response (CAR) describes the increase in cortisol by about 50% within the first 30 minutes after awakening in about 75% of people (Wust et al., 2000). Normally, cortisol levels peak in the morning and reach their nadir (lowest point) around midnight, with smaller peaks induced by meal consumption (Debono et al., 2009). The regulation of circadian cortisol rhythm is controlled by a specific part of the brain (the suprachiasmatic nucleus of the hypothalamus) (Germain & Kupfer, 2008), and it has been suggested that basal resting cortisol may not be the best marker for detecting dysfunction in the HPA-axis response to stress (Wilkinson & Goodyer, 2011). Therefore, the response of the HPA-axis system when challenged by a stressor may be more appropriate for assessing variability in the stress response between people and how this links to depressive/anxious symptoms.

Various types of laboratory tests have been developed to elicit an HPA-axis response using a stressor. Physical tests include subjecting a participant to exercise or cold pressor tests, which are not suitable/safe for all participants, and pharmacological tests involve administering synthetic forms of cortisol to capture how much the HPA-axis is downregulated (via negative feedback mechanisms). Psychological stressors are non-metabolically challenging tasks and arguably more representative of common acute stressors in social settings. Not all psychological stressors are equivalent, and different tasks vary in how robustly they induce increases in cortisol levels.

A comprehensive investigation into the types of psychological laboratory tests that elicit an HPA-axis response found that the combination of both uncontrollable and socially evaluative elements was associated with the largest cortisol and ACTH responses and the longest times to recovery (the most robust and reliable responses)(Dickerson & Kemeny, 2004). With an acute psychological stressor, healthy HPA functioning results in the release of cortisol, reaching peak levels after about 20 minutes and then gradually falls back to the baseline expected for that time of day after the stressor is removed (Kudielka, Schommer, et al., 2004). The most robust response to a psychological stressor was found when measured in the afternoon when the rate of change due to diurnal rhythm is low (Dickerson & Kemeny, 2004), and there was little difference in cortisol response when measured via plasma vs salivary cortisol (Dickerson & Kemeny, 2004). Therefore, psychological stress challenges offer the unique advantage of measuring a robust real-time response to a typical stressor from the brain's perception of threat to endogenous HPA-axis activity and release of cortisol.

2.5.1 Trier Social Stress Test (TSST)

The Trier Social Stress Test (TSST) utilises the combination of uncontrollable and socially evaluative elements and is based on the stress induced by public speaking and mental arithmetic in front of a formal audience (Allen et al., 2017; Kirschbaum, Pirke, et al., 1993). It is widely considered the gold-standard test for inducing and measuring an acute stress response in the laboratory (Dickerson & Kemeny, 2004). Regarding social evaluation, participants are told their public speaking performance is being captured on permanent record (video recorder), and an evaluative audience is present who remain serious/expressionless and non-responsive. These elements represent threats to the social self, one's social esteem or social status. Humans are driven to preserve the social

self and are especially attentive to threats that may negatively impact social evaluation. The following sections will discuss some of the existing gaps in the literature with regard to the use of the TSST to measure the HPA-axis response.

2.5.1.1 Sample size

Despite the valuable information gained from the TSST, it is time-consuming and expensive to conduct; therefore, studies tend to be limited in size, with sample sizes generally ranging between 40-150 participants (Benson et al., 2009; Carnuta et al., 2015; Childs et al., 2010; Elzinga et al., 2008; Engert et al., 2013; Fiksdal et al., 2019; Heim et al., 2002; Huang et al., 2015; Jones et al., 2012; Kirschbaum et al., 1999; Maki et al., 2015; Morris et al., 2012; Petrowski et al., 2010; Petrowski et al., 2013; Rao et al., 2008; Rohleder et al., 2001; Rohleder et al., 2003; Schommer et al., 2003; Wingenfeld et al., 2017). When sample sizes are limited, choices may be made to focus on one sex (Benson et al., 2009; Carnuta et al., 2015; Childs et al., 2010; Engert et al., 2013; Heim et al., 2002; Huang et al., 2015; Maki et al., 2015; Ouellet-Morin et al., 2019; Rohleder et al., 2003; Wingenfeld et al., 2017; Wust et al., 2005), one demographic, e.g. University students (Elzinga et al., 2008; Engert et al., 2013; Fiksdal et al., 2019; Huang et al., 2015; Maki et al., 2015; Powers et al., 2016; Rohleder et al., 2001; Schommer et al., 2003), or clinical samples (Morris et al., 2012; Petrowski et al., 2010; Petrowski et al., 2013; Rao et al., 2008), reducing the generalisability of findings to the rest of the population. Large sample sizes are called for as they have greater power to detect more subtle differences and reduce discrepancies within the TSST literature.

2.5.1.2 Sex differences and sex steroids

One of the most consistent findings in the TSST literature is the significantly larger salivary cortisol response in healthy adult men compared to women, up to twice as high. Males also generally display higher ACTH and blood cortisol responses compared to females (Kirschbaum et al., 1999), although this is not always the case (Rohleder et al., 2001). These differences arise during puberty and are often attributed to the altered sex steroid production at this time. In support of this theory, cortisol responses to stress in women have been shown to vary with oral contraceptive (OC) use and menstrual cycle, although results differ. Cortisol responses have been reported to be higher in the luteal phase (Felmingham et al., 2012; Kirschbaum et al., 1999), in the follicular phase (Huang et al., 2015; Maki et al., 2015) or show no change (Childs et al., 2010). Women using OC

universally display very high blood cortisol levels compared to women not on OC and men (Kirschbaum et al., 1999) and have shown a blunted salivary cortisol response compared to women not on OC (Kirschbaum et al., 1995; Rohleder et al., 2003).

2.5.1.3 Other modifiers

The HPA-axis response to the TSST has been found to differ with age, food or drink consumption, smoking, vigorous exercise, obesity and time of the day (Allen et al., 2017; Kudielka et al., 2007). Some of these are generally controlled for in the implementation of the test. For example, the increases in cortisol after a meal and with exercise are avoided by asking participants to refrain from these activities for at least an hour before arrival at the laboratory. Similarly, tests are conducted at the same time of the day, generally in the afternoon, when the changes in the background diurnal rhythm are low. In terms of age, younger adults have shown higher cortisol (Hidalgo et al., 2015) and ACTH responses to the TSST (Kudielka, Buske-Kirschbaum, et al., 2004). However, whilst many studies may include young adults in their data collection, there are limited large-scale normative data available in an unselected population providing insight into the 'normal' range of stress-induced HPA-axis reactivity.

Long-term smokers generally display a lower or blunted HPA response compared with non-smokers (Childs & de Wit, 2009; Rohleder & Kirschbaum, 2006). There are also conflicting reports on the impact of BMI, with reports of a blunted response with increasing BMI (Jones et al., 2012) and a higher cortisol response with obesity (Benson et al., 2009). In summary, with factors contributing to normal TSST variation, there are discrepancies in results between studies concerning the size and direction of HPA-axis reactivity. There is a need for studies with sufficiently large sample sizes to resolve these and determine normative responses in a young adult population.

2.5.1.4 Measuring salivary cortisol versus blood cortisol and ACTH

Salivary cortisol is a useful biomarker in stress reactivity research and remains the measure of choice in the literature. It allows the collection of samples relatively stress-free without medical staff and in many different environments. This is of particular relevance in children and large samples. However, salivary cortisol may not capture all aspects of HPA-axis reactivity. The different levels of the HPA-axis are modulated by a variety of factors, and dissociations of the salivary cortisol response with both ACTH and plasma cortisol levels have been reported, particularly with sex steroids (Hellhammer et

al., 2009). For example, estrogen upregulates the binding protein CBG, which binds cortisol in the blood; therefore the body produces more cortisol to maintain the free (active) cortisol compartment resulting in extremely high total cortisol results in plasma in females on the oral contraceptive pill, but relatively comparable salivary cortisol levels (Hellhammer et al., 2009). Measurement of ACTH and cortisol in plasma and cortisol in saliva carries different information, and examination of all these different levels of the HPA axis has been described as a best-case scenario (Hellhammer et al., 2009). However, the blood draw or cannulation required for measuring plasma ACTH and cortisol comes with legitimate concerns as it is a more invasive procedure and carries the potential for a cortisol response independent of the TSST. Blood samples also require additional processing (storage, costs), which is not always feasible. To alleviate the effect of the blood collection, studies recommend a rest period of 45 minutes after cannulation before the start of the TSST (Epel et al., 2000; Kirschbaum et al., 1999; Kudielka et al., 2007). Due to some of the issues mentioned above, there remains a shortage of information examining the HPA-axis at the levels of blood ACTH, blood cortisol and salivary cortisol, representing different stages of regulation via the hypothalamus, pituitary and adrenals.

2.5.1.5 Analytical measures selected

A variety of analytical measures are used for assessing HPA-axis reactivity (for example, cortisol concentration at the peak response, the magnitude of change from baseline to peak and the overall cortisol response indicated by the Area Under the Curve (AUC)(Pruessner et al., 2003). In addition, the curves from different participants (or groups of participants) can be directly assessed/compared using repeated measures analysis. In most studies, one or two specific measures are used to assess reactivity, whereas an altered stress response profile is likely to show differences over multiple domains. There are limitations with simply identifying a shift in the curve up or down, and others have recognised the need for dynamic measures such as reactivity and recovery slopes (Fiksdal et al., 2019; Powers et al., 2016). Further research is needed incorporating measurements over multiple parameters to increase the depth of understanding of HPA-axis differences between individuals/groups and to ensure data is not cherry-picked for positive associations. If differences are observed with one parameter, they will likely be reinforced by complementary changes in other parameters.

There have been some classifications of different response ‘patterns’. For example, up to 30% of TSST participants do not show a notable increase in HPA-axis activity and

have been termed non-responders (Kudielka et al., 2007). This distinct attenuated pattern of non-responsiveness has been found in patients with panic disorder (Petrowski et al., 2010) and adverse life events (Elzinga et al., 2008), suggesting this profile may convey risk and warrants further study. People who mount a cortisol response early in anticipation of a stressful event have been termed anticipatory responders (AR) compared with those who respond predictably at the time of the event, the reactive responders (RR) (Engert et al., 2013). Whilst there has been some subgroup analysis in the literature of high and low responders (Schommer et al., 2003) and responders versus non-responders with the menstrual cycle (Maki et al., 2015), there have also been analyses excluding non-responders (Fiksdal et al., 2019). These patterns have not been examined together in association with known modifiers of the HPA-axis response or expression of disease. This method of grouping the reactive stress response represents a different categorical classification/approach, which may complement traditionally used measures.

2.5.1.6 Summary

In summary, the TSST is currently the best available experimental procedure for investigating the acute psychosocial stress response in human participants. Previous work has been limited by sample size and has examined one sex or demographic, reducing generalisability to the general population. Saliva has been the most frequent source alongside a variety of different cortisol outcome measures, and there are discrepancies in the literature about the effect of various modifiers on the TSST.

There is a need for large sample sizes enabling examination of sex differences and clarification of the role of identified modifiers in a community population, which will increase the generalisability of results to the general population. In addition, there is a need to see integration and comparison of the 'pattern of overall response' with traditional validated measures over three levels of the HPA-axis (ACTH, total blood cortisol and salivary cortisol).

2.6 Association of early life stress with adult cortisol reactivity

Having considered the measurement of HPA-axis reactivity, the following section will review the evidence for the association of early life stressors with adult HPA-axis reactivity.

Exposure to early life stressors appears to disrupt regulation of the HPA-axis in both animal and human models. This is proposed to occur through fetal programming effects or mechanisms of biological embedding. The direction of this effect, however, remains controversial as studies have shown relative increases (Charmandari et al., 2003; Hunter et al., 2011) and decreases (Carpenter et al., 2007; Carpenter et al., 2009; Charmandari et al., 2003; Lam et al., 2019) in the production of cortisol with stressful life events compared to controls.

These conflicting results may be due to the timing and/or severity of life stress, sex differences and the timing of the HPA-axis reactivity measurement, which varies between studies (Lupien et al., 2009; Weinstock, 2008). These issues will be discussed in further detail below.

2.6.1 Relative timing of early life stressors

The time during early life in which the stressor occurs is now known to be important as the brain regions regulating the HPA-axis response to stress develop and mature at different stages during development, as discussed in Section 2.3.1.1. In addition, prenatally, the fetus is exposed to maternal stress hormones; however, after birth the infant must rely on their own production of stress hormones and learning regulatory capacity. Therefore, different mechanisms of action may be relevant before birth compared to after birth.

Prenatal stress exposure has been associated with a blunted cortisol stress response in infants and a higher cortisol response on the first day of school (age 5)(Gutteling et al., 2005; O'Connor et al., 2012). Examination of prenatal stress and offspring cortisol reactivity in adulthood is rare. A study examining maternal retrospective accounts of stress during pregnancy and the cortisol response to stress in their 25-year-old children found some evidence for lower baseline cortisol in offspring exposed to one or more stressors in-utero compared to no stressors (Entringer et al., 2009). However, we know little about how the timing of the stressor during pregnancy may impact the HPA-axis. There is some evidence that high maternal cortisol late in gestation is related to a higher stress response to heel prick in the infant (Davis et al., 2011), and that high maternal cortisol early in pregnancy and maternal stress throughout pregnancy are associated with longer time to infant recovery from a stressor (Davis et al., 2011). Different exposure times to cortisol in-utero correspond to different behavioural outcomes in child (Davis & Sandman, 2010). As specific

fetal brain structures and physiological systems develop at different times over gestation, the timing of stress exposure during gestation may be relevant to later life HPA-activity. Unfortunately, there is limited data available regarding the timing of stressor exposure during pregnancy and the impact on adult functioning of the HPA-axis.

Postnatal stress exposures have been examined at different times over childhood with respect to HPA-axis reactivity. Childhood stressors such as neglect/deprivation and being housed in an orphanage are associated with reduced cortisol responses to a stressor in childhood compared to family-reared children (DePasquale et al., 2019; M. Gunnar & K. Quevedo, 2007). Children exposed to bullying before age 12 have shown a blunted response to psychosocial stress compared to controls (Ouellet-Morin et al., 2011). Early life trauma before the age of 18 (maltreatment (incorporating physical, emotional, sexual abuse or neglect), parental conflict or violence, separation from parents, parental mental illness or substance abuse) have shown generally lower cortisol responses to stress, as assessed in a recent meta-analysis of 30 data sets (Bunea et al., 2017). However, a second meta-analysis focusing on the long-term effects of early life stress on HPA-axis reactivity in adulthood found no specific associations (Fogelman & Canli, 2018). Furthermore, sometimes early life trauma has been linked with higher responses to a psychosocial challenge in adolescents (Harkness et al., 2011), adult women (Heim et al., 2002) and adult males (Ouellet-Morin et al., 2019). Thus, inconsistent findings complicate overall interpretations of results. Variation in the time point at which the stressor was experienced may contribute to these inconsistencies. The study of Bosch et al. (2012) supports this idea, where 16-year-olds who experienced high-stress exposures pre/postnatally and during late childhood (age 6-11) showed high cortisol reactivity whilst those with high-stress exposure during early adolescence (age 12-13) and middle adolescence (age 14-15) showed lower cortisol reactivity compared to controls (low-stress exposures) (Bosch et al., 2012). This suggests the timing of the stressor in childhood may contribute to altered cortisol reactivity later in life and is worthy of further investigation.

2.6.2 Severity of stressor

The severity of the stressor may be another factor explaining inconsistencies in the literature. In general, the focus has been on more severe life stressors, including abuse, maltreatment, and trauma. In their meta-analysis, Bunea et al. (2017) found that

maltreatment resulted in a larger effect size for blunted cortisol reactivity compared to other forms of stress. With regard to maltreatment, childhood physical abuse was found to predict attenuated cortisol reactivity in adulthood compared to other forms of maltreatment, such as emotional abuse or neglect (Carpenter et al., 2011). In terms of neglect, low early life maternal care is a reliable proxy measure and, in a study examining early life maternal care and cortisol reactivity in adulthood, low maternal care was associated with blunted cortisol reactivity compared with moderate maternal care (Engert et al., 2010). However, another study did not find cortisol reactivity differences between young adults from high family conflict and low family conflict early life environments (Andreotti et al., 2015). In general, few studies focus on the relationship between less severe stress events and adult cortisol reactivity. Therefore, the impact of more common early life stress events relevant to the general population remains in question.

2.6.3 Sex differences

There is evidence for sex differences in the physiological response to stressors throughout the lifespan (Oyola & Handa, 2017; Rincon-Cortes et al., 2019). In utero, the placenta, like the fetus, is male or female, and sex differences have been found in placental activity of the 11B-HSD2 enzyme that inactivates cortisol leading to variability in vulnerability to maternal cortisol. There are also sex differences in gene expression and gene methylation (epigenetic changes) in response to prenatal stress (DiPietro & Voegtline, 2017; Sutherland & Brunwasser, 2018), which may differentially program the development and reactivity of the HPA-axis. There are postulated evolutionary benefits for sex differences in the long-term programming effects of prenatal stress, reviewed elsewhere (Glover 2012). Whilst girls and boys differ in their exposure to testosterone and estrogens from birth, in puberty, the effects of sex-specific hormones become more apparent as they modulate the activity of the HPA-axis, and sex differences in response to stressors become more pronounced (Gunnar, Wewerka, et al., 2009; Romeo, 2010b).

There is evidence for a differential effect of early life stress on the cortisol response to a stressor in males and females. In their meta-analysis across 30 data sets, Bunea et al. found the effect of early life stress exposure on later life blunted cortisol responses to social stress was more pronounced in females (Bunea et al., 2017). This may point to stress sensitisation effects in females or sex-specific physiological responses to stress.

Whilst sex may be incorporated into statistical models evaluating the relationship of early life stress with adult HPA-axis reactivity as a confounder, it is rarely examined as a moderator, i.e. associations evaluated separately in males and females and tested for differences in strength or direction. Studies examining exposure to early life stress and HPA axis reactivity specifically stratified by biological sex have been called for (Gobinath et al., 2015; Sutherland & Brunwasser, 2018).

2.6.4 Study design

Research into the links between early life stress exposure and adult cortisol reactivity has mostly been characterised by small cross-sectional samples. As discussed in Section 2.3.2, retrospective accounts of early life stress may not be reliable as they are subject to recall bias, and stressors may have occurred before a child's memory fully develops. Exposure to stress is highly correlated over the lifespan (Cole et al., 2006), making it hard to tease out the relative impact of how the timing of stressors impact the offspring's HPA-axis reactivity in later life.

Whilst cross-sectional and retrospective studies are cost-effective, prospective studies and longitudinal analyses using repeated measures of early life stress occurrence through to adulthood in the same individuals have been called for (Miller et al., 2007).

2.6.5 The timing of HPA-axis reactivity measurement

The functioning of the HPA-axis is known to change over the life course. Immediately after birth, the HPA-axis is hyper-responsive to stressors, then a relatively hyporesponsive period emerges over the course of the first year of life in humans throughout childhood (Gunnar & Cheatham, 2003; Lupien et al., 2009). The pubertal transition and release of sex hormones coincide with large shifts in the HPA-axis when it increases in basal activity and reactivity (Romeo, 2010b). There is evidence that adolescence may be a recalibration window for the stress response (Gunnar et al., 2019; Gunnar, Wewerka, et al., 2009), where it reaches a new set point in preparation for the future challenges of adulthood, and measurement of the HPA-axis after this time may be more indicative of HPA-axis function during adulthood. Therefore, whilst there is an adaption of the TSST for children (TSST-C) (Buske-Kirschbaum et al., 1997), childhood HPA-axis reactivity is likely to be a short-term transitory indicator of the stress response.

As such, studies examining early life stress and HPA-axis reactivity after adolescence are likely to be more representative of longer-term adult HPA-axis functioning.

2.6.6 HPA-axis responder category

As discussed in Section 2.5.1.4, there have been responder categories reported in the literature that describe patterns of cortisol response to a psychosocial stressor: Anticipatory responders (AR), reactive responders (RR) and non-responders (NR). While there is evidence that adverse life events in childhood are associated with a blunted cortisol response to a stressor, similar to non-responders (Elzinga et al., 2008; Lovallo et al., 2012), the association of early life stressors with responder category has never been systematically tested alongside other traditional measures of HPA-axis reactivity.

2.6.7 Summary

There is mounting evidence that early life stress exposures are associated with altered HPA-axis reactivity in adulthood, but conflicting results exist in the literature. Inconsistencies in the data may be due to differences in the timing of the stress exposure, the severity of the stressor, the sex of the individual and the age at which cortisol reactivity was measured. Large studies considering these factors may help clarify why these discrepancies exist and discover important new links that improve our understanding of how early life stress exposures are related to an altered response to stress later in life.

Having considered the association of early life stress exposure with HPA-axis reactivity above, the following section will review the evidence for the association of adult HPA-axis reactivity with symptoms of depression and anxiety.

2.7 Association of cortisol reactivity with depression and anxiety symptoms in adults

Symptoms and diagnoses of depression and anxiety are highly related to stress exposure and linked with differential responses to stressors. A high proportion of people with depressive and anxiety disorders show increases or decreases in cortisol reactivity, indicating a dysregulated HPA-axis may increase vulnerability to these disorders or may occur alongside their development. In people diagnosed with depression and anxiety disorders, there have been inconsistent results in the literature with reports of both

increased cortisol reactivity (Heim et al., 2008; Heim et al., 2002), no change (Ciufolini et al., 2014) and blunted cortisol reactivity (Petrowski et al., 2021; Zorn et al., 2017). Investigation of the cortisol response to stress in the general population with dimensional measures of depression and anxiety symptomology incorporating careful consideration of sex differences and TSST measures may aid the interpretation of these conflicting clinical results. These gaps in the literature are discussed further below.

2.7.1 Measuring symptoms in the population

Unsurprisingly, there has been a focus on clinical populations and disorders in this field. Whilst this is useful in understanding the relationship of the HPA-axis with depression and anxiety disorders, it does not examine the progression of the relationship before that point. ie. Does the HPA-axis change alongside or before symptoms of depression and anxiety emerge? For these types of questions, a population sample is required, and the measurement of symptoms on a continuous scale. Examination of dimensional measures of anxiety and depression enables observation of relationships with cortisol reactivity beneath clinical thresholds. Detection of progressive changes in the HPA-axis and the development of a disorder also helps establish whether a dose-response relationship exists. ie. Does HPA-axis reactivity increase or decrease further with increasing depression and anxiety symptoms/severity?

In addition, concerning findings of low/attenuated reactivity of the HPA-axis with depression and anxiety, the question has been raised, does it go up before it goes down? One of the main pathways to hypocortisolism is proposed to be chronic hyperstimulation (Fries et al., 2005; Phillips et al., 2013). In support of this, one study found that recent onset depression was associated with an increased cortisol response whilst patients with chronic depression showed a blunted cortisol response (Booij et al., 2013). We need investigations of symptoms during the development and progression of these disorders to unravel these complexities and ensure results are generalisable to the general population and clinical populations.

The following paragraph summarises the evidence for HPA-reactivity associations with depression/anxiety symptoms in community populations. One large study (n = 725) of an older sample of males and females (55-60 years) showed lower cortisol reactivity in those with mild to severe symptoms compared to those with no symptoms of anxiety/depression (de Rooij et al., 2010). A second study of 196 dating couples (18-21

years) using discussion of an unresolved relationship conflict as the stressor indicated lower cortisol levels with female depressive symptoms but higher cortisol levels with female anxiety symptoms and male depressive symptoms (Powers et al., 2016). Finally, a third study of a community sample of males and females (*n* = 143 age 18-65, mean age 30) found depressive symptoms were associated with steeper slopes of reactivity and recovery, but anxiety symptoms were associated with flatter reactivity/recovery slopes (Fiksdal et al., 2019). There remains a paucity of research examining the associations between HPA-axis reactivity and symptoms of depression and anxiety, and there are inconsistencies in the studies that do exist. Large community samples are required to help clarify these relationships.

2.7.2 Sex differences

Stress-induced reactivity of the HPA-axis varies between the sexes, as discussed in Section 2.5.1.2. These sex differences arise during and after the hormonal changes that occur with puberty (Romeo, 2010b). At a similar time, there is a sharp increase in the prevalence of depression and anxiety disorders, with a disparity emerging between the incidence rates in males and females, whereby about twice as many females are affected as males (Cyranowski et al., 2000). There is increasing evidence to suggest that girls' higher risk for depression at this time may involve an increased sensitivity to stressful life events (Bale, 2006; Oldehinkel & Bouma, 2011) and evidence indicating sex differences in HPA-axis reactivity to acute stress with depression and anxiety disorders (Zorn et al., 2017).

A meta-analysis of cortisol reactivity and MDD showed an overall blunted response in women with diagnosed depression or anxiety and elevated responses in men with these disorders (Zorn et al., 2017). Consistent with this and concerning symptoms, a study of dating couples showed associations of blunted cortisol responses to a stressor with depressive symptoms in females, but higher cortisol responses to a stressor with depressive symptoms in males (Powers et al., 2016). However, they also found higher cortisol responses to stress with anxiety symptoms and diagnoses in females. Additional research examining symptoms has found weak evidence for moderation by sex (Fiksdal et al., 2019) or no evidence for moderation by sex (de Rooij et al., 2010). Others have highlighted biological sex as an important factor influencing the degree of HPA-axis activation (Rincon-Cortes et al., 2019), and there have been calls for future work to examine relationships separately in males and females (Powers et al., 2016; Rincon-Cortes et al., 2019).

2.7.3 Examination of multiple parameters of the TSST

To some extent, the heterogeneity of cortisol stress response measures to the TSST used in the literature has hampered overall interpretation of the field. The most comprehensive review and meta-analysis of cortisol reactivity across psychiatric disorders to date focused on the standardised cortisol outcomes of area under the curve (AUCi, with respect to increase and AUCg, with respect to ground) (Zorn et al., 2017). Larger AUC is generally assumed to incorporate higher baseline cortisol and/or higher peak cortisol during the psychosocial stressor task, although this is not always the case. Measurement over multiple parameters is necessary to disentangle the effects of sex and identify more subtle changes with developing depression and anxiety symptomatology.

Furthermore, depression disorders and panic disorder especially have been associated with attenuated cortisol response to a stressor, similar to that of non-responders, described in Section 2.5.1.4. However, little research has examined the prevalence of this response category in people with depression/anxiety disorders or symptomatology. A study of cortisol reactivity in a community sample and the association with symptoms of anxiety and depression eliminated non-responders from analyses (Fiksdal et al., 2019), highlighting the need to determine whether this responder category is related to symptoms of depression and anxiety. Others point to the period of anticipation before a stressful event as an important window into stress regulation (Pulopulos et al., 2020). Whilst the anticipatory response to a stressor has not been examined in relation to mental health, there are reasons to connect it with social anxiety, as anticipatory responders exhibit significant cortisol release in anticipation of a stressful event (Engert 2013). This response has been postulated to be related to increased subjective stress sensitivity (Engert 2013) and a negative expectation of being able to handle the upcoming situation (Pulopulos et al., 2020). Therefore, examination of responder category alongside traditional validated TSST measures may shed new light on adaptive and maladaptive responses to psychosocial stress in terms of depression and anxiety symptoms.

2.7.4 Timing of measurement

Research examining HPA-reactivity via the TSST and depression/anxiety has been conducted on participants of different ages. However, given that HPA reactivity changes during and after puberty, and depression and anxiety disorders also rapidly increase over

puberty to a maximum prevalence in young adulthood (Ivancic et al., 2014), this appears to be the optimal time to examine the association between the two.

2.7.5 Summary

Whilst a plethora of research exists linking cortisol reactivity with depression and anxiety disorders, results remain conflicting, and there is little data examining depressive/anxiety symptomatology. Conflicting results may stem from inadequate capture of temporal change in cortisol reactivity over time (e.g. from high to low), sex differences and the choice of TSST outcome measurement used. Investigation of a spectrum of symptoms from low to high may help determine if a dose-dependent relationship exists between cortisol reactivity and increasing symptomatology. There is a need for sufficiently powered studies that consider the differential influence of sex, report on multiple TSST outcomes to allow comparison across studies and consider depression and anxiety symptomatology as a continuum rather than a binary outcome.

2.8 Evidence for the involvement of the HPA-axis in the relationship between early life stress exposure and depression/anxiety problems

The preceding sections have reviewed the evidence for HPA-axis/cortisol reactivity being linked to both early life stress and adult depression/anxiety symptoms and disorders, highlighting the HPA-axis as a potential pathway linking early life stress with the development of depression and anxiety. As previously mentioned in Section 2.6.5, basal cortisol levels and cortisol reactivity increase during adolescence and puberty, a time when we see a corresponding increase in the cases of anxiety and depression (Section 2.1.5)(Andersen & Teicher, 2008). The sex differences in the HPA axis response to stress and the prevalence of depression and anxiety disorders also evolve at a similar time, around adolescence. Furthermore, as discussed in Section 2.3.1.1, changes in the brain regions that regulate the stress response have been associated with both exposure to early life adversity and a diagnosis of depression. Using brain imaging and fMRI technology, reductions in hippocampal volume and function have been observed in people exposed to childhood adversity (Frodl et al., 2017) and patients with depression (Videbech & Ravnkilde, 2004).

The following section will discuss a combination of evidence from different research fields that collectively suggest a role for the HPA-axis in this relationship.

Firstly, many of the hallmark symptoms of depression are the same symptoms observed with disturbances in HPA-axis function (changes in mood, appetite, sleep, libido and motivation as well as metabolism and arousal across the day) (Adam et al., 2008). Cushing's syndrome, characterised by prolonged elevations in cortisol, also includes symptoms of irritability, exhaustion and mood swings (Sonino et al., 2010). The reverse problem of hypocortisolemia, seen in Addison's disease, results in fatigue, muscle weakness, anxiety and mood changes, suggesting that too much and too little cortisol can affect mental health.

Secondly, in addition to reactive cortisol responses to psychosocial stress, exposure to ELS is also associated with altered functioning of the HPA-axis in terms of diurnal fluctuations in cortisol levels and long-term cortisol exposure as measured by cortisol in hair. In a 12-year longitudinal study of adults, with an accumulation of chronic stress exposure over the 12 years, diurnal cortisol variation became flatter over time (Herriot et al., 2020). People exposed to both low and high adversity showed higher hair cortisol levels at age 17 compared to moderate adversity (Ouellet-Morin 2018). Similarly, adults with depressive disorders display altered HPA axis function. Depressed adults compared to non-depressed controls exhibited higher 24hr cortisol levels (Linkowski et al., 1985), increased frequency and volume of cortisol released (Mortola et al., 1987; Pfohl, Sherman, Schlechte, & Stone, 1985) and an overall loss of the normal circadian rhythm over time (Christensen et al., 1985; Pfohl, Sherman, Schlechte, & Winokur, 1985). Furthermore, antidepressant effects have been shown to include normalisation of initial HPA-axis dysregulation (Holsboer & Barden, 1996; Pariante et al., 2004).

Thirdly, in depressed individuals, specific genetic polymorphisms linked to altered HPA-axis function, for example, in the glucocorticoid receptor (GR), the mineralocorticoid receptor (MR) and the corticotrophin-releasing factor type 1 receptor (CRF-R1) have been found in disproportionately high numbers (van Rossum et al., 2006) and gene x environment interactions confer additional vulnerability to depressive disorders (El Hage et al., 2009; Heim & Binder, 2012). This suggests that alterations in HPA-related genes are associated with increased susceptibility to developing a depressive disorder, further implicating the HPA-axis in its pathogenesis.

Taken together, these data strongly suggest that the HPA axis may be involved in linking early life exposure to stressors and later life depression and anxiety symptomology.

2.8.1 Other potential mechanisms linking early life stress and depression/anxiety

Although this thesis focuses on the reactivity of the HPA-axis, it is important to acknowledge that this is not the only stress-related mechanism likely to be involved in a link between exposure to early life stress and adult mental health. There are likely multiple mechanisms and systems involved, and below is a brief overview of other potential pathways implicated in this relationship.

The Autonomic Nervous System mediates a series of different responses to stress, including release of hormones, regulation of heart rate and blood pressure, and regulation of immune and digestive processes. As mentioned earlier in Section 2.2.1, traditional definitions of the ANS describe two subsystems: The term 'fight or flight' is used to describe the activation and mobilisation of resources predominantly via the SNS to adequately cope with a stressor and the term 'rest and digest' is used to describe being in the parasympathetic state of the nervous system with the prevailing assumption that the ANS toggles between the two. However, recent work describes a third state of the nervous system akin to 'freeze or collapse'. Polyvagal theory points to this state as being a second parasympathetic nervous system state and an adaptive response to threat if fight or flight is not an option or inadequate (Porges, 2001). The theory describes a hierarchy of autonomic nervous system responses in which the phylogenetically newer circuits react first (Porges, 2001). This state of shutdown represents the most ancient pathway in phylogenetic terms, demonstrated by hiding or death feigning in animals and is linked to immobilisation and energy conservation (Porges, 2021). The ANS is closely connected with the HPA-axis, and they may show complementary changes or divergence from each other as these two systems are increasingly being studied together (Agorastos et al., 2018).

Inflammation, the microbiome composition and sleep disturbances are also implicated in the relationship between stress exposure and depression/anxiety problems. Higher levels of inflammatory markers such as CRP and IL-6, a disrupted/abnormal gut microbiome and sleep/ circadian rhythm changes have been associated with both early life stress and depression/anxiety problems. More detailed information on these pathways is reviewed elsewhere (Agorastos et al., 2019; Lippard & Nemeroff, 2022; Misiak et al., 2020).

Regardless of the exact mechanism(s) at play, it is likely that stress exposures produce physiological alterations via a combination of genetic/epigenetic variation, mRNA and protein expression changes, and neurological changes (Silva et al., 2021).

2.9 Overall summary

Vulnerabilities to depression and anxiety disorders have their origins in early life and are sex-specific. Early life and continuing exposure to stressors have a very strong predictive role to play in the manifestation of these disorders. Evidence suggests that dimensional measures (symptoms) of depression/anxiety offer richer, more reliable information, so there is a need to examine the link between stress exposure and a continuum of symptoms, including those below diagnostic thresholds. In addition, the timing of the stress exposure, in terms of specific windows of vulnerability prenatally and postnatally, appears to be an important factor in determining later life mental health. Questions remain concerning the timing of more common stress exposures over the early life-course and the existence of sex differences.

Furthermore, there are indicators that early life stress exposure may affect an important stress response system, the HPA-axis, a system that has also been shown to be altered in depression and anxiety disorders. The TSST is currently the gold-standard procedure for investigating the acute psychosocial stress response in humans. However, previous TSST work has been limited by sample size and few measurement parameters, and there is a requirement for sufficiently large sample sizes to examine the effects of sex and other modifiers on novel outcome parameters alongside traditional ones.

Whilst there is mounting evidence that early life stress exposures are associated with altered HPA-axis reactivity in adulthood, conflicting results exist in the literature. Large studies measuring the TSST in adulthood, taking into account the timing of the stressor and sex differences, will help clarify the strength and direction of these associations.

Similarly, whilst substantial research exists linking cortisol reactivity with depression and anxiety disorders, findings are conflicting, and there is relatively less data examining depressive/anxiety symptomatology. There is a need for sufficiently powered studies that consider the influence of sex, report on multiple TSST outcomes and consider depression and anxiety symptomatology as a continuum rather than a binary outcome.

Due to the life-course nature of these questions, the use of data from longitudinal population cohort studies is indicated to further knowledge and explore gaps in the current understanding of links between early life stress exposure, regulation of the HPA-axis response to stress and symptoms of depression and anxiety as an adult. The Raine Study[1], in particular, fits this call as it is a longitudinal community cohort that has

1. A large sample size
2. Data on both males and females
3. Repeated measures of life stress events at two timepoints over gestation and eight timepoints throughout childhood and adolescence
4. The largest gold standard TSST study conducted (*n* = 986), critically after puberty
5. Measures of depression and anxiety symptoms in a vulnerable stage of young adulthood
6. Collected many other potentially important confounders

This doctoral thesis includes four published studies presented unaltered in chapters 3-6.

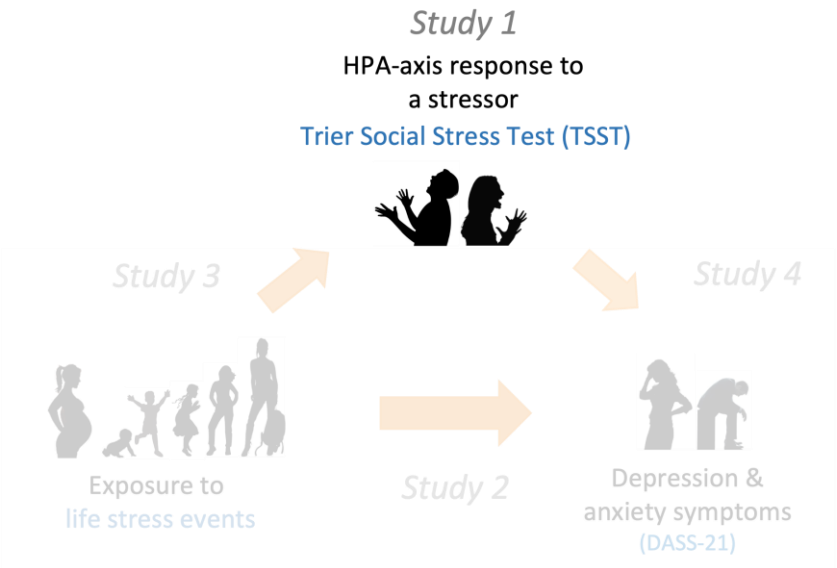
2.10 Studies 1 to 4

The studies contained in this doctoral thesis are summarised below:

1. Study 1 (Chapter 3): examines the Trier Social Stress Test data in the Raine Study cohort at age 18-years, using standard traditional measures and novel responder-category approaches, and examines their relationships with sex, BMI, smoking, oral contraceptive use and menstrual cycle.
2. Study 2 (Chapter 4): defines postnatal trajectories of common life stress exposure from birth to age 17 years and examines the relationship of prenatal stress exposure and postnatal trajectory of stress exposure with symptoms of depression and anxiety at age 20, in males and females.
3. Study 3 (Chapter 5): investigates the relationship between common early life stressors, experienced prenatally or throughout childhood and adolescence, and the HPA-axis response to a stressor at age 18-years, in males and females.
4. Study 4 (Chapter 6): examines the relationship between the HPA-axis response to a stressor at age 18-years and symptoms of depression and anxiety at age 20, in males and females.

[1] http://www.rainestudy.org.au

Chapter 3 Study 1: Characterisation and novel analyses of acute stress response patterns in a population-based cohort of young adults: influence of gender, smoking and BMI



The graphical representation of this doctoral thesis above shows the focus of the following Chapter (Study 1). This study fully characterises the results of the Raine Study Trier Social Stress Test conducted with over 1000 participants at 18 years of age. It examines the HPA-axis hormonal response to a simulated real-life stressor across the three hierarchical levels of plasma ACTH, plasma cortisol and salivary cortisol. It utilises standard traditional measures and a novel responder category approach to examine relationships with sex, smoking, BMI, oral contraceptive use and menstrual cycle phase. While this can be viewed as a stand-alone study, in the context of this thesis, it also paves the way for studies 3 and 4, examining how the HPA-axis is associated with exposure to early life stress events and future symptoms of depression and anxiety.

Publication details

This study was published in the journal *Stress* and is presented here unaltered.

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ORIGINAL ARTICLE

Characterization and novel analyses of acute stress response patterns in a population-based cohort of young adults: influence of gender, smoking, and BMI

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Abstract

Dysregulation of the biological stress response system has been implicated in the development of psychological, metabolic, and cardiovascular disease. Whilst changes in stress response are often quantified as an increase or decrease in cortisol levels, three different patterns of stress response have been reported in the literature for the Trier Social Stress Test (TSST) (reactive-responders (RR), anticipatory-responders (AR) and non-responders (NR)). However, these have never been systematically analyzed in a large population-based cohort. The aims of this study were to examine factors that contribute to TSST variation (gender, oral contraceptive use, menstrual cycle phase, smoking, and BMI) using traditional methods and novel analyses of stress response patterns. We analyzed the acute stress response of 798, 18-year-old participants from a community-based cohort using the TSST. Plasma adrenocorticotrophic hormone, plasma cortisol, and salivary cortisol levels were quantified. RR, AR, and NR patterns comprised 56.6%, 26.2%, and 17.2% of the cohort, respectively. Smokers were more likely to be NR than (RR or AR; adjusted, $p < 0.05$). Overweight and obese subjects were less likely to be NR than the other patterns (adjusted, $p < 0.05$). Males were more likely to be RR than NR (adjusted, $p = 0.05$). In addition, we present a novel AUC measure (AUC_R), for use when the TSST baseline concentration is higher than later time points. These results show that in a young adult cohort, stress-response patterns, in addition to other parameters vary with gender, smoking, and BMI. The distribution of these patterns has the potential to vary with adult health and disease and may represent a biomarker for future investigation.

Keywords

ACTH, cortisol, HPA, hypothalamic-pituitary-adrenal axis, Trier Social Stress Test, TSST, Raine Study

History

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3.1 Introduction

Dysregulation of the biological stress response system via the hypothalamic-pituitary-adrenal (HPA-axis) has been implicated in the development of cardiovascular, metabolic and mental health problems (Arlt & Stewart, 2005; Chrousos & Kino, 2007). However, it is important to first clarify the range of stress responses within a normal population and their relationship to factors contributing to that variation.

In a healthy human volunteer, the normal HPA-axis response following a stress-inducing stimulus involves the release of adrenocorticotrophic hormone (ACTH) from the pituitary into the circulation within minutes. ACTH binds to its adrenal gland receptor resulting in cortisol secretion. The Trier Social Stress Test (TSST), is a standardised laboratory procedure for measuring an individual's response to a psychosocial stress by stimulating reproducible increases in cortisol (Foley & Kirschbaum, 2010; Kirschbaum & Hellhammer, 2000; Kirschbaum, Pirke, et al., 1993). Factors contributing to TSST variation in normal healthy populations include gender, oral contraceptive (OC) use, menstrual cycle phase, smoking and obesity (Kudielka & Wust, 2010), although not all studies are in agreement with the size and direction of the effects.

Males generally display higher ACTH and cortisol responses compared to females not taking OC (Kirschbaum et al., 1999) although this is not always the case (Rohleder et al., 2001). Females taking OC show higher plasma cortisol responses but not salivary cortisol responses to stress compared to females not taking OC (Kirschbaum et al., 1999) though it is rare for studies to measure all three levels of the hormonal stress response. With regard to the menstrual cycle, HPA responses have been reported to be higher in the luteal phase (Felmingham et al., 2012; Kirschbaum et al., 1999), in the follicular phase (Huang et al., 2015; Maki et al., 2015) or show no change (Childs et al., 2010; Duchesne & Pruessner, 2013). Long term smokers generally display a reduced or blunted HPA response compared to non-smokers (Back et al., 2008; Childs & de Wit, 2009; Kirschbaum, Strasburger, et al., 1993; Rohleder & Kirschbaum, 2006). Finally, there are conflicting results on the impact of BMI with studies showing both a higher cortisol response with obesity (Benson et al., 2009) and a blunted cortisol response with increasing BMI (Jones et al., 2012).

Clearly most analyses identify a shift in the response curve up or down. Here we highlight a novel method, dependent instead on the particular shape of the hormonal

stress-response. Recently, there have been separate reports identifying distinct patterns of stress response apparent in the TSST data. For example, up to 30% of TSST participants do not show a notable increase in HPA-axis activity with the TSST and have been termed 'non-responders' (NR) (Kudielka et al., 2007). This distinct pattern of 'non-responsiveness' has been found in patients with panic disorder (Petrowski et al., 2010), premenstrual syndrome (Huang et al., 2015), adverse early life events (Elzinga et al., 2008) and other negative health outcomes (Fries et al., 2005; Phillips et al., 2013) suggesting that this pattern of response may convey risk and warrants further study. People with high baseline HPA hormone levels, who appear to mount an HPA response in anticipation of a stressful event have been termed 'anticipatory-responders' (AR) compared to those who respond at the time of the stressful event, 'reactive-responders' (RR) (Engert et al., 2013). Anticipatory-responder rates of 20-40% have been found in healthy men aged 18-30 in a modified TSST protocol (Engert et al., 2013). The anticipatory-response pattern has been associated with PTSD (Bremner et al., 2003) and may vary with other disease states. Whilst there has been some subgroup analysis in the literature of high- and low-responders (Schommer et al., 2003) and responders vs non-responders in the menstrual cycle (Maki et al., 2015), to our knowledge, no previous study has analysed TSST data in terms of these three stress-response patterns. In addition, we specifically perform TSST measurements in an unselected teenage population to gain insight into the 'normal' range of stress-induced HPA-activation. At this age, after puberty, the HPA axis reaches a new set-point and long term HPA functioning is established (McCormick & Mathews, 2010).

