Correlation of Rodent Behavioural and Physiological Measures Following Different Combinations of Lifetime Stress

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Abstract

There are more than 200 classified forms of mental disorders, with depressive and anxiety disorders as the most prevalent in the world. These disorders represent a societal burden due to the impact on an individual's occupational, social, and personal commitments. Often these depressive and anxiety disorders develop following a stressful life event or series of stressful life events. The diagnosis of depressive and anxiety disorders rely solely on behavioural symptomology despite the fact that stress events also result in physiological symptoms. Furthermore, examination of behavioural and physiological changes in depressive and anxiety disorders is difficult because of the heterogeneity these disorders have in humans due to individual reactions to stress. This heterogeneity can be mitigated in rodent models of depressive and anxiety disorders using a consistent stress as the trigger. Thus, it was hypothesised that there is a connection between the stress-induced changes to behaviour and physiology that can be examined in a rat model. The connection between behaviours and physiological markers to stress can be used in further research to stratify depressive and anxiety disorder diagnosis in humans.

A model combining early and recent life stress was designed to examine the longitudinal changes to behaviour in stress-resistant rats. At the end of the stress challenge post-mortem concentrations in stress hormones and cellular changes in adrenal glands, hippocampus, and hypothalamus were examined, based on the knowledge that stress often modifies these regions. The cumulative stress-induced behaviour and physiology changes were assessed for any associations. Given that stress impacts people differently depending on the circumstances, the model used a stress-resistant rat strain hypothesising that if stress-induced behaviour and physiology modifications occur in stress-resistant animals such changes will also occur in stress-susceptible strains.

The combination of early and recent life stress did result in modification of behaviour and physiology. The observed stress-induced change in rat behaviour was determined as anxiety-like behaviour. Physiologically, there were changes to chronic stress hormones but not acute stress

hormones. There were associations between the stress-induced cumulative behaviour and end-point physiology modifications. Integrating stress-induced physiological symptoms with the behavioural symptoms of depressive and anxiety disorders can further the stratification of disorder diagnosis. Further research is required to expand the behavioural and physiological measures included to better define depressive- and anxiety-like behaviour in rats. This improvement can be used to further the stratification of depressive and anxiety disorder diagnosis, as well as to investigate novel pharmacological treatments for depressive and anxiety disorders in humans.

Declaration

The University of New England

I certify that the substance of this thesis has not already been submitted for any degree or qualification and it is not currently being submitted for any other degree or qualification.

Additionally, I certify that to the best of my knowledge, any help received in preparing this thesis, and all sources used, have been acknowledged accordingly.

Signed: ___  ___ Date: 18/06/2018

Nicarla Sarah Glyde

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List of Abbreviations

5HT	5-hydroxytryptamine (Serotonin)
11-DOC	11-deoxycorticosterone
ABS	Australian Bureau of Statistics
ACTH	Adrenocorticotropic Hormone
AIHW	Australian Institute of Health and Welfare
ANOVA	Analysis of Variance
APA	American Psychologists Association
BDNF	Brain Derived Neurotrophic Factor
BrdU	Bromodeoxyuridine
BOOF	Behavioural Observation Open Field
CBT	Cognitive Behavioural Therapy
CE	Centre Entries
CI	Corner Immobility
CMS	Chronic Mild Stress
CUMS	Chronic Unpredictable Mild Stress
COMT	Catechol-O-Methyl Transferase
CRH	Corticotrophin Releasing Hormone
CT	Computed Tomography
DA	Dopamine
DALYs	Disability Adjusted Life Years
DRD4 VNTR	Dopamine receptor variable number tandem repeat
EEG	Electroencephalography
ELS	Early Life Stress
EPM	Elevated Plus Maze
fMRI	Functional Magnetic Resonance Imaging
FST	Forced Swim Test
FKBP5	FK506 binding protein 5 gene
GC	Glucocorticoid(s)
GH	Growth Hormone
GR	Glucocorticoid Receptor

GRE	Glucocorticoid-Response Elements
HMA	Horizontal Motor Activity
HPA	Hypothalamic-Pituitary-Adrenal
HSP	Heat Shock Proteins
IL	Interleukin
LC-MS	Liquid Column Mass Spectrometry
LH	Learned Helplessness
MANOVA	Multiple Analysis of Variance
MAO	Monoamine Oxidase
MAOA	Monoamine Oxidase A
MAOI	Monoamine Oxidase Inhibitor
MD	Maternal Deprivation
MD/S	Maternal Deprivation/Separation
MDD	Major Depressive Disorder
MDE	Major Depressive Episode
MES	2-morpholin-4-yl ethanesulfonic acid
MRI	Magnetic Resonance Imaging
MRM	Multiple Reaction Monitoring
NA	Noradrenaline
NDRI	Noradrenaline-Dopamine Reuptake Inhibitors
NG	Normal Grooming
NHPs	Non-Human Primates
NHS	National Health Survey
NIH	National Institutes of Health
NIMH	National Institute of Mental Health
NRIs	Norepinephrine Reuptake Inhibitors
O1	Object 1 Investigation
O2	Object 2 Investigation
O3	Object 3 Investigation
O4	Object 4 Investigation
OB	Olfactory Bulbectomy

OFT	Open Field Test
PBS	Phosphate Buffered Saline
PET	Positron Emission Tomography
PFC	Prefrontal Cortex
PND	Post Natal Day
PVN	Paraventricular Nucleus
RBC	Red Blood Cells
SD	Standard Deviation
SEM	Standard Error of the Mean
SG	Stereotypic Grooming
SI	Square Immobility
SNPs	Single Nucleotide Polymorphisms
SNRI	Selective and Noradrenaline Reuptake Inhibitors
SP	Sucrose Preference
SSRI	Selective Serotonin Reuptake Inhibitors
SLC6A4	Serotonin Transporter
STAR*D	Sequenced Treatment Alternatives to Relieve Depression
TCAs	Tricyclic Antidepressants
TG	Total Grooming
TI	Total Immobility
TNF	Tumour Necrosis Factor
TOI	Total Object Investigation
VMA	Vertical Motor Activity
vmPFC	Ventromedial Prefrontal Cortex
dlPFC	Dorsolateral Prefrontal Cortex
WHO	World Health Organisation