The first aim of this study was to investigate factors contributing to TSST variation across three levels of the HPA-axis in a normal young-adult cohort of large sample size to help resolve differences in the literature. These factors include gender, OC use, menstrual cycle, smoking and BMI. Our second aim was to analyse the relationship between individual stress-response patterns (RR, AR and NR) and the same factors contributing to TSST variation. A novel AUC calculation is described, AUC_R (with respect to concentration range) and multiple parameters were examined, essential to our understanding of the stress response and to facilitate comparison to other studies (Juster et al., 2012). We hypothesised that the distribution of these patterns would vary with gender, OC use, menstrual cycle, smoking and BMI.

3.2 Materials and Methods

3.2.1 Study Design and participants

Participants included 1137 males and females from the 18-year follow-up of the Western Australian Pregnancy Cohort (Raine) Study (Newnham et al., 1993). At 18 years of age, participants were invited to attend and complete the TSST. Ethics approval for this study was obtained from the University of Western Australia Human Research Ethics Committee. All procedures were carried out with written informed consent from the participants. Prior to analysis, 116 individuals were sequentially eliminated for the following reasons: unable to complete TSST (*n* = 2); unusable blood/saliva samples (*n* = 3); severe menstrual pain (*n* = 1); pregnancy (*n* = 2); lactation (*n* = 2); type-1 diabetes (*n* = 4); use of exogenous steroids (*n* = 7), neuroactive medications (*n* = 22), anti-depressants (*n* = 19), or other medications affecting the HPA-axis (*n* = 2); fainting (*n* = 15); or younger siblings of other participants violating assumptions of statistical independence of data (*n* = 37). Of the 1021 participants eligible for analysis, 806 had both blood and saliva collected and were used in the initial analyses.

3.2.2 Trier Social Stress Test (TSST) Protocols

The TSST was used to assess participant's stress responses. All subjects arrived between midday and 3pm for participation in the TSST between 1pm and 4pm to minimise the effects of diurnal variation in cortisol. Participants were instructed to refrain from eating or drinking for at least one hour before their appointment and time of last meal/drink was recorded. Participants agreeing to have blood collected had an intravenous cannula inserted by an anaesthetist 15 minutes after arrival. This was followed by a 45 minute rest period during which participants completed a questionnaire relating to any illnesses, medication use, recent physical activity, smoking habits, timing and regularity of menstrual cycles and oral contraceptive (OC) use. Height and weight were measured for calculation of BMI. After resting for 45 min, blood samples were taken just prior to the test (0 minutes), after completing the test (15 minutes) and then at 25, 35, 45, 60, 75 and 105 minutes. Saliva samples were collected at 0, 15, 35 and 105 minutes. The test itself took 15 minutes to complete and consisted of 3 minute preparation time alone, a free speech interview (5 min) and an arithmetic task (5 min) as per established protocols (Kirschbaum, Pirke, et al., 1993; Kudielka et al., 2007). The test

was performed in front of a non-responsive panel audience of three or four adults wearing formal clothing with a dummy video camera and mock audio equipment recording the interview and task. The panel reported on the participant's response to the interview: i) confident during interview, minimal prompting required; ii) some prompting required to complete the interview; iii) significant prompting required to the complete interview; and iv) non-cooperative or not responsive to interview questions. Similarly, all panel members reported on the individual's response to the arithmetic challenge: i) willing to complete the task; ii) mildly anxious while completing the task; iii) became anxious/frustrated after incorrect answers, and iv) non-cooperative or refused to complete task. Following the test, participants were debriefed on the goal of the study and the nature of the stressor. There is variation in the literature with regard to reporting TSST time points. We report time zero as the start of the TSST (Kirschbaum, Pirke, et al., 1993).

3.2.3 Laboratory Analysis of plasma and saliva

Blood was collected in BD Vacutainers containing EDTA (Becton, Dickinson and Company, NJ, USA) and saliva was obtained using Salivette collection devices (Sarstedt, Germany). Biological samples were kept on ice during the test, then centrifuged, aliquoted and frozen at -80C until assayed. Total plasma cortisol and free salivary cortisol were quantified using the GammaCoat^{TM} ^{125}I cortisol radioimmunoassay (RIA) kit (DiaSorin, Stillwater, MN, USA). Concentrations were converted from µg/dl to nmol/L by multiplying by 27.59. Plasma ACTH was measured by ^{125}I immunoradiometric (IRMA) assay (DiaSorin, Stillwater, MN, USA) as per the manufacturer's instructions. All samples were assayed in duplicate against an appropriate standard curve and were repeated with additional dilutions, where required. For all assays, the intra and inter-assay variability was < 10%. Inter-assay coefficients of variation for total and free cortisol, used in the criteria assigning participants to pattern groups, were 6.60% and 4.52% respectively.

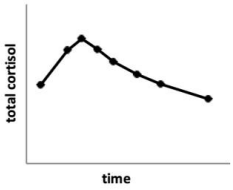
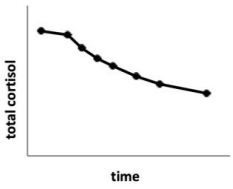
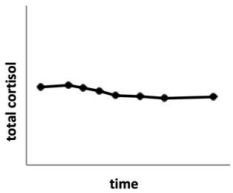
3.2.4 TSST pattern groups

Naming of stress-response patterns is aligned with previous reports of distinct response patterns in the literature (Engert et al., 2013; Schommer et al., 2003). The primary parameter used for stress pattern determination was the plasma total cortisol measured at eight regularly spaced time points. Additional information was derived from the secondary parameters of salivary cortisol and plasma ACTH. A set of criteria for the

grouping of TSST patterns was developed and refined from examination of the literature and response data and is shown in Figure 3.1.

Figure 3.1

Definitions of TSST stress response patterns

Pattern	Example	Definition	Characteristics*
RR Reactive-responder		Plasma cortisol rises immediately after TSST to a noticeable peak. Tail end of profile is consistent with subsequent return to baseline. 2 points of the peak are over 2 x CV of the plasma cortisol assay from baseline OR 1 point of the peak is over 2 x CV of the plasma cortisol assay AND salivary cortisol profile also shows a clear response (defined as peak > 2xCV of saliva cortisol assay).	low C_{BL} high C_{MAX} high C_{RANGE} high Δ1(BL to MAX) low Δ2(BL to MIN) high AUC_R, AUC_I and AUC_G
AR Anticipatory-responder		Absence of a clear response. Plasma cortisol levels consistently fall over first 60 minutes of the study by > 20.2% (2xCV, 13.2% in this study + estimation of drop per hour of 7%, due to circadian rhythm)^	high C_{BL} high C_{MAX} high C_{RANGE} low Δ1(BL to MAX) high Δ2(BL to MIN) high AUC_R, AUC_G negative AUC_I
NR Non-responder		No clear initial plasma cortisol peak, little change in cortisol over the study period. No points above 2 x CV of the plasma cortisol assay OR 1 point only above 2 x CV and salivary cortisol NR or decreasing.	low C_{BL} low C_{MAX} low C_{RANGE} low AUC_R, AUC_I

* For a graphical representation of these parameters, see Supplementary Figure 3.1. CV, coefficient of variation ^ Estimation of 7% plasma cortisol drop per hour due to circadian rhythm in the afternoon (Cizza et al., 2012; Selmaoui & Touitou, 2003); 7% salivary cortisol drop per hour (Heaney et al., 2012; Stone et al., 2001).

Briefly, a reactive-response was defined as an increase in cortisol from baseline of greater than 2x the coefficient of variation (CV) of the cortisol assays (Van Cauter & Refetoff, 1985), in this case 13.2% for the plasma cortisol assay and 9.04% for the salivary cortisol assay. This is in contrast to the use of an absolute increase in cortisol (e.g. 55.2nmol/l and 2.5nmol/l for total and salivary cortisol respectively) used by other studies (Engert et al., 2013; Petrowski et al., 2010), which may not effectively take individual baseline variation into consideration. An anticipatory-response was defined as a drop from baseline of greater than 20.2% within the first 60 minutes; 20.2% was derived from twice the CV of the plasma cortisol assay (13.2%) plus an estimation of afternoon cortisol changes due to diurnal variation (approximately 7% per hour) obtained from the literature for blood (Cizza et al., 2012; Selmaoui & Touitou, 2003) and saliva (Heaney et

al., 2012; Stone et al., 2001). Non-responders did not show reactive or anticipatory responses. A small subset of eight individuals were unable to be categorised and displayed very unusual patterns. Due to the small numbers of participants in this category, it has been excluded from analyses, resulting in a final number of 798 used in the analyses presented.

3.2.5 TSST parameter measures

Cortisol data from the TSST were reported using established definitions including: initial baseline concentration (C_{BL}); peak concentration after stress (C_{MAX}); change in concentration from baseline to maximum ($\Delta 1_{(BL\ to\ MAX)}$ = C_{MAX} minus C_{BL}); area under the curve above zero (with respect to ground)(AUC_G)(Pruessner et al., 2003); and area under the curve above baseline (with respect to increase) (AUC_I) both calculated using the trapezoidal rule (Pruessner et al., 2003). In addition, a number of additional variables were created to describe different aspects of the TSST data which include: minimum concentration (C_{MIN}); concentration range (C_{RANGE} = C_{MAX} minus C_{MIN}); change in concentration between baseline and minimum ($\Delta 2_{(BL\ to\ MIN)}$ = C_{BL} minus C_{MIN}); area under the curve (with respect to range) (AUC_R). This calculation uses C_{MIN} as the lower reference point for the area instead of C_{BL}. See Supplementary Figure 3.1 for a visual representation of these parameters.

3.2.6 Covariates

Gender and oral contraceptive use were combined to create a single variable with three categories: males, females not taking the OC and females taking the OC. BMI was categorised into underweight, normal, overweight and obese as per standard definitions (Cole et al., 2000; Cole & Lobstein, 2012). Smoking status was analysed as a simple dichotomous variable (smoker/non-smoker). In girls who reported regular cycles, menstrual cycle was divided into three categories; early follicular (day 1-8, reflecting low levels of estrogen and progesterone); ovulatory (day 9-19, reflecting high levels of estrogen and lower levels of progesterone) and late luteal (day 20-28, reflecting lower levels of estrogen and high levels of progesterone).

3.2.7 Statistical analysis

Cohort descriptive statistics were compared using t-tests for continuous variables and χ^2 tests for categorical variables. Comparisons of TSST parameters were performed using non-parametric Kruskal-Wallis and Mann-Whitney tests due to the asymmetrical distribution of the data.

General Linear Models (GLMs)/ANOVAs with repeated measures were used to assess differences between specified groups. In these analyses, a normal distribution was obtained through log transformation of ACTH and cortisol concentrations. Values were back-transformed after analysis to produce geometric means. These data are presented in graphical format. Differences between group TSST profiles were calculated using group by time effects consistent with similar studies. Greenhouse-Geisser corrections for sphericity were used where appropriate. Logistic regression models were used to determine the odds of being in a particular response group for analyses of gender group, smoking and BMI. Menstrual cycle was not included as it showed no significant contribution to the univariate or multivariate models and would diminish female group sizes.

3.3 Results

Of the original 2868 participants, 2086 (73%) were eligible to be contacted at the age 18 review; 782 had withdrawn from the study, were lost to follow up or had died. Of these, 1137 adolescents participated in the TSST. This study focuses on 798 individuals with complete information for all three biomarkers (plasma ACTH, plasma cortisol and salivary cortisol). Characteristics of study participants are shown in Table 3.1. Participants were just over 18 years of age with slightly higher numbers of males than females. Males smoked significantly more than females and one third of females were taking OCs.

Table 3.1
Characteristics of Raine Study Trier Social Stress Test participants used in analyses

YOUNG ADULT	**Total** *n* = 798	**Males** *n* = 436	**Females** *n* = 362	
	Mean (SD)	*Mean (SD)*	*Mean (SD)*	***P***[a]
Age *(years)*	18.3 (0.3)	18.3 (0.3)	18.3 (0.3)	0.87
	n (%)	*n (%)*	*n (%)*	***p***[b]
BMI				
underweight	22 (2.8)	5 (1.3)	17 (5.3)	*<0.001*
normal	462 (57.9)	244 (63.4	218 (68.3)	
overweight	137 (17.2)	92 (23.9)	45 (14.1)	
obese	83 (10.4)	44 (11.4)	39 (12.2)	
Smoker				
yes	119 (14.9)	78 (18.1)	41 (11.4)	0.01
no	673 (84.3)	353 (81.9)	320 (88.6)	
OC pill				
yes		NA	139 (38.4)	
no			223 (61.6)	
Cycle phase[c]			48 (27.7)	
early follicular			48 (27.7)	
ovulatory			70 (40.5)	
late luteal			55 (31.8)	

[a]*ANOVA p-value;* [b]*Chi2 p-value, comparisons between males and females. OC = oral contraceptive.*
[c] *Menstrual cycle phase analysed in girls with regular cycles only. 50 girls reported irregular cycles. 94 individuals did not have BMI data. 6 individuals did not have smoking data.*

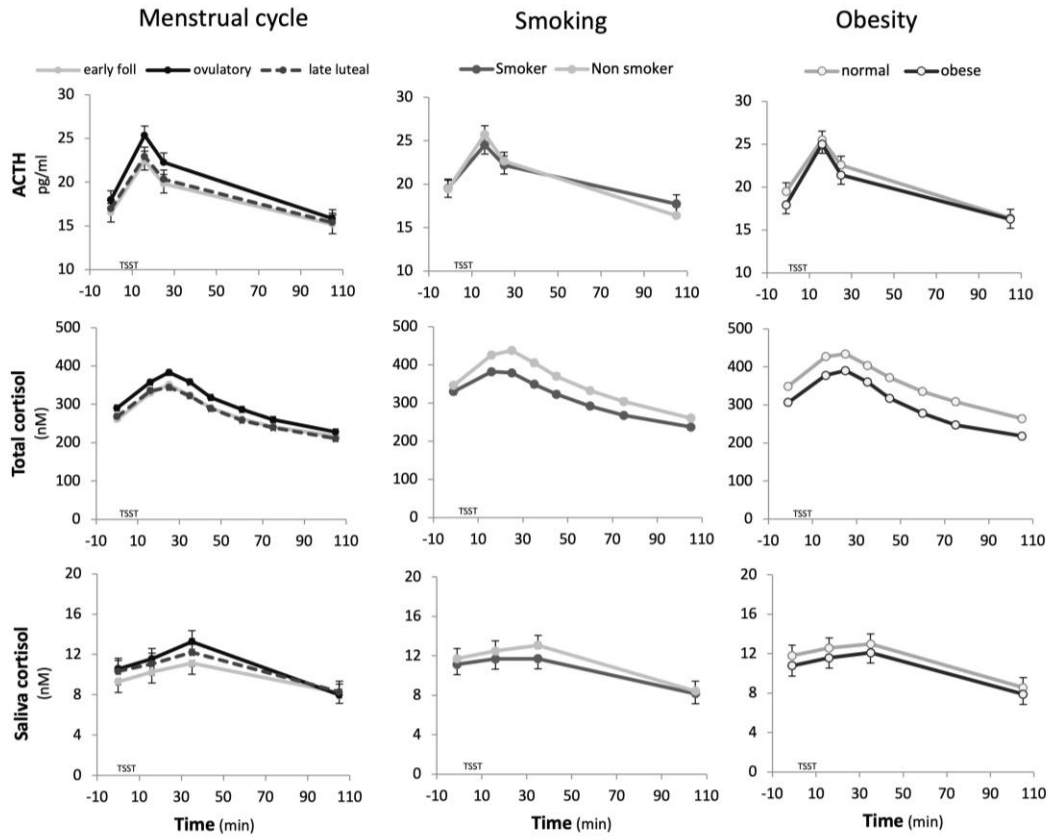
3.3.1 TSST longitudinal analyses

We analysed total results using GLM/ANOVA with repeated measures analysis to allow comparison with previous studies (Figure 3.3a). ACTH levels were significantly different across the three gender groups [F(4.2, 1620.6)=4.11, p=0.002]. ACTH was consistently higher in males than females, and females on OC displayed lower ACTH levels compared to females not on OC. These data are supported by changes in C_{BL}, C_{MAX}, C_{MIN}, C_{RANGE}, AUC_R, Δ1 and Δ2 (Supplementary Table 3.1). In contrast, for total cortisol levels, females on OC had higher levels than males who in turn had higher levels than females not on OC [F(6.2, 2322.9)=9.96, p<0.001]. See Table 3.4 for supporting

information. Salivary cortisol was consistently higher in males than females [F(4.4,1664.4)= 7.24 p<0.001]; there were no differences between females taking and not-taking OC. See Supplementary Table 3.2 for mathematical parameters. With regard to menstrual cycle, the changes in stress response over the early follicular, mid ovulatory and late luteal phases were not significant. See Figure 3.2.

Figure 3.2

TSST profiles with menstrual cycle, smoking and obesity



Mean responses for ACTH, plasma cortisol and salivary cortisol at each timepoint are shown with standard error bars.

The influence of smoking and obesity are also shown in Figure 3.2. In smokers, ACTH and total plasma cortisol TSST profiles were significantly attenuated in comparison to non-smokers [F(2.1,1607.3)=5.4 p=0.004, F(3.1,2305.5)=3.22 p=0.02 respectively]. In addition, smokers showed reductions in mathematical parameters including significantly lower plasma cortisol C_{MAX} [Mann Whitney *U*=7.2, p=0.007], C_{MIN} [U=5.2, p=0.02], AUC_R[U =9.5 , p=0.002], AUC_G [U =8.3, p=0.005] and significantly lower plasma ACTH AUC_R [U =5.2, p=0.02] and C_{RANGE} [U =4.4, p=0.04], compared to non-smokers. We saw no significant difference in salivary cortisol [F (2.24, 1670.9)=1.16 p=0.32] between smoking

groups. We also found significantly reduced total plasma cortisol in obese participants compared to those with normal weight [F(3.08, 1563)=3.18 p=0.02]. ACTH and salivary cortisol levels were not influenced by BMI [F(2.1, 1118.6)=1.25 p=0.29, F(2.25, 1165.6)=0.04 p=0.97 respectively]. There were no significant dose-dependent responses across BMI categories and no significant differences in the mathematical parameters.

3.3.2 TSST stress response patterns

The reactive-responder pattern (RR) was observed in 56.7% of participants, followed by 26.3% anticipatory-responder pattern (AR) and 17.0% with non-responder pattern (NR) of those who had total plasma cortisol measured (Table 3.2). In univariate analyses, the distribution of RR, AR and NR patterns was significantly different between males and females, smokers and non-smokers and across BMI groups. A greater proportion of males than females were RR; conversely more females than males were NR (p=0.002). Compared to non-smokers, fewer smokers were RR and more were AR and NR (p=0.03). As BMI increased, a greater proportion of participants were RR with the highest proportion of NR seen in underweight participants (p=0.01). In reactive-responders, the ACTH peak (15min) preceded total plasma cortisol peak (25min) which preceded the salivary cortisol peak (35min), as expected (Kirschbaum, Pirke, et al., 1993). There were no significant differences in the distributions of response patterns with the panel's report on interview or arithmetic performance, the participant's appointment time or time of last meal (data not shown).

Table 3.2
Distribution characteristics of TSST patterns by sex, OC use, smoking and BMI

	RR *n* = 452 (56.6%) *n (%)*	**AR** *n* = 209 (26.2%) *n (%)*	**NR** *n* = 137 (17.2%) *n (%)*	***p (χ2)***
Sex				
Female	183 (50.6)	102 (28.2)	77 (21.2)	0.003
Male	269 (61.8)	107 (24.6)	59 (13.6)	
Smoking				
non smoker	395 (58.7)	168 (25.0)	110 (16.3)	0.03
smoker	54 (45.4)	39 (32.8)	26 (21.8)	
BMI				
underweight	7 (31.8)	6 (27.3)	9 (40.9)	0.01
normal	255 (55.2)	121 (26.2)	86 (18.6)	
overweight	86 (62.8)	33 (24.1)	18 (9.6)	
obese	55 (66.3)	20 (24.1)	8 (9.6)	
Female OC use				
no OC	118 (52.9)	62 (27.8)	43 (19.3)	0.42
OC	65 (46.8)	40 (28.8)	34 (24.5)	
Female cycle phase				
early follicular	28 (28.6)	7 (18.4)	13 (38.2)	0.39
ovulatory	38 (38.8)	19 (50.0)	13 (38.2)	
late luteal	32 (32.7)	12 (31.6)	8 (23.5)	

Total *n* (RR + AR + NR) = 798.

Using traditional longitudinal analyses (ANOVA repeated measures), the three stress response patterns were significantly different from each other for ACTH [F(4.2, 1591.5)=91.9 p=<0.001)], total plasma cortisol [F(6.9, 2565.9)=159 p<0.001], and salivary cortisol [F(4.9, 1819.2)=149.1 p<0.001].

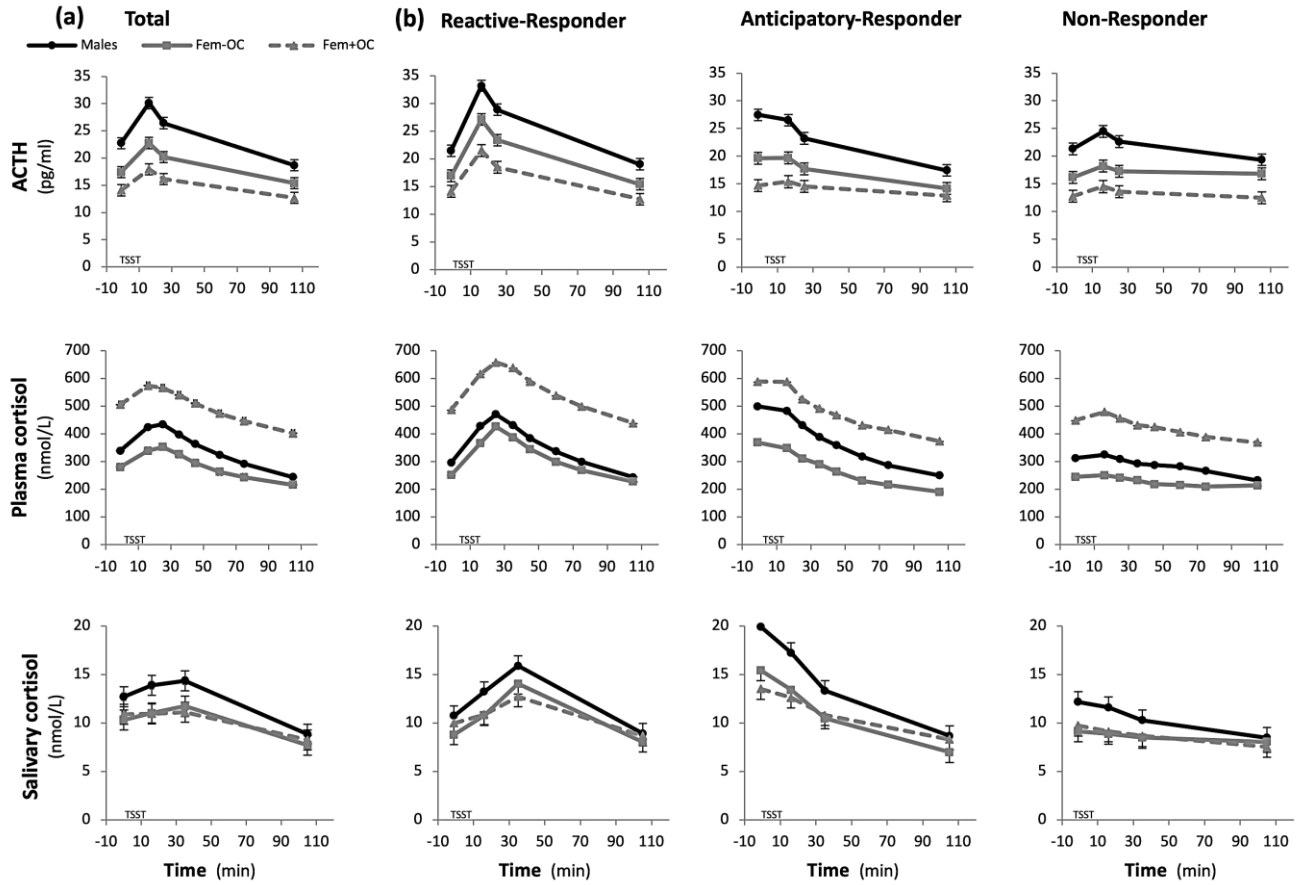
Figure 3.3 presents the ACTH, plasma cortisol and salivary cortisol for the total cohort (Figure 3.3a) and for the individual response groups (Figure 3.3b) stratified by gender group. The influence of gender and OC use can clearly be seen in ACTH, total plasma cortisol and salivary cortisol levels. If all response groups are analysed together (Figure 3.3a), sub-group effects of anticipatory and non-responders are not visible when the study numbers are sufficiently large. Logistic regression models examining the effect of gender, OC use, smoking and BMI on TSST pattern group are presented in Table 3.3.

We modelled the odds of being in NR vs (RR or AR) as both the AR and RR groups appear able to mount a clear HPA stress response. Smokers were more likely to be NRs than RRs/ARs, after adjusting for sex, OC use and BMI. Conversely, overweight and obese individuals were less likely to be NRs than RRs/ARs and more likely to be a RR (after adjusting for gender group and smoking). Similar results were seen when AR was excluded and NR was compared to RR. Males had higher odds of being a RR (and lower odds of being a NR), compared to females not taking OC, after adjusting for smoking status and BMI.

Descriptive data characterising the gender groups and TSST patterns (RR, AR, NR) are presented in Table 3.4 (plasma cortisol), Supplementary Table 3.1 (ACTH) and Supplementary Table 3.2 (salivary cortisol). In this population, C_{MIN} occurred at the end of the test, on the final sample in approximately 70% of cases. This suggests that the initial baseline sample is not the true baseline. For this reason, we describe a new AUC calculation, with respect to range, (AUC_R) instead of increase (AUC_I). AUC_R showed significant differences between gender groups across ACTH, plasma and salivary cortisol, whilst the traditional AUC_I did not. See Table 3.4 and Suppl Tables 1 and 2. Significant differences were found in stress-response patterns across all parameters except for C_{MIN}. See Figure 3.1 for a summary of these differences.

Figure 3.3

TSST response patterns by gender group



Mean responses for ACTH, plasma cortisol and salivary cortisol at each timepoint are shown (a) with standard error bars for the total population and (b) separately for reactive, anticipatory and non-responder patterns. Dark circles with solid lines represent males; grey squares with solid lines represent females not on OC; grey squares with dashed lines represent females taking OC.

Table 3.3
Logistic Regression models

	NR vs RR/AR *OR (95% CI)*	***p***	**NR vs RR** *OR (95% CI)*	***p***	**AR vs RR** *OR (95% CI)*	***p***	**NR vs AR** *OR (95% CI)*	***p***
Gender group								
Fem-OC	1.00		1.00		1.00		1.00	
Fem+OC	1.32 (0.75, 2.32)	0.33	1.49 (0.82, 2.72)	0.19	1.31 (0.85, 2.02)	0.23	1.18 (0.68, 2.06)	0.56
Male	0.67 (0.41, 1.09)	0.10	0.61 (0.36, 1.01)	0.05	0.67 (0.47, 0.97)	0.03	1.01 (0.61, 1.67)	0.98
Smoker								
no	1.00		1.00		1.00		1.00	
yes	1.68 (0.99, 2.86)	0.05	2.09 (1.19, 3.70)	0.01	1.88 (1.24, 2.85)	0.003	0.84 (0.48, 1.46)	0.540
BMI								
underweight	1.58 (0.70, 3.58)	0.27	2.02 (0.81, 5.06)	0.13	1.13 (0.56, 2.31)	0.73	1.26 (0.54, 2.92)	0.60
normal	1.00		1.00		1.00		1.00	
overweight	0.44 (0.23, 0.86)	0.02	0.41 (0.21, 0.81)	0.01	0.88 (0.58, 1.32)	0.54	0.62 (0.33, 1.14)	0.12
obese	0.40 (0.17, 0.90)	0.03	0.40 (0.17, 0.94)	0.04	0.86 (0.52, 1.41)	0.54	0.52 (0.23, 1.15)	0.11

Table 3.4

Total plasma cortisol TSST mathematical parameters by gender and stress-response pattern

PLASMA CORTISOL	Gender group				Stress-response pattern			
	Male *n* = 436 *Median (IQR)*	**Fem-OC** *n* = 223 *Median (IQR)*	**Fem+OC** *n* = 139 *Median (IQR)*	***p KW***	**RR** *n* = 452 *Median (IQR)*	**AR** *n* = 209 *Median (IQR)*	**NR** *n* = 137 *Median (IQR)*	***p KW***
C_{BL}	333.0 (250.5, 453.7)	280.0 (208.3, 366.9)	516.5 (389.6, 644.2)	*	300.2 (221.3, 394.5)	485.9 (353.5, 635.7)	313.7 (230.1, 452.5)	*
C_{MAX}	481.7 (380.0, 608.0)	393.7 (295.8, 514.3)	600.1 (481.2, 798.2)	*	501.9 (393.3, 630.0)	502.3 (363.6, 658.9)	354.5 (270.6, 483.3)	*
C_{MIN}	233.7 (178.5, 294.8)	191.6 (156.7, 243.1)	390.7 (288.6, 517.6)	*	234.8 (177.5, 309.5)	239.8 (175.8, 328.9)	222.6 (173.7, 321.8)	NS
C_{RANGE}	247.4 (167.0, 341.5)	190.6 (128.3, 275.1)	214.6 (147.6, 325.0)	*	255.9 (181.9, 342.4)	242.2 (168.8, 339.9)	118.5 (81.8, 171.3)	*
$\Delta 1(C_{BL}$ to $C_{MAX})$	137.4 (66.3, 224.5)	122.8 (46.1, 232.9)	100.4 (39.8, 183.9)	*	179.4 (113.5, 266.1)	29.8 (10.5, 53.7)	39.2 (22.1, 65.2)	*
$\Delta 2(C_{BL}$ to $C_{MIN})$	110.9 (54.8, 196.0)	97.4 (50.8, 158.8)	123.3 (54.5, 211.5)	*	72.8 (38.6, 122.8)	226.0 (154.8, 322.1)	85.0 (50.3, 118.1)	*
AUC_I	-142 (-6859, 7082)	-224 (-6516, 6198)	-2539 (-8203, 4071)	NS	5606 (539, 10594)	-12775 (-18442,-8521)	-2546 (-4653,-566)	*
AUC_8	11805 (7724, 16537)	8949 (5748, 13165)	10553 (7074, 14894)	*	12317 (8499, 16787)	10351 (7370,15522)	6398 (3735,8613)	*
AUC_G	36342 (28929, 46057)	29877 (22496, 39017)	51711 (39185, 67603)	*	37388 (29586, 48394)	36576 (26696, 48727)	30589 (22359, 41098)	*

Concentration units = nmol/L. * Kruskal Wallis p-value < 0.001.

3.4 Discussion

We have conducted the TSST in a large community-acquired population of young adults and here we report on the effects of gender, OC use, menstrual cycle, smoking and BMI and describe data in terms of traditional and novel TSST parameters. Many of these factors have been investigated in studies with smaller sample sizes that do not examine the HPA axis at three levels (ACTH, plasma cortisol and salivary cortisol) and produce inconsistent results in the literature. Further, we define, characterise and analyse three patterns of stress hormone response; reactive-responders, anticipatory-responders and non-responders and introduce a novel AUC calculation. The application of this approach to TSST data may represent a new biomarker to add to the established descriptive parameters describing TSST data.

When traditional methods of analysis (ANOVA with repeated measures) were used to examine the impact of gender on the TSST, our data were consistent with previous reports of higher ACTH, plasma and salivary cortisol levels in males compared to females not taking OC (Kirschbaum et al., 1999). The potential modulating effects of corticosteroid-binding globulin (CBG) in women (Kirschbaum et al., 1999) and complex interactions between sex steroids and the regulation of glucocorticoid and mineralocorticoid receptors (Kudielka & Kirschbaum, 2005) may contribute to this consistently reported variation.

We found increased total plasma cortisol levels in females taking OC but unchanged salivary free cortisol and decreased ACTH responses to stress compared with women not on OC. The increase in plasma cortisol is in part explained by the up-regulation of CBG by estrogen, resulting in a higher cortisol binding capacity (Kirschbaum et al., 1999). However, the ACTH and salivary cortisol data indicates OC use is potentially linked to HPA alterations at the level of ACTH, suggesting changes to pituitary sensitivity or cortisol feedback mechanisms from the high levels required to saturate CBG and maintain the same biologically active compartment. This is in contrast to previous studies showing no change in ACTH and lower salivary cortisol in women on OC, resulting in the conclusion that young women have a heightened adrenocortical sensitivity to ACTH (Kirschbaum et al., 1999; Kudielka & Kirschbaum, 2005). These differences may be due to the age of the women, length of time on OC or sample sizes. In support of our finding of reduced ACTH

response in females taking OC, Kumsta et al. found that high CBG levels in women were associated with reduced ACTH (Kumsta et al., 2007).

CBG levels may also vary over the menstrual cycle, increasing in response to estrogen (Coolens & Heyns, 1989). However, we did not find significant differences in ACTH, plasma or salivary cortisol across the early follicular, ovulatory or late luteal phases of the menstrual cycle. This suggests CBG does not vary enough to induce a significant change in total cortisol. We would therefore expect salivary cortisol, as a marker of free cortisol, to remain similar across the menstrual cycle. A recent report linking premenstrual syndrome with blunted cortisol stress-reactivity suggests additional confounders may exist within the menstrual cycle data (Huang et al., 2015). We should note that this study was not designed to assess menstrual phase and as such we do not have hormone measures confirming estrogen/progesterone status and must make assumptions about cycle phase. Nonetheless, others who have investigated menstrual cycle using a similar method to us also found no change in ACTH or cortisol but did find alterations in cardiac index and epinephrine responses to the TSST over the menstrual cycle suggesting changes in other aspects of the sympathetic nervous system may be more prominent across the menstrual cycle (Gordon & Girdler, 2014).

Smokers have been shown to display an attenuated response to the TSST compared to non-smokers (Kirschbaum, Strasburger, et al., 1993; Rohleder & Kirschbaum, 2006), a finding we confirm in this study. Nicotine is thought to activate the HPA-axis via stimulation of nicotinic receptors in the brain; however, with chronic exposure to nicotine through smoking, desensitisation occurs (Rohleder & Kirschbaum, 2006). Whilst it is unclear how this attenuated HPA responsiveness is mediated, changes in glucocorticoid receptor level have been proposed (Kirschbaum, Strasburger, et al., 1993; Rohleder & Kirschbaum, 2006).

The relationship between increasing BMI/obesity and response to psychosocial stress has produced contradictory results in the literature (Benson et al., 2009; Jones et al., 2012) In traditional analyses we found obese (but not overweight) participants displayed lower total plasma cortisol but similar salivary cortisol and ACTH responses to stress, compared to participants with normal BMI. In obesity, basal circulating cortisol levels are often normal or low-normal despite an elevation in the cortisol secretion rate (Bjorntorp & Rosmond, 2000). This is likely due to an increase in the rate of removal of

cortisol from the circulation (Lottenberg et al., 1998). In women, cortisol reactivity to acute stress appears to alter with body fat distribution with more pronounced differences in central obesity (Epel et al., 2000). Differing patterns of fat distribution along with study sample sizes may contribute to some extent to the discrepancies in the literature relating TSST results to BMI.

Following the seminal publication of the TSST method in 1993 (Kirschbaum, Pirke, et al., 1993), the majority of TSST studies have utilised approaches using ANOVA with repeated measures analyses to compare groups of interest. In addition, parameters such as areas under the curve (Pruessner et al., 2003) and peak hormone increase relative to baseline have proven very useful and can be used to compare results between publications. In general, differences in the stress response have been shown as a relative shift up or down in the hormone response curve. This study describes a novel approach to examining TSST data, in particular, the shape of the response curve. The three patterns identified include Reactive-Responders (RR), Anticipatory-Responders (AR) and Non-Responders (NR) to be used in conjunction with the previously established parameters.

A response to the TSST is often defined as an increase from baseline above an absolute value (e.g. 2.5mmol/l salivary cortisol). We suggest that a response be designated as the relative increase (twice the coefficient of assay variation), as originally recommended by the commonly cited paper (Van Cauter & Refetoff, 1985) instead of an absolute increase. This method increases transferability across different laboratories and takes assay variation and initial baseline levels into consideration. Others have also recently suggested reconsidering the use of the 2.5mmol/l absolute increase with salivary cortisol (Miller et al., 2013).

We observed reactive-responder, anticipatory-responder and non-responder rates of 56.6%, 26.2% and 17.2%, respectively. In each response group the same stress pattern was generally observed across ACTH, total plasma and salivary free cortisol. This reactive-responder rate is lower than the reported 70-85% for the TSST in adult populations, possibly due to our use of updated response criteria in a younger population and the higher proportion of the study population with the anticipatory-responder pattern. There is some prior indication that the anticipatory-response is much higher in adolescents (13-20 years) than in children (Evans et al., 2013). The presence of this early 'anticipatory' rise in ACTH and cortisol levels may dull/dampen the stress response to the TSST as part of a

ceiling effect in relation to the law of initial value (Block & Bridger, 1962). Both the RR and AR appear able to mount an HPA response, unlike the NR, and combined they account for 83% of our sample, in keeping with TSST responder estimates in adult populations.

When we examined gender using the stress response patterns, we found males were significantly more likely to be RR than NR and more likely to be RR than AR, compared to females not taking OC, after adjustment. This is consistent with males showing a greater HPA response to stress and the suggestion that females may show greater changes in other physiological responses to stress such as corticolimbic reactivity and heart rate (Ordaz & Luna, 2012)

Analysing the stress-response patterns (RR, AR, NR) in logistic regression analyses, we did not find clear relationships between females on and off OC or across the menstrual cycle. However, smokers were more likely to be NR than (RR or AR) and more likely to be AR than RR, after adjustment. This is consistent with the blunted HPA response to acute stress found with chronic nicotine exposure that is similar to the NR pattern (Childs & de Wit, 2009).

With regard to BMI, we found what appears to be a dose-dependent effect with the shape of the TSST response curve. Overweight and obese participants showed increased odds of being RR versus being NR. The relationship with underweight participants was hampered by low numbers but odds ratios tend to support the opposite association; increased odds of being NR versus RR. This is interesting as the majority of analyses do not show a dose-dependent response with BMI, and HPA axis differences are limited to obese vs normal subjects. The increased rate of removal of cortisol from the circulation results in higher basal HPA activity and this may explain the increased RR rates with increasing BMI. In support of this, obese men who reduced their weight have been shown to produce more attenuated responses to the TSST (Therrien et al., 2010).

The stress response patterns described in this study can be characterised using traditional and novel mathematical parameters and are best used in conjunction with these measures as they describe different elements of the HPA-axis response to a psychological challenge. The RR and AR patterns reveal a higher global exposure to glucocorticoids. The AR pattern may convey an increased sensitivity to stress and potentially has a greater exposure to glucocorticoids if the AUC was measured from when

the HPA-response actually began. Unlike Engert et al, we did not see higher cortisol peaks in the AR group (Engert et al., 2013). Nonetheless, the AR pattern is likely to be a form of RR with the HPA response commencing prior to the TSST in anticipation of the challenge. There is evidence to support this hypothesis, with individuals demonstrating increased anticipatory stress effects when they undertake subsequent TSSTs, demonstrating higher plasma cortisol, ACTH and heart rate in the succeeding tests (Schommer et al., 2003). While ARs show an early rise in glucocorticoids, they may also have a reduced recovery time (Juster et al., 2012). Compared to RRs, ARs were more likely to be female and to be a smoker. There is evidence of a relationship between ARs and PTSD (Bremner et al., 2003) and between NRs and panic disorder (Petrowski et al., 2010), premenstrual syndrome (Huang et al., 2015), attempted suicide (Melhem et al., 2015), adverse early life events (Elzinga et al., 2008) and other negative health outcomes (Fries et al., 2005; Phillips et al., 2013). Future studies using the response patterns alongside traditional TSST measures should investigate whether ARs or NRs differ with regard to life stressors, psychological traits such as self-esteem, anxiety and rumination and markers of mental, metabolic and cardiovascular health.

This study involved the creation of a novel AUC calculation. We found that approximately 70% of our participants displayed their lowest stress hormone level (C_{MIN}) at the final point of measurement, 90 min post TSST, and this led us to develop a novel AUC calculation taking C_{MIN} into account. AUC_R (with respect to range) detected significant differences across gender groups and smokers vs non-smokers when compared to AUC_I, which showed no significant changes. This suggests that AUC_R may be a more sensitive measure as it assumes that basal hormone levels approximate C_{MIN} and not $C_{BASELINE}$. This may be useful to researchers when participants commence the TSST with higher hormone levels than subsequent timepoints, as illustrated in anticipatory-responders. Another benefit of AUC_R involves the generation of positive values for ease of interpretation, whilst AUC_I calculations can result in negative areas (Grice & Jackson, 2004).

When the three response patterns were characterised by traditional descriptors of TSST data, the only measure found not to differ between the three groups was C_{MIN}. This also suggests that C_{MIN} may be more indicative of basal cortisol levels than $C_{BASELINE}$. This finding requires replication in future studies but does imply that the three patterns have similar basal resting hormone levels; they may differ only in their response to an

impending challenge. Further, it reiterates that the initial hormone measures in the TSST are often not representative of basal HPA levels as they are affected by the participants' anticipation of the impending challenge (Kudielka et al., 2007). This emphasises the potential value of the novel AUC_R calculation mentioned above.

A strength of this study lies in its evaluation of the TSST assessment in a large number of young adult participants from a well-characterised community population. Further, we have measured the HPA axis at three levels and described a novel 'pattern' approach to analysing TSST results. When interpreting the results of this study, two issues deserve consideration; the absence of a sample taken on immediate arrival, and the requirement for cannulation of participants. The analysis of samples taken prior to the TSST, during the initial rest phase, would have allowed a more comprehensive assessment of arrival/anticipatory dynamics. The change in cortisol in the first hour after arrival has been correlated with the subsequent response to challenge (Balodis et al., 2010). Nonetheless, the TSST protocols we used between 2008-2010 are consistent with those used by others and recommended in the literature (Kirschbaum et al., 1999; Kudielka et al., 2007). Recently a modified TSST protocol to specifically measure the anticipatory response has been developed (Engert et al., 2013) and will be valuable for future studies. The second issue involves measurement of cortisol and ACTH from blood using cannulation which is a more invasive procedure than collecting saliva-only, before and after the TSST. Cannulation carries the potential for a cortisol response independent of the TSST. Our participants had at least 45 minutes rest after cannulation prior to a challenge, consistent with recommendations and other studies (Epel et al., 2000; Kirschbaum et al., 1999; Kudielka et al., 2007). Whilst it is tempting to speculate that there may be more anticipatory responders with cannulation, this does not explain the relative absence of this pattern in the TSST literature. The period of adolescence is associated with many changes in the HPA-axis and it is possible that the reaction to cannulation may be heightened in this age group. It is worth noting that we did not assess cognitive/emotional state at the time of the TSST and it is possible that this may contribute to some of the differences observed. Finally, it's possible that the pulsatile nature of cortisol secretion may impact the response to a stressor (Young et al., 2004).Extensive work in rats suggests that the rising/secretory phase of the pulse yields a corticosterone response to a stressor whilst the HPA axis appears relatively inhibited during the falling phase (Windle et al., 1998). These results differ between rat strains and

it is difficult to extrapolate these data to humans. Others have found similar, although habituating, cortisol responses to the TSST on retesting, which suggests that the pulsatile nature of cortisol release does not significantly interfere with individual response to the TSST (Petrowski et al., 2012).

3.5 Conclusions

We have used a community-based sample population of 798 teenagers to describe in detail the acute response of the HPA-axis to a psychological challenge. In addition to examining traditional measures of the TSST data, we have used novel mathematical data to describe the TSST response and have characterised the three patterns of stress hormone response; reactive-responder, anticipatory-responders and non-responders. Results demonstrate that the stress-response patterns vary with risk factors contributing to TSST variation. The influence of gender, smoking and BMI on stress-response patterns suggests there is the potential for the patterns to vary with health and disease. Therefore, the use of these patterns alongside traditional measures, may give greater insight into the stress-response in future studies.