Chapter 1 – Review of the Literature

1.1 Introduction

Depressive and anxiety disorders are the most common mental disorders worldwide and represent a huge burden on society due to the impact on an individual's ability to maintain occupational, social, and personal commitments (Greenberg, Fournier, Sisitsky, Pike & Kessler, 2015; Kessler, Petukhove, Sampson, Zaslavsky & Wittchen, 2012; Wang *et al.*, 2004; Irwin, 2002). Depressive disorders can occur as early as three years of age and affect people irrespective of ethnicity or gender (WHO, 2017; Ferrari *et al.*, 2013; Whiteford *et al.*, 2013). There are an estimated 322 million people that suffer from depression, accounting for 13% of the global burden of disease (DALYs) (WHO, 2017; Ferrari *et al.*, 2013). Lifetime prevalence of depression ranges from 6.1% in Japan, 11.5% in Germany, to 16.9% in the United States of America (USA) (Ishikawa, Kawakami & Kessler, 2016; Kessler *et al.*, 2003). In Australia, the National Health Survey (NHS) indicated that 17.5% of Australians reported some mental or behavioural condition at some point in the past 12 months, with 8.9% of the population specifying depression or feelings of depression (ABS, 2015).

Depressive disorders represent a large economic burden, with both direct and indirect associated costs. The direct cost of depression in the USA is estimated at US$21.5 billion per year, with 50% of these costs attributed to the workplace (absenteeism and presenteeism) (Greenberg *et al.*, 2015). Most of the remaining direct costs are due to medication, as well as inpatient and outpatient medical services. Finally, approximately 5% of the total costs associated with depressive disorders are due to suicide (Greenberg *et al.*, 2015). In the United Kingdom (UK), depression is estimated to cost the economy approximately £11 billion in lost earnings, health service demands, and drug prescriptions (McCrone, Dhanasiri, Patel, Knapp & Lawton-Smith, 2008). In Australia, the overall cost of mental disorders, including the cost of lost productivity, was estimated at AUD$20 billion per year (ABS, 2009). Depression specifically costs Australian society an estimated AUD$12.6 billion yearly, with lifetime costs of depression

in Australia equating to AUD$213.5 billion in total (LaMontagne, Sanderson & Cocker, 2010). The bulk of this cost was associated with lost productive time and the cost of employee replacement during job turnover rather than health service use and medication cost.

In addition to the economic impact, depression significantly affects physical health and quality of life, depending on the age of onset and the severity of the disorder. In cases of early onset, depression has been associated with lower education due to elevated odds of failure to complete higher level schooling (Kessler, 2012). In adulthood, mental disorders can impair parental functioning, generating negative parenting behaviours that are most prevalent for the parents of young children (Wilson & Durbin, 2010; Lovejoy, Graczyk, O'Hare & Neuman, 2000). Depression is associated with marital dissatisfaction and discord with similar patterns noted in both men and women (Whisman & Uebelacker, 2009; Culp & Beach, 1998). Additionally, depression is both a risk factor and predictor for marital violence, both perpetration and victimisation (Renner, 2009; Lorber & O'Leary, 2004; Riggs, Caulfield & Street, 2000). At its worst, depression correlates with suicidal ideation and suicide, with 15% of those suffering from depression worldwide attempting suicide, and 800,000 lives lost due to suicide every year (WHO, 2017; Oquendo, Currier & Mann, 2006).

Clearer differential diagnosis methods that involve physiological markers, in addition to the behavioural symptoms, would improve early diagnosis of these disorders. This, in turn, would reduce the associated economic costs of depression and anxiety through improved novel treatment development and more efficient treatments following diagnosis. Development of physiological markers for diagnosis of depressive and anxiety disorders can use animal models to elucidate how stress and the associations between stress-induced behavioural and physiological measures trigger these disorders.

1.2 Depression and Anxiety in Humans

1.2.1 Human Behaviours Associated with Depression and Anxiety

In humans, depression is characterised by the main symptoms of depressed mood (or irritable mood in children), and a loss of pleasure or interest in activities once enjoyed. These symptoms can accompany somatic and cognitive alterations that affect an individual's ability to function normally such as: sleep disturbance, fatigue, weight changes, poor concentration, feelings of guilt, and suicidal thoughts (APA, 2013). Behaviourally, depression often results in withdrawal from social situations or support networks because of the two main symptoms of depressive disorders; depressed or irritable mood depending on age and loss of enjoyment in activities (NIMH, 2017). The word depression is a colloquial term used to encompass both mild transient conditions of low mood, as well as more severe clinical depressive sub-classifications that are diagnosed based on the duration and timing of such depressive episodes (AIHW, 2016; APA, 2013).

This colloquial use of "depression" suggests that it is a unitary construct, where the behavioural, biochemical, and neurobiological symptoms are consistent for all individuals diagnosed with a depressive disorder (Bitsika & Sharpley, 2012). This is not the case given the diverse nature of the potential qualifiers for depressive disorders (Wray *et al.*, 2018; APA, 2013). Ostergaard, Jensen and Bech (2011) calculated the possible qualifying symptom combinations for a major depressive episode (MDE), which is a period characterised by the symptoms of major depressive disorder (MDD), to be 1,497 based on the potential symptoms for a depressive episode. This was a conservative estimate since the qualifiers for additional features were not included in the calculation. As a result, depressive disorders are very heterogeneous, with many different symptom combinations in different individuals (Lieblich *et al.*, 2015; Fried & Nesse, 2014; Goldberg, 2011). Depression as a unitary construct is unlikely to explain all the possibilities for development of depressive disorders (Ghaemi & Vohringer, 2011; Kessing, 2007; Luyten, Blatt, Van Houdenhove & Corveleyn, 2006).

When discussing anxiety disorders in a general setting, the terms anxiety and fear are often interchangeable terms, particularly since there is a normal role of anxiety in life, serving as a warning sign of potential danger (Freeman & Freeman, 2012). However, while there is an overlap in these two states they are considered as distinctly different states (APA, 2013). Anxiety is a future-oriented mood state, associated with vigilance against future danger, muscle tension, and any cautious or avoidant behaviours (Freeman & Freeman, 2012; Reiss, 1991). Fear is a response to an immediate threat, associated with fight-flight-freeze responses, autonomic arousal, and escape behaviours (Freeman & Freeman, 2012; Reiss, 1991). Anxiety in humans is characterised by excessive levels of fear and behavioural disturbance related to overly cautious, avoidant or vigilant responses to specific stimuli (APA, 2013). Clinical anxiety is often out of proportion to a given situation, lasting for much longer, with the potential to cause long-term behavioural changes and impair overall functioning (Steimer, 2011).