Acknowledgements

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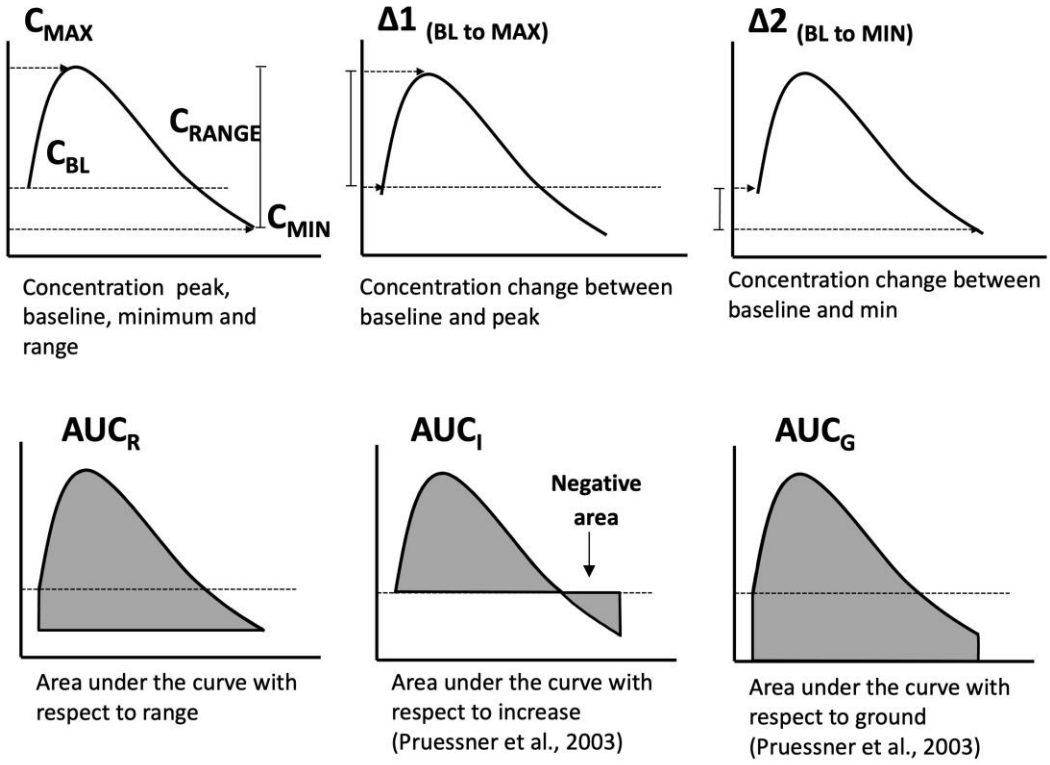
Supplementary Data

Supplementary Figure 3.1

Visual representation of TSST mathematical parameters



Supplementary Table 3.1

ACTH TSST mathematical parameters by gender and stress-response pattern

ACTH	Gender group				Stress-response pattern			
	Male *n* = 436 *Median (IQR)*	**Fem-OC** *n* = 223 *Median (IQR)*	**Fem+OC** *n* = 139 *Median (IQR)*	***p KW***	**RR** *n* = 452 *Median (IQR)*	**AR** *n* = 209 *Median (IQR)*	**NR** *n* = 137 *Median (IQR)*	***p KW***
C_{BL}	22.5 (18.4, 28.5)	17.6 (14.0, 21.9)	13.8 (10.6, 17.1)	*	19.3 (15.2, 24.1)	21.3 (15.4, 30.8)	17.3 (13.2, 22.2)	*
C_{MAX}	31.1 (24.4, 40.0)	23.5 (18.3, 31.0)	18.0 (12.8, 24.4)	*	30.0 (23.2, 40.0)	24.3 (17.6, 32.1)	20.5 (16.1, 25.5)	*
C_{MIN}	18.6 (14.8, 22.3)	14.9 (11.8, 18.0)	11.9 (8.6, 15.1)	*	16.7 (12.7, 21.1)	15.7 (11.3, 19.9)	16.1 (12.0, 19.7)	NS
C_{RANGE}	11.2 (5.8, 20.2)	7.5 (4.4, 13.7)	5.0 (2.6, 10.0)	*	12.6 (7.2, 20.7)	6.4 (3.3, 13.2)	4.5 (2.7, 6.7)	*
$\Delta 1(C_{BL}$ to $C_{MAX})$	7.8 (3.5, 15.3)	5.5 (2.9, 10.8)	4.0 (1.8, 8.4)	*	9.5 (5.4, 16.8)	2.4 (1.1, 4.3)	3.4 (1.8, 5.7)	*
$\Delta 2(C_{BL}$ to $C_{MIN})$	4.6 (2.0, 8.7)	3.1 (1.5, 5.3)	1.9 (0.9, 3.6)	*	3.4 (1.7, 6.1)	5.1 (2.1, 12.1)	2.1 (0.8, 4.6)	*
AUC_I	128 (-160, 498)	125 (-60, 365)	73 (-54, 328)	NS	305 (67, 681)	-202 (-655,-8)	70 (-57,211)	*
AUC_8	549 (248, 926)	348 (181, 631)	223 (100, 526)		AUCR	549 (248, 926)	348 (181, 631)	223 (100, 526)
AUC_G	2591 (2105, 3163)	1993 (1606, 2507)	1552 (1152, 1987)	*	2497 (1890, 3064)	2053 (1588, 2602)	1932 (1493, 2375)	*

Concentration units = nmol/L. * Kruskal Wallis p-value ≤ 0.001, ^ p-value < 0.05

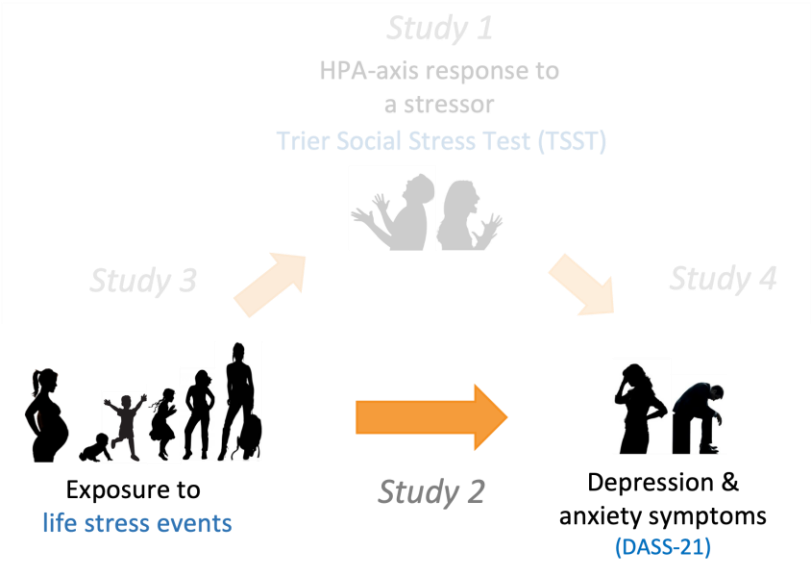
Supplementary Table 3.2

Salivary cortisol TSST mathematical parameters by gender and stress response pattern

SALIVARY CORTISOL	Gender group				Stress-response pattern			
	Male *n* = 436 *Median (IQR)*	**Fem-OC** *n* = 223 *Median (IQR)*	**Fem+OC** *n* = 139 *Median (IQR)*	***p KW***	**RR** *n* = 452 *Median (IQR)*	**AR** *n* = 209 *Median (IQR)*	**NR** *n* = 137 *Median (IQR)*	***p KW***
C_{BL}	11.9 (8.7, 18.3)	9.6 (6.9, 14.4)	10.6 (8.0, 14.3)	*	9.9 (7.5, 13.6)	16.7 (11.5, 25.8)	9.9 (7.6, 14.4)	*
C_{MAX}	16.8 (12.1, 24.7)	13.5 (9.2, 20.7)	12.5 (9.1, 17.4)	*	15.8 (11.3, 22.3)	16.8 (12.3, 26.0)	10.8 (8.2, 15.8)	*
C_{MIN}	8.2 (6.3, 10.7)	6.9 (5.2, 9.5)	8.1 (5.9, 10.7)	*	7.8 (6.2, 10.2)	8.2 (6.0, 10.9)	7.3 (5.5, 9.9)	NS
C_{RANGE}	8.2 (6.3, 10.7)	6.6 (3.2, 11.6)	3.9 (2.8, 6.9)	*	7.5 (4.2, 12.6)	8.9 (5.3, 14.9)	3.5 (2.2, 5.9)	*
$\Delta 1(C_{BL}$ to $C_{MAX})$	4.7 (1.9, 9.4)	4.6 (1.8, 9.0)	2.2 (0.8, 4.3)	*	5.2 (2.6, 9.8)	1.3 (0.5, 3.3)	1.2 (0.5, 2.3)	*
$\Delta 2(C_{BL}$ to $C_{MIN})$	4.4 (2.2, 9.0)	3.6 (1.9, 7.6)	2.7 (1.6, 5.4)	*	2.8 (1.3, 5.0)	8.8 (4.9, 14.7)	2.8 (1.5, 5.4)	*
AUC_I	-42 (-381, 286)	-46 (-315, 261)	97 (-279, 72)	NS	168 (-53, 435)	-570 (-932,-274)	-136 (-285,-35)	*
AUC_8	433 (249, 708)	327 (152, 528)	205 (123, 351)	*	425 (245, 670)	364 (180,666)	158 (97,292)	*
AUC_G	1350 (983, 1832)	1053 (759, 1554)	1088 (754, 1494)	*	1272 (960, 1754)	1253 (871, 1782)	959 (697, 1345)	*

Concentration units = nmol/L. * Kruskal Wallis p-value ≤ 0.001.

Chapter 4 Study 2: The impact of life stress on adult depression and anxiety is dependent on gender and timing of exposure



The graphical representation of this doctoral thesis above shows the focus of the following Chapter (Study 2). This study forms the foundation of the thesis, examining how the occurrence and timing of common early life stress events may impact future mental health. This investigation had the rare ability to examine prenatal stress event timing and took the unique approach of characterising trajectories of stress exposure from birth to age 17. In addition, as the TSST response to a stressor in Study 1 showed marked differences between males and females, and the prevalence of depression and anxiety varies by sex, this study considered the relationship between early life stress and symptoms of depression, anxiety and stress at age 20, separately in males and females.

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The impact of life stress on adult depression and anxiety is dependent on gender and timing of exposure

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Abstract

There is debate about the relative importance of timing of stressful events prenatally and over the life course and risk for subsequent depressive/anxious illness. The aim of this study was to examine the relative roles of prenatal stress and postnatal stress trajectories in predicting depression and anxiety in early adulthood in males and females. Exposure to life stress events was examined in the Western Australian Pregnancy Cohort (Raine) Study during pregnancy and ages 1, 2, 3, 5, 8, 10, 14, and 17 years. At age 20, offspring completed the Depression Anxiety Stress Scale. Prenatal stress and trajectories of stress events from age 1 to 17 were analyzed in linear regression analyses. Five postnatal stress trajectories were identified. In females, medium to high chronic stress exposure or exposure during puberty/adolescence predicted depression and anxiety symptoms while low or reduced stress exposure over the life course did not, after adjustment for relevant confounders. High stress early in pregnancy contributed to male depression/anxiety symptoms independent of postnatal stress trajectory. In females, postnatal stress trajectory was more important than prenatal stress in predicting depression/anxiety symptoms. Interventions focused on reducing and managing stress events around conception/pregnancy and exposure to chronic stress are likely to have beneficial outcomes on rates of depression and anxiety in adults.

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Abstract

There is debate about the relative importance of timing of stressful events prenatally and over the life-course and risk for subsequent depressive/anxious illness. The aim of this study was to examine the relative roles of prenatal stress and postnatal stress trajectories in predicting depression and anxiety in early adulthood in males and females. Exposure to life stress events was examined in the Western Australian Pregnancy Cohort (Raine) Study during pregnancy and ages 1,2,3,5,8,10,14 and 17. At age 20, offspring completed the Depression Anxiety Stress Scale. Prenatal stress and trajectories of stress events from age 1-17 were analysed in linear regression analyses. Five post-natal stress trajectories were identified. In females, medium to high chronic stress exposure or exposure during puberty/adolescence predicted depression and anxiety symptoms while low or reduced stress exposure over the life course did not, after adjustment for relevant confounders. High stress early in pregnancy contributed to male depression/anxiety symptoms independent of postnatal stress trajectory. In females, postnatal stress trajectory was more important than prenatal stress in predicting depression/anxiety symptoms. Interventions focused on reducing and managing stress events around conception/pregnancy and exposure to chronic stress are likely to have beneficial outcomes on rates of depression and anxiety in adults.

Keywords : Raine Study, DASS, mental health, longitudinal

4.1 Introduction

Stress events have consistently been linked to the development of depression and anxiety disorders (Kendler et al., 1999). Increased exposure to and severity of stress events have been associated with poorer clinical outcomes including more severe symptoms (Chapman et al., 2004), earlier onset (Kessler, 1997), recurrence (Francis et al., 2012) and reduced response to treatment (Nemeroff et al., 2003).

When considering the available research, exposure to stressful events during prenatal life, throughout childhood, and in adolescence have all been associated with depressive/anxious illness later in life. However, their relative importance in predicting psychological outcomes remains in question. With regard to the timing of stress exposure, some data suggest that recent life stress events are more relevant predictors of depression than past events. Indeed, up to 80% of depression in the community occurs after a recent major life event (Mazure, 1998). Further, a 2007 meta-analysis of previous research, found that the longer the time since the onset of the stress, the less affect it had on stress hormones (Miller et al., 2007). At the same time, it is clear that not all individuals who experience major life events will go on to develop depression, which suggests that other factors serve to increase risk for depressive symptoms after stress.

The theory has been proposed that sensitisation to later stressors may occur In those who have been exposed to early adverse experiences (Hammen et al., 2000; Heim et al., 2002), such that early adversity increases vulnerability to depression in response to stress in later life (Espejo et al., 2007). Similarly, chronic or cumulative stress sustained over a long period of time may increase the risk of affective illness especially when combined with recent acute stressors , (Hammen et al., 2009; Schilling et al., 2008; Vinkers et al., 2014). An alternative hypothesis suggests that moderate life stress early in life may confer some resilience against the negative effects of later life stressors, similar to an inoculation effect (Liu, 2015). In such a case, the opportunity to develop an endogenous stress response early in life may confer an increased adaptive ability to respond adequately when the stress system is challenged again (Gunnar, Frenn, et al., 2009). It also follows that those deprived of any form of early life stress may be less prepared to respond appropriately to future stress exposures. This concept aligns with the ‘mismatch hypothesis’, that individuals are more likely to suffer disease if a mismatch occurs between the early programming environment and the later life adult environment (Gluckman & Hanson, 2006).

Mechanistically, the timing of stress exposure may be important as brain regions regulating the stress response (via the Hypothalamic-Pituitary-Adrenal (HPA)-axis) develop and mature at different ages (Lupien et al., 2009). The hippocampus attains maturation at the age of two years, the prefrontal cortex matures between 8 and 14 years and finally the amygdala continues to mature slowly until the late twenties (Giedd et al., 1996). Exposure to high levels of stress during these critical windows of development can impair the growth and maturation of these brain regions (Lupien et al., 2009). In utero, the fetus may be exposed to stress hormones via placental transfer from the mother. Prenatal stress exposure in animal models has been shown to affect neurodevelopment and increase the risk for behavioural changes in the offspring (Kofman, 2002; Weinstock, 2001). Studies have associated prenatal stress or anxiety with later symptoms of anxiety and depression (Talge et al., 2007; Van den Bergh et al., 2005) or increased stress reactivity (Davis et al., 2011) in the child. Epigenetic mechanisms have been proposed to mediate these effects (Conradt et al., 2013; Meaney et al., 2007; Pena et al., 2012; Tyrka et al., 2012). In addition, puberty is marked by dramatic changes in stress reactivity and exposure to stressors during this sensitive period can have a lasting impact on the stress response via the HPA-axis (Romeo, 2010a, 2010b). This may contribute to the increase in psychological disorders seen to emerge during adolescence, especially in females. However, stress-provoking events rarely occur in isolation and research shows that life stress events in an individual or family are highly correlated over time. Cole et al. (2006) found that the numbers of adversities experienced in childhood are positively associated with the number of life stress events in adolescence and adulthood (Cole et al., 2006). Most studies examining prenatal stress exposure are unable to control for stress exposure after birth and so it remains to be seen whether the effect of prenatal stress exposure on mental health outcomes is independent of the effects of later life stress. Hence, prenatal stress and early life adversity may be associated with depression predominantly because they are followed by a trajectory of negative life stress events across the lifespan (Hazel et al., 2008). Conversely, a recent life stress event may be more likely to trigger a depressive episode if it is preceded by a certain pattern of stress events from birth. Therefore, a lifecourse approach is required to explore the relative impact of prenatal and postnatal timing of stress exposures on offspring mental health. The developmental pathway of stress exposure may play a role in identifying subgroups of children at higher risk of stress sensitivity (Liu, 2015), optimal

times for interventions (Heim & Binder, 2012) and defining subgroups of depression likely to respond differently to treatment (Heim et al., 2004).

Many previous studies examining the link between stress exposure and mental health problems have been cross-sectional or retrospective in nature, or have not been able to adequately examine confounding factors such as early or recent life stress, socioeconomic status or parental mental health. Very few studies have had the capability to examine stress exposure at different developmental stages in the same individuals and psychological outcomes. As stress events in the same family are highly correlated over time, analyses taking repeated measures from the same person into consideration are vital to our understanding of the relative timing of life stress exposure and psychiatric outcomes.

The Western Australian Pregnancy Cohort (Raine) Study provides a unique opportunity to examine family stress event exposure from the start of pregnancy to 17 years post-birth and subsequent symptoms of depression and anxiety in young adulthood. The first aim of this study was to examine longitudinal trajectories of postnatal stress exposure over the first 17 years of life and their relationship with symptoms of depression, anxiety and stress at age 20 in males and females, adjusting for appropriate covariates. The second aim was to examine the relative contribution of prenatal versus postnatal stress events to depression, anxiety and stress at age 20. We hypothesised, firstly, that trajectories involving high numbers of stress events during early life and adolescence would predict symptoms of depression and anxiety by early adulthood; secondly, that stress exposure from birth to age 17 would be more predictive of adult depression and anxiety symptoms than prenatal stress exposure and finally, that the impact of stress events across the lifespan on adult depression and anxiety would differ between males and females.

4.2 Methods

4.2.1 Participants

The Western Australian Pregnancy Cohort (Raine) Study, established in 1989, is a prospective cohort study of 2868 live births, with longitudinal follow-up from 16 weeks gestation to early adulthood (Newnham et al., 1993). Mothers were recruited from the public antenatal clinic at King Edward Memorial Hospital (KEMH) and nearby private

clinics. After the initial measures at 16-20 weeks gestation, follow-ups were conducted at 34 weeks gestation and ages 1, 2, 3, 5, 8, 10, 14, 17 and 20. Ethics approval for this study was obtained from King Edward Memorial Hospital, Princess Margaret Hospital and The University of Western Australia. All procedures were carried out with parental written informed consent up to the age of 18. Participants provided their own consent at age 20. Racial characteristics were 2473 (88.2%) Caucasian, 124 (4.4%) Chinese, 74 (2.6%) Indian, 67 (2.4%) Aboriginal, other 66 (2.4%) and not reported 64 (2.2%). Of the original 2868 participants, 2076 (72.4%) were retained until the age 20 review (1473 completed the age 20 review, 603 deferred from participating, 519 had withdrawn from the study, 236 were lost to follow up and 37 had died). As with all long-term cohort studies, there has been attrition over time with those lost to follow-up more likely to have higher indices of social disadvantage. This loss of disadvantaged families to follow-up is partially offset in the Raine Study by the fact that the original cohort showed an over-representation of socially disadvantaged families (Robinson et al., 2010).

4.2.2 Measurement of stress events

During pregnancy, at 18 weeks gestation, mothers filled out a questionnaire asking whether any of 11 life stress events had been experienced since becoming pregnant. At 34 weeks gestation, the questionnaire was repeated referring to the last 4 months. From age 1 to 17 primary caregivers reported on the same 11 life stress events occurring in the past 12 months. These life events were selected from the life stress inventory developed by Tennant and Andrews (1976) (Tennant & Andrews, 1976). They included problems with pregnancy, death of a close relative, death of a friend, marital problems, separation or divorce, problems with children, own job loss (involuntary), partner's job loss (involuntary), financial hardship, residential move and 'other' stressful events, as used in other studies (Robinson et al., 2011; Tearne et al., 2015). We examined high stress during pregnancy (the sum of 18wk and 34wk events dichotomised as 3+ events vs <3 events) and also looked at high stress early in pregnancy and late in pregnancy (3+ events vs <3 events at 18wk and 34wk). Longitudinal stress exposure, composed of the total number of family stress events experienced at each age, was used in trajectory analysis (see below). Additional information detailing the number and distribution of stress event data at each time point can be found in Supplementary Figure 4.1.

4.2.3 Measurement of Depression/Anxiety

Symptoms of anxiety, depression and stress were measured using the Depression, Anxiety and Stress Scale (DASS-21) answered by the Raine young adults at age 20. The DASS-21 is a 21-item self-report questionnaire producing a total score and three subscale scores designed to measure the negative emotional states of depression, anxiety and stress over the past week (Lovibond & Lovibond, 1995). It is a shorter version of the original 42-item DASS, more suitable for research purposes in which final scores are multiplied by two to give the final values. The DASS-21 has been shown to produce similar results to the full DASS, has the same factor structure and excellent internal consistency and reliability with Cronbach's alpha values > 0.9 for the DASS total score (Crawford & Henry, 2003; Sukantarat et al., 2007). We report the total DASS score as there is strong support for the derivation and use of the DASS-21 total scale score rather than independent scores (Osman et al., 2012), in acknowledgement of extreme co-morbidity between depression, anxiety and stress. We use DASS severity ratings (normal, mild, moderate, severe, extremely severe) to interpret the clinical significance of changes in DASS scores by stress exposure (Lovibond & Lovibond, 1995).

4.2.4 Covariates

We examined a number of covariates that may confound the relationship between the occurrence of life stress events and young adult mental health problems.

Baseline covariates: Maternal education (12 or more years of education vs. less than 12 years of education); family income (total income ≤ 24 000 AUD per annum vs. > 24 000 AUD per annum, in accordance with the poverty line at the time the data were collected); smoking during pregnancy (no cigarettes smoked vs any cigarettes smoked); father living at home during pregnancy (living at home vs not living at home); length of gestation (<37 weeks vs ≥ 37 weeks); and parity (0, 1 and 2 or more children). Birthweight and maternal age during pregnancy were used in their continuous form. The Socioeconomic Indices for Areas (SEIFAs) are relative summary measures of socioeconomic disadvantage created by combining information collected from the Australian Census data. We used the Index of Relative Socio-economic *Disadvantage* (IRSD) in this study as a continuous socio-economic indicator where lower numbers represent greater disadvantage (Statistics, 2006). This index incorporates many different

measures of social and financial status and we found it to account for more variability in our models than any other single socio-economic variable.

Age 20 covariates: Living arrangements (living with family or relatives vs living alone, with a partner, child or flatmate); smoking at age 20 (no cigarettes smoked vs any cigarettes smoked); BMI (continuous); alcohol consumption (standard drinks per day); the IRSD (see above); and vigorous exercise, moderate exercise and walking in the form of number of hours per week were tested in their continuous form.

Parental mental health was measured at the age 14 and 17 reviews where the mother and father of the child were asked if they had ever been treated for an emotional or mental health problem (other than post-natal depression). A three-category parental mental health measure at each age was created for analyses (neither parent, one parent or both parents). This measure is likely to incorporate both genetic and environmental components. The age 14 and 17 parental mental health measures were highly correlated and both yielded the same overall results when used interchangeably in regression models. The age 14 measure was used in final analyses to minimise data loss as n = 1790 compared to n = 1404 at age 17.

4.2.5 Statistical Analyses

We used a latent class growth analysis (LCGA) to derive trajectories of life stress events from age 1 to age 17 using PROC TRAJ within SAS (Jones et al., 2001) and we only included individuals in the analysis if they had ≥ 3 measures of stress events over the time period (n = 2497). This method is designed to identify clusters of individuals who have followed a similar developmental trajectory (Nagin & Odgers, 2010) and assigns each person to a group depending on the probability of membership. We systematically compared a number of different models from 2 to 7 trajectory classes with linear and quadratic functions for each group. To determine the best model we used the Bayesian Information Criterion (BIC) statistic, probability diagnostics and the practical value of the grouping with respect to the research question. The final groupings and the posterior probability diagnostics were exported to SPSS for linear regression analyses.

Covariates were included from baseline measures (pregnancy/birth) and at the time of the outcome (age 20), with the exception of the longitudinal measure of parental mental health. These covariates were examined as predictors of DASS outcomes in

univariate analyses before multivariate linear regression models were constructed to examine the associations between stress exposure trajectory and young adult depression, anxiety and stress. Those covariates that predicted outcomes significantly ($p<0.05$) were included in the final multivariate models. Final linear regression models were weighted by the probability that each individual belonged to that particular trajectory, essentially giving greater weight to more accurate results. All analyses were conducted in SPSS Statistics version 19 and alpha was set at $p<0.05$.

4.3 Results

4.3.1 Trajectories of stress events: Descriptive results

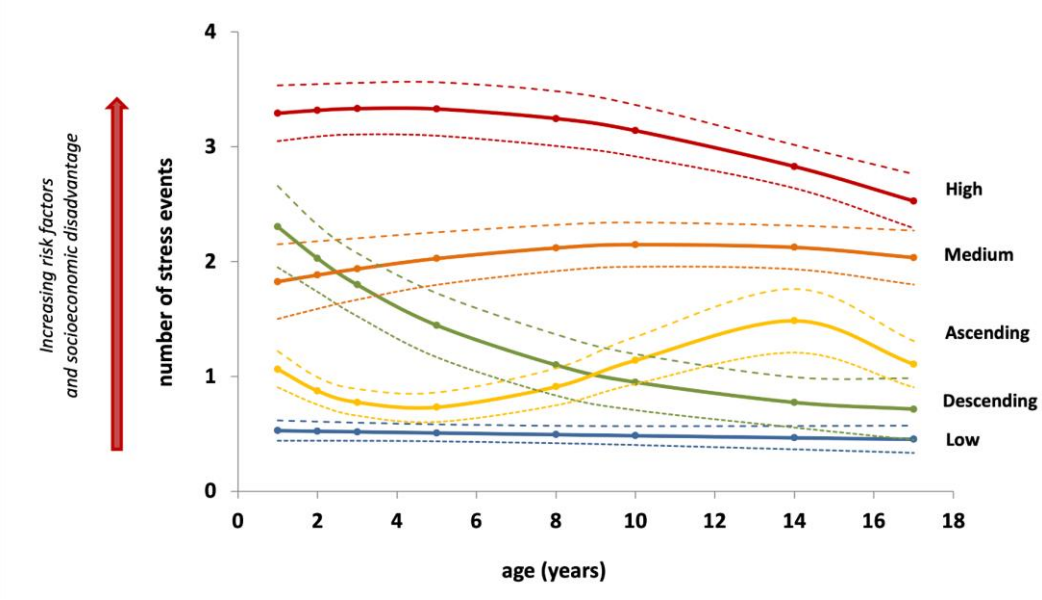
A five class model was selected as best describing the trajectories of stress exposure over 17 years. The five class model revealed the highest BIC and both an ascending and descending trajectory with meaningful numbers of individuals. This model also demonstrated good model fit based on posterior probabilities of membership to each trajectory group > 70%. The five classes (Figure 4.1) involved three relatively consistent trajectories and two trajectories that changed over the life course. The Low, Medium and High trajectories displayed consistently low, intermediate or high levels of stress exposure over 17 years.

The Ascending trajectory showed relatively low stress exposure in early life, increasing in the early teens representing an adolescent onset of stress exposure. The Descending trajectory showed relatively high stress exposure in early childhood, decreasing over time to low levels during adolescence, representing early-life-stress only. Cohort percentages were as follows; Low (16.7%), Medium (30.7%), High (17.2%), Ascending (27.5%) and Descending (7.8%).

The characteristics of individuals classified into these trajectories are shown in Table 4.1. There were approximately equal proportions of males and females in each trajectory. Focusing initially on the three stress trajectories that remained consistent over time, in the increasing order of Low, Medium to High trajectories, there were increases in the proportions of low-income families, maternal smoking and stress events during pregnancy and the percentage of parents treated for a mental health problem. Conversely, there were decreases in maternal age and education and fathers present in the family home in early life.

Figure 4.1

Trajectories of stress exposure across the life-course



The five trajectories of stress exposure and 95% confidence intervals.

Similarly, in the offspring at age 20, with higher levels of chronic stress, there were increases in BMI and smoking and more young people were living outside the family home (comparing the Low to the Medium/High trajectory groups). In line with these observations, we observed lower socioeconomic status (SES) with higher chronic stress over the 20 years, as indicated by lower SES-disadvantage scores both before birth and at age 20.

Focusing on the trajectories that change over time, the Descending stress trajectory clearly displays higher numbers of stress events during pregnancy and the early years post-birth, at levels similar to the Medium trajectory, while the Ascending curve displays low early life stress events, increasing around ages 10-14. Both these trajectories show similar maternal age and education, maternal smoking, proportion of fathers in the family home, and parental mental health, suggesting a similar socioeconomic status. However, the SEIFA scores at birth and age 20 indicate that the Descending trajectory lies near the Medium trajectory in terms of SES whilst the Ascending trajectory SES is higher, lying between the Low and Medium trajectories. Further, we note that mothers of children on the Ascending stress trajectory had a higher chance of already having other children than the Descending trajectory and the children exposed to the Ascending (adolescent-onset) stress are more likely to be smokers at age 20 (Table 4.1).

Table 4.1

Characteristics of participants in the five trajectories of stress exposure

	Trajectory					
	Low	**Medium**	**High**	**Ascending**	**Descending**	
	%	*%*	*%*	*%*	*%*	***P* value***
Pregnancy/birth risk factors						
n (%) at birth	418 (16.7	767 (30.7	430 (17.2)	686 (27.5	196 (7.8)	
Gender						
female	49	48.8	47.9	49	50.5	0.98
male	51	51.2	52.1	51	49.5	
Family income						
Low<$24K	19.9	46.5	64.3	33	39.2	<0.001
≥$24K	80.1	53.5	35.7	67	60.8	
Maternal education						
< y12	46.2	64	73.3	53.6	54.6	<0.001
≥ y12	53.8	36	26.7	46.4	45.4	
High stress 18wk						
3+	1.7	17.1	32.0	5.6	11.8	<0.001
<3	98.3	82.9	68.0	94.4	88.2	
High stress 34wk						
3+	1.4	13.8	25.5	4.4	10.7	<0.001
<3	98.6	86.2	74.5	95.6	89.3	

	Trajectory					
	Low *%*	**Medium** *%*	**High** *%*	**Ascending** *%*	**Descending** *%*	***P* value***
Maternal smoking						
yes	20.7	42.3	53.4	35.8	37.9	<0.001
no	79.3	57.7	46.6	64.2	62.1	
Father at home						
no	2.2	14.8	23.1	8.1	7.7	<0.001
yes	97.8	85.2	76.9	91.9	92.3	
Gestational age						
≥37w	89.7	87.7	87.6	92.4	86.2	0.016
<37w	10.3	12.3	12.4	7.6	13.8	
Parity						
0	45.2	48.1	56.2	44.3	55.6	0.009
1	33	30.2	25.3	28.9	27.6	
2+	21.8	21.6	22.1	26.8	16.8	
Parental Mental Health						
0 parents	86.1	59.8	43.9	73.7	74.2	<0.001
1 parent	12.7	34.8	48.1	24.2	23.9	
2 parents	1.2	5.5	8	2	1.8	
SES Disadv *mean (SD)*	1041.6 (77.8)	1013.2 (78.0)	990.2 (81.6)	1026.6 (78.1)	1006.1 (74.0)	<0.001
Maternal age *mean (SD)*	30.2 (4.9)	27.2 (5.8)	25.5 (5.8)	28.8 (5.4)	27.9 (6.4)	<0.001

	Trajectory					
	Low *%*	**Medium** *%*	**High** *%*	**Ascending** *%*	**Descending** *%*	***P* value***
Young adult risk factors						
n (%) at age 20	246 (20.3)	355 (29.2)	173 (14.3)	329 (27.1)	111 (9.1)	
Smoking						
yes	8.7	20.1	16	14.9	8	0.001
no	91.3	79.9	84	85.1	92	
Living with family						
yes	82.2	74.9	68	78	74.1	0.013
no	17.8	25.1	32	22	25.9	
SES Disadv *mean (SD)*	1066.2 (56.6)	1037.2 (72.4)	1019.9 (78.9)	1055.5 (64.5)	1038.8 (63.8)	<0.001
BMI *mean (SD)*	23.6 (4.1)	25.1 (5.5)	25.8 (6.1)	23.7 (4.4)	24.6 (5.2)	<0.001

Data shown as column % unless otherwise specified

*ANOVA p-value for continuous data, differences between groups; or χ2 p-value for categorical data, differences between groups.

In the final subset analysed with mental health outcomes and covariates (*n* = 769) maternal education, prenatal stress, gestational age and parity did not reach statistical significance < 0.05.

4.3.2 Relationship of stress trajectories to depression/anxiety symptoms

A total of 1214 participants had stress trajectory data and completed the DASS at the age 20 review. The characteristics of the DASS scores by trajectory and gender are presented in Table 4.2. In the total population, DASS scores were higher in the High, Medium and Ascending stress trajectories compared to the Low trajectory. When we examined gender differences, the DASS scores were significantly higher in females than males across all trajectories except the Descending trajectory where there were no gender differences in DASS scores. It is interesting to note that the range of DASS scores over the five life stress trajectories was approximately 4 points in males (15.15-19.23) and around 12 points in females(16.41-28.33), suggesting that a history of developmental stress may have a greater discriminating value in predicting depression/anxiety in females than males.

The Medium, High and Ascending stress trajectories predicted significantly increased total DASS scores at age 20, with an increase of 5.4-6.9 in total DASS score compared to the Low trajectory (see Table 4.3). These figures remained significant after adjustment for early life, socio-economic and parental mental health factors, alcohol and cigarette exposure. Upon further investigation by gender, this relationship was only found in girls with predicted increases in DASS scores of 5.8 -9.5 for females in the Medium and High or Ascending trajectories. There was a trend to significance for increased DASS score in boys in the Medium trajectory. Formally testing for differences between males and females using a trajectories x sex interaction term in the model indicated that there was a significant difference between males and females in the High trajectory with depression/anxiety symptoms at age 20 (high stress x female 8.15 (0.77, 15.54) p=0.03). Other trajectory x sex comparisons were not significant and may be limited by small group sizes. Although the Descending trajectory commenced with high numbers of stress events in the years after birth (levels similar to the Medium trajectory), stress exposure reduced with time (to levels similar to the Low trajectory) by adolescence and there was no relationship with DASS scores at age 20.

Table 4.2

Depression, Anxiety and Stress symptoms by trajectory of stress exposure and gender

	DASS total score				
	Total *n* = 1214 *Mean+ (SD)*	**Females** *n* = 648 *Mean+ (SD)*	**Males** *n* = 566 *Mean+ (SD)*	***p****	**Difference in means**
Trajectory					
Low	17.10 (14.95)	18.75 (15.60)	15.15 (13.96)	0.06	3.60
Descending	17.10 (14.12)	16.41 (14.44)	17.85 (13.87)	0.6	-1.44
Ascending	22.22 (20.74)	25.36 (21.43)	18.94 (19.54)	0.005	6.41
Medium	23.47 (21.19)	27.28 (23.05)	19.23 (18.04)	<0.001	8.06
High	23.98 (19.96)	28.33 (22.00)	17.72 (14.59)	<0.001	10.62

*ANOVA p-values assessing differences between males and females

+ANOVA assessing effect of stress trajectory on DASS $F(4, 1209) = 6.44$ $p < 0.001$

Table 4.3

*Trajectory of stress exposure and association with depression and anxiety symptoms – adjusted models**

	Total *B (95%CI)*	***p***	**Females** *B (95%CI)*	***p***	**Males** *B (95%CI)*	***p***
Trajectory						
Low	0.00		0.00		0.00	
Descending	2.28 (-2.82, 7.39)	0.38	0.68 (-6.53, 7.89)	0.85	4.69 (-2.49, 11.86)	0.20
Ascending	5.49 (1.89, 9.08)	0.003	7.52 (2.21, 12.83)	0.006	3.44 (-1.38, 8.26)	0.16
Medium	5.35 (1.68, 9.02)	0.004	5.82 (0.41, 11.24)	0.035	4.59 (-0.32, 9.50)	0.07
High	6.93 (2.36, 11.50)	0.003	9.47 (2.99, 15.95)	0.004	3.62 (-2.78, 10.03)	0.27

*final *n* = 769, adjusted for socioeconomic disadvantage at enrolment, gender, parental mental health and smoking.
Low income, father living with family, maternal age and education, birthweight, gestational age, smoking in pregnancy, parity, age 20 BMI, alcohol consumption, exercise and living with family were also examined but were removed from models due to lack of statistical significance and therefore limited contribution to the model). Analyses were weighted by the probability that each individual would fall into the allocated trajectory however weighted and unweighted analyses produced similar results.

4.3.3 Relationship of prenatal stress to depression/anxiety symptoms

High prenatal stress throughout pregnancy (0-34wks) showed a trend to predicting depression/anxiety symptoms in the total population after adjustment for gender, SES, parental mental health and smoking at age 20 (B=3.17 (-0.26, 6.60) p=0.07) but was not statistically significant and the association reduced when examined separately in males (B=3.47 (-1.39, 8.34) p=0.16) and females (B=2.56 (-2.29, 7.42) p=0.30). However, when we examined the effect of timing of stress exposure in pregnancy, high stress early in pregnancy (0-18wks), significantly predicted higher DASS scores in the total population and in both males and females after adjustment for SES, parental mental health and smoking at age 20 (see Table 4.4a). In contrast, high stress later in pregnancy (18-34wks), was not associated with DASS scores after adjustment in the total population (B= -0.29 (-4.82, 4.23) p=0.90), in females (B= -1.14 (-7.19, 4.90) p=0.71), or in males (B= -0.14 (-7.14, 6.86) p=0.97). Testing for gender differences in the total model by using a sex x prenatal stress interaction term did not identify a significant difference, suggesting these results require replication for confirmation.

4.3.4 Relative contribution of prenatal stress and postnatal stress trajectories to depression/anxiety symptoms

In order to test whether there was a contribution of both prenatal stress and postnatal stress trajectories to depression/anxiety symptoms, we examined these together in adjusted regression models with total DASS scores as the outcome (Table 4.4b). In the total population, there was a significant contribution of early prenatal stress, and the Medium, High and Ascending postnatal stress trajectories to depression/anxiety symptoms in adulthood, after adjustment. However, when we separated the results by gender, in boys prenatal stress but not postnatal stress trajectory was significantly related to adult DASS score whereas the reverse was true in girls; postnatal stress trajectory but not prenatal stress predicted adult DASS score.

Table 4.4

Models for relative contribution of prenatal and postnatal stress trajectory to DASS score

	Total *B (95%CI)*	***p***	**Females** *B (95%CI)*	***p***	**Males** *B (95%CI)*	***p***
(a) Modelling prenatal stress alone						
High prenatal stress 18w*	**5.73** (1.67, 9.78)	0.006	**5.71** (-0.26, 11.68)	0.06	**5.71** (0.28, 11.15)	*0.04*
(b) modelling prenatal stress with postnatal stress						
High prenatal stress 18w*	4.93 (0.78, 9.09)	0.02	3.87 (-2.28, 10.01)	0.22	5.**83** (0.29, 11.38)	0.04
Postnatal stress						
Low	0.00		0.00		0.00	
Descending	1.80 (-3.40, 7.00)	0.50	0.34 (-7.08, 7.77)	0.93	4.15 (-3.08, 11.38)	0.26
Ascending	5.38 (1.70, 9.05)	0.004	7.53 (2.05, 13.02)	0.007	3.37 (-1.52, 8.27)	0.18
Medium	4.41 (0.57, 8.24)	0.02	5.08 (-0.62, 10.78)	0.08	3.58 (-1.49, 8.66)	0.17
High	4.91 (0.02, 9.80)	0.049	8.16 (1.16, 15.17)	0.02	1.07 (-5.72, 7.85)	0.76

*adjusted for socioeconomic disadvantage, gender, parental mental health and smoking. Analyses were weighted by the probability that each individual would fall into the allocated trajectory however weighted and unweighted analyses produced similar results.

4.4 Discussion

In this study we identified five post-natal trajectories of life stress events to measure the longitudinal impact of stress exposure over eight time points from age 1 to 17. These trajectories, consisting of the number and timing of life stress events, predicted depression, anxiety and stress symptoms in early adulthood, especially in girls. In addition, high prenatal stress events early, but not late, in pregnancy also predicted affective symptoms. However, when considering both pre- and post-natal stress exposure, the effect of prenatal stress appeared to be more important in boys and postnatal stress more important in girls, although these results require replication in an independent sample. The results of this study provide evidence that the relative impact of pre and postnatal stress on later life mental health may be gender specific.

4.4.1 Trajectories of life stress exposure and adult affective symptoms

We examined postnatal stress exposure by identifying five different life stress event trajectories. In the trajectories that were consistent throughout childhood and adolescence (Low, Medium and High trajectories), medium to high numbers of stress events predicted increased depression/anxiety symptoms in girls whilst there was only a trend to significance with medium stress events in boys. In contrast, consistently low numbers of stress events showed no significant associations with symptoms of depression/anxiety.

To put these changes into perspective, in this general population, whilst we see 85.6% participants displaying normal to mild symptoms and 14.4% showing moderate to extremely severe symptoms, a positive shift of 5 in DASS scores (as seen in this cohort with moving from Low to Medium stress) would result in 15% of the population shifting into a higher category of severity and 5% of the population moving from normal or mild symptoms into moderate to extremely severe symptoms of depression and anxiety. In the girls, we see 82.1% in the normal or mild category and 17.9% in the moderate to extremely severe category. A positive shift of 10 in DASS scores, as we see in the High vs Low trajectory in girls, would result in almost 10% (9.6%) of the female population moving from normal to mild symptoms into moderate to extremely severe symptoms.

Our results are reinforced by other cross-sectional or retrospective work showing that the chronic accumulation of stress exposures across the lifespan increases

depression risk (Vinkers et al., 2014), particularly in women (Hammen et al., 2009). Families in the High/Medium trajectories are more likely to continue experiencing high stress, and this may be due to the compounding effects of socioeconomic disadvantage (Goodman et al., 2003). Indeed, we show here that high chronic stress is associated with lower maternal age and education, reduced family income and SES score.

Unlike responses to acute stressful events that are protective and adaptive in nature, chronic stress elicits physiological changes that may have deleterious consequences on higher brain functioning. This is supported by structural and functional MRIs measuring activity and volume of different brain regions after exposure to chronic stress (Ansell et al., 2012; Frodl & O'Keane, 2013; Seo et al., 2014). Repeated exposure to stressful life events have been suggested to predispose individuals to depression and anxiety via increased allostatic load, or increased 'wear and tear' on the stress system (Juster et al., 2011) and affective disorders may develop when the adaptive capacity of the stress system reaches its limit. Therefore, it is not only a matter of the numbers of stress events experienced over the lifespan, but also their consistency that contribute to affective disorders in adulthood. Public health policies that focus on poverty-reduction and education-initiatives are likely to have a positive impact on the cost of chronic mental health problems that track across the lifespan.

In comparison with consistent stress exposures, the Ascending trajectory predicted depression, anxiety and stress symptoms in girls but not in boys. These results parallel those of Boardman (2011) who identified four stress event trajectories in black and white US adolescents from age 11-21. The two trajectories at greatest risk of depression were the *chronic* (increasing life stress events from age 11 peaking at age 14 with a second peak at age 21) and the *peak at 15* (increasing life stress events from age 13, peaking at 15) but not the *peak at 17* or *minimal* stress trajectories (Boardman & Alexander, 2011). Puberty is marked by dramatic changes in neuroendocrinology, most notably via sex hormone production but it is also associated with profound shifts in stress reactivity via the Hypothalamic-Pituitary-Adrenal (HPA) axis (Romeo, 2010a, 2010b). Maturation of the HPA axis occurs during this period and long term levels of HPA activity are established that may impact vulnerability to future depression and anxiety disorders (Goel & Bale, 2009; McCormick & Mathews, 2010). Much evidence points to this window of development as being particularly sensitive to stress exposure.

Therefore, certain events such as death of a close relative or marital problems may be experienced more intensely at this age.

Given these results, adolescence appears to be the ideal time to examine exposure to stress in order to predict future depression/anxiety, especially in girls. However, to adequately capture an adolescent-onset trajectory it is necessary to consider adolescent stress relative to early childhood exposure. Nonetheless, these results draw attention to a critical period in adolescence for the effects of stress impacting longer term mental health outcomes, justifying the need for targeted assessment and interventions between the ages of 10 and 14.