Due to the multifaceted nature of depression and anxiety, there is a high comorbidity between the symptomology of these two disorders (Figure 1.1) (Berton & Nestler, 2006; Mineur, Belzung & Crusio, 2006; Bhugra & Mastrogianni, 2004). In the USA, approximately 50% of individuals with a depressive disorder also have a history of one or more anxiety disorders, such as: panic disorder, social phobia, generalised anxiety, and post-traumatic stress disorder (Kessler *et al.*, 2003; Kaufman & Charney, 2000). Another study from the Netherlands stated that depressive and anxiety comorbidity was associated with higher severity, greater chronicity, increased disability and more treatments required (Hofmeijer-Sevink, 2012). In Australia, about 85% of people with depression suffer from symptoms of anxiety, and 90% of patients with anxiety disorders have comorbid depressive symptomology (Tiller, 2012).

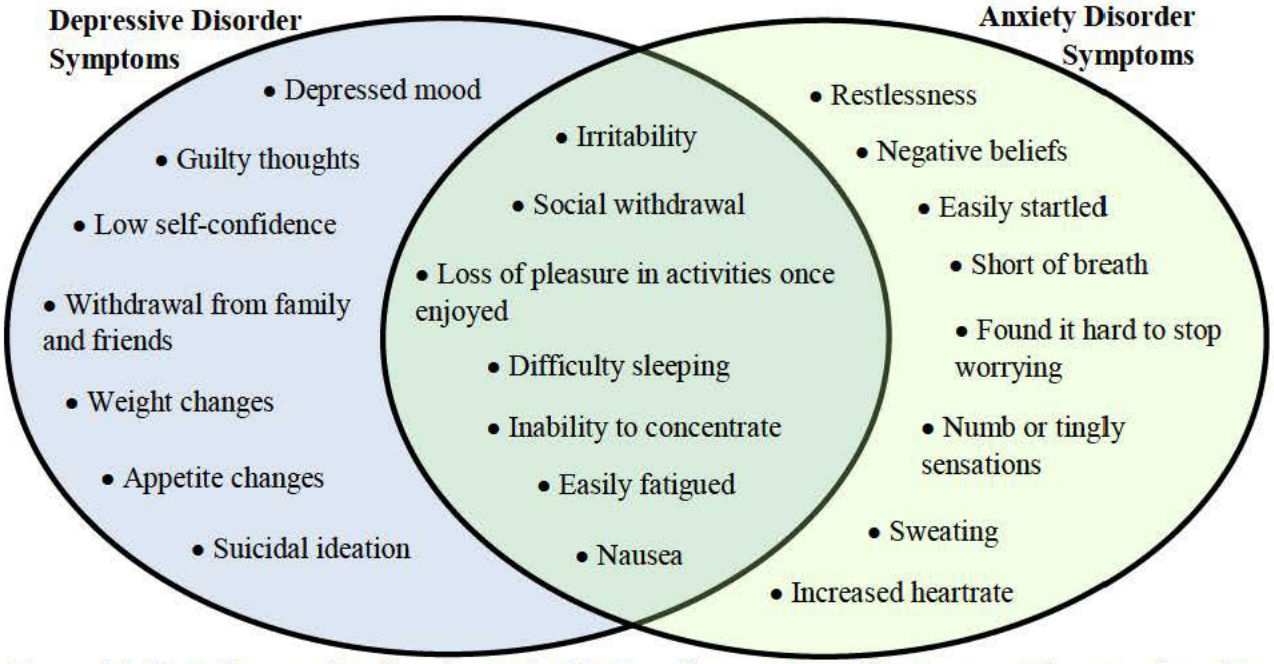

Figure 1.1: Venn diagram detailing the overlap between the symptoms of depressive and anxiety disorders (constructed based on diagnostic criteria APA, 2013; WHO, 1992).

1.2.2 Genetic and Familial Factors Associated with Depression and Anxiety

The symptom overlap in depressive and anxiety disorders implies a shared aetiology, supported by family, twin and adoption studies examining the familial aggregation of these disorders (Najman, *et al.*, 2017; Taporoski *et al.*, 2015; Lohoff, 2010; Smoller, Block & Young, 2009). Specifically, family studies indicated a twofold to threefold increase in lifetime risk for MDD among first-degree relatives, while twin studies suggest 40% to 50% heritability of MDD (Lohoff, 2010; Sullivan, Neale & Kendler, 2000). In anxiety disorders, the estimated heritability is 20% to 40% (Smoller *et al.*, 2009). A recent study in high-risk patients for depression reported a heritability of 67% for MDD and 49% heritability for anxiety (Guffanti *et al.*, 2016). Additionally, the same study noted that sequential comorbidity of anxiety and MDD, where anxiety precedes MDD development, possessed a heritability of 53% in the same high-risk patient group. Another study noted that, when anxiety and depressive symptoms are assessed with the Hospital Anxiety and Depression Scale (HADS), 66% of the total genetic variance for anxiety symptoms was shared with depressive symptoms, though HADS does not fully assess specific depressive or

anxiety disorders (Taporoski *et al.*, 2015). Collectively, this informs the important role genetic factors play in susceptibility to depressive and anxiety disorders.

There have been as many as 151 candidate genes for depression (mostly MDD), with a larger number of possible polymorphism variants that have been associated with other depressive disorders (Flint & Kendler, 2014; Nair, Nair & Moochhala, 2015; Kao, Fang, Zhao & Kuo, 2011). A number of single nucleotide polymorphisms (SNPs) of genes have been identified to be associated with depression or anxiety. Some genes with a shared association between depressive and anxiety disorders include: brain-derived neurotrophic factor (BDNF), catechol-O-methyl transferase (COMT), the variable number tandem repeat in the dopamine receptor (DRD4 VNTR), FK506 binding protein-5 gene (FKBP5), monoamine oxidase A (MAOA), and the serotonin transporter (SLC6A4). The main findings for these genes are summarised in Table 1.1. Collectively, such genes are often described as genetic susceptibility or vulnerability markers, since the presence of these alleles does not indicate that a depressive or anxiety disorder will occur, only that there is a higher potential risk for development.

Table 1.1: Summary of overlapped candidate gene polymorphisms in depression and anxiety.