Our data suggest that the Descending stress trajectory is not associated with depression and anxiety at age 20: The implications of this demonstrate the potential benefit of interventions to reduce exposure to stress in children. Despite starting life with relatively high early life stress and relatively low SES, a reduction in exposure to stress over the lifecourse was protective against depression, anxiety and stress symptoms. The benefit of stress reduction is further highlighted when we directly compare this trajectory to the Medium trajectory where this decrease in stress exposure did not occur. Girls, in particular, were at higher risk of depression, anxiety and stress symptoms in adulthood if they followed the Medium trajectory. In addition, the Descending trajectory showed low exposure to stress around the time of puberty and adolescence. The absence of an association with depression/anxiety suggests, firstly, that in girls early life stress alone may not be enough to increase the risk of affective illness in adulthood. Secondly, these data suggest that interventions aimed at reducing the impact of life stress events in childhood, especially around puberty, are likely to have beneficial outcomes on rates of depression and anxiety in adult women.

The Ascending and Descending stress trajectories had very different associations with depression/anxiety symptoms; this raises questions about early life stress exposure and resilience. Whilst girls in the Ascending trajectory experienced relatively few early life stress events, they were at increased risk of depression/anxiety symptoms as adults. Conversely, girls in the Descending trajectory, who experienced higher numbers of stress events early in life and lower numbers in adolescence, were at decreased risk of depression/anxiety symptoms in adulthood. It is possible that the experience of a moderate stress early in life provides an opportunity to develop resilience against future

stress exposure (Sapolsky, 2015). The development of an endogenous stress response early in life may confer an increased adaptive ability to respond adequately when the system is challenged again (Gunnar, Frenn, et al., 2009). Indeed, mild to moderate stress in utero has been associated with greater motor and cognitive development (DiPietro et al., 2006). Further, while severe early life stress has been found to increase HPA axis reactivity, moderate early life stress reduced HPA axis reactivity compared to control children with relatively low early life stress (Gunnar, Frenn, et al., 2009). There is some evidence that those children who have never experienced how to respond to stress may be more sensitive to the negative effects of stress exposure later in life, especially during vulnerable windows such as puberty, as seen in the Ascending trajectory here (Seery et al., 2013).

4.4.2 Prenatal stress and adult affective symptoms

Whilst many studies have found a relationship between prenatal stress or anxiety and offspring mental health problems (Davis et al., 2011; de Bruijn et al., 2009; Kofman, 2002; Li et al., 2010; Mueller & Bale, 2008; O'Connor et al., 2003; Talge et al., 2007; Van den Bergh et al., 2008; Weinstock, 2001), the relative contribution of prenatal stress on a child's long term psychopathology is still under scrutiny due to the limited number of long term studies in human populations (Glover & Hill, 2012). When we examined prenatal stress without adjusting for stress exposure after birth, we found a significant contribution of high stress exposure early in pregnancy (first 18 weeks), but not stress exposure late in pregnancy (18-34 weeks) to affective disorder symptoms at age 20. This effect was not observed when we combined the two pregnancy time-points (high stress exposure between 0-34 weeks).

The placental enzyme, 11B-HSD2, which inactivates cortisol, is expressed and active from early pregnancy. The expression of 11B-HSD2 in the placenta rises more than 12-fold over the course of pregnancy; at the same time there is a 3-4-fold increase in maternal cortisol levels (Konstantakou et al., 2017). These physiological changes protect the developing fetus from exposure to high levels of glucocorticoids; however, this is only a partial barrier (Davis & Sandman, 2010). In the early stage of pregnancy, high (stress-induced) maternal cortisol combined with relatively lower expression of 11B-HSD2 may render the fetus more vulnerable to the effects of glucocorticoid overexposure whereas later in pregnancy the large increase in placental 11B-HSD2 potentially offers the

developing fetus more protection. In addition, the maternal HPA-axis becomes less responsive to stress towards the end of pregnancy, corresponding to rising cortisol levels and this may have a relative protective effect compared to stress exposures in early pregnancy (Entringer et al., 2010).

Our results are comparable to one study finding maternal emotional complaints during first trimester to be associated with behavioural problems in boys (de Bruijn et al., 2009); however, the authors also found emotional complaints during the third trimester were associated with behavioural problems in girls. Our results also contrast those of O'Connor et al who found maternal anxiety during late pregnancy to be predictive of child emotional and behavioural problems (O'Connor et al., 2003). Given that specific fetal structures and physiological systems develop at different times during gestation, it is likely that the timing of environmental insult may be relevant, affecting those systems undergoing rapid change at that time. It has been suggested that maternal stress and anxiety may operate via different mechanisms throughout gestation (Van den Bergh et al., 2005) or may impact different stress response pathways at different times during pregnancy (eg.HPA-axis vs sympathetic nervous system) (Vedhara et al., 2012). Our results suggest that interventions designed to reduce controllable stressors and the impact of stress around conception and early in pregnancy may prove beneficial for offspring mental health later in life.

4.4.3 Prenatal stress exposure relative to postnatal stress exposure and affective symptoms

The data from this longitudinal study suggest that prenatal and postnatal stress have different contributions to adult depression, anxiety and stress symptoms in males and females. In females, after taking postnatal stress trajectory into account, prenatal stress exposure was no longer significantly related to DASS. In contrast, in males we found that the contribution of prenatal stress did not change after adjusting for postnatal stress trajectory indicating the effect is independent of postnatal stress exposure. In other words, these results show that prenatal stress appears to be more important in males, whilst stress exposure after birth appears to be more important in females in terms of predicting affective symptoms in adulthood. Whilst our findings require replication, some animal studies are consistent with these results, where stress early in gestation increased behavioural and physiological stress responses in males. Epigenetic studies in

these animals showed methylation differences in stress related genes in the amygdala and hippocampus but only in male offspring (Mueller & Bale, 2008), suggesting a gender specific response to the fetal environment. In addition, early life stress causing altered HPA functioning has previously been associated with depression in females but not males (Van den Bergh et al., 2008). This may indicate that stress exposure manifests differently in males and there are reports that boys are more likely to show other changes including learning and memory (Glover & Hill, 2012), ADHD (Li et al., 2010; Rodriguez & Bohlin, 2005) and schizophrenia (Levine et al., 2014). This is consistent with the large study of Laceulle et al (2014) which found increased sensitivity to the effects of stressful life events on psychological difficulties during pre-adolescence in girls (Laceulle et al., 2014).

4.4.4 Gender specific effects

The most frequently proposed mechanism to explain the impact of prenatal stress or anxiety on the future health of the child is in the way elevated maternal glucocorticoids cross the placental barrier and affect development of the fetal HPA-axis. The placenta bears the same genetic information as the fetus (XX or XY) and whilst often overlooked there are clear gender differences in placental size, shape, and function including bloodflow, gene expression, action of the 11B-HSD2 enzyme, sensitivity to hormones and responses to environmental stimuli (DiPietro & Voegtline, 2017). These and other mechanisms are elegantly reviewed elsewhere and may explain some of the gender differences seen in response to prenatal stress (Beijers et al., 2014). Early in postnatal life, the stress response forms in association with experience of physical and mental stressors which are modified/ameliorated by primary caregivers/attachment (Beijers et al., 2014). Later in adolescence, relationships with peers become more important although also in a sex-specific manner. In this study, we measured the family's exposure to life stress events. The effect on the child may be mediated by the parental response to stress, altered parenting quality and family functioning, all of which may impact girls more than boys. There is also clear evidence that gender differences exist in the HPA-axis physiological response to a psychosocial stress. That is, the release of ACTH from the pituitary and the subsequent secretion of cortisol from the adrenal glands. Adult male cortisol responses to the well-validated Trier Social Stress Test are reproducibly higher than female responses (Herbison et al., 2016; Kudielka & Kirschbaum, 2005). Male

and female sex hormones interact with receptors in the brain and the adrenal glands resulting in gender differences in stress regulation.

4.4.5 Strengths and limitations

This study has a number of strengths, including sample size, prospective longitudinal design and the use of the same stress event measures across ten time-points from pregnancy to age 17. We have evaluated stress at two time-points during gestation and examined gender differences. Further, we measured depression and anxiety at an age where Australian youth (18-24 years old) have the highest prevalence of mental illness than any other age group (Statistics, 2009) and the population sample we use is not clinical, therefore findings are relevant to general Caucasian population.

It is worth mentioning that whilst we have delineated five different stress trajectories, not everyone will fit directly into a specific trajectory. To take this into account we weighted our analyses by the probability that an individual would belong to their designated trajectory. Our measures of stress also focus on the more common stress exposures in a population, and we do not examine severe trauma, maltreatment, abuse and neglect in childhood which may have a different impact on the occurrence of affective disorders. Not all children are affected in the same way by prenatal or postnatal stress, many remain unaffected and many of the symptoms displayed by participants in this study are in the subclinical range. Our measures of prenatal stress precluded the ability to examine mid-pregnancy and late-pregnancy separately. In addition, there are other factors, such as onset of puberty, individual coping mechanisms and changes in socioeconomic disadvantage, that were not tested in this study which may impact the mental health of the participants. There is also the potential for participants' mental health conditions to develop and change in future years, possibly influenced by their experience of life stress events in the past. It is highly likely that the pathways of stress exposure over the life course interact with individual genetic and epigenetic predisposition and gene x environment studies will be important for future investigation.

4.5 Conclusions

These results suggest that both prenatal stress and the pattern of postnatal stress exposure can influence adult mental health; further there appear to be gender differences in the timing of stress exposure and impact on depression and anxiety

symptoms. This is important as stress exposure is a potentially modifiable factor and may have generational effects (Gluckman et al., 2005).

Acknowledgements

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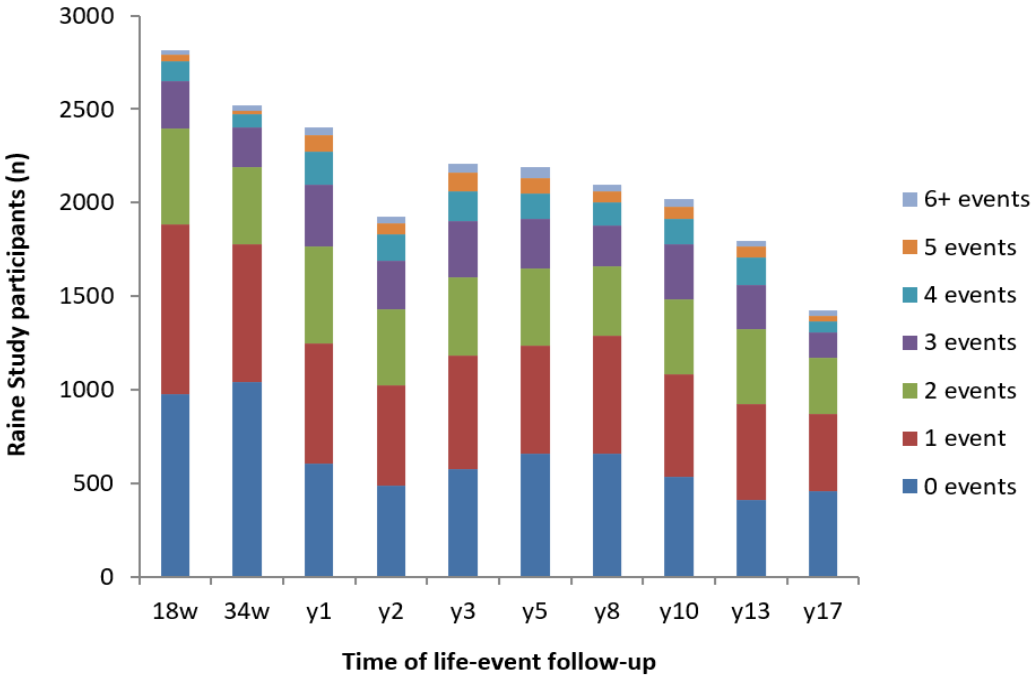
At a population level these results have the potential to guide interventions in two different ways. The first is in driving pre-emptive strategies focused on stress-management and reducing controllable stress events in early pregnancy and throughout early childhood. The second is in guiding the delivery of mental health support for adolescents at risk due to recent or prolonged stress exposure. These strategies are likely to have beneficial outcomes on rates of depression and anxiety in adults and reduce long term mental health issues.

Supplementary Data

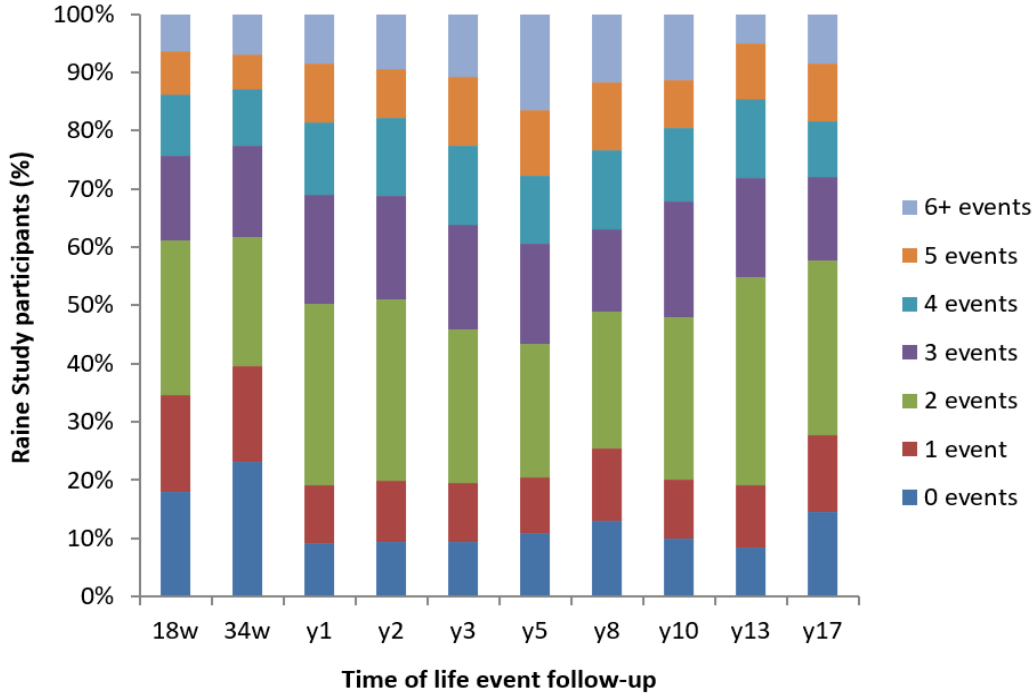
Supplementary Figure 4.1

Raine Study life stress event data for each follow-up

(a) Longitudinal life stress event data in the Raine Study (by number)

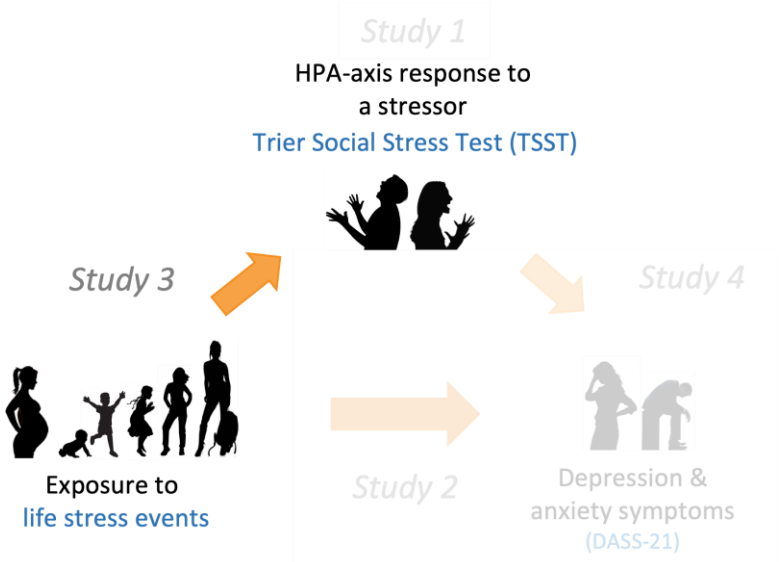


(b) Longitudinal life stress event data in the Raine Study (by percentage)



NOTE: The timepoints of 18w and 34w refer to the prenatal follow-ups and y1-y17 refer to the postnatal follow-ups. y2 was not a complete follow-up, therefore numbers are lower than y1 and y3.

Chapter 5 Study 3: Prenatal and childhood stress exposure and the sex specific response to psychosocial stress in adulthood



The graphical representation of this doctoral thesis above shows the focus of the following Chapter (Study 3). The results presented in the previous chapter suggest that exposure to common early life stress events is associated with symptoms of depression and anxiety at age 20 with sex specific differences. One potential mechanism proposed to explain this relationship is the altered functioning and regulation of the HPA-axis or the physiological stress response. Therefore, Study 3 aimed to investigate whether common early life stressors, experienced prenatally or throughout childhood and adolescence, play a role in the dysregulation of the HPA-axis in early adulthood. This involved the early life stress measures from Study 2 and the TSST outcome measures from Study 1 and once again assessment separately for males and females.

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Prenatal and childhood stress exposure and the sex specific response to psychosocial stress in adulthood

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ABSTRACT

Background: Early life stress exposures may cause dysregulation of the Hypothalamic Pituitary Adrenal (HPA)-axis and cortisol production, with timing and sex-specific effects. Studies examining the impact of early life stress on cortisol responses to stress have focused on severe trauma and have produced inconsistent results. The aim of this study was to investigate whether common early life stressors, experienced prenatally or throughout childhood and adolescence, play a role in the dysregulation of the HPA-axis in early adulthood.

Methods: Exposures to common life stress events were examined prenatally and as longitudinal trajectories of stress exposure from birth to age 17 in males and females from Gen2 of the Raine Study. At age 18 years, 986 participants were assessed for their salivary cortisol response to a psychosocial stressor - the Trier Social Stress Test (TSST).

Results: In males there was an association between high prenatal stress exposure at 18 weeks gestation and a heightened TSST response. We found evidence for sex-specific associations with increasing stress exposure during adolescence (the ascending trajectory) whereby males were more likely to be non-responders to the TSST and females were more likely to be responders.

Conclusion: Our results point to sex differences in how stress exposure in-utero and exposure increasing during adolescence may affect regulation of the HPA-axis later in life. However, overall common life stress events experienced in-utero, during childhood and adolescence show limited impact on the HPA-axis stress response in early adulthood.

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5.1 Introduction

Early life stress has been linked to a lifelong increased risk of adult psychopathology, cardiovascular disease and obesity (Cuijpers et al., 2011; Norman et al., 2012). Therefore, early life stress represents a common risk factor during development, particularly within vulnerable windows of brain plasticity, as the brain regions regulating the stress response develop and mature at different times, with sex specific differences (Agorastos et al., 2018; Lupien et al., 2009). These windows include prenatal, early childhood (0-2 years), and adolescence as times of vulnerability to exposure to stress (Agorastos et al., 2018; Glover & Hill, 2012; Romeo, 2010b; Xiong & Zhang, 2012). The human hippocampus is fully developed by two years of age and during adolescence there is an important increase in frontal cortex volume and the amygdala is still developing (Lupien et al., 2009). Adolescence is also marked by an increase in the activity of the HPA-axis, often coinciding with increases in sex-specific steroids and changes in behaviour (Romeo, 2010b).

Through fetal programming effects or mechanisms of biological embedding, early life stress exposures are hypothesised to dysregulate the stress response system, particularly the hypothalamic pituitary adrenal (HPA)-axis (Megan Gunnar & Karina Quevedo, 2007). Cortisol mobilises energetic resources to meet the increased physiological demands required to cope with a stressor (Gunnar, 2015). Prenatally, the fetus is exposed indirectly to the maternal stress hormones (Seckl & Holmes, 2007), whilst after birth the infant must rely on their own developing hormonal responses. An inappropriate HPA-axis response, involving production of too much or too little cortisol may produce negative effects in the long term. High cortisol responses have been associated with hypertension (Hamer & Steptoe, 2012) and low or blunted cortisol responses have been associated with depression, anxiety, panic disorder, attempted suicide, obesity and other poor health outcomes (Melhem et al., 2015; Petrowski et al., 2010; Phillips et al., 2013; Turner et al., 2020).

HPA-axis reactivity via the Trier Social Stress Test (TSST) or equivalent serves as a proxy measure for the response to stressful real-life situations and tests involving uncontrollable social-evaluative elements are a very effective method for stimulation and measurement of the cortisol response (Allen et al., 2017; Dickerson & Kemeny, 2004; Kirschbaum, Pirke, et al., 1993). The relationship between early life stress and HPA-axis regulation has been examined with exposures and outcomes at different ages in different

populations. Substantial evidence implicates prenatal programming of the fetal HPA-axis in the development of poor health in adulthood, with time of stress exposure during gestation and sex as modifiers of this relationship (Carpenter et al., 2017; Howland et al., 2017).

Evaluation of prenatal stress with cortisol reactivity in adulthood, representing long term HPA-axis modifications are rare. A unique retrospective account of maternal exposure to common negative life events during pregnancy and the blood cortisol response to the TSST in 30 adult children at age 25 found some evidence for lower baseline blood cortisol in offspring exposed to one or more life stress event in utero compared to those not exposed to any life stress events but did not examine salivary cortisol, timing of exposure during pregnancy or sex differences (Entringer et al., 2009).

A meta-analysis of 30 studies of postnatal stress exposures showed support for the association between early life trauma over childhood and adolescence and blunted salivary cortisol response to social stress (Bunea et al., 2017). They identified larger cortisol effect sizes in women compared to men and in adults compared to children and adolescents, possibly because the HPA-axis is relatively under-reactive before sexual maturity. Alternatively, effects detected in adulthood may represent more chronic, long term consequence of early life stress.

A meta-analysis of 24 studies investigating the association between early life stress and cortisol reactivity to an acute stressor in adulthood identified no significant associations (Fogelman & Canli, 2018). Focussing on long term effects by evaluating outcomes in adult populations, their results challenge current dogma that exposure to early life stress affects cortisol response in adulthood. The contrasting results of these meta-analyses are likely due to a combination of differences in study selection (type, severity and time of exposure), type of cortisol measured (salivary vs plasma) and the quality of the studies included. Neither of these reviews incorporated prenatal stress.

It remains to be seen whether 1) HPA-axis dysregulation is found in those with exposure to common life stress events or if dysregulation is limited to more extreme forms of trauma in early life and 2) HPA-axis dysregulation is more evident with high stress exposure at different periods during early life eg. prenatal, childhood or adolescence. Retrospective accounts of early life stress may not be reliable. Prospective

studies measuring early life stress occurrence, and following participants over time from early life to adulthood, are required.

There is evidence for sex-specific HPA-axis responses to early life stress exposure. In prenatal life, the placenta contains the same genetic information as the fetus (male or female) and there are sex differences in placental size and function including gene expression, activity of the 11B-HSD2 enzyme, sensitivity to hormones and blood flow (DiPietro & Voegtline, 2017). Later during puberty, the production of sex-specific hormones modulates the reactivity of the HPA-axis (Romeo, 2010b). In their meta-analysis, Bunea et al found that those studies with a greater proportion of female participants showed a larger effect size for blunted cortisol activity (Bunea et al., 2017). In a meta-analysis of cortisol stress reactivity across psychiatric disorders, males and females displayed opposing cortisol responses to a stressor (Zorn et al., 2017). Therefore, the HPA-axis response to early life stress exposure should be examined separately in males and females.

In the Raine Study, a Western Australian pregnancy cohort, we identified that associations between exposure to higher numbers of stress events over prenatal, childhood and adolescent periods, and symptoms of depression and anxiety in early adulthood, differed by sex (Herbison et al., 2017), with males showing stronger associations with exposure during the prenatal period and females during the postnatal periods. This may be due to a sex-dependent effect of stress exposure on later life HPA-axis reactivity. Few studies have the ability to examine stress exposure at different developmental stages and subsequent effects on HPA-axis reactivity, in the same individuals. The Raine Study represents a unique opportunity to examine this question. In this study we aimed to investigate whether common early life stressors, experienced prenatally or throughout childhood and adolescence, play a role in the dysregulation of the HPA-axis in early adulthood.

We hypothesised that i) high prenatal stress and ii) postnatal stress trajectories involving high exposure to stress during childhood and around adolescence (that we have previously shown to be associated with poor mental health outcomes) would be associated with a decreased cortisol response to stress at age 18 years, and that these associations would differ in magnitude between males and females.

5.2 Material and methods

5.2.1 Participants

The Raine Study commenced in 1989 when pregnant women [Gen1] were recruited at 16-20w gestation via the antenatal clinic at King Edward Memorial (public) Hospital and nearby private clinics (Newnham et al., 1993). This prospective study of 2868 live births [Gen2] involved data collection at 18 and 34 weeks gestation, ages 1, 2, 3, 5, 8, 10, 14, 17 and 18 years. Participant numbers have gradually decreased over time from 2819 eligible/2446 participating at age 1 to 2352 eligible/1726 participating at age 17 and a detailed profile of the cohort is available elsewhere (Straker et al., 2017). Ethics approval was obtained from King Edward Memorial Hospital, Princess Margaret Hospital and the University of Western Australia. All procedures were carried out with parental informed consent up to the age of 18, after which participants provided their own consent. The cohort is broadly representative of the general Western Australian population (Straker et al., 2017).

5.2.2 Stress event measures

The same panel of life stress events were measured at 10 different time points (18 weeks, 34 weeks gestation, ages 1, 2, 3, 5, 8, 10, 14 and 17). At 18 weeks gestation, the questionnaire referred to the time period since becoming pregnant, at 34 weeks it referred to the last 4 months and at age 1-17, it referred to the past 12 months. Mothers or primary care givers reported on problems with pregnancy, death of a close relative, death of a close friend, marital problems, separation or divorce, problems with children, own job loss (involuntary), partner's job loss (involuntary), financial hardship, residential move and 'other' stressful event. This stress event list was selected from the previously developed life stress inventory (Tennant & Andrews, 1976). Prenatal stress was examined in four categories; low stress during pregnancy (0-2 events); high stress (3+ events) at 18weeks; high stress (3+ events) at 34 weeks and high stress at both 18 and 34 weeks (n = 933). Eighty six were missing data at either 18w or 34w and therefore could not be accurately categorised. We chose to dichotomise in this way to examine timing effects and previous research in this pregnancy cohort has associated 3+ stress events with higher risk of depression and anxiety in adulthood (Herbison et al., 2017) and child behaviour problems (Robinson et al., 2011). Longitudinal exposure to life stress events

over 17 years post-birth was examined using a latent class growth analysis with PROC TRAJ in SAS. Only participants' who had data over 3 or more time points were used and the creation and profile characteristics of each stress trajectory have been reported elsewhere (Herbison et al., 2017). Briefly, five trajectories of stress exposure from birth to age 17 years were identified and are shown in Figure 5.1a. The low, medium and high trajectories displayed consistently low, intermediate and high levels of stress exposure over 17 years. The ascending trajectory displayed relatively low stress exposure in early life, increasing around adolescence and the descending trajectory displayed relatively high stress in the early years, decreasing to low levels during adolescence. The distribution of the trajectories in the cohort were; low (16.7%), medium (30.7%), high (17.2%), ascending (27.5%), descending (7.8%) and males and females were equally distributed across trajectories (Herbison et al., 2017).

5.2.3 Trier Social Stress Test

At age 18, participants were invited to complete the TSST. Details of this study and relationships with sex, BMI, smoking, oral contraceptive use and menstrual cycle can be found elsewhere (Herbison 2016). Briefly, 1137 males and females provided informed consent and completed this test. All testing was conducted in the afternoon and participants were asked to restrict eating and drinking in the hour prior to their appointment. All subjects arrived between midday and 3 p.m. for participation in the TSST between 1 p.m. and 4 p.m. to minimize the effects of diurnal variation in cortisol. A cannula was inserted by an anaesthetist with agreement from the participant and questionnaires were completed during a 45 min rest period. Blood samples were taken just before (0min) and after the test (15min) and then at the 25, 35, 45, 60, 75 and 105 min timepoints. Saliva samples were collected at 0, 15, 35 and 105min. In participants declining cannulation, saliva samples were taken at all 8 time points. The TSST itself involved a free speech interview and arithmetic challenge (5 min each) in front of a non-responsive, serious panel of well-dressed adults. A dummy video camera and mock audio equipment was set up to record the tasks and participants were debriefed afterwards. Prior to analyses, 79 individuals were eliminated for the following reasons; unusable samples, severe menstrual pain, pregnancy, lactation, type 1 diabetes, use of steroids/neuroactive medications/antidepressants and other medications known to impact the HPA-axis and fainting). A small number of participants had saliva-only

collected due to failed cannulation (*n* = 39) and were also excluded. Of the 1019 participants eligible for analysis, 830 had both blood and saliva collected and 189 had saliva-only collected. In this study we report on salivary cortisol as this is the most common measure in the literature (Hellhammer et al., 2009; Levine et al., 2007).

5.2.4 Measurement of salivary cortisol

Saliva was collected using Salivette collection devices (Sarstedt, Germany) and kept on ice until the final sample for each participant was obtained. Samples were spun, aliquoted and frozen at -80C until assayed. Free salivary cortisol was quantified using the GammaCoat TM 125I immunoradiometric assay (RIA) kit (DiaSorin, Stillwater, MN), as per the manufacturer's instructions. Samples were measured in duplicate against an appropriate standard curve and repeated as required. All intra- and inter-assay CV's were < 10%. Salivary cortisol was measured in nanomoles per litre (nmol/L) = nM.

5.2.5 Covariates

Socioeconomic status (SES) - household income as a five class category, measured at recruitment was used as an indicator of SES (<$7000, $7000-$11999, $12000-$23999, $24000-$35999, $36000+). Small numbers of missing values were imputed from family income recorded at 1 year after birth.

Smoking and BMI - Participants reported whether they were a smoker or non-smoker at the time of the test. Height and weight were measured for the calculation of BMI (used in continuous form). For BMI, small numbers of missing values were imputed from BMI recorded at age 17.

Saliva-only – a total of 189 participants declined cannulation and received saliva-only collection. This was used as a dichotomous variable blood and saliva =0, saliva only = 1.

Oral contraceptive use in girls was not adjusted for in this study because (in contrast to plasma cortisol) salivary cortisol does not show differences between girls taking and not taking oral contraceptives (Herbison et al., 2016; Kirschbaum et al., 1999). The birth measures of delivery method, birthweight and gestational age were tested in univariate analyses but as there was no evidence for association with dependent or independent variables, and thus no potential for confounding, we did not include these in our models.

5.2.6 Statistical analyses

Prenatal stress as a four category variable and postnatal stress as a five category variable were considered in separate regression models and the outcome (the TSST) was considered in three different ways; summary measures, response curves and responder category. We reported separately by sex based on prior evidence for sex differences in fetal programming (Carpenter et al., 2017; Glover & Hill, 2012; Sandman et al., 2013), cortisol response (Herbison et al., 2016; Kirschbaum, Wust, & Hellhammer, 1992) and risk associations between early life stress and mental health (Herbison et al., 2017). For all models sex-specific results were estimated by inclusion of an interaction term between sex and the stress event measure under investigation. All regression models were adjusted for family income at recruitment, smoking and BMI at age 18 years. We also included an indicator variable denoting TSST blood and saliva collected vs saliva-only in all models to eliminate this as a potential source of confounding.

We also tested for prenatal stress adjusted for postnatal stress and vice versa in models for all TSST outcomes.

The summary measures of salivary cortisol over the TSST included concentration at baseline (C_{BL}), Concentration at peak (C_{MAX}), concentration at minimum (C_{MIN}), concentration range (C_{RANGE}), area under the curve with respect to ground (AUC_G) and area under the curve with respect to range (AUC_R). These measures are summarised in Figure 5.1b. We have used AUC_R in place of area under the curve with respect to increase (AUC_I) as it is more effective in detecting changes when a proportion of participants' have a high C_{BL}. AUC_R is not subject to issues of negative area, unlike AUC_I (Herbison et al., 2016). The associations of prenatal stress (four-category variable described above) and postnatal trajectory of stress (five category variable described above), with these summary measures of salivary cortisol, were analysed using linear regression models (stress exposure as the independent variable of interest and TSST summary measures as the dependent variable).

For response curves, salivary cortisol response to the TSST over time was examined using a linear mixed model. Raw salivary cortisol data was natural log transformed.

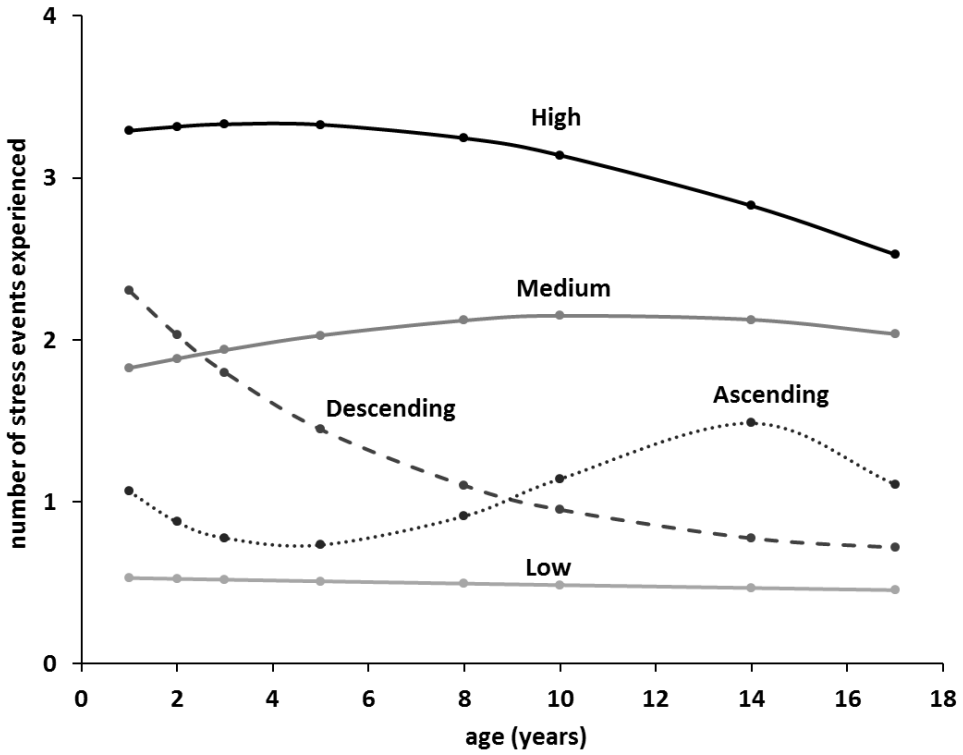
The group definitions used for responder-category identification have previously been described (Herbison et al., 2016). Briefly, responders were defined as either

Reactive-Responders (where an increase in plasma cortisol was observed immediately after the TSST to a noticeable peak before decreasing back to baseline) or Anticipatory-Responders (the absence of a clear upward response and plasma cortisol levels fell over the first 60 min of the study by >20% indicating the cortisol response commenced before the TSST). Non-responders showed no clear plasma cortisol peak and little change in cortisol over the study period. This data subset included participants with both blood and saliva *n* = 830, as the criteria for group membership involved both the TSST plasma cortisol and salivary cortisol response curves (Herbison et al., 2016). Logistic regression models were used to determine the odds of being a 'Non-Responder' vs being a 'Responder'.

Figure 5.1

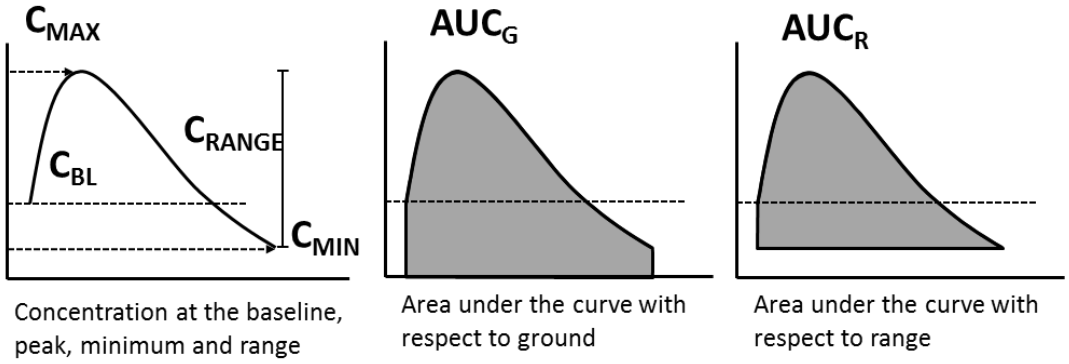
Measurement of postnatal stress exposure and cortisol parameters

(a) Trajectories of stress exposure from birth to age 17



Life stress events were measured at eight time points over 17 years. Each point refers to number of life stress events experienced within the previous 12 months in that trajectory. Further details in methods and elsewhere (Herbison et al, 2016). [Adapted with permission from *Development and Psychopathology* 29 2017 ©Cambridge University Press]

(b) Visual summary of TSST summary measures



[Adapted with permission from *Stress* 29 2017 ©Taylor and Francis] (Herbison et al 2017)
All models were checked for the absence of influential outliers by visual examination of scatterplots and residual diagnostics, and absence of collinearity via assessment of variance-inflation factors'. Participants missing from final models were determined to be missing completely at random according to Little's MCAR test. Statistical analyses were performed using IBM SPSS Statistics for Windows, version 25 (IBM Corp., Armonk, N.Y., USA) and Stata/IC 15.0 for Windows (StatCorp LLC, College Station TX USA). Regression coefficients and odds ratios are presented with accompanying 95% confidence intervals and p-values.

5.3 Results

5.3.1 Sample characteristics

The characteristics of this sample including exposure and outcome variables and covariates are presented in Table 5.1, separated by sex. There was a strong association between prenatal stress exposures and postnatal stress trajectories (Pearson χ^2=162.68 df 12 p<0.0001) with a pattern of high postnatal stress trajectories being more likely to occur in conjunction with high prenatal stress. See Supplementary Table 5.1.

Table 5.1
Participant characteristics

	Total	Females	Males
Exposures			
	n (%)	*n (%)*	*n (%)*
Prenatal stress			
Low	750 (82.2)	353 (80.8)	397 (83.6)
High 18w	77 (8.4)	39 (8.9)	38 (8.0)
High 34w	50 (5.5)	30 (6.9)	20 (4.2)
High both	35 (3.8)	15 (3.4)	20 (4.2)
Postnatal stress trajectory			
Low	210 (21.1)	110 (23.0)	100 (19.4)
Ascending	280 (28.2)	119 (24.9)	161 (31.3)
Descending	97 (9.8)	49 (10.3)	48 (9.3)
Medium	279 (28.1)	137 (28.7)	142 (27.6)
High	127 (12.8)	63 (13.2)	64 (12.4)
Covariates			
	mean (SD)	*mean (SD)*	*mean (SD)*
Age *(years)*	18.3 (0.3)	18.3 (0.3)	18.3 (0.3)
BMI	23.7 (4.8)	23.8 (5.3)	23.6 (4.2)
	n (%)	*n (%)*	*n (%)*
Smoker	142 (14.3)	52 (10.9)	90 (17.5)
Saliva-only	186 (18.7)	113 (23.6)	73 (14.2)
Income (at recruitment)			
<$7000	49 (4.9)	27 (5.6)	22 (4.3)
$7000-11999	56 (5.6)	31 (6.5)	25 (4.9)
$12000-23999	221 (22.3)	100 (20.9)	121 (23.5)
$24000-35999	279 (28.1)	133 (27.8)	146 (28.3)
$36000+	388 (39.1)	187 (39.1)	201 (39.0)

	Total	Females	Males
Outcomes			
	mean (SD)	*mean (SD)*	*mean (SD)*
Summary measures			
C_{BL} (nM)	13.5 (9.6)	12.2 (9.0)	14.7 (9.9)
C_{MAX} (nM)	17.7 (10.9)	15.9 (10.3)	19.3 (11.1)
C_{MIN} (nM)	8.4 (4.1)	7.8 (3.7)	8.9 (4.4)
C_{RANGE} (nM)	9.3 (8.4)	8.1 (8.0)	10.4 (8.6)
AUC_G	1335 (722)	1205 (668)	1455 (750)
AUC_R	454 (413)	382 (380)	520 (430)
	median (IQR)	*median (IQR)*	*median (IQR)*
Response curve			
t0min (nM)	10.7 (7.9, 15.5)	9.7 (7.3, 14.1)	11.7 (8.6, 17.5)
t15min (nM)	11.8 (8.7, 16.8)	10.5 (7.7, 14.1)	13.2 (9.6, 18.2)
t35min (nM)	12.4 (8.7, 18.2)	10.9 (7.7, 15.8)	14.2 (9.9, 19.6)
t105min (nM)	8.2 (6.2, 11.0)	7.9 (5.9, 10.6)	8.6 (6.5, 11.4)
	n (%)	*n (%)*	*n (%)*
Responder Category			
Responders	667 (83.4)	286 (79.2)	381 (86.8)
Non-responders	133 (16.6)	75 (20.8)	58 (13.2)

Characteristics are detailed for postnatal stress trajectory models, with the exception of prenatal stress exposures at the top. Total adjusted numbers for prenatal stress linear regression *n* = 912 and Responder category logistic regression *n* = 739
Total adjusted numbers for postnatal stress trajectories linear regression *n* = 995 and Responder category logistic regression *n* = 800

5.3.2 Prenatal stress

The association of summary measures of salivary cortisol with the four group variable representing prenatal stress is presented in Table 5.2a and the results are presented graphically in Figure 5.2. Using the summary measures of salivary cortisol, we found some evidence for an association of exposure to high stress at 18w but not at 34w or at both time points in males only. Male offspring with exposure to 3 or more stress events at 18w pregnancy, compared to low stress exposure, showed an increase of C_{MAX} by 4.28nM (nmol/L) (95%CI: 0.59, 7.96, p=0.02), an increase in C_{RANGE} of 2.94nM (95%CI: 0.08, 5.79 p=0.04), an increase in AUC_G of 287 (95%CI: 44, 531 p=0.02) and an increase in

AUC_R of 143 (95%CI:-3, 283 p=0.04), Table 5.2a and Figure 5.2. Males with high stress at 18w also showed higher cortisol summary measures than the other two stress exposure groups (high stress at 34w and high stress at both time points).Table 5.2a and Figure 5.2. After adjusting for postnatal stress trajectories, estimates were similar.

Trier response curves from mixed models adjusted analyses for prenatal stress are shown in Figure 5.3a for males and females. There was no evidence for a differential pattern of change in salivary cortisol over time by prenatal stress group in either males (p=.21) or females (p=.19). However, there were differences between prenatal stress groups in levels of salivary cortisol pooled over time periods in males (p=.03) but not in females (p=.83), with cortisol values for males with high stress exposure at 18w being higher than the other three groups. Figure 5.3a. These estimates remained similar after adjustment for postnatal stress trajectory membership.

Table 5.2

Stress exposures with salivary cortisol TSST summary measures in males and females

(a) Prenatal stress exposures

	Females			Males		
	EMM	***B (95% CI)***	***p***	**EMM**	***B (95% CI)***	***p***
C_{BL}						
Low	10.87	(REF)	*0.89#*	13.48	(REF)	*0.48*
High 18w	11.90	1.03 (-2.24, 4.30)	0.54	15.55	2.06 (-1.25, 5.37)	0.22
High 34w	11.88	1.00 (-2.63, 4.64)	0.99	11.83	-1.65 (-6.02, 2.71)	0.46
High both	10.75	-0.12 (-5.32, 5.08)	0.96	12.32	-1.16 (-5.82, 3.50)	0.62
C_{MAX}						
Low	14.41	(REF)	*0.90*	18.02	(REF)	*0.04*
High 18w	15.13	0.72 (-2.95, 4.39)	0.70	22.29	**4.28 (0.59, 7.96)**	**0.02**
High 34w	15.71	1.30 (-2.78, 5.39)	0.53	15.27	-2.74 (-7.65, 2.16)	0.27
High both	15.24	0.83 (-5.02, 6.67)	0.78	15.44	-2.57 (-7.56, 2.41)	0.31
C_{MIN}						
Low	7.31	(REF)	*0.92*	8.36	(REF)	*0.05*
High 18w	7.79	0.48(-0.91, 1.86)	0.50	9.70	1.34(-0.05, 2.73)	0.06
High 34w	7.23	-0.08 (-1.62, 1.46)	0.92	7.07	-1.30 (-3.15, 0.56)	0.17
High both	7.26	-0.05 (-2.26, 2.15)	0.96	7.19	-1.25 (-3.13, 0.64)	0.19

	Females			Males		
	EMM	***B (95% CI)***	***p***	**EMM**	***B (95% CI)***	***p***
C_{RANGE}						
Low	7.10	(REF)	*0.84*	9.65	(REF)	0.14
High 18w	7.34	0.24 (-2.60, 3.09)	0.87	12.59	**2.94 (0.08, 5.79)**	***0.04***
High 34w	8.48	1.38 (-1.78, 4.55)	0.39	8.20	-1.45 (-5.25, 2.35)	*0.45*
High both	7.98	0.88 (-3.65, 5.41)	0.70	8.33	-1.33 (-5.19, 2.54)	*0.50*
AUC_G						
Low	1098	(REF)	*0.99*	1345	(REF)	0.04
High 18w	1122	24 (-218, 267)	0.84	1632	**287 (44, 531)**	***0.02***
High 34w	1142	44 (-226, 314)	0.75	1157	-188 (-512, 136)	*0.25*
High both	1121	23 (-363, 409)	0.91	1163	-182 (-511, 147)	*0.28*
AUC_R						
Low	333	(REF)	*0.79*	470	(REF)	0.18
High 18w	297	-36 (-176, 103)	0.61	613	**143 (3, 283)**	***0.04***
High 34w	398	64 (-91, 219)	0.42	411	-59 (-245, 128)	*0.54*
High both	355	21 (-201, 243)	0.85	425	-45 (-234, 144)	*0.64*

#The p value against the Low stress category represents the overall test for differences between the 4 groups. EMM=Estimated marginal mean. Linear regression models presented were adjusted for income, BMI, smoking and blood vs saliva-only collected and results were similar before adjustment. Results for males high at 34w or high both compared to high18w (REF) were as follows; C_{MAX} high34w: 7.02nM (95%CI: 1.09, 12.95, p=0.02), C_{MAX} high both: 6.85nM (95%CI: 0.88, 12.81, p=0.02); C_{MIN} high34w: 2.63nM (95%CI: 0.40, 4.87, p=0.02), C_{MIN} high both: 2.58nM (95%CI: 0.33, 4.83, p=0.02); AUC_G high 34w: 465 (95%CI: 84, 867 p=0.02), AUC_G high both: 469 (95%CI: 76, 863 p=0.02), . Results for prenatal stress x sex interaction C_{BL} (F=0.39 p=0.76), C_{MAX} (F=1.48 p=0.22), C_{MIN} (F=0.85 p=0.47), C_{RANGE} (F=1.28 p=0.28), AUCg (F=1.48 p=0.22), AUCr (F=1.59 p=0.19).

(b) Postnatal stress trajectory

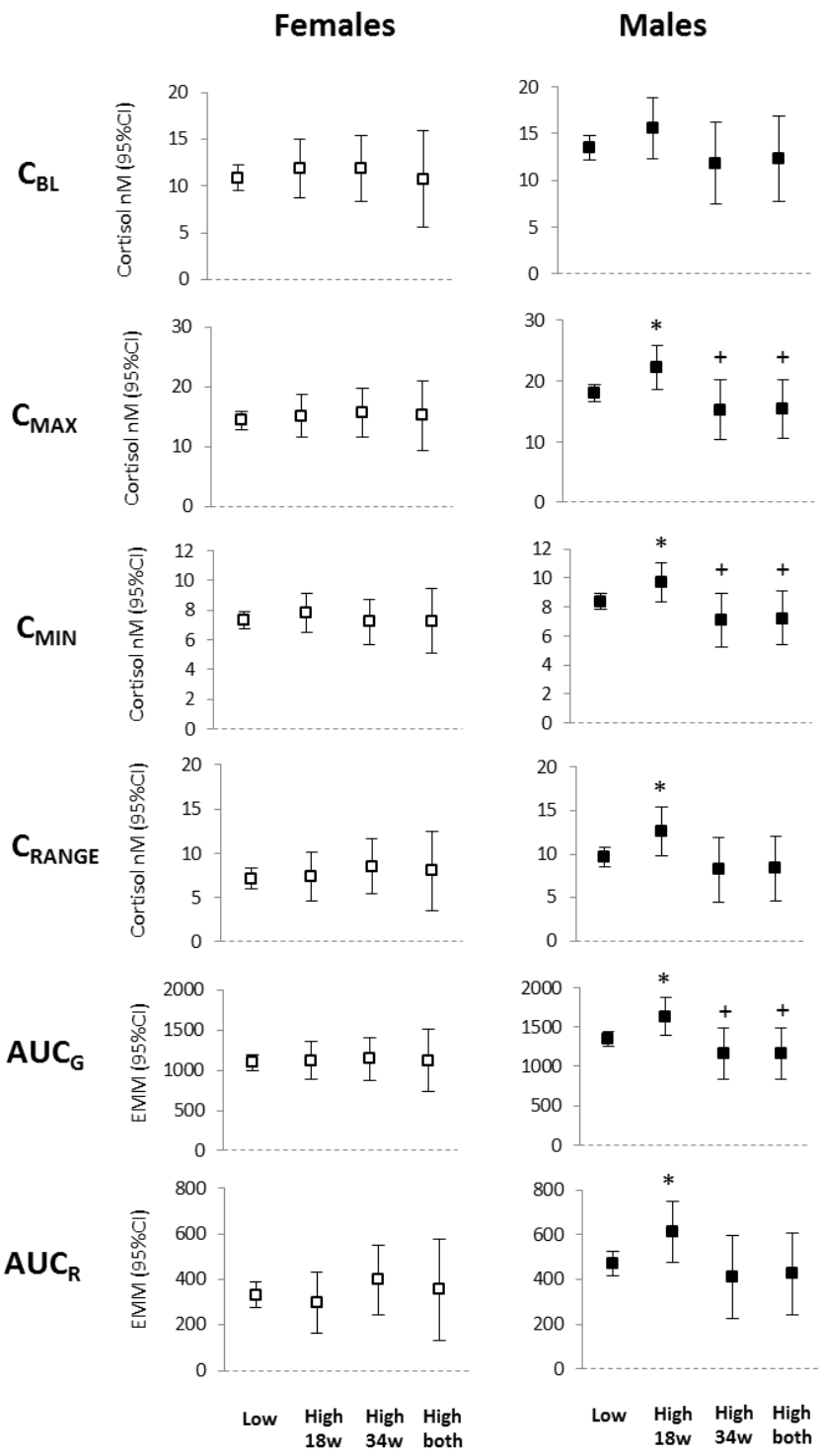
	Females			Males		
	EMM	***B (95% CI)***	***p***	**EMM**	***B (95% CI)***	***p***
C_{BL}						
Low	12.81	(REF)	*0.53#*	14.85	(REF)	*0.72*
Ascending	11.44	-1.37(-4.44,1.69)	0.38	14.30	-0.55(-3.22,2.12)	0.69
Descending	13.71	0.89(-3.00,4.78)	0.65	13.22	-1.63(-5.35,2.09)	0.39
Medium	11.01	-1.81(-4.67,1.05)	0.21	15.38	0.53(-2.25,3.31)	0.71
High	12.61	-0.21(-3.81,3.39)	0.91	13.74	-1.10(-4.70,2.50)	0.55
C_{MAX}						
Low	16.26	(REF)	*0.45*	19.57	(REF)	*0.89*
Ascending	14.49	-1.77(-5.20,1.66)	0.31	18.38	-1.19(-4.19,1.80)	0.43
Descending	17.33	1.07(-3.30,5.44)	0.63	17.64	-1.93(-6.11,2.24)	0.36
Medium	14.05	-2.21(-5.41,0.98)	0.17	19.03	-0.54(-3.66,2.58)	0.73
High	15.67	-0.60(-4.64,3.45)	0.77	18.68	-0.89(-4.89,3.11)	0.66
C_{MIN}						
Low	8.41	(REF)	*0.09*	8.38	(REF)	*0.34*
Ascending	7.79	-0.62(-1.91,0.68)	0.35	9.37	0.98(-0.15,2.11)	0.09
Descending	8.81	0.41(-1.24,2.05)	0.63	8.53	0.14(-1.43,1.72)	0.86
Medium	7.04	-1.37(-2.58,0.16)	0.03	8.92	0.54(-0.64,1.71)	0.37
High	8.18	-0.23(-1.75,1.30)	0.77	8.24	-0.15(-1.66,1.36)	0.85

	Females			Males		
	EMM	***B (95% CI)***	***p***	**EMM**	***B (95% CI)***	***p***
C_{RANGE}						
Low	7.86	(REF)	*0.81*	11.19	(REF)	*0.40*
Ascending	6.70	-1.16(-3.81,1.49)	0.39	9.01	-2.17(-4.48,0.14)	0.06
Descending	8.52	0.67(-2.71,4.04)	0.70	9.11	-2.07(-5.30,1.15)	0.21
Medium	7.01	-0.84(-3.31,1.63)	0.50	10.11	-1.08(-3.49,1.33)	0.38
High	7.49	-0.37(-3.49,2.75)	0.82	10.44	-0.75(-3.83,2.34)	0.64
AUC_G						
Low	1263	(REF)	*0.23*	1417	(REF)	*0.93*
Ascending	1165	-98(-328,131)	0.40	1469	52(-148,251)	0.61
Descending	1359	96(-195,387)	0.52	1371	-46(-324,232)	0.74
Medium	1073	-190(-403,23)	0.08	1463	46(-162,254)	0.67
High	1218	-45(-314,224)	0.74	1411	-6.5(-273,260)	0.96
AUC_R						
Low	370	(REF)	*0.87*	551	(REF)	*0.66*
Ascending	347	-22(-155,110)	0.74	476	-75(-190,40)	0.20
Descending	426	56(-112,224)	0.51	465	-86(-247,75)	0.30
Medium	339	-31(-154,92)	0.62	516	-35(-155,86)	0.57
High	351	-19(-175,137)	0.81	542	-9(-163,145)	0.91

#The p-value against the low trajectory is for overall differences between groups. Regression models presented were adjusted for income at recruitment, smoking, BMI at age 18 and blood vs saliva-only collected and results were similar before adjustment. EMM=Estimated marginal mean. Results for postnatal stress trajectory x sex interaction C_{BL} (F=1.01 p=0.35), C_{MAX} (F=0.68 p=0.61), C_{MIN} (F=2.28 p=0.06), C_{RANGE} (F=0.41 p=0.80), AUCg (F=1.29 p=0.27), AUCr (F=0.52 p=0.72).

Figure 5.2

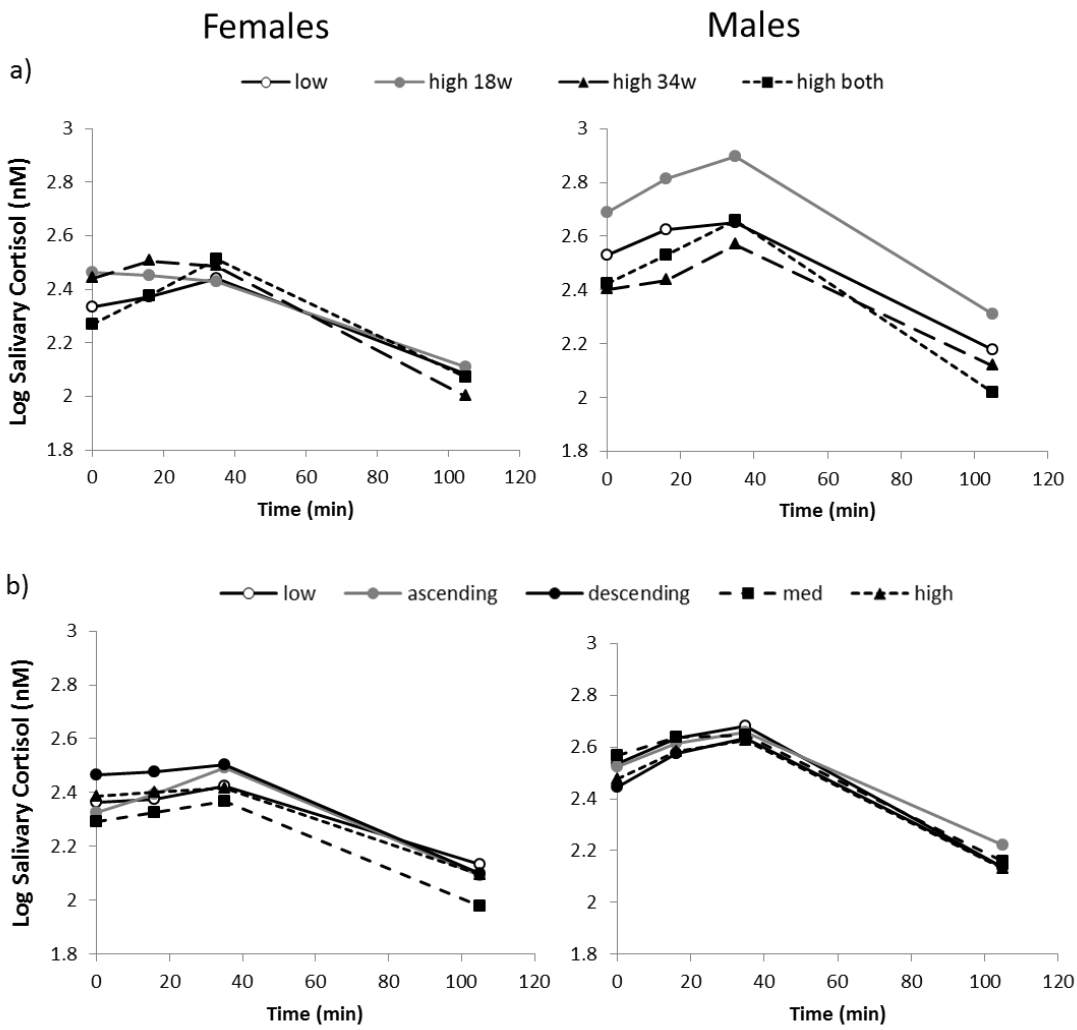
Salivary cortisol estimated marginal means (EMM) with prenatal stress exposure in males and females



Estimated Marginal Means (EMM) are plotted with 95% CI. * p<0.05 compared to low prenatal stress, + p<0.05 compared to high stress at 18w.

Figure 5.3

Salivary cortisol response curves to the TSST in males and females for (a) prenatal stress exposure and (b) postnatal stress trajectories



The TSST was conducted at 1-15min. Males with high stress at 18w had a significantly higher TSST response than the other 3 groups with differences as follows; Low stress at 18w and 34w: 0.18log nM (0.04,0.33) p=.01; High stress at 34w: 0.30log nM (0.07, 0.53) p=.01; High stress at 18w and 34w: 0.27log nM (0.04, 0.50) p=.02

For responder-category analyses, logistic regression models showed no differences in the odds of being a Non-Responder vs being a Responder across prenatal stress groups in males (p=0.15) and females (p=0.47). See Table 5.3. Estimates were very similar after adjustment for postnatal stress exposure (males (p=0.22), females (p=0.39)).

Table 5.3

Prenatal stress and postnatal stress trajectory by TSST responder category

	Females				Males			
	NR *n (%)*	**R** *n (%)*	**NR vs R** *OR (95% CI)*	***p***	**NR** *n (%)*	**R** *n (%)*	**NR vs R** *OR (95% CI)*	***p***
Prenatal Stress								
Low	59/270 (21.9)	211/270 (78.1)	(REF)	*0.47#*	44/343 (12.8)	299/343 (87.2)	(REF)	*0.15#*
High 18w	6/31 (19.4)	25/31 (80.6)	0.78 (0.30, 2.04)	*0.61*	4/27 (14.8)	23/27 (85.2)	1.15 (0.37, 3.54)	*0.37*
High 34w	1/20 (5.0)	19/20 (95.0)	0.21 (0.03, 1.59)	*0.13*	2/19 (10.5)	17/19 (89.5)	0.74 (0.16, 3.34)	*0.70*
High both	3/13 (23.1)	10/13 (76.9)	1.06 (0.28, 4.03)	*0.94*	5/16 (31.3)	11/16 (68.8)	3.81 (1.19, 12.21)	*0.02*
Postnatal Stress								
Low	24/83 (28.9)	59/83 (71.1)	(REF)	*0.14#*	6/86 (7.0)	80/86 (93.0)	(REF)	*0.07#*
Asc	9/77 (11.7)	68/77 (88.3)	**0.33 (0.14, 0.76)**	***0.01***	27/142 (19.0)	115/142 (81.0)	**3.12 (1.22, 7.98)**	***0.02***
Desc	7/36 (19.4)	29/36 (80.6)	0.63 (0.24, 1.65)	*0.35*	4/40 (10.0)	36/40 (90.0)	1.42 (0.37, 5.41)	*0.60*
Med	24/114 (21.1)	90/114 (78.9)	0.67 (0.34, 1.31)	*0.24*	12/120 (10.0)	108/120 (90.0)	1.43 (0.51, 4.06)	*0.50*
High	11/51 (21.6)	40/51 (78.4)	0.77 (0.32, 1.84)	*0.56*	9/51 (17.6)	42/51 (82.4)	2.61 (0.84, 8.08)	*0.10*

#The p-values against the low prenatal stress exposure category are for the overall group contrast. Logistic regression models (odds of being NR vs R) adjusted for income at recruitment, smoking and BMI at age 18 and results were similar before adjustment. NOTE: these data include those participants with both blood and saliva collected. Prenatal stress final adjusted total *n* = 739. Postnatal stress trajectory final adjusted total *n* = 800. Prenatal stress x sex interaction (p=0.383). Postnatal stress trajectory x sex interaction (p=0.0084)

5.3.3 Postnatal stress trajectories from birth to age 17

Using the summary measures of salivary cortisol, we found no evidence of an association across postnatal stress exposure groups in males and females. In post hoc analyses, males with an ascending stress trajectory showed a reduced C_{RANGE} -2.17nM (-4.48, 0.14) p=0.06 using the Low trajectory as the reference group, Table 5.2b, and this estimate was similar after adjustment for prenatal stress (C_{RANGE} -2.74nM (-5.21, -0.28) p=0.03).

Trier response curves from mixed models adjusted analyses for postnatal stress trajectories are shown in Table 5.3b for males and females. There was no evidence for a differential pattern of change in salivary cortisol over time by postnatal stress trajectory group in either males (p=.62) or females (p=.29), and no evidence for differences between postnatal stress trajectory groups in levels of salivary cortisol pooled over time periods in either females (p=0.24) or males (p=0.87). Results were similar after adjustment for prenatal stress.

For responder-category analyses, logistic regression models showed some weak support for differences in the odds of being a Non-Responder vs being a Responder across postnatal stress trajectory groups in males (p=0.07) but not females (p=0.14). Post hoc analyses identified a sex specific effect of being in the ascending stress trajectory. Females in the ascending trajectory had a reduced risk of being a Non-Responder (0.33 (0.14, 0.76) p=0.01) whereas males in the ascending trajectory had an increased risk of being a Non-Responder (3.12 (1.22, 7.98) p=0.02). See Table 5.3. After adjusting for prenatal stress, estimates were similar whereby females in the ascending trajectory had a reduced risk of being a Non-Responder (0.27 (0.11, 0.69) p=0.01) and males in the ascending trajectory had an increased risk of being a Non-Responder (2.81 (1.08, 7.27) p=0.03).

With the numbers available, it was not possible to test for effect modification between pre and postnatal stress by inclusion of an interaction term, due to sparse data in some cross-tabulated categories.

5.4 Discussion

This study examined the relationships between pre and postnatal early life stress exposure and cortisol response to an acute social stressor at age 18 years. Using three

methods to assess the stress response (salivary cortisol summary measures, response curves and responder category) we found no strong associations between pre or postnatal stress exposure and the salivary cortisol response to stress in females. However, we did find interesting results in males exposed to high stress early in pregnancy and in adolescence. These findings highlight vulnerable time periods in males during which exposure to common life stress events may impact HPA-axis regulation in adulthood.

Using the TSST, we found evidence for a heightened salivary cortisol response in males who had high stress exposure at 18w pregnancy (but not 34w) compared to low stress exposure. Specifically, C_{MAX}, C_{MIN}, C_{RANGE}, AUC_G and AUC_R measures in males with exposure to 3 or more stress events at 18w were 12-20% higher than males with low stress exposure during pregnancy. The reproducibility of this increase over multiple parameters increases its reliability. These results suggest there may be sex-specific prenatal stress regulation of the HPA-axis and that in males, high stress in the first half of pregnancy may confer a greater risk than high stress in the latter half of pregnancy.

There is a biological explanation for early pregnancy vulnerability: the placental enzyme 11B-HSD2 inactivates cortisol in order to protect the developing fetus from glucocorticoid exposure and the expression of 11B-HSD2 rises more than 12-fold during a normal pregnancy but it is only a partial barrier (Davis & Sandman, 2010; Konstantakou et al., 2017). Low expression of 11B-HSD2 early in pregnancy combined with higher maternal cortisol levels due to increased stress exposure may render the fetus sensitive to higher glucocorticoids. Towards the end of pregnancy 11B-HSD2 expression is high and maternal cortisol levels have risen 2-3 fold. The HPA-axis also becomes less responsive to stress and this has been proposed to have a protective effect in late pregnancy (Entringer et al., 2010). This relative protective effect may explain why participants with high stress exposures at 34 weeks and at both time points did not show elevations in salivary cortisol reactivity. Our results differ from those of Entringer et al 2009 who investigated prenatal stress involving one or more negative life event during the whole pregnancy and ACTH/plasma cortisol response to the TSST at mean age 25 years (Entringer et al., 2009). Their study sample contained five (16%) males so would have been underpowered to detect the male-specific changes found in this sample. Also in contrast to our results, high prenatal maternal stress late in gestation has been associated with higher cortisol reactivity by AUCi but not AUCg at age 2 (Yong Ping et al.,

2015). These results may differ from ours due to sample size, age and methological differences. Others have suggested that the specific pattern of or change in stress exposure over pregnancy may be more related to adverse outcomes than exposure at any individual gestational period (Alen et al., 2020; Glynn et al., 2008). This could explain why we saw an association of high stress at 18w (descending stress over pregnancy) and not high stress at both 18w and 34w (constant high) compared with low stress at 18w and 34w (constant low) with the TSST. Our findings are consistent with animal studies whereby stress exposure early in gestation was found to alter behaviour and increase corticosterone production in response to stress, but only in males. Further studies in these male animals revealed changes in the expression and methylation of stress related genes in the hippocampus and amygdala (Mueller & Bale, 2008), indicating a sex-specific response mechanism to changes in the fetal environment.

Our results may join the large body of evidence suggesting that the male fetus is at increased vulnerability to maternal and environmental exposures, with negative in utero exposures more likely to lead to mortality or morbidity than female fetus' (DiPietro & Voegtline, 2017; Glover & Hill, 2012; Sandman et al., 2013). This may be because the male fetus puts a higher investment into physical growth, rendering them less adaptable to fluctuating conditions in utero, and more susceptible to poorer developmental outcomes (DiPietro & Voegtline, 2017). From a different viewpoint, these differences found in males exposed to stress in early gestation, may represent an adaptation to improve coping in an environment that is predicted to be hostile, by upregulation of cortisol stress reactivity.

The most frequent mechanism by which stress is proposed to impact the fetus is via maternal cortisol crossing the placental barrier and affecting the development of the HPA-axis. Whilst often overlooked, the fetal placenta is formed from the same embryonic tissue as the fetus and is either male or female. The sex differences in placental size and function including gene expression, activity of the 11B-HSD2 enzyme, sensitivity to hormones and blood flow may be involved (DiPietro & Voegtline, 2017).

In our analysis of postnatal trajectories of stress exposure, we found that males in the ascending trajectory had an increased risk of being a non-responder whilst females in the ascending trajectory of life stress events had a reduced risk of being a non-responder, compared to the low trajectory. This result in males is supported by the

reduced cortisol C_{RANGE} in the ascending trajectory compared to the low trajectory. The ascending trajectory was the most common trajectory in males in this sample at 31%. Given the widespread finding that male cortisol reactivity profiles are more robust and consistently higher than female cortisol reactivity profiles (Herbison et al., 2016; Kirschbaum et al., 1999; Stephens et al., 2016), the absence of a cortisol response in 19% of those males in the ascending trajectory is notable. Also of note, this effect was not in all males with exposure to stress events around adolescence but specifically those who experienced a low level of stress exposure in their past. One hypothesis suggests that the experience of moderate stress early in life may facilitate the development of an adaptive endogenous stress response in preparation for future exposures (Gunnar, Frenn, et al., 2009; Liu, 2015). It may be that participants deprived of early life stress may be less prepared to respond appropriately when the system is challenged in adolescence. It will be interesting to see whether these individuals are more at risk of stress-related health conditions in the future.

In contrast to males, females with the same pattern of stress exposure (the ascending trajectory), were less likely to be non-responders, highlighting the potential for divergent sex responses to the same level of stress exposure. Adolescence represents a time of transition incorporating the changes associated with the production of sex-specific gonadal hormones. Sex hormones interact with receptors in the brain and adrenal glands that help regulate the HPA-axis. This time is also associated with major changes in HPA-axis activity as it increases in resting and reactive states after a relative period of hyporeactivity during childhood (Romeo, 2010a). There is discussion of adolescence as a second sensitive period in development (Hostinar et al., 2018) and evidence that adolescence may be a time of HPA-axis recalibration (Gunnar et al., 2019). The modulation of the HPA-axis at this time may reprogram it for new challenges in adulthood but may also shed light on the vulnerability of this period of development. In support of this, recent fMRI research found that adolescent maltreatment (age 13 -15) was associated with an increased amygdala response, in contrast to maltreatment at age 4 (Zhu et al., 2019). Our results suggest that males exposed to stress around adolescence with relatively low experience of life stress in the past, may be more at risk for a blunted response to stress or a non-responder profile at age 18, whilst females exposed to a similar stress trajectory are more likely to have a responder profile.

With the exception of the above results, we did not find clear associations between common family life stress events from birth to age 17 and HPA-axis dysregulation in adulthood. This is in line with the meta-analysis by Fogelman and Canli, which also focused on outcomes measured in adults aged 18 years and over (Fogelman & Canli, 2018). In their meta-analysis of 24 studies they did not identify a significant correlation between early life trauma and cortisol reactivity (9/24 studies) or the three other outcomes examined: Cortisol Awakening Response (CAR), baseline cortisol and non-stressed cortisol over time. They also found no modification by sex. In contrast, Bunea et al examined 30 studies and did find a positive association between severe early life trauma and blunting of cortisol reactivity with a moderate effect size (g=-0.39)(Bunea et al., 2017), and that the percentage of female participants was a positive predictor of the effect size. It is interesting to note that the effect size was moderate across the studies that focussed on maltreatment and small in those including other early life adversities. Therefore, the effect on HPA dysregulation may be more pronounced with severe maltreatment in early life and this may explain why we did not see the same effect measuring less severe but more common life stress events.

We have previously reported that for males in this same cohort, high prenatal stress at 18w predicted higher depression/anxiety symptoms at age 20 (Herbison et al., 2017), and it may be that this association is partially mediated by HPA-axis reactivity. From a developmental programming point of view, whereby the conditions in utero may confer epigenetic/regulatory changes to prepare the fetus for their external environment, prenatal stress may play a role by adjusting HPA-axis sensitivity in anticipation of a stressful environment (Glover & Hill, 2012). We also previously reported higher levels of depression, anxiety and stress at age 20 in female participants in the medium, high and ascending trajectories (Herbison et al., 2017) and taken together with these results it indicates that this relationship is unlikely to be mediated by changes in HPA-axis reactivity at age 18. Other potential pathways for mediation of this relationship need to be considered, and are reviewed elsewhere (Beijers et al., 2014).

A number of limitations with this study deserve discussion. We did not examine severe cases of childhood trauma and abuse. As a prospective study we focused on the more common stress-inducing life events that are relevant to the general population but are still linked to psychopathology later in life (Herbison et al., 2017). We did not find

strong associations of early life stress with cortisol reactivity at age 18 and it is possible that HPA-axis changes may be more evident with more severe trauma. We used parent report for the same 10 life stress events across the lifespan which is an indirect measure of stress exposure for the child and may be affected by parent coping strategies, parenting methods, family functioning etc. Although we were able to adjust prenatal stress for postnatal stress and vice versa, with the numbers available we were unable to statistically estimate interactions between them. It may be that the prenatal group differences reported in this study may differ according to levels of postnatal stress, or vice versa. Although we reported results separately for sex, with the numbers available there was no statistical evidence for differences in associations by sex, with the exception of the association between postnatal trajectory group membership and TSST responder category. Investigations by sex were exploratory in nature as the inconsistent directional findings by sex in the literature limited our ability to form a directional hypothesis. We did not adjust for multiple tests due to recent calls to abandon reliance on strict significance thresholds (Wasserstein et al., 2019). However, because of the multiple tests conducted there is a possibility some of the findings may be spurious. We conducted the TSST on the cusp of adulthood after the impact of puberty has taken effect and at an age where the prevalence of mental health disorders is at a peak (Statistics, 2009). However, it is possible that the link between early life stress and cortisol stress reactivity may manifest more reliably in older populations. Carpenter et al showed that with exposure to early life emotional abuse, the older age group (36-61y) had a significantly lower cortisol response than the younger age group (18-31y), compared to controls (Carpenter et al., 2009).

Furthermore, a commonly held hypothesis is that blunted cortisol responses may develop as an adaptation to a constantly hyper-activated stress system. It is possible that our TSST measurements were performed during a window where adaptation was occurring and the shift in reactivity may become more evident with time, showing greater HPA-axis dysregulation in older populations. This may also be sex-dependent as brain regions regulating the HPA-axis mature at different times in males and females (Fish et al., 2020). Prospective longitudinal stress reactivity measurements are needed to address this.

Despite some limitations, it is rare to find a prospective cohort study from in-utero to adulthood with over 950 individual TSSTs performed. We examined windows of developmental vulnerability using prenatal stress exposure and postnatal trajectories of stress exposure over 17 years in males and females. Ultimately, the HPA-axis changes manifesting with exposure to commonly occurring early life stress events are subtle and it may be the interaction of these exposures with additional genetic and environmental risk factors that is relevant to health and disease.

5.5 Conclusions

Early gestation and adolescence represent vulnerable windows during which common life stressors can affect functioning of the HPA-axis later in life, especially in males. Overall, however, common life stress events experienced in-utero, during childhood and adolescence show limited impact on the HPA-axis stress response in early adult life.

Acknowledgements

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Supplementary Data

Supplementary Table 5.1

Distribution of prenatal stress exposure category and postnatal stress trajectory

	Postnatal stress trajectory *n* (%)					
	Low	**Ascending**	**Descending**	**Medium**	**High**	**Totals**
Prenatal stress exposure						
low	191 (24.9)	237 (30.9)	72 (9.4)	203 (26.5)	63 (8.2)	766 (100)
High 18w	2 (2.5)	12 (15.0)	10 (12.5)	33 (41.3)	23 (28.7)	80 (100)
High 34w	3 (5.8)	8 (15.4)	7 (13.5)	24 (46.2)	10 (19.2)	52 (100)
High both	0 (0.0)	4 (12.1)	1 (3.0)	6 (18.2)	22 (66.7)	33 (100)
Totals	**196 (21.1)**	**261 (28.0)**	**90 (9.7)**	**266 (28.6)**	**118 (12.7)**	**931 (100)**

Supplementary Table 5.2

Univariate analysis of covariates

(a) With TSST summary measures

	TSST salivary cortisol summary measure					
Covariate	**C_{BL}** *n* = 1003 *B (95% CI)*	**C_{MAX}** *n* = 1012 *B (95% CI)*	**C_{MIN}** *n* = 1012 *B (95% CI)*	**C_{RANGE}** *n* = 1012 *B (95% CI)*	**AUC_G** *n* = 1011 *B (95% CI)*	**AUC_R** *n* = 1011 *B (95% CI)*
Categorical						
Sex (*n* = 1019)						
Male	(REF)	(REF)	(REF)	(REF)	(REF)	(REF)
Female	-2.70 (-3.90, -1.51)**	-3.66 (-5.00, -2.32)**	-1.16 (-1.66, -.65)**	-2.50 (-3.53, -1.47)**	-263 (-351, -175)**	-144 (-194, -94)**
Collection (*n* = 1019)						
Blood+Saliva	(REF)	(REF)	(REF)	(REF)	(REF)	(REF)
Saliva only	-2.29 (-3.83, -0.75)*	-1.91 (-3.64, -0.18)*	-1.22 (-1.87, -.57)**	-0.69 (-2.02, .65)	-213 (-328, -99)**	-78 (-143, -13)*
Smoker (*n* = 1013)						
No	(REF)	(REF)	(REF)	(REF)	(REF)	(REF)
Yes	-1.36 (-3.06, 0.35)	-2.27 (-4.19, -0.35)*	-0.60 (-1.32, 0.12)	-1.67 (-3.16, -0.19)*	-137 (-263, -9)*	-67 (-140, 5)
Continuous						
Income (*n* = 1013)	-0.12 (-0.66, 0.41)	0.04 (-0.56, 0.64)	0.08 (-0.15, 0.30)	-0.03 (-0.49, 0.43)	8 (-31, 48)	1 (-22, 23)
BMI (*n* = 1007)	-0.15 (-0.28, -0.03)*	-0.05 (-0.19, 0.09)	-0.07 (-0.13, -0.02)*	0.02 (-0.09, 0.13)	-6 (1256, 1707)	1 (-4, 7)

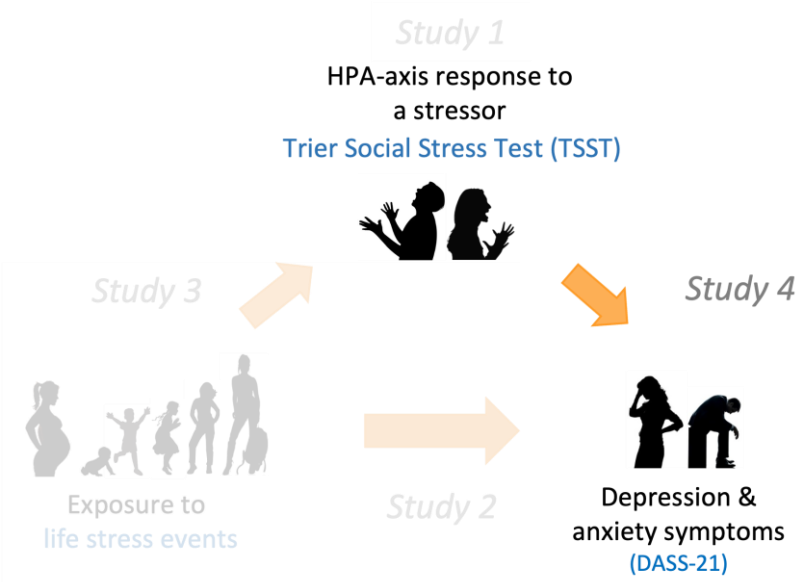
* p<0.05, **p<0.001

(b) With responder category

Covariate	NR vs R *OR (95% CI)*
Categorical	
Sex (*n* = 1019)	
Male	(REF)
Female	0.58 (0.40, 0.84)*
Collection (*n* = 1019)	
Blood+Saliva	(REF)
Saliva only	Not included
Smoker (*n* = 1013)	
No	(REF)
Yes	1.42 (0.88, 2.30)
Continuous	
Income (*n* = 1013)	0.91 (0.78, 1.07)
BMI (*n* = 1007)	0.93 (0.89, 0.98)*

* p<0.05, **p<0.001

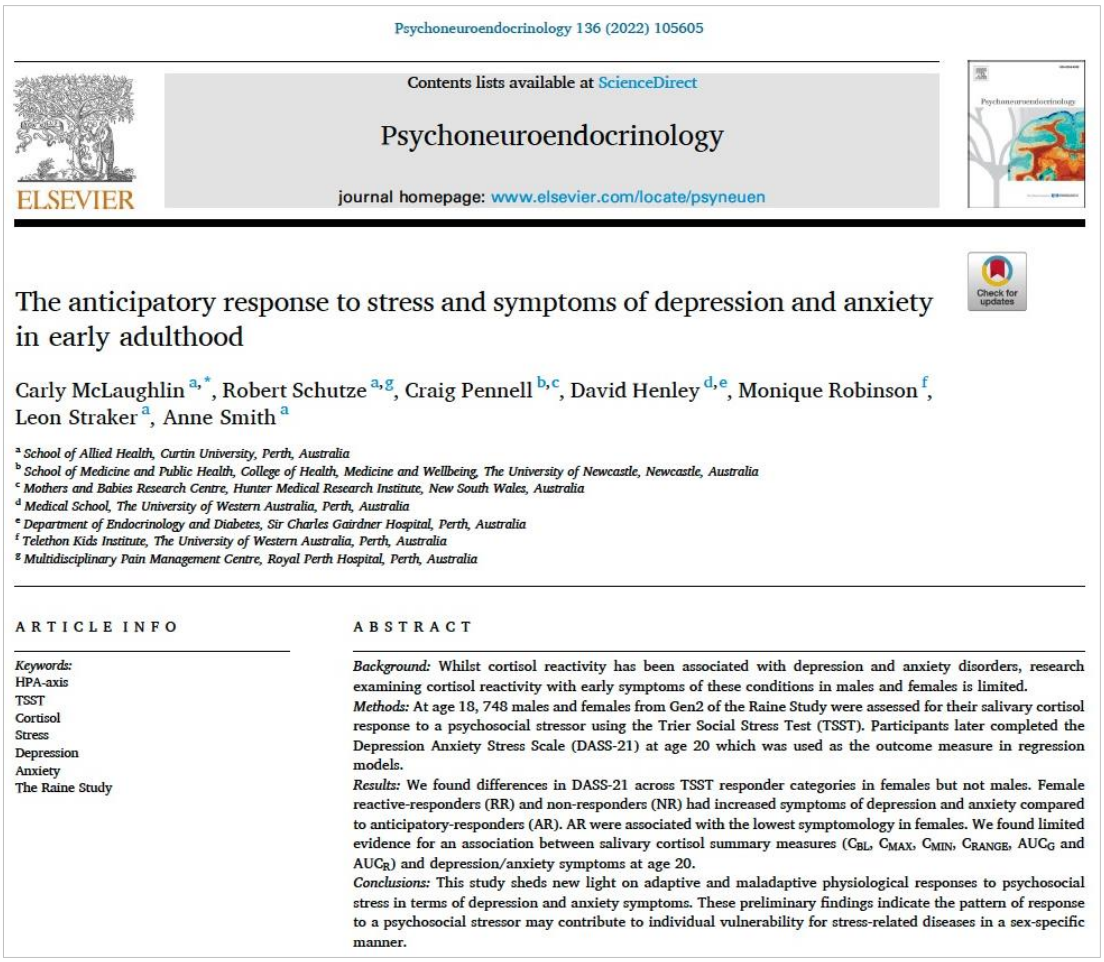
Chapter 6 Study 4: The anticipatory response to stress and symptoms of depression and anxiety in early adulthood



The graphical representation of this doctoral thesis above shows the focus of the following Chapter (Study 4). The results presented in Study 2 suggest that exposure to common early life stress events is associated with symptoms of depression and anxiety at age 20 with sex specific differences. On investigating whether functioning of the HPA-axis may be a mechanism contributing to this relationship, Study 3 found that the specific timing of early life stressors was associated with different TSST outcome measures in males and females. Building on these results from the previous chapter, Study 4 aimed to examine the final side of the triangle and the relationship between the stress response to the TSST at age 18 years and depression/anxiety symptoms at age 20 both in males and females.

Publication details

This study was published in the journal *Psychoneuroendocrinogy* and is presented here unaltered.



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The anticipatory response to stress and symptoms of depression and anxiety in early adulthood

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ABSTRACT

Background: Whilst cortisol reactivity has been associated with depression and anxiety disorders, research examining cortisol reactivity with early symptoms of these conditions in males and females is limited.
Methods: At age 18, 748 males and females from Gen2 of the Raine Study were assessed for their salivary cortisol response to a psychosocial stressor using the Trier Social Stress Test (TSST). Participants later completed the Depression Anxiety Stress Scale (DASS-21) at age 20 which was used as the outcome measure in regression models.
Results: We found differences in DASS-21 across TSST responder categories in females but not males. Female reactive-responders (RR) and non-responders (NR) had increased symptoms of depression and anxiety compared to anticipatory-responders (AR). AR were associated with the lowest symptomology in females. We found limited evidence for an association between salivary cortisol summary measures (C_{BL}, C_{MAX}, C_{MIN}, C_{RANGE}, AUC_G and AUC_R) and depression/anxiety symptoms at age 20.
Conclusions: This study sheds new light on adaptive and maladaptive physiological responses to psychosocial stress in terms of depression and anxiety symptoms. These preliminary findings indicate the pattern of response to a psychosocial stressor may contribute to individual vulnerability for stress-related diseases in a sex-specific manner.

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6.1 Introduction

Depression and anxiety disorders are common, typically arise in young adult life and are very strongly linked with exposure to stress (Heim et al., 2008; Herbison et al., 2017; Kessler et al., 2005). Up to 80% of depression in the community has been shown to occur after an adverse life event (Mazure, 1998) and both high stress exposure and depression show high comorbidity with many other chronic health problems (Hughes et al., 2017; Moussavi et al., 2007). Physiologically, the response to stress involves hormone release to assist the individual to cope with the challenging situation. The dysregulation of one of these stress response systems, the Hypothalamic Pituitary Adrenal (HPA)-axis, and resultant release of the end product cortisol, has been associated with depression and anxiety (Herbert, 2012; Zorn et al., 2017) and more generally is assumed to play an important role in the relationship between stress exposure and psychopathology (Heim et al., 2008). Here we focus on the acute reactive response to a psychosocial stressor measured via the Trier Social Stress Test (TSST) or similar laboratory model (Allen et al., 2017; Kirschbaum, Pirke, et al., 1993). These test situations effectively model a real life social-evaluative threat involving higher order neurological circuits and are very effective for eliciting a cortisol response (Dickerson & Kemeny, 2004).

An inappropriate response involving too much or too little cortisol release, may have detrimental effects in the long term and may increase vulnerability to, or occur concurrently with, the development of depression and anxiety. Previous investigations have predominantly focused on clinical populations and have produced inconsistent results with reports including both hyper-activity (Ciufolini et al., 2014) and hypo-activity (Petrowski et al., 2021) of the HPA axis in patients with these conditions (Zorn et al., 2017).

Few studies have investigated the association of reactive cortisol response with symptoms of depression and anxiety in community populations. Investigation of symptomology is important to detect smaller changes in the development of a disease and establish whether a dose-response relationship exists. One large scale study (n = 725) of an older population (55-60 years) found reduced cortisol reactivity in those with mild to severe symptoms compared to those with no symptoms of anxiety or depression (de Rooij et al., 2010). A second study of a community sample (n = 143, age 18-65, mean age 30) reported that depressive symptoms were associated with steeper reactivity and recovery slopes and anxiety symptoms were associated with flatter reactivity and

recovery slopes in those 50% of participants who mounted a response to the TSST challenge (Fiksdal et al., 2019).

A recent meta-analysis of studies examining cortisol reactivity in people with current major depressive disorder (MDD) and social anxiety disorder found that women with these conditions displayed blunted cortisol responses whilst men with these conditions showed elevated responses (Zorn et al., 2017). Therefore, there is evidence that the relationship of cortisol reactivity with depression and anxiety may differ by sex. In support of this, sex hormones modulate reactivity of the HPA axis (Juster et al., 2016; Stephens et al., 2016), sex differences are observed in all measures of the TSST (Herbison et al., 2016; Kudielka & Kirschbaum, 2005; J. J. W. Liu et al., 2017) and the prevalence of depression and anxiety symptoms and disorders varies by sex with both being notably higher in females after adolescence (Ivancic et al., 2014). Therefore, it is critical to examine these relationships separately in males and females.

It is now recognised that a participant's response to a psychosocial stressor can be grouped into categories depending on the overall shape of the cortisol response curve. 'Non-responders' do not show a noticeable reactive response to the TSST (Fiksdal et al., 2019; Herbison et al., 2016). Participants who demonstrate a cortisol response in anticipation of the TSST stressor, with an early response to the TSST or high baseline cortisol concentration, have been termed 'anticipatory-responders' and are in contrast to the 'reactive-responders' who demonstrate a reactive response only to the actual acute TSST stressor (Engert et al., 2013; Herbison et al., 2016). The pattern of cortisol responsiveness has been associated with negative health outcomes including anxiety and depression-related disorders. Non-responsiveness has been associated with panic disorder (Petrowski et al., 2010) and pre-menstrual syndrome (Huang et al., 2015) and a type of anticipatory-response has been associated with PTSD (Bremner et al., 2003). These responder-categories have not been examined with respect to symptoms of depression and anxiety and the anticipatory (high-baseline) response is rarely examined as a unique category. We have previously characterised these responses in over 800 young adults in the Raine Study, at the levels of adrenocorticotropic hormone (ACTH), plasma cortisol and salivary cortisol (Herbison et al., 2016). In related work, we have also explored the relationship between early life stress events and responses to the TSST (McLaughlin et al., 2021).