Polymorphism	Gene Purpose	Function in Depression	Function in Anxiety	References
BDNF Val66Met	Neurogenesis in psychiatric disorders	Met carrier stress exposure associated w/ ↑ depression, ↓ grey matter in hippocampus	Val homozygote associated w/ ↑ anxiety	Gatt *et al.*, 2009
COMT Val158Met	Degrades catecholamines	Met allele associated w/ ↑ risk for MDD	Met homozygote significantly associated with panic disorder	Won & Ham, 2016; Gratacos *et al.*, 2007
DRD4 VNTR	Dopamine receptor subtype D4	Short allele repeat sequence associated w/ ↑ risk for depression	Long allele associated w/ PTSD symptom severity. Long allele associated w/ adolescent anxiety	Perez-Edgar *et al.*, 2014; Dragan & Oniszczenko, 2009; Lopez-Leon *et al.*, 2005
FKBP5	Binding protein in glucocorticoid-mediated stress responses	Functional and structural changes in areas involved with emotional perception, inhibition associated w/ T allele carriers	Associated w/ post-traumatic stress disorder severity in victims of child abuse	Tozzi *et al.*, 2016; Yaylaci *et al.*, 2016; Binder *et al.*, 2008; Lekman *et al.*, 2008
MAO-A	Catalyses oxidation of monoamines	Conflicted reports – both high and low activity variants associated w/ MDD	Reduced MAOA methylation associated w/ anxiety disorders	Won & Ham, 2016; Ziegler *et al.*, 2016; Shimada-Sugimoto, Otowa & Hettema, 2015
SLC6A4	Transports serotonin from the synaptic cleft	Conflicted reports - The *s* allele supposed to ↑ risk for MDD development w/ stress exposure	Association of the *s* allele with anxiety specifically in men	Chang *et al.*, 2016; Nair *et al.*, 2015; Shimada-Sugimoto *et al.*, 2015

Despite the importance of genetic factors, there has been limited success in studies examining the genes that underlie the vulnerability to depressive and anxiety disorders for use as potential diagnostic or screening tests for these disorders in clinical settings (Otowa *et al.*, 2016; Demirkan *et al*, 2011). However, a recent genome-wide association study by Wray *et al.* (2018) noted 153 significant risk variant genes, of which 44 loci were found to be statistically significant for MDD, independent of any other signals. These risk variant genes were also noted to have high genetic correlations with other comorbid psychiatric disorders, particularly anxiety disorders (comorbid correlation with anxiety disorders = 0.8). The multifaceted nature of depressive and anxiety disorders means that no single gene is sufficient to explain the development of these disorders (Bosker *et al.*, 2011). Thus, depressive and anxiety disorders manifest via the small effect of many susceptibility genes together (Wray *et al.*, 2018; Flint & Kendler, 2014; Norrholm & Ressler, 2009). The recent study by Wray *et al.* (2018) concluded that the genetic risk for MDD is continuous and normally distributed and that non-genetic factors (environmental experiences) play critical roles in protection from or predisposition towards these disorders. The complex genetic diversity in depressive and anxiety disorders results in multiple potential overlapping sets of susceptibility genes that can predispose an individual to, or protect them from, manifesting the symptoms of depressive or anxiety disorders (Wray *et al.*, 2018; Lohoff, 2010). Each gene may contribute a small fraction to the total estimated genetic variance (~20 – 50%) associated with depressive or anxiety disorder development, the remaining estimated variance for the development of these disorders is attributed to environmental events (50 – 80%) experienced by the individual (Lubke *et al.*, 2012; Smoller *et al.*, 2009). Moreover, this suggests that environmental events can influence any given set of susceptibility genes, and potentially change the manifestation of identifiable depressive or anxiety disorder symptoms based on individual experiences.

1.2.3 Role of Environmental Influence in Depression and Anxiety

The importance of environmental influence in the development of depressive and anxiety disorders has been extensively documented (Koolhaas *et al.*, 2011; Mathew, Pettit, Lewinsohn, Seeley & Roberts, 2011; McVicar & Clancy, 2011; Kinney & Tanaka, 2009; Shanahan, Copeland, Costello & Angold, 2008). As mentioned, after accounting for potential genetic inheritability, the remaining variance in the development of depressive and anxiety disorders involves the influence of environmental stressors and significant life events. Therefore, approximately 50% to 60% of the total variance for depressive disorders and 60% to 80% of the total variance for anxiety disorders is due to environmental stress or stressful life events (Kendler & Aggen, 2017; Taporoski *et al.*, 2015; Peyrot *et al.*, 2013; Hettema, Neale, Myers, Prescott & Kendler, 2006). Life events that can precede the development of depressive and anxiety disorders include (but are not limited to): spousal death, divorce or separation, marital discord, loss of job (redundancy or retirement), occupational harassment or bullying, unwanted pregnancy, rape, childhood trauma, social isolation, war, and major accidents (Mathew *et al.*, 2011; Shanahan *et al.*, 2008; Brook & Schmidt, 2008; Boomsma, van Beijsterveldt & Hudziak, 2005).

When the role of environmental stress in the development of depressive and anxiety disorders is discussed, it should be noted that stress is defined as a negatively perceived harmful event, attack, or threat (APA, 2013; Koolhaas *et al.*, 2011; Mello, Mello, Carpenter & Price, 2003). In this case, the perception of any given individual is what defines a positive or negative life event (Raffaelli *et al.*, 2016; Sutin, Costa, Wethington & Eaton, 2010). However, positively perceived environmental stressors and life events are generally events such as: marriage, the birth of a child, occupational success, availability of social supports, achieving financial independence or other financial support, can improve the outcome of depressive and anxiety disorders (Disabato, Kashdan, Short & Jarden, 2016; Ozbay *et al.*, 2007). These positively geared events appear to confer some form of protection against the development of, or improve remission, for these disorders (Bergin & Paenham, 2016; Disabato *et al.* 2016; Ozbay *et al.*, 2007).

The types of environmental influences divide into common and unique environmental influences in twin and family studies (Sullivan *et al.*, 2000). Common influences are the result of shared environmental factors such as being raised together and unique influences involve events that impact one individual, rather than both twins. When the contribution of environmental influences on the development of depressive and anxiety disorders are analysed with respect to the genetic heritability, it has been found that, in many cases, unique environmental influences contribute more to development of these disorders than common environmental influences (Kendler & Halberstadt, 2013; Subbarao *et al.*, 2008; Hettema *et al.*, 2006; Sullivan *et al.*, 2000).

The discussion of the manifestation of depressive and anxious behaviours in humans is often expressed using the term "gene by environment" interaction. The previous section (section 1.2.2) discussed genetic factors that result in a genetic susceptibility or vulnerability, while the current section (section 1.2.3) noted that the response to environmental events, both negative and positive influences manifestation of symptoms based on the genetic factors (Dunn *et al.*, 2015; Wray *et al.*, 2012; Lee, Jeong, Kwak & Park, 2010). This gene-environment interaction plays a role in the pathophysiology of depressive and anxiety disorders under a diathesis-stress model of disorder development, based on the concept of nature vs nurture (Franck *et al.*, 2016; Lopizzo *et al.*, 2015). The phenotype of depressive or anxiety behavioural symptoms (depressed mood or loss of pleasure etc.) manifests through a diathesis component, which corresponds to genetic or nature influences, and the genetic component is influenced and acted upon by stress events, which correspond to environmental or nurture events (Halldorsdottir & Binder, 2017; Chang, Yu, Chang & Hirsch, 2016). However, gene by environment interactions (where a physical response to the environment changes based on the underlying genetics) and epigenetic modifications (such as children inheriting alterations to DNA formed through stress-induced methylation or histone modification) in depression and anxiety development are beyond the scope of this thesis and will not be further discussed.

1.2.4 Pathophysiology of Depression and Anxiety in Humans

There have been many studies in humans and animals that have attempted to elucidate the pathophysiology of depressive and anxiety disorders, beyond the behavioural symptoms used for disorder diagnosis. However, the precise mechanism(s) by which these disorders develop is yet to be completely understood (Hasler, 2010; Holtzheimer III & Nemeroff, 2006). Part of this difficulty is due to the heterogeneity of depressive disorders and the high degree of comorbidity that depressive and anxiety disorders possess (APA, 2013; Bitsika & Sharpley, 2012; Brigitta, 2002). Some prominently recognised mechanisms involved in the pathophysiology of depressive and anxiety disorders that will be discussed, aside from the genetic and environmental influences already described, include: changes in neural circuitry and dysregulation of stress response systems (Fekadu, Shibeshi & Engidawork, 2017; Thapar, Collishaw, Pine & Thapar, 2012; Brigitta, 2002). Other mechanisms associated with depressive disorder pathogenesis include: changes in neurotransmitter concentrations, endocrine factors, and inflammatory markers (Fekadu, *et al.*, 2017; Brigitta, 2002). These mechanisms will be discussed in the following sections given their connection to what is known about the pathophysiology of depressive and anxiety disorders. Many studies in animal models demonstrate similar modifications in comparative systems to those that will be discussed in humans here (Ellenbroek, Angelucci, Husum & Mathe, 2016; Gass *et al.*, 2016; Kato, Kasahara, Kubota-Sakashita, Kato & Nakajima, 2016; Mehta-Raghavan, Wert, Morley, Graf & Redei, 2016; Menard, Hode & Russo, 2016; Berton *et al.*, 2012; Skelin, Kovacevic & Diksic, 2011). Furthermore, measurement of these neurological and biochemical changes in human subjects should be noted as being based on indirect non-invasive neuro-imaging (neuroanatomical changes) or minimally invasive phlebotomy (neurotransmitter, neuroendocrine, and immunological factors) (Fekadu, *et al.*, 2017).

1.2.4.1 Neurological Modification in Stress-Induced Depressive Disorders

Within the brain, the hippocampus is involved in declarative memory and spatial learning, the amygdala is the primary emotional regulator, and the prefrontal cortex (PFC) assists in working memory, attention, impulse control and executive function (Nestler & Carlezon, 2006; Drevets, 2001). Neuroimaging and post-mortem studies report abnormalities in the areas of the brain responsible for mood regulation, reward response, and executive function in individuals with depressive disorders when compared to healthy controls (Fekadu, *et al.*, 2017; Drevets, Price & Furey, 2008). Examples of types of neuroimaging often used in preclinical and clinical research include but are not limited to: magnetic resonance imaging (MRI), computed tomography (CT) scans, positron emission tomography (PET), and electroencephalography (EEG) (NINDS, 2017; Savitz, Rauch & Drevets, 2013; Kloppel *et al.*, 2012). Such neuroimaging techniques examine changes in grey-matter volume and glial density specifically in the hippocampus, prefrontal cortex (PFC), and amygdala (Figure 1.2) in addition to examining changes in functional activity in these regions (Wise, Cleare, Herane, Young & Arnone, 2014; Hasler, 2010; Krishnan & Nestler, 2008).

Figure 1.2: Overview of brain regions of interest in depressive disorders (Joy, NIMH, NIH, 2017.).

The hippocampus mediates the development of behavioural responses to stress as a negative feedback site for the stress response (Drevets *et al.*, 2008; Karten, Olariu & Cameron, 2005). The hippocampus has been a target for many regions of interest studies for depressive disorders in humans (Arnone, McIntosh, Ebmeier, Munafò & Anderson, 2012; Drevets *et al.*, 2008). A reduction in hippocampal volume remains the most replicated finding in depressive disorders

from structural MRI studies, as indicated by several meta-analyses (Buddeke *et al.*, 2017; Wise *et al.*, 2014; Nugent, Davis, Zarate & Drevets, 2013; Sacher *et al.*, 2012). However, healthy individuals with a family history of depressive disorders also possess smaller hippocampal volumes, indicating that a volume reduction may reflect genetic risk rather than current depressive symptoms (Amico *et al.*, 2011). There were smaller hippocampal volumes in unmedicated patients when compared to medicated patients receiving antidepressant treatment (selective serotonin reuptake inhibitors (SSRI), serotonin and noradrenaline reuptake inhibitors (SNRI), or noradrenaline-dopamine reuptake inhibitors (NDRI)) (Huang *et al.*, 2013). A smaller baseline hippocampus volume was associated with patients that experienced an increase in depressive symptoms over eight years of follow up (Buddeke *et al.*, 2017). Similarly, functional MRI (fMRI) studies have reported that hippocampal activity can be used to predict response to pharmacological treatment as individuals with poor pharmacological treatment response show lower activation in the hippocampus in the left hemisphere when memorising positive word pairs (Toki *et al.*, 2014; Fu, Steiner & Costafreda, 2013). Collectively, these studies indicate that hippocampal volume and function in depression are potential markers that can predict antidepressant treatment non-responders (Hasler, 2010).