The Raine Study Gen2 cohort presents a valuable opportunity to examine the association between reactive cortisol response and symptoms of depression and anxiety due to a uniquely large sample size and prospectively collected data from a normal population up to young adulthood, when risk of mental health disorders is high. In Australia, youth (18-24 years old) have the highest prevalence of mental illness of any age group (Ivancic et al., 2014).

We aimed to examine whether the salivary cortisol response to the TSST at age 18 was associated with symptoms of depression, anxiety and stress at age 20 in males and females by investigating both TSST standard summary measures and responder category. We hypothesised firstly, that lower TSST summary measures would be associated with higher symptoms of 1] depression and 2] anxiety at age 20. Secondly, that TSST non responders would be associated with higher symptoms of 1] depression and 2] anxiety at age 20 compared to responders and finally, that these associations would be stronger in females.

6.2 Material and Methods

6.2.1 Participants

Participants were from the Raine Study, a prospective cohort study of 2868 live births [Gen2] with longitudinal follow-up from pregnancy to adulthood (Newnham et al., 1993). This study used data from the Trier Social Stress Test (TSST) conducted at age 18 years and mental health at the age 20 follow-up. Participant numbers have decreased over time from 2819 eligible/2446 participating at age 1 to 2313 eligible/1462 participating at age 20. However, this cohort remains broadly representative of the Western Australian population without significant perinatal selection bias and more detailed profiles and analyses are available elsewhere (Straker et al., 2017; White et al., 2017). All procedures were carried out with participant informed consent. Ethics approval was obtained from King Edward Memorial Hospital, Princess Margaret Hospital and the University of Western Australia.

6.2.2 Trier Social Stress Test

Raine Study participants were invited to take part in the TSST challenge at age 18 as part of the ‘Challenge Me’ study. Cohort participants were informed that the study

would 'measure what happens to your stress hormones when we give you a challenge that will make you feel under stress'. Cohort participants knew they would be subject to a 'challenge' that would create a stress response but did not know the details. The information sheet also stated researchers would like to take blood and saliva samples. Recruitment appointments were made by telephone and these conversations stated the option of saliva-only if participants chose not to have blood taken. Detailed information of this study and the associations of plasma ACTH/plasma cortisol and salivary cortisol with sex, BMI, smoking and oral contraceptives can be found elsewhere (Herbison et al., 2016). Briefly, 1137 participants completed the TSST which was conducted in the afternoon between 1pm and 4pm to minimise the effect of diurnal variation on cortisol and subjects were instructed to refrain from eating or drinking for at least 1h before their appointment. After arrival and with participant agreement, a cannula was inserted by an anaesthetist and during a 45-minute rest period, a brief questionnaire was completed to collect information regarding illnesses, medication use, recent physical activity, consumption of food/drink, smoking habits, timing and regularity of menstrual cycles and OC use. Height and weight were measured for calculation of BMI. Blood samples were taken at 0 min (just before the test) and at 15 min (just after the test) and then at 25, 35, 45, 60, 75 and 105 minutes. Saliva samples were collected at 0, 15, 35 and 105min with the exception of people declining cannulation, in which case saliva samples were collected at all 8 time points. As per established protocols, the test consisted of a 5 minute preparation time before a free speech interview (5 min) and an arithmetic challenge (5 min) in front of a non-responsive panel of 3-4 formally dressed men and women (Kirschbaum, Pirke, et al., 1993; Kudielka et al., 2007). A dummy camera and mock audio equipment were set up as if to record the tasks and participants were debriefed afterwards.

A number of participants (n = 118) were excluded from analyses due to fainting, pregnancy, lactation, severe menstrual pain, type 1 diabetes, unusable samples, failed cannulation, use of steroids/neuroactive medications/antidepressants and other medications known to affect the HPA-axis. Of 1019 participants eligible for analysis, 830 had both blood and saliva collected and 189 had saliva-only collected. In this study we report on salivary cortisol as this is the most commonly used measure in the literature (Achenbach, 1991; Hellhammer et al., 2009; Levine et al., 2007).

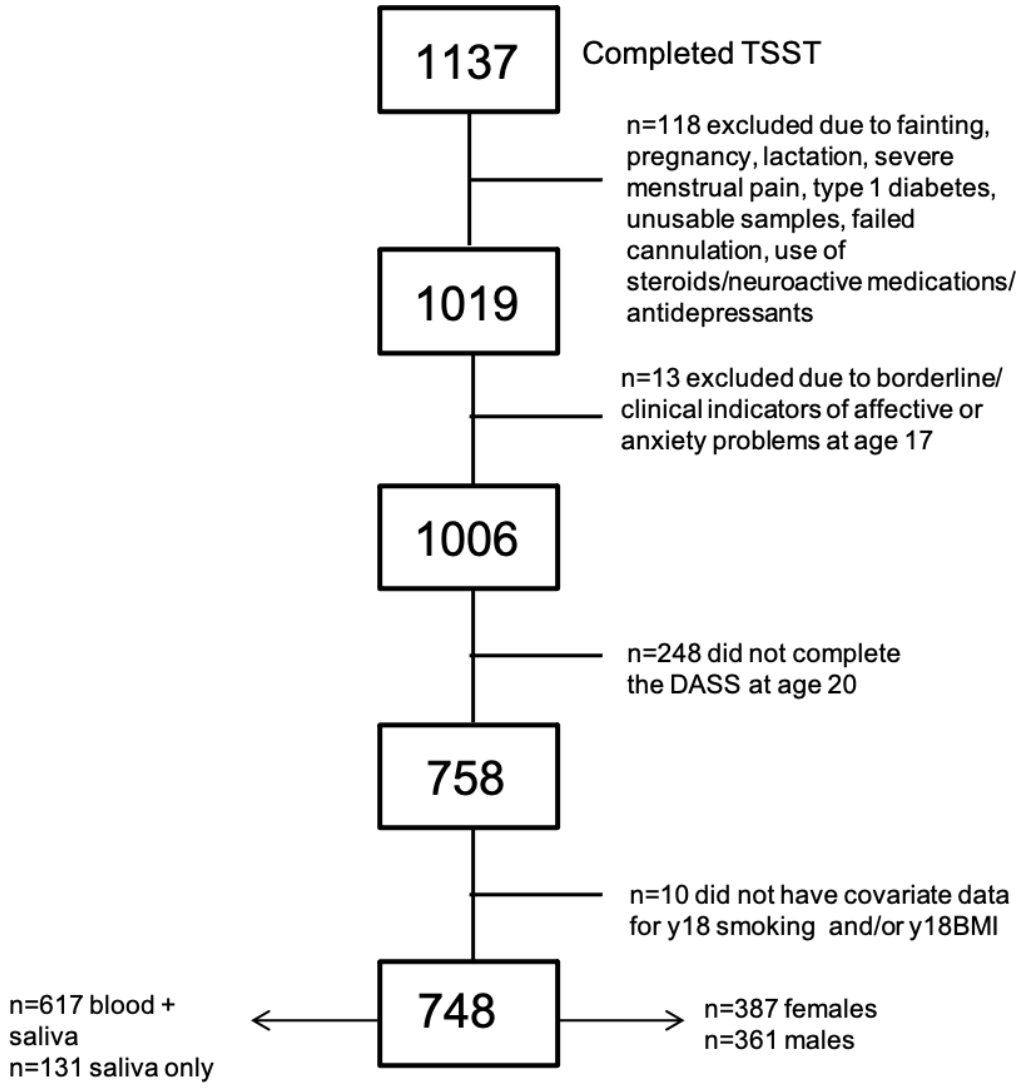
6.2.3 Measurement of Salivary Cortisol

Saliva was obtained using Salivette collection devices (Sarstedt, Germany) and kept on ice until processed. Samples were spun, aliquoted and frozen at -80C until assayed. Free salivary cortisol was quantified using the GammaCoat TM 125I immunoradiometric assay (RIA) kit (DiaSorin, Stillwater, MN), as per the manufacturer's instructions. Samples were measured in duplicate against an appropriate standard curve and repeated as required. The intra- and inter-assay variability was < 10%. Salivary cortisol was measured in nanomoles per litre (nmol/L) = nM.

6.2.4 Identification and exclusion of participants with clinical anxiety and/or depression

Participants taking medication for depression and/or anxiety at the time of the TSST were eliminated from analyses, as detailed above. In the absence of depression and anxiety evaluation at the time of the TSST, we identified participants with borderline/clinical anxiety and or depression using the Youth Self Report form (YSR), assessed at the age 17 cohort follow-up (Achenbach, 1991). The YSR/11-18 is a 118-item widely used child-reported measure that assesses problem behaviours along the broadband scales of Internalising and Externalising behaviours. It also scores eight empirically based syndromes and DSM-oriented scales (from the Diagnostic and Statistical Manual of Mental Disorders). We used the DSM-IV scales, incorporating the borderline range, for affective problems (items rated as consistent with Dysthymia and Major Depressive Disorder; scoring 8 or above in boys and 10 or above in girls) and anxiety problems (items rated as consistent with Generalised Anxiety Disorder, Separation Anxiety and Specific Phobia; scoring 6 or above in boys and 7 or above in girls). The YSR/11-18 has good test-retest reliability and internal consistency (Achenbach & Dumenci, 2001; Bordin et al., 2013). We identified ten and three individuals within the borderline-clinical range for affective problems and anxiety problems respectively. These 13 participants were removed from analyses. A flow chart for the available data is shown in Figure 6.1.

Figure 6.1
Flow chart for available data



6.2.5 Measurement of Depression and Anxiety Symptoms at age 20

Symptoms of depression and anxiety at age 20 were measured using the Depression, Anxiety and Stress Scale (DASS-21), a shortened version of the original DASS-42. The DASS-21 is a 21 item self-report questionnaire designed to measure the emotional states of depression, anxiety and stress over the past week, using a 4-point Likert scale for each item ranging from 0 to 3 (Lovibond & Lovibond, 1995). Final scores have been doubled to equate to the DASS-42 therefore the total DASS scores range from 0-126 and the subscales range from 0-42.

We report on the total DASS score (DASS-Total) and the subscales of depression (DASS-Dep) and anxiety (DASS-Anx) and stress (DASS-Stress) to further investigate whether HPA-reactivity at age 18 is associated with symptoms of depression, anxiety and stress. Cronbach's alpha for the DASS Dep, Anx and Str scales were 0.88, 0.74 and 0.85 respectively.

6.2.6 Covariates

Smoking and BMI - At the time of the TSST, participants reported whether they were a smoker or non-smoker and height and weight were measured for the calculation of BMI (used in continuous form). For BMI, where possible missing values were imputed from BMI recorded at age 17.

Saliva-only – a total of 189 participants chose to receive saliva-only collection. This was used as a dichotomous variable: blood and saliva =0, saliva only = 1. Univariate analyses of the association of covariates with DASS score and TSST responder category are shown in the Supplementary Data tables.

The menstrual cycle phase in females was tested in univariate analyses but as there was no evidence for differences in salivary cortisol between phases (Herbison et al., 2016) and no association with dependent or independent variables in this study, and thus no potential for confounding, we did not include this in our models.

6.2.7 Statistical Analyses

TSST responder category as a three-category variable and TSST summary measures as continuous variables were considered as independent variables in separate linear regression models with the outcome measures of the DASS-21 (total and subscales).

TSST responder categories have previously been defined and described (Herbison et al., 2016). Briefly, responders were defined as either reactive-responders (RR, where an increase in plasma cortisol was observed immediately after the TSST to a noticeable peak before decreasing back to baseline) or anticipatory-responders (AR, where initial cortisol levels were high, there was the absence of a clear upward response and plasma cortisol levels fell over the first 60 min of the study by >20% indicating the cortisol response commenced before the TSST). Participants who showed no clear plasma cortisol peak and little change in cortisol over the study period were termed non-responders (NR). See

Figure 6.2. The summary measures of salivary cortisol over the TSST included concentration at baseline (C_{BL}), Concentration at peak (C_{MAX}), concentration at minimum (C_{MIN}), concentration range (C_{RANGE}), area under the curve with respect to ground (AUC_G) and area under the curve with respect to range (AUC_R). We have used AUC_R in place of area under the curve with respect to increase (AUC_I) as it is more effective in detecting changes when a proportion of participants have a high C_{BL}. AUC_R is not subject to issues of negative area, unlike AUC_I.

We reported separately by sex based on prior evidence for sex differences in HPA reactivity (Herbison et al., 2016; Juster et al., 2016; Kirschbaum, Wust, & Hellhammer, 1992), mental health outcomes (Ivancic et al., 2014) and the associations between them (Zorn et al., 2017). For all models sex-specific results were estimated by inclusion of an interaction term between sex and the TSST measure under investigation. All regression models were adjusted for smoking, BMI and whether both blood and saliva were collected vs saliva-only collected to eliminate these as a potential source of confounding. Bias-corrected and accelerated 95% CI's for the cortisol summary measures were generated using 1000 bootstrap replications to account for the deviation from normality of the distribution of DASS-21 outcome measures. For responder category, we report B coefficients and estimated marginal means. For the summary measures, to facilitate interpretation as the range and units differ, we multiplied the B coefficient by the standard deviation (X-standardisation) and report expected change in DASS for a 1 standard deviation increase in cortisol summary measure. All models were checked for the absence of influential outliers and linearity of association. Statistical analyses were performed using IBM SPSS Statistics for Windows, version 25 (IBM Corp., Armonk, N.Y., USA) and Stata/IC 15.0 for Windows (StatCorp LLC, College Station TX USA).

6.3 Results

6.3.1 Sample Characteristics

Participant characteristics of this sample including TSST measures, covariates and outcome measures are presented in Table 6.1, separated by sex. The mean age of study participants was 18.3 years (SD 0.3 years). Participants excluded from analyses had a slightly higher BMI, were more likely to be a smoker and more likely to be male than our final analytical sample (Supplementary Data tables). DASS profiles of severity in this

sample, univariate association of covariates with the DASS or TSST responder category, differences in summary measures between the responder categories and differences in AR profiles by collection method are available in the Supplementary Data. Briefly, female sex, saliva-only collection and smoking were associated with higher DASS scores; B(95% CI) Female sex 5.84 (3.26, 8.41) $p<0.001$; Saliva-only 4.23 (0.82, 7.64) $p<0.05$; Smoker 8.84 (4.67, 13.00) $p<0.001$. There were no differences in the distribution of responder categories in participants choosing saliva-only vs blood collection. All TSST summary measures except C_{MIN} differed across responder categories. AUC_G and AUC_R were similar between ARs and RRs however male ARs had higher C_{MAX} and C_{RANGE} than male RRs. ARs who had blood taken had higher salivary cortisol profiles than ARs who chose saliva only. See Supplementary Data.

Table 6.1
Participant characteristics

	Total	Females	Males
TSST			
	mean (SD)	*mean (SD)*	*mean (SD)*
Summary measures			
C_{BL} (nM)	13.5 (9.6)	12.0 (8.9)	15.1 (10.1)
C_{MAX} (nM)	17.5 (10.8)	15.8 (10.2)	19.4 (11.1)
C_{MIN} (nM)	8.4 (4.1)	7.8 (3.7)	9.0 (4.5)
C_{RANGE} (nM)	9.1 (8.3)	8.0 (8.0)	10.4 (8.5)
AUC_G	1329 (708)	1201 (644)	1465 (747)
AUC_R	447 (397)	379 (370)	519 (412)
	n (%)	*n (%)*	*n (%)*
Responder Category			
Anticipatory-responders	189 (25.6)	100/381 (26.2)	89/356 (25.0)
Reactive-responders	425 (57.7)	200/381 (52.5)	225/356 (63.2)
Non-responders	123 (16.7)	81/381 (21.3)	42/356 (11.8)
Covariates			
	mean (SD)	*mean (SD)*	*mean (SD)*
BMI *(kg/m2)*	23.5 (4.7)	23.7 (5.3)	23.3 (4.0)
	n (%)	*n (%)*	*n (%)*
Smoker	80 (10.7)	35 (9.0)	45 (12.5)
Saliva-only	131 (17.5)	84 (21.7)	47 (13.0)
Outcomes			
	mean (SD)	*mean (SD)*	*mean (SD)*
DASS-Total* *(0-126)*	20.0 (18.1)	22.8 (19.4)	17.0 (16.1)
DASS-Dep *(0-42)*	6.5 (7.4)	7.3 (7.8)	5.6 (6.8)
DASS-Anx *(0-42)*	4.8 (5.3)	5.5 (5.9)	4.1 (4.5)
DASS-Stress *(0-42)*	8.7 (7.7)	10.0 (7.9)	7.3 (7.1)

6.3.2 Responder category and DASS

The three different categories for responses to the TSST for males and females are shown in Figure 6.2. The associations of TSST responder category with total DASS, DASS-Dep, DASS-Anx and DASS-Stress subscales are shown in Table 6.2. We found evidence for differences in DASS-Total and DASS-Dep scores across TSST responder categories in females but not in males with results as follows: [DASS-Total testing for differences in females (F 3.80 p=0.02) and males (F 0.83 p=0.44)]; [DASS-Dep testing for differences in females (F 4.58 p=0.01) and males (F 1.44 p=0.24)]; [DASS-Anx testing for differences in females (F 2.84 p=0.06) and males (F 0.18 p=0.84)]; [DASS-Stress testing for differences in females (F 1.83 p=0.16) and males (F 1.31 p=0.27)]. In a formal test of moderation by sex, there was evidence for the presence of an interaction between sex and responder category in DASS-Dep only. See Table 6.2 footnotes. In females, reactive-responders were estimated to score 4.26 points higher (95% CI 0.04, 8.49, p=0.05); and non-responders 7.02 points higher (95% CI 1.87, 12.16, p=0.01) compared to anticipatory-responders on the DASS-Total score. When we examined the subscales, this association was most notable with the DASS-Dep subscale where reactive-responders were estimated to score 2.06 points higher (0.31, 3.82) p=0.02; and non-responders 3.13 points higher (1.00, 5.26) p=0.004 compared to anticipatory-responders, and to a lesser degree with the DASS-Anxiety subscale where reactive-responders scored 1.12 points higher (-1.12, 2.37) p=0.08; and non-responders scored 1.77 points higher (0.26, 3.30) p=0.02, compared to anticipatory-responders. These relationships are shown graphically in Figure 6.3. In other words, an anticipatory response in females is associated with a lower DASS-total, DASS-Dep and DASS-Anx score at age 20 than a reactive or non-response. We found no association of TSST responder category with the DASS in males.

6.3.3 Salivary cortisol summary measures and DASS

In formal tests of moderation by sex, there was no evidence for the presence of an interaction between sex and summary measures. See Supplementary Data for details. The association of the TSST summary measures of salivary cortisol with the DASS in males and females is presented in Table 6.3. To facilitate interpretation, these data show the expected change in DASS for a 1 standard deviation increase in cortisol summary measure. We found some evidence for a small negative association between salivary cortisol C_{BL}, C_{MIN}, AUC_G and DASS-Anx at age 20 in females only: with 1 SD increase in C_{BL},

DASS is lower by 0.53(-1.07, -0.09) p=0.02; C_{MIN}, DASS is lower by 0.44(-0.88, 0.011) p=0.06; AUC_G, DASS is lower by 0.644(-1.29, 0.00) p=0.06.

Figure 6.2

Salivary cortisol response to the TSST by responder category in males and females

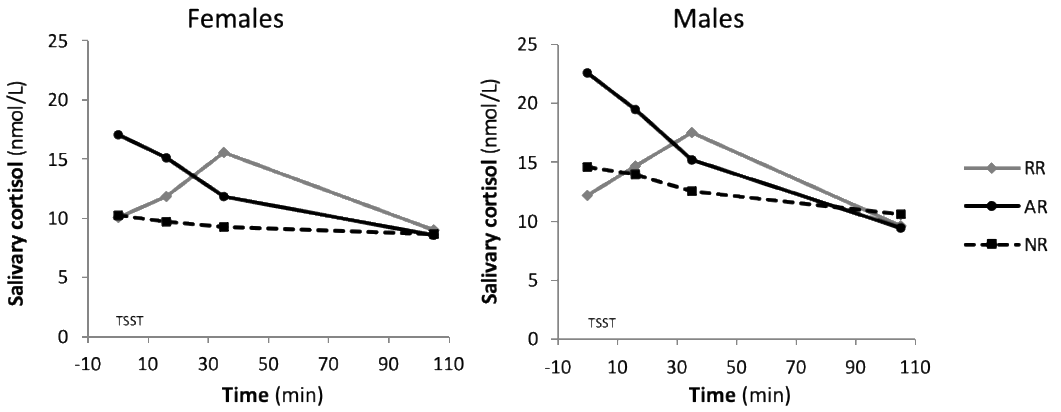
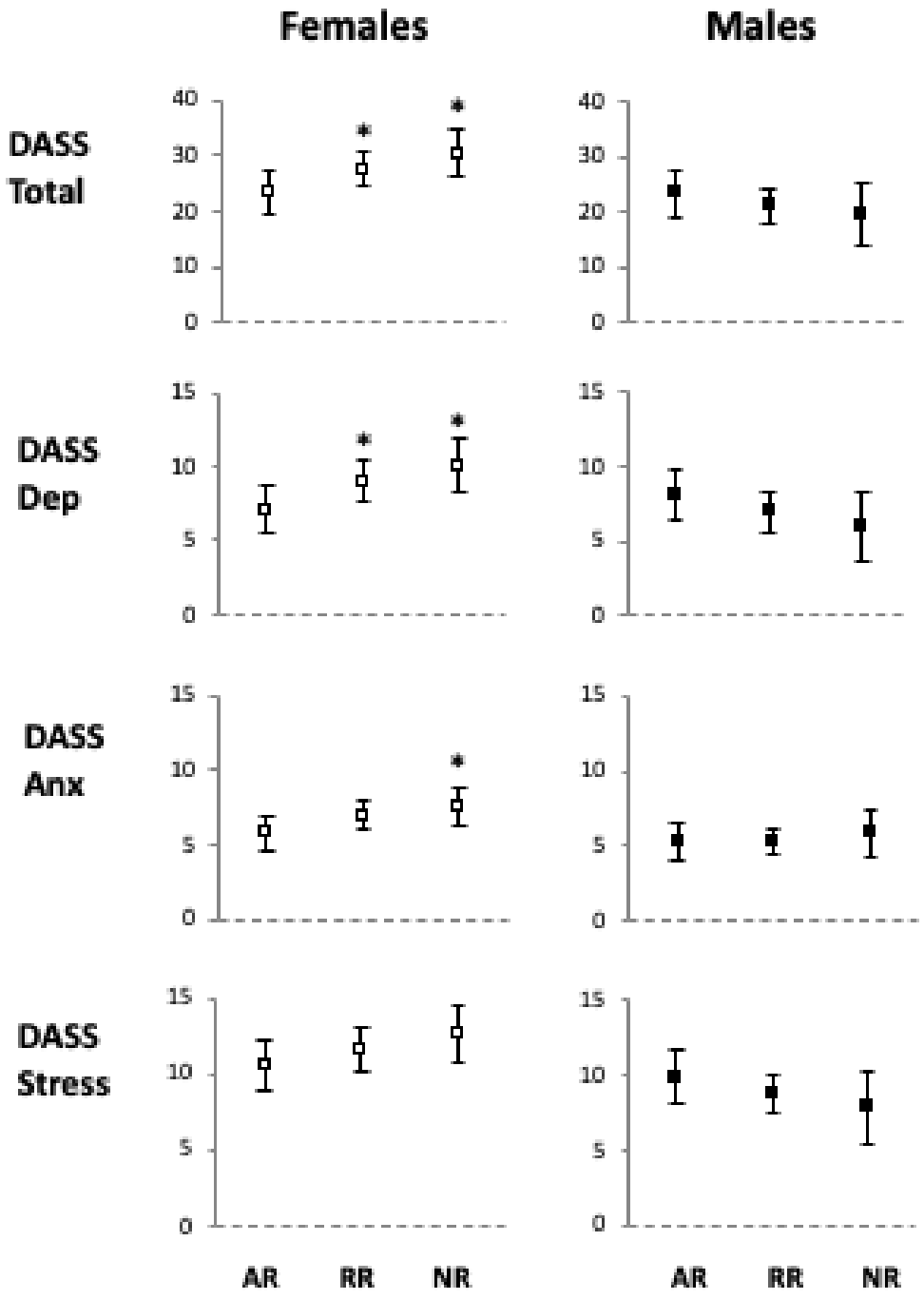


Figure 6.3

Estimated marginal means of DASS Total and subscales by responder category in males and females



* Indicates p<0.05 compared to AR.

Table 6.2

TSST Responder category with DASS at age 20 in males and females

Responder category	Total DASS			DASS-dep			DASS-anx			DASS-str		
	EMM	**B (95% CI)**	***p***	**EMM**	**B (95% CI)**	***p***	**EMM**	**B (95% CI)**	***p***	**EMM**	**B (95% CI)**	***p***
Females (*n* = 381)												
Anticipatory (AR) (*n* = 100)	23.26	REF		6.97	REF		5.80	REF		10.50	REF	
Reactive (RR) (*n* = 200)	27.53	4.26 (0.04, 8.49)	0.05	9.03	2.06 (0.31, 3.82)	0.02	6.92	1.12 (-0.12, 2.37)	0.08	11.58	1.08 (-0.71, 2.86)	0.24
Non responders (NR) (*n* = 81)	30.28	7.02 (1.87, 12.16)	0.008	10.10	3.13 (1.00, 5.26)	0.004	7.58	1.77 (0.26, 3.30)	0.02	12.61	2.11 (-0.06, 4.28)	0.06
Males (*n* = 356**)**												
Anticipatory (AR) (*n* = 89)	23.37	REF		8.15	REF		5.31	REF		9.91	REF	
Reactive (RR) (*n* = 225)	20.98	-2.35 (-6.63, 1.92)	0.28	6.93	-1.22 (-3.01, 0.56)	0.18	5.31	-0.003 (-1.27, 1.27)	0.99	8.75	-1.16 (-2.98, 0.66)	0.21
Non responders (NR) (*n* = 42)	19.70	-3.56 (-9.97, 2.86)	0.28	6.05	-2.10 (-4.76, 0.56)	0.12	5.82	0.51 (-1.39, 2.40)	0.60	7.84	-2.07 (-4.78, 0.64)	0.13

Estimated marginal means (EMM) shown for Total DASS and subscales; DASS-Depression; DASS-Anxiety and DASS-Stress. Final models adjusted for BMI, Smoking status and blood+saliva vs saliva-only collection. Results for responder category x sex interaction for DASS-Total F=1.39 p=0.24, for DASS-Dep F=4.79 p=0.009, for DASS-Anx F=0.65 p=0.52 and for DASS-Str F=2.48 p=0.08. There were no differences between RR and NR (See Supplementary Data).

Table 6.3

Change in DASS with 1 SD change in cortisol summary measure in males and females

Summary Measure	Total DASS		DASS-dep		DASS-anx		DASS-str	
	B (95% CI)	*p*	B (95% CI)	*p*	B (95% CI)	*p*	B (95% CI)	*p*
Females (*n* = 387)								
C_{BL}	-0.80 (-2.83, 0.60)	0.36	-0.14 (-0.89, 0.49)	0.70	-0.52 (-1.06, -0.11)	0.02	-0.17 (-0.97, 0.50)	0.67
C_{MAX}	-0.89 (-2.88, 0.74)	0.28	-0.26 (-0.97, 0.37)	0.46	-0.42 (-0.91, 0.02)	0.08	-0.21 (-1.08, 0.53)	0.58
C_{MIN}	-0.32 (-2.16, 1.56)	0.71	-0.088 (-0.78, 0.92)	0.82	-0.44 (-0.89, 0.01)	0.06	0.03 (-0.71, 0.79)	0.92
C_{RANGE}	-0.99 (-2.74, 0.38)	0.21	-0.37 (-0.98, 0.15)	0.24	-0.35 (-0.80, 0.09)	0.14	-0.28 (-1.10, 0.41)	0.45
AUC_G	-0.79 (-2.35, 0.91)	0.32	-0.13 (-0.86, 0.52)	0.69	-0.45 (-0.95, 0.05)	0.06	-0.20 (-0.91, 0.47)	0.56
AUC_R	-1.06 (-2.64, 0.51)	0.18	-0.33 (-0.96, 0.27)	0.28	-0.36 (-0.82, 0.10)	0.16	-0.37 (-1.09, 0.36)	0.31
Males (*n* = 361)								
C_{BL}	0.54 (-0.85, 2.12)	0.51	0.41 (-0.33, 1.29)	0.25	0.03 (-0.36, 0.41)	0.88	0.11 (-0.50, 0.76)	0.79
C_{MAX}	0.47 (-1.12, 2.32)	0.55	0.41 (-0.31, 1.21)	0.31	0.03 (-0.39, 0.54)	0.92	-0.03 (-0.55, 0.78)	0.93
C_{MIN}	1.07 (-0.43, 3.05)	0.24	0.57 (-0.12, 1.57)	0.18	0.33 (-0.11, 0.92)	0.17	0.17 (-0.49, 1.06)	0.68
C_{RANGE}	0.04 (-1.33, 1.76)	0.94	0.24 (-0.43, 1.00)	0.52	-0.14 (-0.51, 0.28)	0.47	-0.05 (-0.68, 0.67)	0.89
AUC_G	0.44 (-0.97, 2.44)	0.62	0.40 (-0.31, 1.32)	0.31	0.10 (-0.30, 0.52)	0.65	-0.05 (-0.65, 0.65)	0.85
AUC_R	-0.27 (-1.61, 1.31)	0.72	0.15 (-0.47, 1.00)	0.65	-0.16 (-0.54, 0.24)	0.38	-0.26 (-0.82, 0.34)	0.42

Final models adjusted for BMI, Smoking status and blood+saliva vs saliva-only collection. B represents the expected change in DASS for a 1 standard deviation increase in the cortisol summary measure. 95% CIs obtained using bootstrapping. Final n females=390. Final n males=365. Results for summary measures x sex interaction for DASS-Total and subscales DASS-Dep, DASS-Anx and DASS-Stress are shown in Supplementary Data.

6.4 Discussion

We examined the relationship between the cortisol response to a psychosocial challenge at age 18 and symptoms of depression and anxiety two years later. The novelty of this study lies in the examination of responder categories, the profile of cortisol response to a psychosocial stress. Anticipatory-responders have an early cortisol peak before the TSST, reactive-responders show a cortisol peak after the TSST and non-responders do not show a definitive cortisol response. Using analyses of responder categories, we found that RR and NR had higher DASS-Total, DASS-Dep and DASS-Anx scores than AR, but only in females. Using TSST summary measures we found some evidence for reduced salivary cortisol C_{BL}, C_{MIN} and AUC_G with higher DASS-Anx with a small effect size, but once again, only in females. These results suggest that TSST responder category may be associated with varying degrees of predisposition to depression/anxiety symptomatology.

In females, we found TSST non-responders and reactive-responders showed higher total depression, anxiety and stress symptoms compared to anticipatory-responders. In terms of the size of these differences, compared to AR, we observed a 4.3-point higher DASS-Total score in RR and 7.0-point higher DASS-Total score in NR (where higher scores represent more or worse symptoms). Although modest, these may represent important differences as they are broadly comparable with the differences in DASS-Total scores between the known risk factors, sex (5.8) and smoking status (8.8), that were observed in this study. These positive shifts in DASS scores may see at-risk females with subclinical scores move closer to or over a clinical threshold and normal asymptomatic females move into mild symptoms of depression and anxiety. Furthermore, these preliminary findings help shed light on determining adaptive versus maladaptive physiological responses to stress.

In females, non-responders showed higher symptoms of depression compared to anticipatory-responders. These results are reinforced by the clinical literature where a meta-analysis of 7 studies of MDD patients vs controls found evidence that depression was associated with blunted stress reactivity and that this was more pronounced with age and depression severity (Burke et al., 2005). A later meta-analysis of 14 studies using standardised AUC methodology showed current and remitted MDD to be predominantly associated with a blunted cortisol response in females compared to controls (Zorn et al.,

2017). Our results also align with those of de Rooij et al in their population-based study of older (age 55-60) men and women. They report reduced HPA-axis responses to a psychosocial stressor in participants with depression symptoms, despite their perceived stress over the test being high (de Rooij et al., 2010).

In contrast, Fiksdal et al (2019) report steeper cortisol reactivity and recovery slopes (ie a more vigorous response, akin to a reactive-responder) with symptoms of depression. These differences may be due to the fact that the authors' analyses examined 'responders' only (removing NRs) (Fiksdal et al., 2019). In summary, our results indicate that in females, TSST non-responsiveness (20% of our sample) may be a risk phenotype for symptoms of depression.

In females, we also found that non-responders displayed increased anxiety symptoms compared to anticipatory-responders. This finding is supported by the clinical literature showing reduced or blunted cortisol reactivity to a psychosocial stressor in anxiety disorders (Petrowski et al., 2021; Zorn et al., 2017), although comorbidity of up to 70% has been described in patients with depression and anxiety (Cummings et al., 2014). In terms of population studies, these results are also consistent with the work of Fiksdal et al (Fiksdal et al., 2019) reporting flatter reactivity and recovery slopes with symptoms of anxiety in their sample with a mean age of 30. Although Fiksdal et al (2019) did not report statistical significance at $p<0.05$, their data show weak evidence for moderation by sex ($p=0.07$), which is comparable to our findings of differences across responder categories in females. Our results are also consistent with those of de Rooij et al (2010) who found cortisol responses decreased with increasing symptoms of anxiety in older adults (age 55-60), although they did not find evidence that sex moderated this association.

It is of note that, in this study, NR did not display increased symptoms of depression and anxiety compared to RR. This was unexpected and may be due to the delineation of the separate anticipatory category from standard responders.

We also found weak evidence that higher salivary cortisol summary measures were associated with less anxiety symptoms. However, a 1 SD change in salivary cortisol C_{BL}, C_{MIN} and AUC_G was associated with very small reductions in DASS that are unlikely to be clinically relevant. Nonetheless, our results indicate that in females, non-responsiveness may also be a risk phenotype for symptoms of anxiety.

In contrast, we found that female ARs had reduced symptoms of depression, anxiety and stress in comparison to RR and NR, leading us to question whether the anticipatory response may be protective in the short term. We found no differences in AUC between ARs and RRs during the time of the TSST, however, we acknowledge that in ARs, the rise in cortisol occurred prior to the TSST, likely resulting in higher global exposure to cortisol compared to RR participants. Male ARs displayed higher cortisol peak and range compared to RRs, supporting this notion. There is also evidence that those who mount an early response resolve their response (return to baseline) earlier (Juster et al., 2012).

Whilst it could be assumed that anticipatory-responders, with a more vigilant HPA response, are more prone to anxiety, this is not supported by these preliminary results. Although we note that the DASS-anxiety scale detects more severe, acute forms of anxiety over the past week and other instruments may be better placed to assess more generalised behavioural manifestations of anxiety. It's possible this response may be beneficial in evolutionary terms, representing increased vigilance to protect from harm in the short to medium term. Specifically, an adaptive calibration allowing preparations in cognitive and behavioral strategies which ensure survival in a changing or potentially challenging environment (Karatsoreos & McEwen, 2013).

In sports medicine, an anticipatory rise in cortisol has been identified prior to competition and is thought to exert a beneficial priming effect to prepare participants for the psychological and physical challenge ahead (van Paridon et al., 2017). Together these results suggest that, in females, the anticipatory response to a psychosocial stressor may be adaptive and protective against the development of depression and anxiety symptoms.

We have previously characterised a distinctive high baseline cortisol response in participants, who appear to have mounted an HPA-axis response in anticipation of an unknown, potentially stressful event and have also called this group 'Anticipatory responders' (Herbison et al., 2016). This pattern has also been termed 'high baseliners' (Dalile et al., 2021). However, determination of a proper baseline for anticipatory stress-sensitive participants is problematic. Engert et al (2013) describe a revised protocol to better differentiate the anticipatory response to the TSST (after initial briefing) from the reactive response itself (during the interview/arithmetic challenge) (Engert et al., 2013), but in the current study, the source and timing of the anticipatory response remain

undefined as the participants already had elevated cortisol at the time of first measurement. Indeed, the anticipation of either the stress challenge or potential venesection may have acted as the stressor. In their study Engert et al reported that ARs had an elevated cortisol baseline even after 30 minutes of rest before saliva sampling and called for future studies to investigate whether an elevated TSST baseline is a consistent finding with ARs (Engert et al., 2013). It is interesting to note a relative absence of this AR pattern in the TSST literature. Peripubertal adaptations likely contribute to a protracted period of heightened social stress sensitivity and reactivity (Miller & Prinstein, 2019). The AR may be predominant in adolescence/youth and there is some evidence of a higher anticipatory response in adolescents (13-20 years) (Evans et al., 2013). However, high baseline samples (such as those seen with AR) may be eliminated from results (Evans et al., 2013; Fiksdal et al., 2019), may be unreported and diluted by other results or may be classified as a NR (Dalile et al., 2021). Our rationale for classifying ARs was the absence of a clear response coupled with cortisol levels consistently falling over 20% in the first hour, substantially greater than the ~7% per hour decrease expected in the afternoon due to circadian rhythm (Herbison et al., 2016). It is possible that the high levels of AR in this study are due to the nature of the recruitment process in which 18-year-old participants were informed they would be given a challenge in order to measure stress hormones and they may feel under stress for a short period of time. They were told that researchers would like to collect blood and saliva but were also given the option of saliva-only.

Dalile et al (2021) have suggested that 'high baseliners', such as the anticipatory-responders here, experienced a vasovagal reaction to cannulation (Dalile et al., 2021). However, in our study we also observed this response in individuals who had saliva-only taken and no venepuncture, and distribution of the responder categories did not differ between collection methods, suggesting the AR pattern was not dependent on cannulation in this sample. Participant choice to have saliva-only taken, was associated with an increased DASS score so it is possible that fear of venepuncture is a marker for a depressive/anxious phenotype. However, choice of saliva-only was not associated with increases in the numbers of ARs. Moreover, medical staff monitored participants throughout and those who experienced a vasovagal reaction (including fainting, feeling faint, dizzy, cold, clammy, sick or looking pale $n = 15$) were excluded from further analyses (Herbison et al., 2016). These numbers are comparable with rates of vasovagal reactions

among phlebotomy patients (Deacon & Abramowitz, 2006), whereas the AR prevalence was 25%. Therefore, we feel the anticipatory response in this study is unlikely to be due to a vasovagal reaction (Dalile et al., 2021).

The anticipatory response may be an interim phenotype that changes over time and longitudinal TSSTs would be required to evaluate this. Currently, our understanding of the anticipatory dynamics of the stress response is limited. However, our results indicate that differences in stress regulation may occur before the stressful encounter and high baseline readings may indicate an anticipatory response to stress.

We can speculate that the mechanism by which depression and anxiety varies with responder category likely involves varying exposure to allostatic load (wear and tear) over time (Guidi et al., 2021). Responder categories may also vary in their psychological thinking style and behavioural coping responses (eg avoidant/dissociative), habituation to the TSST (with successive TSSTs: decline, sensitization or no change (Wust et al., 2005)), exposure to early life trauma/adversity (McLaughlin et al., 2021) and other stress sensitive parameters.

This study identified evidence that the association between cortisol reactivity and symptoms of depression was modified by sex. This is supported by others in clinical populations (Zorn et al., 2017) and non-clinical populations (Powers et al., 2016). These findings are perhaps not surprising given that males and females respond differently to the TSST (Herbison et al., 2016; Kudielka & Kirschbaum, 2005; J. J. W. Liu et al., 2017), sex hormones modulate reactivity of the HPA axis (Juster et al., 2016; Stephens et al., 2016) and the prevalence of adult depression and anxiety symptoms and disorders varies by sex.

Notably, in this sample we found no associations in males between stress response and symptoms of depression/anxiety. Exposure to stress is likely to manifest differently in males with more externalising symptoms, risk taking behaviour and substance use (Martel, 2013). There is a potential benefit for males and females to develop different physiological and behavioural responses to stress exposure, given their distinctive roles from an evolutionary point of view in hunter-gatherer communities (Glover & Hill, 2012).

In the current study, examination of responder category was more informative than other summary measures for identifying associations between TSST response and symptoms of depression/anxiety. In this way, reducing the ‘dimensionality’ of the data

down from six different summary measures to one categorical variable representing groups of individuals with similar response profiles is more easily interpretable yet discriminative of differences in symptomatology. The lack of associations of TSST summary measures with DASS is notable and may indicate that the background variability is too great for detection of the same consistent change in single measures in different individuals. Analyses using responder category may prove to be a useful addition to future studies examining the relationship between stress response and mental health.

This study has a number of limitations. We had higher numbers of males missing or excluded from analyses and due to recent calls to abandon reliance on strict significance thresholds (Wasserstein et al., 2019), we did not adjust for multiple tests. Therefore, in the context of null hypothesis testing, there is a possibility some of the findings may be spurious. Investigations by responder category were exploratory in nature as the lack of data in the literature limited our ability to form a directional hypothesis. In addition, we did not collect saliva samples on arrival at the lab. Baseline samples on arrival and during the resting phase would have allowed for a better understanding of the anticipatory response, although this was not standard practice when these studies were conducted. Salivary cortisol is now recognised as the method of choice and we consider it a future directive that all stress reactivity studies collect additional early saliva samples before the TSST.

The DASS-21 represents a short-term snapshot of mental health over the last week and therefore may not be representative of long-term mental health, although it has been shown to have relatively good consistency over 3 or more years (Lovibond, 1998). In addition, symptoms of depression and anxiety were not screened for at the time of the TSST and although the DASS was measured two years later, the design of this study precludes comments on the direction of the association or causality. Similarly, we measured the TSST only once and it has been suggested that the stress response may change over time. For example, Booij et al found a higher cortisol response to stress in participants with recent psychiatric symptoms and blunted response in those with chronic ongoing symptoms, compared to controls (Booij et al., 2013). They and others have proposed that HPA axis stress adaptation may involve a period of hyperactivity (metabolically costly) followed by downregulation after a period of time to hypoactivity (metabolically conservative)(Miller et al., 2007), suggesting responder category may change over time. This in itself may explain the lack of definitive associations between

TSST summary measures and depression/anxiety symptoms in this study. If we are measuring people at different stages of this journey, it negates the detection of a consistent effect. Longitudinal studies with repeated measures of TSST and mental health symptoms are needed to establish changes over time and address issues of causation versus co-emergence. In addition, the sample of this study was Caucasian youth, with relatively high SES and these findings require external validation in different populations.

Despite some limitations, this study has unique strengths including the prospective nature of the study, large sample size for studies utilising the TSST, analysis by responder category in addition to summary measures, and consideration of sex differences. This study was also conducted at an age of high risk for the development of symptoms or conditions of depression and anxiety.

6.5 Conclusions

In conclusion, we found differences in depression/anxiety symptoms across TSST responder categories in females but not males. Female reactive responders (RR) and non-responders (NR) had increased symptoms of depression and anxiety compared to anticipatory responders (AR). Stress responder categories may be a factor in determining individual vulnerability for stress related diseases in a sex-specific manner.

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Supplementary Data

Supplementary Table 6.1
Participants included vs those excluded from analyses

	Included (*n* = 748)	**Excluded** (*n* = 264)
TSST		
	mean (SD)	*mean (SD)*
Summary measures		
C_{BL} (nM)	13.5 (9.6)	14.1 10.0)
C_{MAX} (nM)	17.5 (10.8)	18.6 (11.4)
C_{MIN} (nM)	8.4 (4.1)	8.6 (4.1)
C_{RANGE} (nM)	9.1 (8.3)	10.0 (8.8)
AUC_G	1329 (708)	1383 (767)
AUC_R	447 (397)	483 (455)
	n (%)	*n (%)*
Responder category		
Anticipatory-responders	189 (25.6)	71 (27.3)
Reactive-responders	425 (57.7)	150 (57.7)
Non-responders	123 (16.7)	39 (15.0)
Covariates		
	mean (SD)	*mean (SD)*
BMI *(kg/m2)*	23.5 (4.7)	24.5 (5.1)*
	n (%)	*n (%)*
Smoker	80 (10.7)	63 (24.4)*
Saliva-only	131 (17.5)	56 (21.2)
Sex		
Female	387 (51.7)	97 (36.7)*
Male	361 (48.3)	167 (63.3)

*p<0.05 for the difference between those included and those excluded from analyses.