The amygdala is the second most researched area in region-of-interest studies in depressive disorders, due to its role in emotional regulation (Arnone *et al.*, 2012). Structural MRI studies of the amygdala have reported inconsistent findings regarding volume change in depressive disorders (Arnone *et al.*, 2012; Gotlib & Hamilton, 2008). Functional MRI studies noted increased activation in response to negative emotional stimuli, such as sad facial expression and negative connoted images (Arnone *et al.*, 2012; Peluso *et al.*, 2009; Abler, Erk, Herwig & Walter, 2007). Similarly, reduced activity is associated with amygdala processing in response to positive stimuli, such as monetary gains or positive connoted images (Liu *et al.*, 2017; Wise *et al.*, 2014). These functional changes are also associated with pharmacological treatment response, with normalisation in reactivity to negative and positive stimuli in the amygdala noted following treatment with SSRI antidepressants (Arnone *et al.*, 2012; Victor, Furey, Fromm, Öhman &

Drevets, 2010; Anand, Li, Wang, Gardner & Lowe, 2007). Similar responses are noted following psychological treatment with cognitive behaviour therapy (CBT) as well, indicating similar potential mechanisms of action (Fu *et al.*, 2013; Ritchey, Dolcos, Eddington, Strauman & Cabeza, 2011).

The prefrontal cortex (PFC) is associated with depressive disorders due to its role in executive function and behavioural regulation (Treadway *et al.*, 2015). As with the hippocampus, MRI studies have noted reduced volume in the PFC in depressed patients when compared with healthy controls (Arnone *et al.*, 2012; Hasler, 2010). In terms of functional specialisation, there are two subregions of the PFC: the ventromedial PFC (vmPFC), and the dorsolateral PFC (dlPFC) (Koenigs & Grafman, 2009). The vmPFC is involved in emotional regulation, particularly negative emotions, given its dense connections to the amygdala, as well as processing reward and motivational cues (Liu *et al.*, 2017; Koenigs & Grafman, 2009). The dlPFC is associated with cognitive and executive functioning, regulating intention formation, working memory and goal-directed action (McEwen & Morrison, 2013; Marvel & Paradiso, 2004). Functional neuroimaging studies noted opposite changes in at-rest activity levels of these two sub-regions in depressed patients, hyperactivity in the vmPFC and hypoactivity in the dlPFC, which suggests a region-specific impact on the development of depressive symptoms (Liu *et al.*, 2017; Greicius *et al.*, 2007). This difference in activity appears to reverse in response to antidepressant treatment with SSRIs or psychotherapy treatments, resulting in hypoactivity in the vmPFC and hyperactivity in the dlPFC (Fales *et al.*, 2009; Keedwell *et al.*, 2009; Koenigs & Grafman, 2009). This suggests that the activity imbalance between these two regions may contribute to depressive disorders (Koenigs & Grafman, 2009). However, when compared with never-depressed controls, patients with remitted depressive disorders had reduced dlPFC reactivity to reward loss, which suggests that altered PFC responses to reward loss may be useful as a prospective marker of MDD vulnerability (Schiller, Minkel, Smoski & Dichter, 2013).

1.2.4.2 Dysregulation of Stress Response Systems

Stressful events (as defined in Section 1.2.3) contribute a large portion of the overall variance for depressive and anxiety disorder development, often because stressful situations regularly occur prior to the development of these disorders (Young & Dietrich, 2015; Cummings, Caporino & Kendall, 2014; McLaughlin & Hatzenbuehler, 2009). As a result, the hypothalamic-pituitary-adrenal (HPA) axis, which acts as a central control and regulatory centre in the stress response, is hypothesised to play a role in the incidence of depressive disorders (Fekadu, *et al.*, 2017; Bet *et al.*, 2008; Kudielka & Kirschbaum, 2005). Depressive individuals tend to demonstrate hypersecretion of corticotrophin releasing hormone (CRH), adrenocorticotrophic hormone (ACTH), and cortisol, in addition to dysfunctional glucocorticoid feedback mechanisms such as glucocorticoid receptor insensitivity (Raglan, Schmidt & Schulkin, 2017; Tofoli, Baes, Martins & Juruena, 2011).

Under normal conditions, internal or external stressors trigger sympathetic stimulation to the amygdala and the hypothalamus (Figure 1.3-A), stimulating the paraventricular nucleus (PVN) of the hypothalamus (McVicar & Clancy, 2011). In response to these stimulatory inputs, CRH is secreted, it binds to receptors on the anterior pituitary which stimulates the release of ACTH, in turn, ACTH stimulates the production and release of glucocorticoids from the adrenal cortex, specifically cortisol (McVicar & Clancy, 2011; Leonard, 2005). The steroid hormone cortisol generates a negative feedback on glucocorticoid receptors in the PVN to reduce secretion of CRH, and consequently ACTH (Ortsater, Sjoholm & Rafacho, 2012). In addition to acting directly on the hypothalamus, cortisol also stimulates the hippocampus, promoting indirect inhibitory outputs to the hypothalamus (Figure 1.3) (McVicar & Clancy, 2011; Lanfumey, Mongeau, Cohen-Salmon & Hamon, 2008).