Supplementary Table 6.2

DASS Profiles of severity in this sample population

	Total *n*=748 *n* (%)	**Females** *n*=387 *n* (%)	**Males** *n*=361 *n* (%)
Severity DASS - Dep			
normal	546 (73.0)	272 (70.3)	274 (75.9)
mild	84 (11.2)	43 (11.1)	41 (11.4)
moderate	76 (10.2)	44 (11.4)	32 (8.9)
severe	19 (2.5)	14 (3.6)	5 (1.4)
ex. severe	23 (3.1)	14 (3.6)	9 (2.5)
Severity DASS - Anx			
normal	575 (76.9)	280 (72.4)	295 (81.7)
mild	53 (7.1)	30 (7.8)	23 (6.4)
moderate	78 (10.4)	46 (11.9)	32 (8.9)
severe	21 (2.8)	15 (3.9)	6 (1.7)
ex. severe	21 (2.8)	16 (4.1)	5 (1.4)
Severity DASS - Str			
normal	611 (81.7)	303 (78.3)	308 (85.3)
mild	64 (8.6)	36 (9.3)	28 (7.8)
moderate	42 (5.6)	27 (7.0)	15 (4.2)
severe	23 (3.1)	15 (3.9)	8 (2.2)
ex. severe	8 (1.1)	6 (1.6)	2 (0.6)

Supplementary Table 6.3
Univariate analysis of covariates

(a) Univariate analysis of covariates with DASS-Total

Covariate	NR vs R *OR (95% CI)*
Categorical	
Sex	
Male	(REF)
Female	5.84 (3.26, 8.41)**
Collection	
Blood+Saliva	(REF)
Saliva only	4.23 (0.82, 7.64)*
Smoker	
No	(REF)
Yes	8.84 (4.67, 13.00)**
Continuous	
BMI	0.22 (-0.06, 0.50)

* p<0.05, **p<0.001

(b) Univariate analysis of covariates with responder category

Covariate	AR *n* = 189 *OR (95% CI)*	RR *n* = 425 *OR (95% CI)*	NR *n* = 123 *OR (95% CI)*	***p***
Categorical				
Sex				
Male	100/381 (26.2)	200/381 (52.5)	81/381 (21.3)	0.001
Female	89/356 (25.0)	225/356 (63.2)	42/356 (11.8)	
Collection				
Blood+Saliva	154/613 (25.1)	353/613 (57.6)	106/613 (17.3)	0.55
Saliva only	35/124 (28.2)	72/124 (58.1)	17/124 (13.7)	
Smoker				
No	164/659 (24.9)	391/659 (59.3)	104/659 (15.8)	0.02
Yes	25/78 (32.1)	34/78 (43.6)	19/78 (24.4)	
Continuous				
BMI	23.07 (3.83)	24.02 (5.22)	22.24 (3.80)	0.001

P-values from Chi2 or ANOVA tests.

(c) Univariate analysis of collection method with responder category in males and females

Covariate	AR *n (%)*	RR *n (%)*	NR *n (%)*	***p***
Females	*n = 1*00	*n* = 200	*n* = 81	
Collection				
Blood+Saliva	76/300 (25.3)	156/300 (52.0)	68/300 (22.7)	0.40
Saliva only	24/81 (29.6)	44/81 (54.3)	13/81 (16.0)	
Males	*n = 8*9	*n* = 225	*n* = 42	
Collection				
Blood+Saliva	154/313 (25.1)	353/313 (57.6)	106/313 (17.3)	0.86
Saliva only	11/43 (25.6)	28/43 (65.1)	4/43 (9.3)	

(d) Univariate analysis of TSST summary measures with responder category in males and females

	AR *Mean (SD)*	RR *Mean (SD)*	NR *Mean (SD)*	***p***
Total	*n = 1*89	*n* = 425	*n* = 123	
C_{BL} (nM)	19.7 (13.7)*	11.2 (6.2)	11.7 (7.5)	<0.001
C_{MAX} (nM)	20.3 (13.9)*	17.7 (9.4)	13.0 (8.5)	<0.001
C_{MIN} (nM)	8.6 (4.3)	8.4 (3.7)	8.0 (5.1)	0.42
C_{RANGE} (nM)	11.7 (11.2)*	9.3 (7.2)	5.0 (4.9)	<0.001
AUC_G	1381 (798)	1381 (660)	1077 (687)	<0.001
AUC_R	480 (465)	495 (383)	236 (250)	<0.001
Females	*n = 1*00	*n* = 200	*n* = 81	
C_{BL} (nM)	17.1 (13.5)*	10.1 (5.2)	10.3 (6.4)	<0.001
C_{MAX} (nM)	17.7 (13.6)	16.6 (8.7)	11.5 (7.3)	<0.001
C_{MIN} (nM)	8.1 (4.0)	7.9 (3.4)	7.3 (3.8)	0.40
C_{RANGE} (nM)	9.6 (10.8)	8.7 (6.9)	4.2 (4.8)	<0.001
AUC_G	1249 (790)	1277 (583)	962 (537)	<0.001
AUC_R	394 (460)	450 (349)	191 (208)	<0.001
Males	*n = 8*9	*n* = 225	*n* = 42	
C_{BL} (nM)	22.6 (13.4)*	12.2 (6.9)	14.6 (8.6)	<0.001
C_{MAX} (nM)	23.1 (13.6)*	18.6 (9.8)	15.8 (10.0)	<0.001
C_{MIN} (nM)	9.2 (4.5)	8.9 (3.9)	9.2 (6.8)	0.78
C_{RANGE} (nM)	13.9 (11.2)*	9.8 (7.4)	6.6 (4.9)	<0.001
AUC_G	1528 (785)	1473 (709)	1299 (875)	0.26
AUC_R	577 (455)	536 (407)	322 (299)	0.003

P values represent differences across all three groups ***** Comparison of AR vs RR p<0.05

Supplementary Table 6.4

TSST Responder category with DASS at age 20 in males and females NR vs RR

Responder category	Total DASS			DASS-dep			DASS-anx			DASS-str		
	EMM	**B (95% CI)**	***p***	**EMM**	**B (95% CI)**	***p***	**EMM**	**B (95% CI)**	***p***	**EMM**	**B (95% CI)**	***p***
Females (*n* = 381)												
Reactive (RR) (*n* = 200)	27.53	REF		9.03	REF		6.92	REF		11.58	REF	
Non responders (NR) (*n* = 81)	30.28	2.75 (-1.84, 7.34)	0.24	10.10	1.06 (-0.84, 2.97)	0.27	7.58	0.65 (-0.70, 2.01)	0.34	12.61	1.03 (-0.91, 2.97)	0.30
Males (*n* = 356)												
Reactive (RR) (*n* = 225)	20.98	REF		6.93	REF		5.31	REF		8.75	REF	
Non responders (NR) (*n* = 42)	19.70	-1.28 (-7.05, 4.50)	0.66	6.05	-0.88 (-3.27, 1.52)	0.47	5.82	0.51 (-1.20, 2.22)	0.56	7.84	-0.91 (-3.35, 1.53)	0.46

Final models adjusted for BMI, Smoking status and blood+saliva vs saliva-only collection.

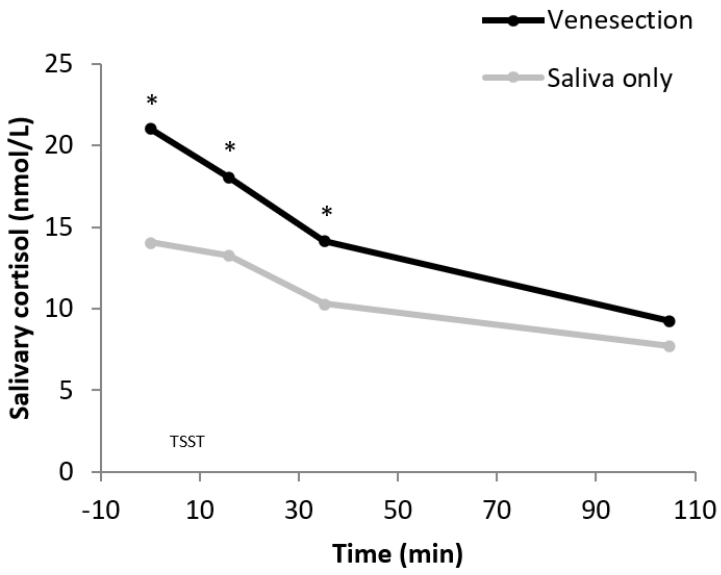
Supplementary Table 6.5

Test of interaction between sex and summary measures

Summary Measure	Total DASS		DASS-dep		DASS-anx		DASS-str	
	F	*p*	F	*p*	F	*p*	F	*p*
C_{BL}	0.63	0.53	0.42	0.66	2.12	0.12	0.29	0.75
C_{MAX}	0.86	0.42	0.74	0.48	1.96	0.14	0.48	0.62
C_{MIN}	0.63	0.53	0.69	0.50	2.32	0.10	0.20	0.82
C_{RANGE}	0.84	0.43	0.69	0.50	1.68	0.19	0.46	0.63
AUC_G	0.91	0.40	0.56	0.57	2.20	0.11	0.77	0.46
AUC_R	1.26	0.28	0.62	0.54	1.92	0.15	1.22	0.30

Supplementary Figure 6.1

Anticipatory Responders by collection method



Salivary cortisol response curves for anticipatory responders who had blood taken via venesection or saliva-only. The TSST was conducted at 0-15 min. Linear mixed models analysis showed evidence for a differential response in terms of higher overall salivary cortisol levels in AR blood participants (p=0.02), specifically at timepoints 0 (p=0.004), 15 (p=0.02) and 35 (p=0.05) minutes but not 105 minutes (p=0.59). There was no evidence for interactions by sex (p=0.84).

Chapter 7 Discussion

This thesis represents a comprehensive investigation of the associations between exposure to common early life stressors, the cortisol response to a stressor as an adult and symptoms of depression and anxiety in adulthood, in the same individuals from the Raine Study. The thesis is comprised of 4 studies, the first of which characterizes the hormonal response to a psychosocial stressor, the TSST, at age 18 and the remaining three investigate links between early life stress exposure, the cortisol response to a stressor and adult depression/anxiety symptoms. In this chapter, each of the studies will be summarized, followed by a discussion of the main findings and the work as a whole, integrating the results and highlighting the overall significance and contribution to the field. Strengths, limitations and potential directions for future research will also be examined.

7.1 Summary of studies included in this thesis

Study 1 (Chapter 3): *Characterisation and novel analyses of acute stress response patterns in a population-based cohort of young adults: influence of gender, smoking and BMI*

The first study of this thesis aimed to characterise the HPA-axis response to a psychosocial laboratory stressor in a large number of young adults and the association with sex, smoking, BMI, OC use and menstrual cycle using 1] standard traditional methods and 2] novel responder category analyses. Previous work has been limited by sample size and there are discrepancies in the direction of the effect of various modifiers. In addition, studies report on select measures only and there is a need to integrate and compare multiple measures over different levels of the HPA-axis. This study reported on traditional repeated measures analysis and summary measures such as maximum and minimum concentrations or area under the curve alongside the pattern of response, termed responder category, to extend our understanding of how these different parameters can interact and complement each other.

Using traditional measures, males consistently showed higher responses than females to the TSST across ACTH, plasma and salivary cortisol, with the

exception of plasma cortisol in females using OC, where high levels of estrogens increase CBG binding proteins and cause upregulation of total cortisol in the plasma. Compared to females not taking OC, females taking OC showed much higher plasma cortisol, similar salivary cortisol but reduced ACTH. There were no notable differences over the menstrual cycle in our sample. However, smokers and obese participants displayed a blunted response at the plasma cortisol level.

Noting reports of different patterns of cortisol stress response in the literature, we found three stress response patterns in our sample; Anticipatory-Responder (AR), Reactive-Responder (RR) and Non-Responder (NR). Whilst there has been the occasional examination of 'high responders' versus 'low responders' (Schommer et al., 2003), these three response patterns have never been systematically examined in a large population. Using novel responder category analyses we found variations of RR, AR and NR across sex, smoking and BMI.

Therefore, the key contribution to the literature from this study is the novel way of examining cortisol reactivity data, complementary to traditional measures. Variation of responder category with known risk factors establishes the potential for responder categories to vary with health and disease.

Study 2 (Chapter 4): *The impact of life stress on adult depression and anxiety is dependent on gender and timing of exposure*

The second study in this thesis aimed firstly, to develop postnatal trajectories of stress exposure from birth to age 17 and examine the relationship of prenatal stress exposure and postnatal trajectories of stress exposure with symptoms of depression and anxiety at age 20 in males and females. The second aim was to examine the relative impact of prenatal and postnatal stress exposure on depression and anxiety symptoms in young adult males and females.

Previous research on the relationship between stress exposure and depression/anxiety has focused on more severe trauma and clinical populations at limited time points during development and there has been inadequate investigation of sex differences in these relationships.

This study made an important contribution to the literature, based on the most extensive investigation into common early life stress events in a large

community population at 10 time points over 17 years, concluding that both prenatal stress and the pattern of postnatal stress exposure can influence adult mental health. Results indicated that the early stages of pregnancy may represent a more vulnerable window to the impacts of stress than the later stages of pregnancy, with high stress exposure at 18 weeks pregnancy being associated with higher depression and anxiety symptoms. In addition, participants with stress exposures increasing around adolescence or chronic exposure to stress over childhood and adolescence showed a higher risk for depression and anxiety symptoms in adulthood. This reinforces the transition time of adolescence as a vulnerable window for stress exposure.

Furthermore, this study provided evidence that the relative impact of prenatal and postnatal stress could be sex-specific. The impact of prenatal stress may be more important in boys and exposure to stress during childhood and adolescence may be more important in girls, in terms of predicting depression and anxiety in young adults.

The presence of a descending stress trajectory showed that despite high exposure to stress early in life, reductions in life stress events across childhood and adolescence were protective against future depression/anxiety symptoms, highlighting the potential benefits of early life interventions to reduce stress exposure. Therefore, from a translational point of view, this study provides evidence that interventions and public health policies designed to reduce controllable stressors and the impact of stress around conception, pregnancy and adolescence may prove beneficial for offspring mental health later in life.

Study 3 (Chapter 5): *Prenatal and childhood stress exposure and the sex-specific response to psychosocial stress in adulthood*

The third study in this thesis aimed to examine whether common life stressors, experienced prenatally, during childhood and adolescence were associated with a dysregulation of HPA-axis reactivity in response to a stressor. Research examining the impact of early life stress on cortisol responses to stress has focused on more severe trauma and has produced inconsistent results. In this study, early life stress was measured at ten time points (as mentioned in Study 2),

cortisol reactivity to psychosocial stress was assessed using traditional and novel measures (as developed in Study 1) and relationships were examined in both males and females.

Overall, common life stress events experienced prenatally, during childhood and adolescence showed relatively limited consistent impact on HPA-axis reactivity in early adulthood. However, results showed evidence for a sex-specific association where high prenatal stress early in pregnancy resulted in increased cortisol reactivity in adult males. Furthermore, with increasing stress exposure during adolescence males and females showed divergent responses to stress, whereby males were more likely to be a non-responder and females were more likely to be responders.

The key contributions to the literature with this large prospective study of stress exposure from prenatal life to age 17 are that exposure to common stressors in pregnancy and during adolescence can have sex-specific effects on the cortisol response to stress post-puberty.

Study 4 (Chapter 6): *The anticipatory response to stress and symptoms of depression and anxiety in early adulthood*

The fourth and final study in this thesis aimed to examine the associations between the cortisol response to psychosocial stress (traditional and novel analyses) and symptoms of depression and anxiety in a young adult population. Previous research has focused on depression and anxiety disorders rather than symptoms and research examining cortisol activity with symptomatology in males and females is limited. This study found an association of depression/anxiety symptoms across responder category in females but not males. In females, NR and RR displayed higher depression/anxiety symptoms than AR, with moderate effect sizes comparable to those for sex and smoking status. The highest symptomatology was found in female NR, consistent with the blunted responses found in patients with depression/anxiety disorders. Interestingly, in females, AR showed the lowest symptoms of depression and anxiety. While it could be assumed that AR would be more prone to anxiety, this was not supported by these results, which may underscore the need for a better understanding of adaptive versus maladaptive

responses to stressors. The key contribution to the literature is that the novel responder category analyses enabled the identification of nuanced and sex-specific associations with symptomatology.

7.2 Stress responder category as a novel and useful way of examining stress response data

For many years cortisol reactivity has been assessed and compared using overall 'output' (e.g. area under the curve) or summary measures such as cortisol concentration at baseline and peak. The responder category method presented in this thesis summarises the overall pattern of a participant's stress response. This thesis represents the first time responses have been analysed using the three defined categories of RR, AR and NR. One advantage of this method is that it reduces the 'dimensionality' of the data from multiple different summary measures to one categorical variable representing groups of individuals with similar response profiles, potentially making results more easily interpretable. Study 1 (Chapter 3) showed that each pattern has defined characteristics according to several summary measures. For example, in RR's, the baseline cortisol concentration is low, the maximum and range of cortisol concentrations are high and the AUCs are all high; for AR's, the baseline, maximum and range of cortisol concentrations are high, AUC_R and AUC_G are all high, but AUC_I is negative. In NR's the baseline, maximum and range of cortisol concentrations are low, and AUC_R and AUC_I are also low. See Figure 3.1. Furthermore, as seen in Figure 3.3, these specific patterns of stress response were generally observed across all three levels of the HPA-axis: ACTH, plasma cortisol and salivary cortisol, indicating a level of consistency in hormonal responses from the pituitary to adrenals and between plasma and salivary cortisol.

Responder categories may offer unique insight into HPA-axis dynamics that other methods cannot.

The results of Study 1 (Chapter 3) showed for the first time that the association of responder category with sex, smoking and BMI gives us additional and different information compared to traditional summary measures. In Study 3 (Chapter 5), a sex-specific association was detected with responder category and a specific pattern of childhood stress exposure, specifically with increasing stress exposure during the vulnerable window of adolescence, whereby males in the ascending stress trajectory

were more likely to be non-responders and females in the ascending stress trajectory were more likely to be responders. Given that males have a more robust cortisol response to stressors than females and are more likely to be RR, the absence of a cortisol response in 20% of males in the ascending stress trajectory was notable. This observation was not identifiable by standard curve analysis or summary measures and offers evidence that responder category may offer novel insights into the relationship between early life stress exposure and altered HPA-axis dynamics. Then in Study 4 (Chapter 6), evidence of a sex-specific association was found between responder category and symptoms of depression and anxiety two years later, where female NR showed the highest and female AR displayed the lowest depression and anxiety symptoms, respectively. However, with traditional summary measures, we found limited evidence of an association with depression and anxiety symptoms in males or females. Therefore, responder category analyses were again able to identify differences in this sample more effectively than standard traditional techniques.

The statistical method of latent class analysis has also been used to identify categories of stress responses in this data (Paananen et al., 2015). However, that method is more likely to identify separate groups based on a shift up or down of the entire response curve than differing shapes of response, particularly the high baseline anticipatory response. In recognition of the need to look at the response to stress in different ways, others have investigated additional parameters such as the reactivity slope (suggesting speed or strength of response) and recovery slope (suggesting efficiency of negative feedback, shut down of the response) (Fiksdal et al., 2019; Kuhlman et al., 2015). In terms of responder category, a steep reactivity slope would indicate a reactive-responder, and a blunted/flat reactivity slope would suggest a non-responder. Similarly, a steep recovery slope would indicate a responder (RR or AR), and a shallow recovery slope would again suggest a non-responder. Whilst we found the highest symptoms of depression and anxiety in female NRs and the lowest symptoms in female ARs, Fiksdal et al. (2019) found blunted reactivity and recovery slope with anxiety symptoms but steeper reactivity and recovery slopes for depression symptoms with no reported sex differences. These differences may, in part, be because non-responders were eliminated from analyses, a point which is debated further in the following section. Although there is some evidence of complementarity between responder category and reactivity/recovery slopes, once again, the main profile not captured by slope analysis is

the anticipatory response which would have no real reactivity slope as the baseline values are already high. As a final comment, it should be noted that the TSST conducted with the Raine Study participants is the largest study of its kind, and one disadvantage of responder category analyses for future studies may be a requirement for larger sample sizes to obtain adequate power for meaningful results when using categorical variables (Royston et al., 2006).

In summary, responder category, curve or slope analyses and summary measures represent complementary ways of examining the data that each tell us something slightly different. Hence, it is recommended that multiple measures are assessed in adequately powered studies for transparency and a more nuanced understanding of the relationships involving the stress response. In the following sections, we will take a closer look at the neglected categories of non-responders and anticipatory responders.

7.2.1 Thresholds and the importance of Non-Responders

TSST experiments resulting in a higher proportion of non-responders than the standard 30% described in Trier (Kudielka et al., 2007) may result in assumptions that the stress induction was less effective and participants who do not show a clear response to the TSST (non-responders) are sometimes removed from analyses. Our data indicate that non-responders may show a relationship with depression/anxiety symptoms, sex and smoking and that removal of NR is not recommended and may bias results. This is supported by other data showing an association between an attenuated response to stress with negative health outcomes such as burnout, panic disorder, depression, chronic fatigue, fibromyalgia and chronic pelvic pain (Heim et al., 2000; Petrowski et al., 2010). Collectively, this points to the non-responder profile as being a non-normative or maladaptive state; therefore, it's important to look at what defines a response compared to no response. In the TSST literature, an increase from baseline to peak cortisol concentration above a set threshold has generally been used to indicate a response and assess the effectiveness of the stress induction. Cortisol is secreted in a pulsatile fashion, and early data indicated that a typical secretory episode increased total plasma cortisol levels by 55.2 nmol/l (Weitzman et al., 1971). As salivary cortisol levels are estimated to be approximately 2% to 5% of plasma cortisol levels, it was reasoned that a 2.5-nmol/l salivary cortisol increase should be indicative of a secretory episode (Kirschbaum, Wust, & Strasburger, 1992; Van Cauter & Refetoff, 1985). More recently, this absolute proxy

measure has been reassessed, and increases of 1.5mmol/L or 15.5% for salivary cortisol were proposed as effectively being able to discriminate responders from non-responders (Miller et al., 2013). In Study 1 (Chapter 3), the response threshold was defined by a relative (%) increase incorporating the variability of the assays (2 x the coefficient of variation), which addresses the main limitation of using absolute value thresholds and has been recommended by others (Van Cauter & Refetoff, 1985). It was advantageous to utilise both plasma and salivary cortisol profiles for the determination of responder category in this study, and threshold estimates are comparable with the definitions of Miller et al. (2013). The results of studies 1 and 4 show that NR are more likely to be female and/or smokers and that female NR are at higher risk of depression and anxiety symptoms.

It is of note that if a response is only defined by an absolute increase, then the anticipatory response will not be detected. It is interesting that visually, the non-responders in the study by Fiksdal et al. (2019) look to be an amalgamation of the NR and AR patterns, although the AR is not currently recognised as a separate responder category in the literature. This will be discussed further in the following section.

7.2.2 Are Anticipatory Responders a 'real' and important category?

Results from Study 1 (Chapter 3) showed the presence of a third category of responder with high baseline cortisol concentrations, the absence of a clear peak and cortisol decreasing after the TSST more than the predicted decrease due to circadian rhythm. This pattern was termed anticipatory responder as participants demonstrated a cortisol response before the laboratory stressor (Engert et al., 2013). Engert et al. (2013) p.1239, write *"anticipatory and reactive hormone surges are rarely distinguished",* and it is of note that there remains a relative absence of recognition of this pattern in the TSST literature. However, there are several reasons why there may be few representations of AR in the literature or, alternatively a higher proportion of ARs in this sample and these will be discussed below.

Firstly, this AR discrepancy may be due to differences in classification and analyses. The classic textbook responses are those of a reactive responder (Kirschbaum et al., 1999) or non-responder and responses that deviate from those patterns may be deemed aberrant (Dalile et al., 2021). High baseline samples may be eliminated from

analyses (Evans et al., 2013; Fiksdal et al., 2019). As Evans et al. (2013) p.3 write, *"The first cortisol pre-task value was excluded from the analyses as it was generally higher, thus most likely reflecting anticipatory stress to a greater degree than the second measurement".* High baseline samples may be classified as a NR in the absence of a clear reactive peak (Dalile et al., 2021; Fiksdal et al., 2019). The non-responder profile described by Fiksdal et al. (2019) shows a remarkably steep decrease from baseline, suggesting it may be an amalgamation of NR and AR. Furthermore, the AUC_G measure of overall output does not differentiate AR from RR, effectively obscuring the AR response, and when high baseline responses are combined with other results using repeated measures curve analyses, they are effectively diluted out. This is demonstrated in Figure 3.3 in Chapter 3, where response curves are shown for separate responder categories compared to when they are combined.

Secondly, the high prevalence of ARs in this sample may be due to the age of the Raine Study population (18 years) compared to measures from TSSTs performed in children or older adults. During puberty, coinciding with changes in multiple neuroendocrine hormones, significant shifts in stress reactivity are seen. The period of adaptation and recalibration of the HPA-axis that occurs around puberty is likely to cover a protracted period of heightened stress sensitivity and reactivity (Gunnar et al., 2019; Miller & Prinstein, 2019). The AR subgroup may be predominant in youth, and there is some evidence of a higher anticipatory response in adolescents (age 13-18) (Evans et al., 2013) and sensitisation to social evaluation in adolescents compared to adults (Somerville, 2013).

Thirdly, the high numbers of ARs in this sample may be due to the specific protocols used in the Raine Study TSST. In the recruitment process, the 18-year-old participants were informed by information sheet mailout and during the recruitment telephone discussion, that they would be given a challenge to measure stress hormones, and they may feel under stress for a short period of time. Participants also knew that researchers wanted to take blood and saliva samples but that they could choose to have saliva-only collection if they wished. Venepuncture is associated with a cortisol response in some people independent of the TSST and, for this reason, after cannulation participants had a period of rest for at least 45 minutes before the TSST, consistent with recommendations and previous studies (Epel et al., 2000; Kirschbaum

et al., 1999; Kudielka et al., 2007). It is acknowledged that some authors now feel that ideally, this waiting time should be increased to 2-3 hours to completely eliminate any effect of venesection (Dalile et al., 2021; Weckesser et al., 2014), a recommendation that would increase participant burden and generally preclude the use of this procedure in large studies.

Dalile et al. (2020) have suggested the anticipatory response to the TSST can be largely attributed to a vasovagal response to venepuncture, however, we believe this is unlikely to be the case for participants in the current study, as the AR pattern was observed equally in participants choosing blood or saliva-only, suggesting the AR pattern was not dependent on venepuncture in this sample. Furthermore, medical staff monitored participants throughout, and those who experienced symptoms of a vasovagal reaction (including fainting, feeling faint, dizzy, cold, clammy, sick or looking pale, *n* = 15) were excluded from analyses. These numbers are comparable with reports of vasovagal responses in phlebotomy patients (Deacon & Abramowitz, 2006), whereas the ARs comprised 25% of the sample. Therefore, it would appear unlikely that the ARs in this study are due to a vasovagal reaction.

It is controversial whether AR should be recognised as a separate unique type of response to a stressor. The section below will argue that the AR shows potential for being a separate stress response that differentiates a subset of participants, whilst also pointing out the disadvantages of distinguishing ARs as a separate classification group in research studies.

Anticipatory responders appear to be distinct from RRs. Anticipatory responders certainly have the ability to mount a clear HPA response and therefore form a subset of responders. However, despite consistent recruitment for the TSST and laboratory treatment between participants, these individuals show a cortisol response profile starting well before the procedure instead of when the procedure begins. Whilst we cannot know exactly what participants were reacting to, data from Study 4 (Chapter 6) indicated it was not due to blood collection. The results showed that separating the AR and RR categories was more informative than collapsing them into one category, or using traditional summary measures, for discerning associations between TSST response and symptoms of depression and anxiety. Anticipatory responders display high baseline cortisol similar to their peak cortisol levels, moderate to high cortisol concentration

range, high AUC_G and AUC_R and low AUC_I. In this way, reducing the *dimensionality* of the data down from six different summary measures to one categorical variable representing a profile of anticipatory stress responses proved to be efficient and interpretable, yet discriminatory of differences in symptomatology.

Study 4 (Chapter 6) found that in females, the anticipatory response at age 18 was associated with reduced depression and anxiety symptoms at age 20, compared to RR and NR response types. This relationship was especially noticeable with symptoms of depression. If an absent or attenuated HPA-axis response is associated with a higher risk for depression, as found here and elsewhere (Zorn et al., 2017), then the anticipatory response could be viewed as the opposing end of the response spectrum representing extreme HPA-axis vigilance. It's possible the anticipatory response may be beneficial in evolutionary terms, representing increased vigilance to protect from harm in the short to medium term. In sports medicine, the anticipatory rise in cortisol is thought to prime and prepare participants for the psychological and physical challenges ahead (van Paridon et al., 2017). There is also evidence that anticipatory responders shut down their stress response more efficiently after the stressor, in terms of cortisol and blood pressure recovery (Juster et al., 2012). In contrast to the results of Study 4, one study reported that higher anticipatory stress cortisol levels predicted modest increases in depressive symptoms six months later in 36 never-depressed university students (Morris et al., 2012). These different results from Morris et al may stem from the use of a single cortisol concentration before the laboratory procedure as a proxy measure for anticipatory stress, therefore higher values could represent RR or AR, depending on the trajectory of their response curve. Their study is one of several studies linking cortisol hyper-reactivity instead of hypo-reactivity with depression. Regarding symptoms of anxiety, whilst it may seem reasonable to assume that ARs are more prone to anxiety, this is not supported by the results of Study 4 (Chapter 6), and a direct link between anticipatory cortisol reactivity and anxiety symptoms is missing from the literature. It could also be assumed that ARs would be more fearful of venesection, however the anticipatory response was not associated with the choice of saliva-only or blood collection, suggesting this is not the case. Therefore, in females the anticipatory response may be adaptive and protective against depression/anxiety symptomatology, and this warrants further investigation.

There are a couple of disadvantages to separating out AR from other responders; the first of which involves additional time and calculations and the second is the requirement for larger sample sizes to obtain adequate power for meaningful results when using categorical variables (Royston et al., 2006). Therefore, this method may not be appropriate for smaller studies.

In summary, our understanding of the anticipatory dynamics of the stress response is limited. Results from Study 4 (Chapter 6) suggest that differences in stress regulation may occur before the stressful encounter and may differentiate females at lower risk for depression. If it is confirmed that the AR is linked with reduced risk for depression, it may also be linked with positive psychological and cognitive constructs such as increased excitement, adaptive coping, positive expectation, active problem solving or self-efficacy in regard to challenges. Rather than dismiss the anticipatory response as aberrant and increase the waiting time in the laboratory to diminish the presence of this profile (Dalile et al., 2021), it may prove useful to harness the additional information obtained from this response, with regard to future anxiety and depression symptomatology and conditions.

7.3 The timing of stress exposure is important for later life repercussions

There may be critical time windows for the effect of stress exposure on biological systems. The importance of timing stems from the premise that the brain regions and other organ systems regulating the stress response develop and mature at different ages. It is postulated that high exposure to stressors at critical windows of development may alter stress sensitivity or HPA-axis regulation such that future HPA-axis reactivity is affected and vulnerability to depression and anxiety problems (Koss & Gunnar, 2018).

Stress-provoking events rarely occur in isolation and are highly correlated over time, although studies with repeated measures of stress exposure over childhood are rare. Whilst serious childhood stressors increase the risk for adult depressive and anxious conditions, the relative contribution of common prenatal and childhood stressors on a child's long-term psychopathology is still under scrutiny. This is due to the limited number of long-term studies (Glover & Hill, 2012) and a tendency to consider a broad window of all exposures before the age of 18 (Gardner et al., 2019).

The timing of the stress exposure in childhood may explain divergent results concerning regulation of the HPA-axis (Bosch et al., 2012; Pesonen et al., 2010). However, there are no studies examining adult HPA reactivity that are also able to separate out different timeframes of stress exposure in childhood. The elegant study of Bosch et al (2012) did examine HPA axis reactivity in late childhood with different exposure timeframes but one broad window measure is generally considered up to the age of 15 or 18 as summarised in a recent meta-analysis (Bunea et al., 2017).

This PhD examined stress exposure at multiple time points in utero, childhood and adolescence. The same 11 common life stress events over the first 17 years of life were considered, including pregnancy as a separate construct, with a latent class trajectory approach enabling the identification of different patterns over time. This thesis offers evidence that the timing of stress exposure is important, and may differ between sexes. Results show high prenatal stress early in pregnancy contributed to adult depression and anxiety symptoms, especially in males. Whilst in females, the medium, high and ascending postnatal stress trajectories contributed to depression and anxiety symptoms. In addition, males with high prenatal stress early in pregnancy displayed heightened cortisol reactivity; males in the ascending postnatal stress trajectory were more likely to be non-responders to the TSST whilst females in this trajectory were more likely to be responders. Furthermore, the descending trajectory with relatively high stress exposure at birth reducing over time showed no association with symptoms of depression/anxiety in either sex or alterations in cortisol reactivity compared to low stress exposure. This suggests that stress exposure during prenatal and adolescent life in particular are relevant to HPA-axis regulation and depression/anxiety in later life and that interventions that reduce exposure to life stress events after conception and throughout adolescence are likely to have beneficial outcomes. Below is a discussion of the prenatal window results followed by the adolescent window results, in relation to the literature and potential biological mechanisms.

7.3.1 Prenatal stress exposure

The association of high prenatal stress exposure with adult depression and anxiety symptoms identified in Study 2 (Chapter 4) align with findings from studies of the Dutch Hunger Winter where children exposed to famine as a stressor before birth show higher rates of depressive conditions as an adult (Brown et al., 2000). However, this prenatal

stress exposure may be confounded by the malnutrition experienced and other long-term studies examining adult mental health are not available. With respect to the HPA-axis, the association of high prenatal stress with increased cortisol reactivity in males aligns with the only other study examining stress exposure during gestation and HPA-axis reactivity in adulthood (post-puberty) (Entringer et al., 2009), where they found higher numbers of prenatal stress events were associated with a higher cortisol reactivity compared to no prenatal stress events. Similarly, prenatal stress exposure via the Quebec ice storm of 1998 was associated with elevated offspring cortisol reactivity at age 13 (Yong Ping et al., 2020). However, with low participant numbers, these studies were unable to examine associations by sex. Concerning the impact of timing during pregnancy on later life outcomes, results from Study 2 (Chapter 4) showed that stress exposure early in pregnancy was associated with higher adult depression and anxiety symptoms, especially in males. Previous studies using prenatal anxiety, psychological distress or emotional complaints as a proxy for stress exposure have produced inconsistent results. Aligning with the results from Study 2, stressors in *early* pregnancy have been associated with increased internalising behaviours and negative emotionality in boys (de Bruijn et al., 2009; Martin et al., 1999) and more behavioural problems and depressive symptoms in girls (O'Connor et al., 2003; Van den Bergh et al., 2008). However, in contrast to these results, emotional complaints in *late* pregnancy have also been associated with more behavioural problems in boys and girls (de Bruijn et al., 2009; O'Connor et al., 2003). Data on the timing of stressors during pregnancy and HPA reactivity as a young adult are not available, therefore, the effect of stressor timing in pregnancy remains unclear. However, the findings in this thesis complements other studies suggesting a sex-specific effect of prenatal stress on adult mental health outcomes and HPA-axis regulation (de Bruijn et al., 2009; Martin et al., 1999; Mueller & Bale, 2008; O'Connor et al., 2003; Weinstock, 2008).

7.3.2 Mechanisms for the impact of prenatal stress exposure

There are proposed biological mechanisms for the prenatal period as a critical window of vulnerability (Van den Bergh et al., 2005). Disparities in results examining stressor timing in pregnancy may indicate that different mechanisms are operating at different stages of gestation as stress-sensitive brain and regulatory systems develop and mature at different stages. The first mechanism involves the transmission of high levels of maternal stress hormones across the placenta to the fetus. The placenta normally acts

as an effective barrier to maternal stress hormones via the placental enzyme 11B-HSD2, which inactivates cortisol, however, it is only a partial barrier (Davis & Sandman, 2010) and the expression of this enzyme and amount of cortisol produced varies across pregnancy. The second mechanism involves abnormal blood flow in the uterine arteries with increased maternal stressors or anxiety. In this way, a greater resistance to blood flow (such as found with intrauterine growth restriction or preeclampsia) potentially impairing oxygen and nutrient supply may act as a stressor to the fetus (Van den Bergh et al., 2005). Placental cells bear the same genetic information as the fetus (XX or XY) and sex-specific effects can be explained by gender differences in placental size, shape and function including blood flow, gene expression, action of the 11B-HSD2 enzyme and responses to environmental stimuli (Beijers et al., 2014; DiPietro & Voegtline, 2017). The results from studies 2 and 3 in this thesis (chapters 4 and 5) found that males with high stress exposures early in pregnancy showed higher adult symptoms of depression and anxiety and higher cortisol reactivity, respectively. These results are consistent with a body of evidence that suggests the male fetus is at increased vulnerability to negative exposures in utero (DiPietro & Voegtline, 2017; Glover & Hill, 2012).

7.3.3 Adolescent stress exposure

Moving on to postnatal stress exposure, the results from Studies 2 and 3 (chapters 4 and 5) suggest adolescence as a second critical window for stress exposures. Results showed that the ascending stress exposure trajectory, with stress exposure increasing over adolescence, was associated with higher adult depression/anxiety symptoms and divergent cortisol reactivity in males (more likely to be a non-responder) and females (more likely to be a responder). This parallels data from a US study between the ages of 11-21 years where trajectories of stress events increasing from age 11 to 14 or from age 13 to 15 were associated with higher risk for depression than minimal stress events or those peaking at age 17 (Boardman & Alexander, 2011). These higher risk times incorporate puberty which also marks the point at which depression and anxiety symptoms increase more in females compared to males (Cyranowski et al., 2000). The cortisol reactivity data from Study 3 (Chapter 5) is supported by results from the Tracking Adolescents' Individual Lives Survey study, which showed that stressors between the age of 6-11 were associated with higher cortisol reactivity while stressors between the age of 12-15 were associated with lower cortisol reactivity at age 16 (and stressors before

the age of 5 showed no association with cortisol reactivity), suggesting adolescence as a transition period of the HPA-axis (Bosch et al., 2012). This contrasts with results from an older sample (average age 63.5 years) which points to early childhood as a time of vulnerability (Pesonen et al., 2010). Children separated from their parents between the ages of 2-7 years in WWII showed higher cortisol and ACTH reactivity in later life compared to those not separated. Authors report that separation before the age of 2 or after the age of 7 was associated with lower cortisol reactivity compared to those separated between the age of 2 and 7 (Pesonen et al., 2010). However, it is hard to compare these results, firstly relative to the adolescent window as stress exposures later in adolescence were not examined, and secondly relative to the cortisol response in youth as the HPA-axis reactivity changes over the lifespan. It is of note that sex differences were not examined in these studies. The thesis finding that males in the ascending trajectory are more likely to be cortisol non-responders aligns with a study reporting a significant blunted response in individuals with a history of adverse events compared to no adverse events, and although no empirical data were reported, the authors state that 'This finding appeared to be driven primarily by men' (Elzinga et al., 2008). In contrast, in Study 3 (Chapter 5), females in the ascending trajectory were more likely to be responders, highlighting the potential for divergent stress responses in males and females to identical stress exposures.

7.3.4 Mechanisms for the impact of adolescent stress exposure

In terms of biological mechanisms, adolescence incorporates the sex hormone changes that occur with puberty and represents a time of transition. The rise of symptoms of depression is temporally associated with menarche in females suggesting a hormonal mechanism (Patton et al., 1996). Sex hormones interact with receptors in the brain and adrenal glands that affect regulation of the HPA-axis. This time is associated with dramatic changes in HPA-axis reactivity as it increases in resting and reactive states after a period of relative hyporeactivity during childhood (Romeo, 2010a, 2010b). There is evidence that this is a time of maturation and recalibration of the HPA-axis in preparation for the challenges of adulthood (Gunnar et al., 2019; Hostinar et al., 2018). The increased sensitivity to stress at this time may be accentuated by higher expression of glucocorticoid receptors in the brain (Andersen & Teicher, 2008) and increased psychosocial pressures for both sexes. However, there are sexually dimorphic hormonal

profiles, brain maturation, production and pruning of synapses in brain regions that regulate the HPA-axis. Therefore, stress exposures at this time may result in sex-specific modulation of the HPA-axis, impacting long-term reactivity, sensitivity to stressors and vulnerability to psychopathology.

These findings suggest that exposure to common life stress events during prenatal life and adolescence can have long-term effects on stress reactivity and symptoms of depression and anxiety. Therefore, interventions to reduce prenatal stress exposures and improve maternal stress coping strategies and targeted assessments and interventions to reduce stress exposures and their impact around adolescence are likely to benefit long-term depression and anxiety outcomes.

7.4 Interpretation as a whole: connecting three sides of the triangle

When interpreting the thesis results as a whole, at first glance there appears to be a paradox, and studies 2, 3 and 4 (Chapters 4, 5 and 6) present potentially discrepant findings concerning the associations in females between life stress exposure, non-reactivity of the HPA axis, and depression/anxiety symptomatology. In females, although a high-stress exposure trajectory was associated with higher levels of symptoms in adulthood, the high-stress exposure trajectory had a reduced risk of being a NR, while paradoxically, NRs had higher levels of symptoms. This appears problematic for interpretation unless we view increased reactivity as being on the pathway to reduced reactivity, with both states being different presentations of the systemic response to threat.

For over 70 years, cortisol has been studied as a primary mechanism for the link between higher stress exposure and adverse health outcomes. Traditionally, most models posited that in response to early life trauma, increased cortisol exposed tissues and biological systems to increased allostatic load and ‘wear and tear’ leading to health consequences (McEwen, 1998). In contrast, a handful of theories incorporated declines in cortisol output as the mechanism (Gunnar & Vazquez, 2001; Heim et al., 2000; Hellhammer & Wade, 1993). Across the literature, studies support associations of both cortisol hyper-reactivity and hypo-reactivity with early life trauma and conditions of anxiety and depression. So, it has been unclear how these findings can be integrated to arrive at a general unified consensus on how stress exposure impacts the HPA-axis and

future mental health. Towards this goal, it has been proposed that HPA-axis cortisol reactivity may go up before it goes down (Fries et al., 2005; Miller et al., 2007), and there is evidence for basal cortisol changes across time. Firstly, in a meta-analysis of 107 studies, cortisol levels were higher compared to normal control levels when they were measured close to the stress event, but with more months elapsed since the stressor event, cortisol levels decreased to below normal control levels (Miller et al., 2007). However, when chronic stressors were still present, cortisol output was higher than if the stressor was only in the past (Miller et al., 2007). Secondly, in a longitudinal study of abused girls from age 6 to 30 compared to non-abused controls, there was evidence of higher cortisol levels in childhood changing to lower levels by early adulthood, with adolescence as the transition point (Trickett et al., 2010). Thirdly, in participants with a history of maltreatment, higher cortisol responses to stress have been found in individuals with mild/moderate depression symptoms, and lower cortisol responses have been found in people with more severe symptoms compared to participants with low/minimal symptoms (Harkness et al., 2011), suggesting a time-dependent mechanism. Collectively, this data supports the notion that one type of HPA-axis response to stress may, over time, transition into another. It indicates that exposure to stressors can increase and decrease cortisol activity, which helps explain many conflicting results in the literature.

The work in this thesis is consistent with this concept. Results from Study 3 (Chapter 5) showed that females exposed to recent or chronic stress in adolescence had a greater tendency to be responders (AR/RR) than NR. Therefore, this may reflect an adaptation to upregulate the responsiveness of the HPA axis to cope with a recent or continuing threat. From the perspective of the transitions described in the previous paragraph, it may be a step towards higher cortisol reactivity before potentially rebounding to lower cortisol reactivity. Study 4 (Chapter 6) showed that females with a NR stress response had higher symptoms of depression than RR/AR profiles, suggesting that the low NR cortisol response is associated with more severe depression, consistent with Harkness et al. (2011). The attenuation of the stress response over time can be considered an adaptive strategy to cope with a stressful environment. Down-regulation of the stress system enables the individual to avoid chronic arousal and energy expenditure, eventually leading to negative health consequences (Agorastos & Chrousos, 2022; Susman, 2006).

Therefore, while early life stress shows clear associations with adult depression and anxiety symptoms, it shows the potential to both increase and decrease HPA-axis reactivity over time in relation to these symptoms. The polyvagal model of the stress response, whilst focusing on the autonomic nervous system, is useful for conceptualising what appears to be occurring with the HPA-axis regarding the influence of early life stress exposures and adult mental health, and this will be discussed below.