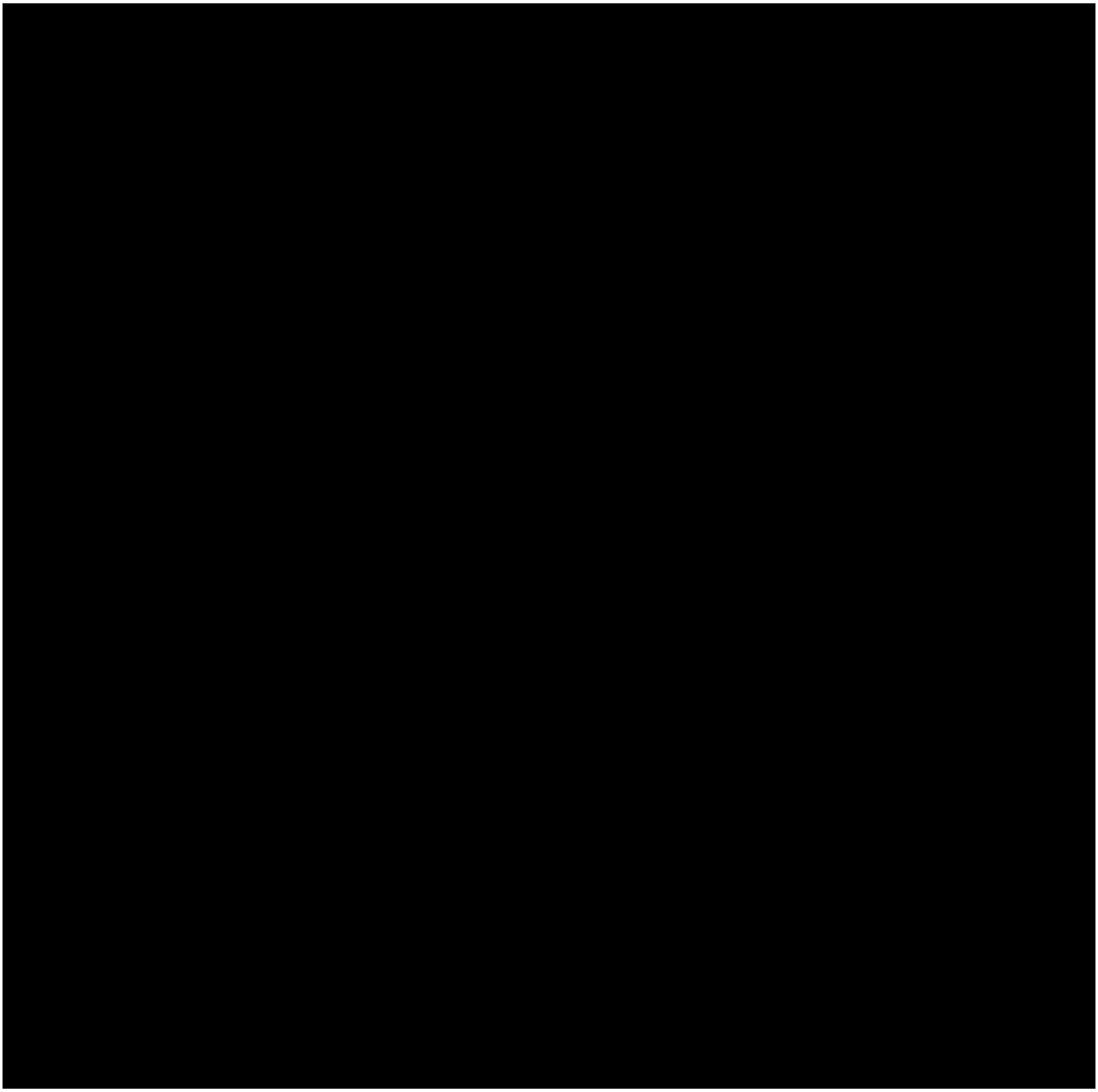

Figure 1.3: Stress system activation and (A) normal glucocorticoid feedback mechanism and (B) diminished feedback during prolonged activation from chronic stress. Dashed arrows indicate inhibitory actions that generate negative feedback (adapted from McVicar & Clancy, 2011).

The HPA axis maintains homeostasis during stressful situations, however, when under prolonged activation, such as during recurrent or continuous exposure to stress, dysregulation of HPA axis function occurs (Bellavance & Rivest, 2014; Trevino, Uhelski, Dougall & Baum, 2012). This dysregulation is a result of diminished negative feedback control of cortisol (Figure 1.3-B) through a mechanism of reduced glucocorticoid receptor sensitivity or reduction in receptor density (McVicar & Clancy, 2011; de Kloet, Sibug, Helmerhorst & Schmidt, 2005). The overall impact is inadequate inhibitory signals from glucocorticoid receptor binding in the hippocampus and hypothalamus, with continued stimulatory signals from the amygdala, which in turn further

increases the secretion of CRH and cortisol. Hypercortisolism, a condition involving elevated levels of cortisol in the body, is a consequence of this diminished feedback, with patients with depressive disorders noted to possess significantly higher levels of cortisol in plasma and saliva samples when compared with healthy controls (Raglan *et al.*, 2017; McVicar & Clancy, 2011; Tofoli *et al.*, 2011). However, hypocortisolism, a condition representing reduced levels of cortisol, has also been associated with depressive symptoms and is thought to arise following prolonged hypercortisolism (Maripuu, Wikgren, Karling, Adolfsson & Norrback, 2017; Maripuu, Wikgren, Karling, Adolfsson & Norrback, 2016; Waller *et al.*, 2016; Maripuu *et al.*, 2014). This suggests that change in cortisol level, increased or decreased, may contribute to depressive or anxiety disorder development following chronic stress exposure depending on the total duration of stress, though the mechanisms behind this are yet to be elucidated (Maripuu *et al.*, 2017).

Glucocorticoids, such as cortisol in humans or corticosterone in rodents, mediate the stress response in both acute and chronic stress situations. Periods of prolonged stress often result in elevated cortisol that can be measured in blood, urine, saliva, hair, fingernails, and faecal matter samples (Danzter & Kalin, 2016; Nejad, Ghaseminezhad, Sung, Hoseinzadeh & Cabibi, 2016; Izawa *et al.*, 2015; Brydges *et al*, 2014; Hueston & Deak, 2014; Accorsi *et al.*, 2008; Tops, Riese, Oldehinkel, Rijsdijk & Ormel, 2008). Cortisol is synthesised in the zona fasciculata of the adrenal glands from cholesterol through pregnenolone, the common precursor for all steroid hormones (Figure 1.4) (Trevino *et al.*, 2012; Hu, Zhang, Shen & Azhar, 2010).