7.4.1 Reinterpretation of the stress response data using polyvagal theory

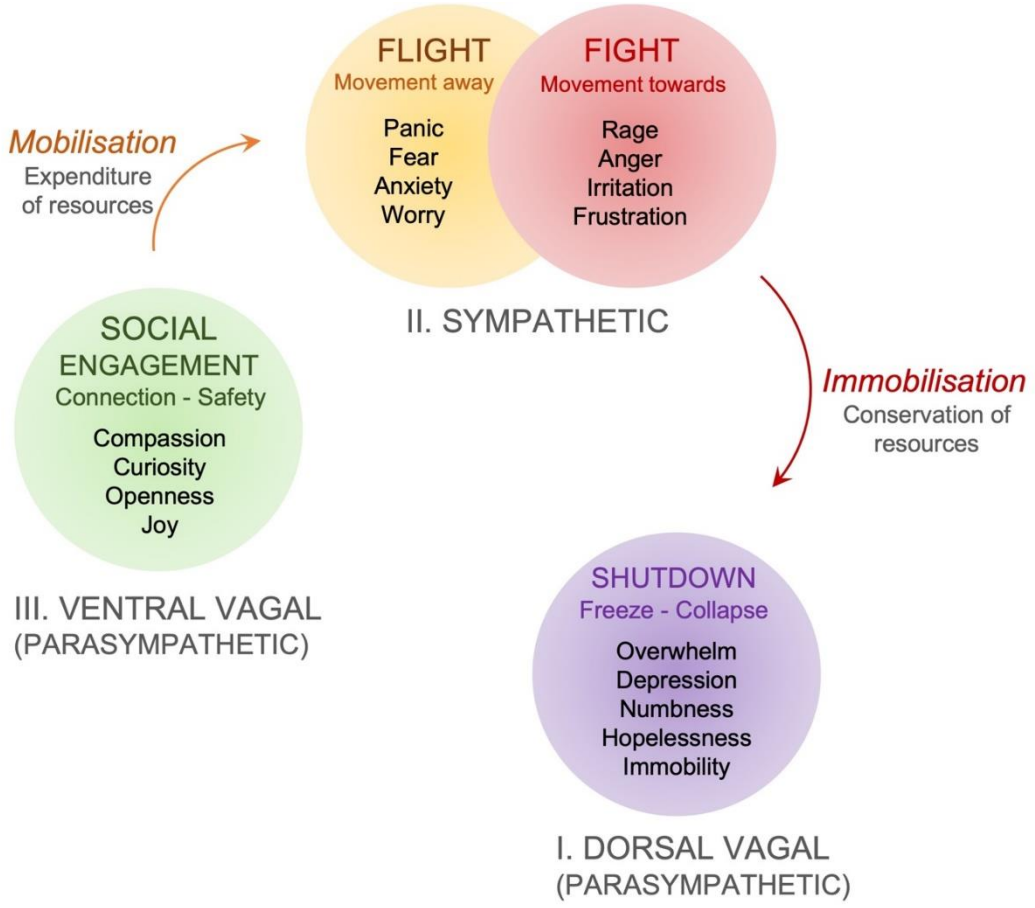
Polyvagal theory can be used as a framework for interpreting the results from this thesis as it describes hyperactivity and mobilisation of resources as potentially being on the path to hypoactivity and immobilisation, with successive or greater threats to safety (Porges, 2007). Although the ANS and HPA-axis are often studied separately, they are inextricably linked. They differ temporally, with the ANS responding to a threat within milliseconds and the HPA-axis hormonal response occurring within 20 minutes but having longer-lasting effects. However, the ANS and HPA-axis are highly coordinated and physically interconnected (Glier et al., 2022; Rotenberg & McGrath, 2016) and recent work suggests a dynamic interplay between them in responding to external stressors (Agorastos & Chrousos, 2022; Glier et al., 2022; Nederhof et al., 2015; Rotenberg & McGrath, 2016).

Traditional definitions of the ANS can be recognised within the framework of polyvagal theory, with the term ‘rest and digest’ used to describe being in the parasympathetic state and the term ‘fight or flight’ used to describe the sympathetic state. The transition to an activated state in the TSST can be seen by the increase of ACTH and cortisol with the laboratory stressor. Both RR and AR represent activated states. Polyvagal theory, however, describes a third state of the nervous system akin to ‘freeze or collapse’ as being a second parasympathetic state and an adaptive response to threat if fight or flight is not an option or is inadequate (Porges, 2001). The two parasympathetic pathways depend on which branch of the vagus nerve is triggered, with the resting state described as Ventral Vagal and the freeze/collapse state described as Dorsal Vagal. The theory is based on the concept that organisms developed different physiological states as they evolved. Mammals, especially humans, have three physiological states: social engagement and safety, fight or flight and freeze/collapse/shutdown. For a graphical representation of these states, see Figure 7.1. This model describes hierarchical adaptive

responses to stressors with more recently evolved pathways used first, before defaulting to older evolved responses to threats in attempts for survival (Porges, 2001). This is the most ancient pathway in phylogenetic terms, represented by hiding or death feigning in animals and linked to immobilisation and conservation of energy (Porges, 2021). In humans, if the sympathetic state is activated over many years, such as with chronic stress exposures or if it is inappropriate/inadequate to cope with acute trauma, the nervous system may default to the dorsal vagal parasympathetic state. In terms of the HPA-axis response, this freeze/collapse state is best represented by the NR. In support of this, hypocortisolism has been found in individuals living under conditions of chronic stress (Heim et al., 2000). Moreover, a form of depression characterised by blunted HPA-axis activation and more profound fatigue reportedly represents a phenotype that is particularly common in females (Kuehner, 2017). This aligns with the proposed dorsal vagal state and the results from Study 4 (Chapter 6), showing that in females, NR showed the highest symptoms of depression. From this perspective, changes in the HPA-axis may be one part of a much larger systemic response to threat. Nonetheless, this thesis allows comment on potential HPA-axis involvement in the relationship between early life stressors and later life mental health, and this will be discussed below.

Figure 7.1

Graphical representation of the different physiological response states according to polyvagal theory (S. W. Porges, 2001; Stephen W. Porges, 2021)



7.4.2 Assessing HPA-axis involvement in the relationship between stress exposure and depression/anxiety

Whilst many allostatic load models have proposed that early life stressors cause changes in the regulation of the HPA-axis stress response, leading to increased vulnerability to conditions of depression and anxiety, the majority of studies have only examined one part of this relationship. The main limitation with advancing knowledge in this area has been the lack of long-term longitudinal studies examining all three components of this pathway. This thesis examined the timing of early life stress, the reactivity of the HPA-axis at age 18 and adult symptoms of depression and anxiety in the same community population cohort over 20 years. Studies 1-4 present evidence that relationships between common early life stressors, HPA-axis reactivity, and adult symptoms of depression and anxiety can be detected by age 20.

In Study 2 (Chapter 4), although prenatal stress exposure and chronic or increasing stress exposure around adolescence were linked to adult depression and anxiety in this sample, the relatively weak relationships detected in Study 3 (Chapter 5), between early life stress exposure and adult HPA-axis reactivity, and in Study 4 (Chapter 6), between adult HPA-axis reactivity and symptoms of depression and anxiety, suggest that the HPA-axis is unlikely to be a strong mediator of this relationship. This means that the effect of early life stress on depression and anxiety symptomatology in early adulthood is likely to involve pathways other than HPA-axis dysregulation. However, this conclusion needs to be tempered with regard to the limitations of the thesis data; studies 1-4 examined only common life stress events, the TSST was assessed at one point in time only, at age 18 years, and depression and anxiety symptoms were assessed over a short time frame by self-report questionnaire at age 20 only. The associations with the HPA pathway may be stronger with more severe early life trauma and HPA-axis reactivity measurement in an older population, as dysregulation of cortisol reactivity may occur gradually over many years. Associations may also be stronger with more severe depression and anxiety symptomatology, and fluctuations in symptoms may occur over the lifespan. Nonetheless, these results join others (discussed in the paragraph below) to challenge a prevailing narrative that dysregulations of the HPA-axis may explain this stress-disorder association (McEwen, 1998).

Although rare, a handful of studies have examined HPA-axis reactivity as a potential mediator between exposure to stressors and depressive/internalising symptoms, and results have pointed to increases and decreases in HPA-axis reactivity as a moderator but not a mediator of the relationship.

In a study of 156 men (age 18-35 years), child maltreatment was associated with a higher risk of depressive symptoms in participants with a higher TSST cortisol response to stress compared to those with a lower response (Cantave et al., 2019). Another study of 166 students (age 9-15 years) showed an interaction of greater stress exposure over the past year with lower levels of cortisol reactivity to predict elevations in depressive symptoms over time (Badanes et al., 2011). More life stress events were related to greater internalising problems with higher cortisol reactivity compared to those with lower cortisol reactivity in 15-year-olds (*n* = 100) (Steeger et al., 2017), and childhood maltreatment was related to greater internalising problems among 18-22-year-old

participants with higher cortisol reactivity, compared to those with low cortisol reactivity in (*n* = 88) (Hagan et al., 2014). A study of 121 youth (9-16-year-olds) showed that exposure to emotional abuse and trauma was associated with more internalising symptoms when youth showed higher cortisol reactivity. In contrast, in the same study, exposure to physical abuse was associated with higher internalising symptoms when youth had blunted cortisol reactivity (Kuhlman et al., 2018). Interestingly, these interaction effects between early life stressors and cortisol reactivity were also detected in studies examining links with externalising behaviours (Busso et al., 2017; Hagan et al., 2014; Kuhlman et al., 2018; Steeger et al., 2017), suggesting a similar effect for other mental health outcome measures. Therefore, the strength of the association between early life stressors and later life depression and anxiety symptoms appears to vary across levels of HPA-axis reactivity. High and low cortisol reactivity may be a marker of previous stress exposure, a marker of the ability of the individual to actively manage life stressors (Badanes et al., 2011) and a marker of risk/vulnerability for current or future mental health challenges.

7.4.3 Other biological pathways in addition to the HPA-axis

The results from this thesis suggest that pathways and mechanisms other than the HPA-axis are involved in the relationship between exposure to early life stress and the development of depression and anxiety symptoms in adulthood. Indeed, the HPA-axis cascade of hormone release is only one part of the body's response to a stressor. As mentioned in Section 7.4.1 above, the ANS response to stress is closely connected with the HPA-axis, and these two systems are increasingly being studied together. Whilst complementary patterns of reactivity are expected under normal circumstances (Glier et al., 2022), emerging evidence suggests the ANS and HPA-axis may show divergence from each other after early life stress or trauma (Agorastos et al., 2018; Nederhof et al., 2015). This progressive disconnect between the two main limbs of the stress response system represents another potential pathophysiological pathway between exposure to early life stress and negative health outcomes in later life (Pervanidou, 2008).

Other biological pathways that may also play a role in the relationship between stress exposure and poor mental health include the immune system and inflammation; the gut-brain axis and the microbiome; sleep and circadian rhythm; and genetic/epigenetic regulation. They are briefly outlined below, and a more detailed

discussion of the proposed mechanisms underpinning these pathways is reviewed elsewhere (Agorastos et al., 2019; Lippard & Nemeroff, 2022)

With regard to the immune system, increases in the inflammatory markers, CRP and IL-6 have been found in individuals with a history of early life trauma and individuals with mood disorders (Y. Z. Liu et al., 2017). In addition, some improvements in depressive symptoms have been reported in Major Depressive Disorder patients treated with anti-inflammatory drugs and anti-depressant medications have been associated with decreases in pro-inflammatory cytokines (Slavich & Irwin, 2014). Stress exposure and depression have also been related to changes in the intestinal microbiota, including diversity and quantities of specific bacterial strains (Agorastos et al., 2019). Specific gut microbes secrete neurotransmitters, now recognised as relevant to mental health, and can also limit the availability of serotonin precursors (Misiak et al., 2020). In addition, sleep disturbances are common with exposure to stress and in conditions of depression and anxiety, and alterations in the circadian rhythm, which may occur over years, have been shown to impact stress response and sensitivity to stressors (Agorastos et al., 2019). In terms of genetics, specific candidate genes and stress-induced epigenetic modifications appear to increase susceptibility to stressors, and their interaction with environmental factors likely contributes to vulnerability to poor mental health (Agorastos et al., 2019; Martins et al., 2022).

It is of note that all these pathways overlap and are interconnected with the ANS/HPA-axis stress response systems. Both increased stress reactivity/hypercortisolism and reduced stress reactivity/hypocortisolism induce secondary changes in a spectrum of factors that will impact the immune system, gastrointestinal environment, sleep, metabolism and food intake and reproductive hormones and some of these relationships are bidirectional. Given this potential for overlap, it will be useful to consider multisystem integration approaches to further understand how early life and continuing stress increases the risk of psychopathology (Koss & Gunnar, 2018).

7.5 Evidence for sex differences in all aspects of this work.

The notion that stress exposure in early life predisposes individuals to later depression and anxiety conditions, and the fact that women show double the prevalence of these conditions compared with men, suggests there are sex-specific pathways

between exposure to stressors and the development of symptoms of depression and anxiety. As men and women also differ in their physiological response to a stressor (via HPA-axis/cortisol reactivity (Kirschbaum et al., 1999), the HPA-axis has been investigated as one of these pathways. However, whilst the potential confounding by sex has been controlled for in statistical models evaluating the relationships between [1] early life stress and adult depression and anxiety symptoms, [2] early life stress and HPA-axis reactivity and [3] HPA-axis reactivity and adult depression and anxiety symptoms, sex is rarely examined as a moderator of these relationships, i.e. associations evaluated separately in males and females and tested for differences in strength or direction. Studies have mostly been small, particularly those incorporating the HPA-axis reactivity to a stressor (n<150), as discussed in Section 2.5.1.1, so they may have been underpowered to detect sex differences. Therefore, there has been a call for studies examining these pathways to stratify analyses by biological sex (Gobinath et al., 2015; Hyde & Mezulis, 2020; Sutherland & Brunwasser, 2018).

This PhD examined sex-specific interrelationships between early life exposure to common life stressors prenatally to age 17, cortisol reactivity to a social stressor using the gold-standard TSST at age 18 and symptoms of depression, anxiety and stress using the DASS-21 at age 20 in a large well characterised community cohort with final analysis numbers ranging from 700-1000. This PhD thesis offers further evidence that there are sex differences in every aspect of this research. Below is a brief discussion of each study concerning sex differences in relation to the literature, followed by potential biological, cultural and evolutionary mechanisms.

7.5.1 Study 1 sex differences

Study 1 (Chapter 3) examined sex differences in the HPA-axis response to a psychosocial stress ; results showed higher HPA-axis reactivity to a social stressor in males compared to females. These results align with a recent meta-analysis examining salivary cortisol as the preferred measure in the literature, which found cortisol reactivity to a social stressor was higher in males compared to females, particularly at the peak cortisol concentration (J. J. W. Liu et al., 2017). However, Study 1 extends these data to show that plasma cortisol and ACTH reactivity following a social stressor are also higher in males compared to females (not taking OC), consistent with other smaller studies (Kirschbaum et al., 1999). Furthermore, males were more likely to be a RR, and less likely

to be a NR than females. These results have since been supported by other reports of a greater proportion of responders being male and more blunted responses to stress in females with the TSST (Fiksdal et al., 2019). In summary, this indicates that on average, adult males and females show consistently different responses to a psychosocial stressor with respect to the secretion of ACTH and cortisol.

7.5.2 Study 2 sex differences

Study 2 (Chapter 4) investigated sex differences regarding the associations between stress exposures in early life and adult symptoms of depression and anxiety; in males, high prenatal stress was associated with higher adult depression and anxiety symptoms, whereas in females, the medium, high and ascending postnatal stress trajectories were associated with higher adult depression and anxiety symptoms. These sex-specific findings align with other research indicating that for boys, exposure to prenatal stressors in the first trimester was associated with higher internalising behaviours at or before five years of age (de Bruijn et al., 2009; Martin et al., 1999), and evidence that in girls, stress exposures around adolescence were associated with increases in depressive symptoms (Laceulle et al., 2014; Oldehinkel & Bouma, 2011). For a discussion of sex differences regarding the timing of stress exposure prenatally or over adolescence, see Section 7.3. Overall, the literature is not unanimous on whether the effects of early life stress on depression and anxiety in later life differ between males and females (Gallo et al., 2018). However, there is some evidence from a meta-analysis that the effect size of childhood abuse on adult major depression and generalised anxiety is larger in women than men (Gallo et al., 2018). In addition, lower associations of early life stress with depression in males than females may be observed because, in males, the impact of early life stressors manifests differently, potentially more in the development of externalising conditions, conduct disorders and substance use, all of which are more prevalent in men (Eaton et al., 2012). In summary, Study 2 (Chapter 4) indicates that there are clear sex differences in the relationship between exposure to common early life stress events and adult depression and anxiety, which may be related to the timing of the stressor exposure.

7.5.3 Study 3 sex differences

Study 3 (Chapter 5) examined sex differences in the association between early life stress exposures and cortisol reactivity to a stressor at age 18; in males but not females,

high prenatal stress was associated with heightened cortisol reactivity, as discussed in Section 7.3.1.

Males in the ascending stress trajectory were more likely to be NRs to the TSST than females, whilst females in this trajectory were more likely to be responders than males. This is evidence for a divergent stress response in males and females experiencing a similar pattern of increasing stress exposure around adolescence. However, it is important to note that the prevalence of the NR pattern in the ascending trajectory was relatively low in both sexes (19% in males and 12% in females). The results of this study are in contrast to the results of a recent meta-analysis of 30 studies that found larger effect sizes for more blunted cortisol reactivity (similar to NR) in females than males after early life adversity (Bunea et al., 2017). However, in that meta-analysis, stress exposures focused on more severe traumas, and multiple exposures were collapsed into one measure, which misses nuances of timing. They report a greater effect size with; [1] maltreatment compared to other adversities and [2] adult stress response compared to stress response in children/youth. Therefore, the impact of stress exposure on the HPA-axis in males and females may differ with the timing of stress exposure or measurement of the stress response and with more severe trauma. This may explain why Study 3 did not find the same effects, measuring more common life stress events at age 18 and separating out timing issues. A second meta-analysis found no significant relationship between early life trauma and cortisol reactivity (9 studies) and no modification by sex (Fogelman & Canli, 2018), although they also considered other types of cortisol measurement in addition to the TSST. In summary, Study 3 points to sex as a potential moderator of the relationship between exposure to early life stressors and the cortisol response to a stressor in early adulthood.

7.5.4 Study 4 sex differences

Study 4 (Chapter 6) consisted of an enquiry into sex differences in the association between cortisol reactivity to a stressor and adult symptoms of depression and anxiety; results showed that in females, TSST responder category was associated with symptoms of depression and anxiety with NRs having the highest symptoms and ARs having the lowest symptoms. No associations were found in males. The potential protective effect of the AR against depression is discussed in Section 7.2.2. The finding of higher symptomatology in NRs in the female sample of this study is supported by a recent meta-

analysis of 14 studies showing that MDD and anxiety disorders were associated with a blunted cortisol response in females but not males compared to controls (Zorn et al., 2017). A study in an older community sample (age 55-60 years) found similar reduced cortisol responses to stress with higher depression or anxiety symptoms but did not differentiate by sex (de Rooij et al., 2010). In contrast, Fiksdal et al. (2019) report increased cortisol reactivity and no sex differences with symptoms of depression in a student sample, although this difference may be because the authors removed 'non-responders' from analyses. Interestingly, Study 4 (Chapter 6) showed no association of cortisol reactivity with depression and anxiety symptoms in males. This contrasts with the meta-analysis of Zorn et al. (2017), which reported that males with MDD or anxiety disorders had an elevated cortisol response to stress as measured by AUCs. There are several points to discuss concerning this inconsistency. Firstly, diagnosis of a clinical disorder indicates a higher severity level than detection of symptoms; therefore, associations in males may emerge with more severe symptoms/conditions. Secondly, in Study 4 (Table 6.2), there is evidence for a sex interaction with stress responder category and the DASS-Depression scale, and it is interesting to note that while the estimates for NR and DASS-Dep are positive in females (10.10 NR vs 9.03 RR and 6.97 AR), they are negative in males (6.05 NR vs 6.93 RR and 8.15 AR), suggesting the reverse association; lower depression/anxiety symptoms in male NRs and higher depression/anxiety symptoms in male responders which would be consistent with the results of Zorn et al. (2017) and Fiksdal (2019). One caveat to consider with this interpretation is that the 95% CIs are wide for the male results, and it cannot be ruled out that minimal differences exist. Thirdly, the lack of association in males of stress response with depression and anxiety in Study 4 may be partially explained by the generalised tendency of females towards expressing internalising symptoms and males towards expressing more externalising symptoms/behaviours (Eaton et al., 2012) as discussed in reference to the findings of Study 2 (Chapter 4) above. Therefore, a different outcome measure may be more relevant for detecting associations between cortisol stress response and mental health in males. In support of this, a blunted cortisol response has also been associated with persistent antisocial behaviour (Susman, 2006). In summary, Study 4 suggests that the pattern of response to a psychosocial stressor may convey differing vulnerability to depression and anxiety in males and females.

7.5.5 Potential mechanisms for sex differences

In terms of the mechanisms involved in the sex differences observed between exposures to stress, the physiological response to stressors and the manifestation of depression and anxiety, it is recognised that there are biological, cultural, psychological and potentially evolutionary effects at play. Biological mechanisms for the sex-specific impact of exposure to prenatal stress and increasing stress around adolescence are discussed in Sections 7.3.2 and 7.3.3. As an overview, in-utero there are sex-specific patterns of fetal adaptation, growth and development with exposure to stressors, related to the response of the placenta, whereby in response to stress exposure the female fetus adjusts growth but the male fetus does not (Howland et al., 2017). It is proposed that the female placenta may be more sensitive and responsive to changes in maternal cortisol levels than the male placenta (Howland et al., 2017). The male fetus appears to put a higher investment into physical growth, potentially rendering them less adaptable to fluctuating conditions in utero and more susceptible to poorer developmental outcomes (DiPietro & Voegtline, 2017). Later in childhood, the production of sex hormones at puberty coincides with large changes in HPA-axis activity (Romeo, 2010b) and emerging differences in the prevalence of depression and anxiety in males and females (Cyranowski et al., 2000), highlighting this time as a window of vulnerability.

In terms of other biological variations, there are reported sex differences in the CRF receptor binding affinity and signalling process, affecting the downstream production of ACTH (Bangasser & Valentino, 2014) and sex differences in biological and behavioural responses to stress in animal models are well established (Hodes, 2018; Hodes & Epperson, 2019). Furthermore, male and female brains show differing patterns of growth and maturation over development (Giedd et al., 1996), and there are proposed sex differences in emotional responses to stressors, including suggestions that females may experience negative emotions with more intensity than men. In support of this, neuroimaging studies have shown greater activation of cortico-limbic circuits (involving the hippocampus, prefrontal cortex and amygdala) in response to stressors in females (Bangasser & Valentino, 2014). These are the same neural circuits underlying emotional responses and signalling to the HPA-axis. Therefore, chronic stressor exposure could have a greater impact on females than males in terms of mental health via this

mechanism, as found in Study 2 (Chapter 4) where females with medium to high chronic exposure to stressors showed increased symptoms of adult depression and anxiety.

Whilst this thesis focuses on biological mechanisms for sex differences, it is acknowledged that there is an ongoing and complex interplay between biological, cultural and psychological factors. Male and female children are generally treated differently from birth. In traditional patriarchal cultures, there are different gender roles/societal expectations placed on girls and specific qualities that are valued in girls compared to boys. The treatment of females and the characteristics selected for (including self-sacrifice, nurture, and submissiveness) may have, in part, led to systematic psychological differences in the female sense of self and tendencies towards low self-esteem, poor self-judgement, negative interpretations of events and rumination (Nolen-Hoeksema, 1990), all of which may increase sensitivity to negative life events/stressors and vulnerability to depression and anxiety. This may also contribute to sex differences in the interpretation of a situation as threatening, with males and females reportedly more sensitive to threats to achievement and social relationships, respectively (Taylor et al., 2000).

From an evolutionary perspective, there may be advantages for males and females to develop different patterns of stress reactivity in reaction to signals of stress from their environment during development and/or tendencies towards internalising conditions. Stress exposure may affect males and females differently as they evolved with specific societal roles conducive to the survival of the individual, offspring, tribe and community. Female stress responses have theoretically been honed to 'tend and befriend'; tend to children to reduce risk and maximise safety, and befriend others for social support and help (Taylor et al., 2000). Male stress responses, in contrast may have been honed more for 'fight or flight' behaviours involving aggression and mobilisation to fight off predators and other threats. From an evolutionary point of view, exposure to stressors resulting in the development of symptoms of depression and anxiety and changes in the stress response system may have been adaptive in a stressful environment. For example, anxiety and forms of HPA-axis hyperreactivity may be associated with increased vigilance and alertness to danger; in depression, the sadness, emotional withdrawal and fatigue, also associated with HPA-axis hyporeactivity, may lead to support from others and conservation of energy (Glover, 2011). Regarding developmental programming, conditions in utero may confer sex-specific epigenetic changes to prepare the fetus for

the external environment, so prenatal stress may adjust HPA-axis sensitivity in anticipation of a stressful environment differentially in males and females (Glover & Hill, 2012). For example, the association of high prenatal stress with increased cortisol stress reactivity in males, reported in Study 3 (Chapter 5), may represent an adaption to improve coping in an environment that is predicted to be hostile via the upregulation of cortisol reactivity. Whilst adolescence is identified as a point of recalibration of the HPA-axis, exposure to stressors throughout childhood will result in an accumulation of adaptive calibrations in cognitive and behavioural strategies to ensure survival in a changing or potentially challenging environment (Karatsoreos & McEwen, 2013). These adaptations are likely to differ in males and females with the varying social and cultural environments.

To conclude this section, this thesis provides evidence of sex-specific vulnerability to early life stress exposure, modifications of adult HPA-axis reactivity and susceptibility to symptoms of depression and anxiety. Accordingly, the experience of stressors in the early environment may lead to different stress reactivity in males and females and varying stress-sensitive outcomes. There are biological, cultural and evolutionary mechanisms contributing to these differences that are potentially relevant to other stress-related conditions. From a practical point of view, these sex differences have implications for future studies in the field. They emphasise the importance of considering and reporting results stratified by sex to detect different relationships in males and females. Studies maximising power to identify sex differences are recommended, and as large samples are challenging for studies of the stress response, meta-analyses specifically testing sex-specific differences may be needed.

7.6 Strengths and limitations of this thesis

This body of research examines exposure to early life stress, the cortisol response to stress and adult depression and anxiety symptoms in the same cohort of participants over 20+ years. This long-term design is the primary strength of this thesis, as it is vital for examining the role of the HPA-axis in the relationship between early life stress exposure and later life mental health symptoms. Collectively, studies 2, 3 and 4 address a large gap in the literature as most studies can only address two components of this relationship in isolation. The thesis facilitates a number of conclusions, not only about the HPA-axis involvement but regarding the timing of stressor exposure and differences

between males and females in these relationships. The following sections summarise and comment on the various strengths and limitations of the studies contained within this thesis in the following order: the Raine Study cohort, the life stress event exposures, the TSST measure of the HPA-axis response to stress, the measurement of depression and anxiety symptoms and the statistical analyses used.

7.6.1 The Raine Study Cohort

One strength of this doctoral thesis is the utilisation of the Raine Study cohort, a large well characterised community-based population. As such, the four studies contained in the thesis may offer better generalisability to the general population than case-control and clinical population studies. When Raine Study participant characteristics were compared to those of similarly aged youth in the general population at the 8-year, 14 and 17-year and 20-year follow-ups (using corresponding census data from 2001, 2006 and 2011 surveys), the Raine Study sample was found to be widely representative of the general population, over a large range of variables (Straker et al., 2017). The Raine Study data have been prospectively collected, and this prospective longitudinal design minimises participant recall bias as data is collected before outcomes manifest. In addition, the Raine Study has collected detailed information on many risk factors, enabling the adjustment for multiple confounding factors in statistical analyses. Long-term pregnancy cohorts of this nature are rare and, therefore, increasingly valuable for examining the early life origins of adult stress responses and conditions of depression/anxiety.

There are also limitations to the use of cohort studies that deserve acknowledgement. The Raine Study cohort has seen incomplete or interrupted follow-up, with selective attrition of participants over time, similar to other long-term cohort studies (de Graaf et al., 2013; Oldehinkel et al., 2015; Radler & Ryff, 2010; Satherley et al., 2015). In the Raine Study at the Gen-2-20 year follow up, there were 2313 eligible participants contacted, and 1462 participated (51% of the original Gen2 participants) in data collection (Straker et al., 2017). As with other long-term cohort studies, there has been greater attrition of socially disadvantaged participants (White et al., 2017). Considering the link between low socioeconomic status and higher levels of stress exposure, it's possible this cohort may have seen attrition of participants with the highest levels of stress exposure. Furthermore, the Raine study participants are a predominantly

Caucasian population from one location, limiting the generalisability of the results to different populations. Nonetheless, cohort studies employing longitudinal methods provide a comprehensive approach to life-course research that allows understanding of the degree and direction of change in measures of interest over time (Caruana et al., 2015).

7.6.2 Early life stress event exposure

With regard to the stress exposure data, strengths include the number of collection times and consistency of life stress events examined. The same life stress events were measured at ten timepoints over 17 years (including two timepoints over pregnancy), enabling examination of the timing of stress exposure prenatally, over childhood and adolescence. Moreover, this work examined common life stress events, including items such as separation and divorce, death of a close relative or friend, job loss and financial problems, ensuring results are relevant and translatable to the general population.

With regard to the limitations of the stress exposure data, early life exposure to the same stressor can affect two individuals differently, and parent report is an indirect measure of stress exposure for the child. Nonetheless, the presence or absence of specific negative life events in the past year (or four months during pregnancy) is less subjective than other retrospective assessments of stress appraisals and symptoms. The effects of life stress events may be ameliorated or buffered by parental coping strategies, parenting style, family functioning, nurture and support, although it was not within the scope of this thesis to assess this. In addition, whilst five different stress trajectories were delineated in Study 2 (Chapter 4), not everyone will fit directly into a specific trajectory and intermediate trajectories will exist. Also, these studies did not examine severe cases of childhood maltreatment, trauma and abuse and it's possible that relationships between childhood stress exposure and adult HPA-axis reactivity (examined in Study 3, Chapter 5) may be more evident with more severe maltreatment (Bunea et al., 2017).

7.6.3 HPA-axis response to stress

In assessing the stress response, one major strength of the HPA-axis data collection at age 18 is the use of the gold standard Trier Social Stress Test. This represents the largest TSST conducted in any population (n = 1137), allowing a comprehensive investigation of normative data in early adulthood, sex differences and concurrent

measurement over the three levels of the HPA-axis; plasma ACTH, plasma cortisol and salivary cortisol. As salivary cortisol is now recognized as the method of choice and the costs associated with blood measures can be prohibitive, Study 1 (Chapter 3) represents a unique collection of data. Another advantage is the timing at which the TSST was performed, after the critical transitions of puberty when the HPA-axis reaches a new set point, more indicative of long-term functioning in adulthood (DePasquale et al., 2021), compared to TSSTs conducted in childhood, when the HPA-axis is relatively hypo-reactive (Gunnar & Cheatham, 2003). A further strength of this TSST study involved the introduction of a novel way of examining patterns of stress response data. Study 1 defined, characterized and analysed 3 categories of hormonal stress response: Reactive Responders, Anticipatory-Responders and Non-Responders and compared them with a number of standard traditional hormone reactivity measures. This method proved to be a useful way to examine the relationship between early life stress exposure and adult cortisol reactivity in Study 3 (Chapter 5) and the relationship between cortisol reactivity and adult symptoms of depression and anxiety in Study 4 (Chapter 6).

In terms of limitations in the assessment of stress response using the TSST, researchers did not take a sample on arrival at the laboratory. Baseline samples on arrival would have allowed a better understanding of the anticipatory response, although this was not standard practice when these studies were conducted. In addition, whilst there are benefits to having the detailed ACTH and cortisol information from blood, the use of cannulation may have impacted the stress response of some individuals. Nonetheless, participants knew in advance that they would be given a choice of blood versus saliva collection on the test day, and the rest period between cannulation and the beginning of the test was consistent with other studies and recommendations in the literature (Kirschbaum et al., 1999; Kudielka et al., 2007). It should also be noted that this TSST, like most others in the literature, captures the HPA-axis reactivity to a social stressor at only one point in time There is evidence that the stress response may change over time in response to chronic activation, transitioning from the metabolically costly hyperactivity to the metabolically conservative hypoactivity (Booij et al., 2013; Carpenter et al., 2009; Miller et al., 2007). This transition may explain why studies 3 and 4 did not find stronger relationships between HPA-axis function and early life stress/symptoms of depression and anxiety. If the TSST is measured at a single point in time, yet participants are at different stages of this transition, detection of a consistent effect will be less likely and

result in reduced ability to discern true associations between HPA axis function and early life stress or mental health. Therefore, TSSTs conducted in an older population after the main window of adolescent adaptation may show different results. On the other hand, this transition from hyperactivity to hypoactivity may equally occur in older populations, depending on current life stressors, and may represent a limitation with all single timepoint TSST studies. Longitudinal studies with repeated stress exposure, TSST and mental health measures would be required to address these issues.

7.6.4 Depression and anxiety outcome measure

With regard to the mental health outcome measure, a further strength of this work is the use of symptoms of depression, anxiety and stress as compared to a categorical diagnosis. Evidence suggests that dimensional measures (examining a continuum instead of a binary diagnosis) offer richer and more detailed information and examining symptoms is important as their expression below diagnostic thresholds is very common (Solis et al., 2021). In addition, the use of the DASS-21 enabled both an overview of total symptoms and a more detailed look at the individual depression, anxiety and stress subscales, where indicated.

In regard to the limitations of the mental health outcome measure, the DASS-21 represents a short term measure of mental health over the last week and is not an indicator of long-term mental health, although it has been shown to have relatively good consistency over three years (Lovibond, 1998). Furthermore, self-report instruments like the DASS-21 are subject to various biases such as social desirability, poor recall and limited participant attention or insight which may impact its validity (DeVellis & Thorpe, 2021). While this thesis examined depression and anxiety symptoms at age 20, there is the potential for participants' symptoms to increase or decrease after this date which may affect associations with early life stress and HPA-axis reactivity in future. In addition, mental health symptoms were not assessed at the time of the TSST, so this work does not incorporate concurrent data on HPA-reactivity and mental health symptomatology.

7.6.5 Statistical analyses

Robust statistical methods have been used in the thesis, including latent class analysis for the identification of stress trajectories. Multivariate regression analyses with consideration and adjustment for potential confounders underpinned all major findings.

For example, poverty and low socioeconomic status are highly correlated with increased exposure to stressors and poor mental health (Ridley et al., 2020; Turner & Avison, 2003) so are essential to consider as potential confounders in the relationships between early life stress and both HPA-axis reactivity and symptoms of depression and anxiety.

Furthermore, sex was investigated as a modifier in all analyses. Although many studies incorporate sex into their models, the work in this thesis consciously and consistently examines relationships in males and relationships in females, between stress exposure, stress reactivity and symptoms of depression and anxiety. This evaluation of sex differences is based on a strong biological and evidence-based rationale and has been called out as a neglected area of research in the field (Gobinath et al., 2015; Koss & Gunnar, 2018; Powers et al., 2016; Raymond et al., 2018; Rincon-Cortes et al., 2019).

With regard to the limitations of the statistical analyses used in this thesis, investigations by sex and responder category were often exploratory by nature due to the limited and/or inconsistent findings in the literature impeding specific directional hypotheses. Furthermore, findings were reported separately for sex even if there was no statistical evidence for differences in associations by sex. This decision was based on biological plausibility and prior evidence for sex differences in early life programming (Carpenter et al., 2017; Glover & Hill, 2012; Sandman et al., 2013), the cortisol response to a stressor (Allen et al., 2017; Kirschbaum, Wust, & Hellhammer, 1992) the prevalence of depression and anxiety (Cyranowski et al., 2000) and associations between cortisol reactivity and mental health (Zorn et al., 2017). The analyses in this thesis were not adjusted for multiple testing, therefore, we acknowledge that there is the possibility some of the findings may be spurious in the context of null hypothesis testing. Due to recent calls from leading statistical associations to abandon the dichotomisation of p-values and reliance on strict significance thresholds (Amrhein et al., 2019; Anderson, 2019; Wasserstein et al., 2019), estimates were transparently reported along with the size and certainty of tested associations, incorporating 95% confidence intervals and associated p-values as continuous, descriptive statistics providing complementary information regarding the strength of the evidence against null associations, as recommended by the American Statistical Association (Wasserstein et al., 2019). Furthermore, despite the richness of the Raine Study data, residual confounding factors may exist.

Finally, whilst this is longitudinal data and the triangular diagrammatic representation of the studies may suggest mediation analysis as a useful approach to assess the role of the HPA axis in mediating the effect of life stress events in pregnancy, childhood and adolescence on adult mental health, this was not conducted as part of the work of this thesis. The combination of an absence of any strong associations between life stress events and HPA axis functioning identified in Study 3 (Chapter 5), differing sex-specific associations in all studies, and the limitations discussed above concerning the long time period between measures of the three constructs meant that a single mediation analysis would be inappropriate and uninformative. However, the thesis does provide direction for the design of future mediation studies as to the role of the HPA-axis as a mediator between stress and mental health, as discussed below in Section 7.7.

7.7 Directions for future research

The research in this thesis serves as a guide for the design of future studies examining this topic. Whilst suggestions for future research have been partially covered at the end of each study, the main points are summarised here. Firstly, it is recommended that future research consider better capture of the spectrum of stress exposures, from common life stress events to more severe forms of trauma. Secondly, examine relationships in both males and females which requires sufficiently large sample sizes and sex-specific analyses. Thirdly, consider assessment of change over time of HPA-axis function by measuring cortisol reactivity at more than one time point after the onset of puberty. Finally, consider capture of adult mental health over a longer time window, preferably in close proximity to measures of HPA-axis reactivity to address temporal issues.

A further suggestion for studies employing the TSST is to use multiple parameters for the assessment of HPA-axis reactivity. HPA-axis or cortisol reactivity is described by researchers using many different measures but they do not all assess the same thing. A significant part of this thesis involved the assessment of HPA-axis reactivity over multiple parameters and results from Studies 1, 3 and 4 (Chapters 3, 5 and 6) showed the benefits of using responder category together with traditional summary measures. Therefore, it is recommended that multiple TSST measures be used alongside each other as each parameter will provide different nuanced information and the reliability of the results is increased if the effect is seen across different parameters. This is a viewpoint endorsed by others (Koss & Gunnar, 2018).

The thesis findings of a link between responder category and depression symptoms suggests future research could consider whether having a NR profile may predispose individuals to other stress-related conditions; and whether the NR category is associated with other aspects of health and disease. An attenuated cortisol response, as seen with NR, has been associated with negative health outcomes such as externalising disorders (Kuhlman 2018), panic disorder (Petrowski et al., 2010), chronic fatigue, fibromyalgia and chronic pelvic pain (Heim et al., 2000). Therefore, examining links between an individual's stress response profile and current or future health could be the subject of further research. The Raine Study for example has data on other relevant health measures including psychological, inflammatory, sleep, pain and addiction outcomes (Straker et al., 2017).

Furthermore, this thesis points to the involvement of other factors, in addition to stress exposure and HPA-axis response, influencing risk for adult depression and anxiety problems that could be considered in future research. Parenting style for example has been associated with adolescent mental health where high maternal control and high maternal care are associated with higher and lower rates of offspring depression and anxiety problems respectively (Eun et al., 2018). Parenting style may therefore interact with stress exposure to better predict mental health problems. Similarly, the presence and availability of another person at times of threat reduces ANS and HPA-axis reactivity (Gunnar, 2017) and depression (Braithwaite et al., 2017) and may therefore buffer the impact of stress exposure.

From a psychological perspective, certain coping strategies and cognitive styles may mediate the relationship between childhood adversity and later life depression (Panagou & MacBeth, 2022). Passive coping, repression and denial have been associated with more depressive symptoms (Cantave et al., 2019) and also with hypocortisolism (Heim et al., 2000). So, in addition to coping styles interacting with stress exposures to modify the risk of depression and anxiety problems, they may also be associated with stress responder categories. Future research could therefore examine whether the NR stress response, in particular, is associated with more negative styles of cognition and coping.

Whilst this thesis investigated one stress response pathway, the HPA-axis, future work needs to look at integrating multisystem measures. Current evidence suggests the HPA-axis and ANS response to a stressor are intricately connected and generally

coordinated, yet this is not always the case and asymmetry between the SNS and HPA-axis may also arise (Agorastos et al., 2018; Glier et al., 2022). The interaction between ANS and HPA-axis measures has been found to explain significantly greater variance in perceived stress (Rotenberg & McGrath, 2016), children's internalising symptoms (El-Sheikh et al., 2011) and adolescent depressive symptoms (Lucas-Thompson et al., 2018) than either stress system alone. Therefore, it may be the interaction of these responses combined that is more relevant to health and disease than any one response system (Buss et al., 2018; Glier et al., 2022). Furthermore, without taking into account the interconnected nature of these systems, studies may misattribute underlying mechanisms to the wrong system and in this way an incomplete framework may compound inconsistencies across studies (Glier et al., 2022). Heart rate variability (the variation in interval between heart beats) and respiratory sinus arrhythmia (the naturally occurring variation in heart rate that occurs during the breathing cycle) are used to assess parasympathetic activation or withdrawal and salivary alpha-amylase or skin conductance level can assess sympathetic activation (Glier et al., 2022; Karemaker, 2022), suggesting the potential for future research to specifically incorporate concurrent assessment of the ANS and HPA-axis systems.

7.8 Significance and implications

This work highlights the relevance of commonly occurring early life stress exposures to the experience of poor mental health in early adulthood. Whilst there remains a lack of understanding of mechanisms, and the exact pathways have not been fully elucidated, the findings of this thesis support the importance of strategies and interventions in early life, to reduce the negative impacts of stress exposure on mental health and wellbeing. Results highlighted the prenatal and adolescent windows of development as times of heightened vulnerability to the effects of stress exposure. The prenatal results support the need for pre-emptive strategies to manage and reduce controllable stress events around pregnancy and early life, interventions to improve the quality of maternal emotional health in pregnancy and beyond, and social support to buffer the impacts of stressors (Glover, 2014). The adolescent results support the need for targeted assessment and interventions (delivery of mental health and social support) during adolescence, around age 10-14, especially in those children suffering from chronic or recent stressors. Given the sex-specific associations identified in this thesis it is

recommended that evaluation of detection and intervention strategies is performed in both young males and females. In terms of detection of high stress exposure environments, there have been recommendations for screening questionnaires delivered by midwives and obstetricians (Glover, 2014) and screening of Adverse Childhood Experiences (ACE) in pediatric and medical settings (Gilgoff et al., 2020). These would provide clear opportunities for early detection of high stress exposure, intervention, and treatment. Early life intervention programs focused on the child and parents' wellbeing have proven successful in promoting healthy child development and reductions in depression and anxiety (Baughman et al., 2020; Davaasambuu et al., 2020; Izett et al., 2020).

Childhood stress exposure is notably prevalent in the general population with over 50% of adults reporting at least one form of childhood stressor (Green et al., 2010). Recent evidence suggests that the impacts of stress exposure may also be transferred to future generations (Hammen et al., 2012; Moog et al., 2022). A review and meta-analysis indicates that a 10-25% reduction in child maltreatment could potentially prevent 30-80 million depression and anxiety cases worldwide (Li et al 2016). Other analyses indicate investment in treatment of depression and anxiety will result in higher returns in terms of economic productivity and health (Chisholm et al., 2016). Therefore, the implementation of targeted and universal interventions, as mentioned above, have significant potential to improve mental health globally, given the prevalence and burden of stress exposure and mental health problems.

7.9 Conclusions

In summary, this body of research in a large prospective cohort study characterised the HPA-axis response to a psychosocial stress at age 18; showed how common life stress exposure in-utero, during childhood and adolescence is linked with later life depression/anxiety; showed how common life stress exposure in-utero and during adolescence may affect the regulation of the HPA-axis later in life and revealed how the type of cortisol response to a stressor in early adulthood is associated with symptoms of depression and anxiety. Notably, results indicated that prenatal stress exposures may have long-term consequences on depression and anxiety symptoms even after accounting for stress exposure after birth up to the age of 17 and highlight adolescence as a second window of vulnerability for common life stress exposures. The studies of this

thesis indicated sex-specific differences in how males and females respond to stress exposures physiologically, encouraging future analyses to stratify by sex. While this research pointed to some involvement of the HPA-axis in the relationship between common early life stress exposures and adult depression/anxiety symptoms, the lack of strong associations also suggests that other stress-related pathways are involved.

In conclusion, this thesis has advanced the understanding of links between early life stress exposure, the HPA-axis stress response and adult symptoms of depression and anxiety. This work has enabled better characterisation of physiological stress responses via responder category, and results support targeting interventions during pregnancy and adolescence, and further exploration of mechanisms using an integrated multi-pathway approach to future research.

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