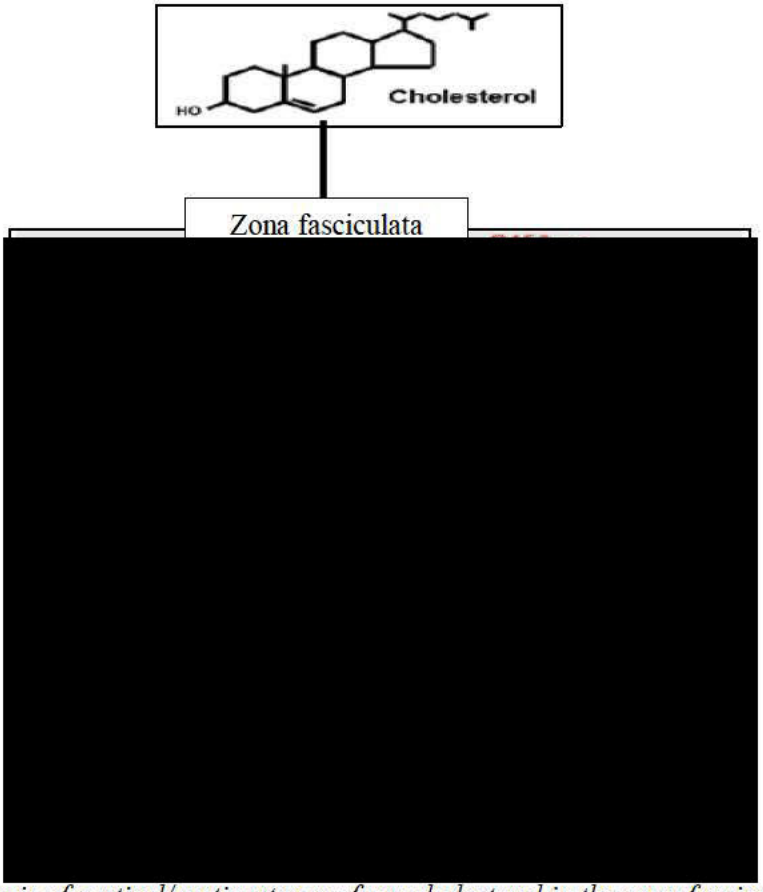

Figure 1.4: Biosynthesis of cortisol/corticosterone from cholesterol in the zona fasciculata of the adrenal gland (adapted from Ortsater, Sjoholm & Rafacho, 2012).

Cortisol is the main hormone associated with the physiological stress response in humans (Trevino *et al.*, 2012). Additionally, cortisol is vital to the circadian rhythms, glucose metabolism, and provides anti-inflammatory and immunosuppressive effects at high concentrations (Sacta, Chinenov & Rogatsky, 2016; Ortsater *et al.*, 2012; Trevino *et al.*, 2012). Cortisol in the bloodstream after secretion is predominantly (90-95%) in a 'bound' form with corticosteroid-binding protein or serum albumin until reaching a target tissue, the remainder is unbound and biologically available to bind to the glucocorticoid receptor (GR) in any potential target tissues (Bellavance & Rivest, 2014; Trevino *et al.*, 2012; Kudielka & Kirschbaum, 2005). Under situations of severe or prolonged stress, the level of cortisol production can increase beyond the capacity of carrier proteins, resulting in the elevated free cortisol levels and enhancing the impact of cortisol activity in the body (Trevino *et al.*, 2012; Zen *et al.*, 2011).

A main mechanism of action for cortisol and other glucocorticoids is to bind to the GR found in the cytoplasm of target cells (Bellavance & Rivest, 2014; Zen *et al.*, 2011). The GR

exists in an inactive complex with molecular chaperones and co-chaperones, the most prominent of which are heat shock proteins (HSP) 70 and HSP-90 (Bellavance & Rivest, 2014; Ortsater *et al.*, 2012). When cortisol binds to the GR the chaperone complex dissociates leaving the cortisol-GR complex active (Ortsater *et al.*, 2012; Trevino *et al.*, 2012). The active complex then dimerises, combining with another cortisol-GR complex and enters the nucleus of the cell (Sacta *et al.*, 2016; Ortsater *et al.*, 2012; Zen *et al.*, 2011). This dimer molecule binds to the promoter regions of genes containing glucocorticoid-response elements (GRE) or negative-GRE, to trigger trans-activation and trans-repression of the genes that contain these response elements, many of which involve anti-inflammatory and pro-inflammatory protein respectively (Bellavance & Rivest, 2014; Trevino *et al.*, 2012; Zen *et al.*, 2011). This system maintains homeostasis during stressful situations. Chronic activation of the stress response can disrupt homeostasis resulting in persistent activation of the HPA axis, impaired neurogenesis, the loss of muscle tissue, and deterioration of immunological tissue (Sacta *et al.*, 2016; Trevino *et al.*, 2012; McVicar & Clancy, 2011; Zen *et al.*, 2011).

The catecholamine adrenaline, also known as epinephrine, triggers many of the physiological responses to stress, specifically during acute stress (Ranabir & Reetu, 2011; Eisenhofer, Kopin & Goldstein, 2004; Goldstein, 2003). Adrenaline is synthesised indirectly from the essential amino acid phenylalanine or directly from the non-essential amino acid tyrosine in the adrenal medulla (Figure 1.5) (Kvetnansky, Sabban & Palkovits, 2009). As with cortisol, signals from the HPA axis trigger the secretion of adrenaline (Trevino *et al.*, 2012; Tsigos & Chrousos, 2002). The synthesis of adrenaline precursors is stimulated by ACTH via enhancing the activity of two key enzymes, tyrosine hydroxylase and dopamine β-hydroxylase (Serova, Gueorguiev, Cheng & Sabban, 2008). Adrenaline is secreted by the adrenal glands during acute stress specifically, unlike cortisol, which is continuously released throughout any exposure to stress, acute or chronic (Ranabir & Reetu, 2011). Adrenaline is a marker for assessment of acute stress levels due to the short biological half-life of 2 minutes, allowing analysis of this

catecholamine to determine if an individual is undergoing acute stress, while high cortisol levels are generally more indicative of chronic stress.

Figure 1.5: Biosynthesis of Catecholamines (adapted from Kvetnansky, Sabban & Palkovits, 2009).

As discussed in section 1.2.3, environmental influence plays an important role in the development of depressive or anxiety disorders. Specifically, the physiological response to environmental stressors, a significant life stress event or series of events (spousal death, loss of employment, marital discord, trauma from accidents, social isolation, or being subjected to bullying or harassment) develops over a longer period than just the duration of the stress (Mathew *et al.* 2011; Shanahan *et al.*, 2008; Brook & Schmidt, 2008). These types of environmental stressors or life events can contribute to depressive and anxiety disorder development through either continuous exposure to the stressor (marital discord or harassment), recurrent stresses that occur because of a specific stress event (recovery from a major accident) or any series of stress events that are unconnected but occur within a similar timeframe (Young & Dietrich, 2015; Cummings *et al.*, 2014). The continuous or recurrent stress exposure results in consistent activation of the stress system, which in turn leads to dysregulation and diminished feedback control of the hormones (Bellavance & Rivest, 2014). This altered feedback control changes the

secretion of CRH, ACTH and cortisol in individuals with depressive and anxiety disorders (Raglan *et al.*, 2017; Tofoli *et al.*, 2011).

1.2.4.3 Neurotransmitters in Depression and Anxiety

Some neurotransmitters of interest in the development of depressive and anxiety disorders are serotonin (5-hydroxytryptamine, 5HT), noradrenaline (NA) and dopamine (DA) (Ruhe, Mason & Schene, 2007; Berton & Nestler, 2006). These neurotransmitters are chemical messengers namely monoamines, and collectively the reduced level of these specific neurotransmitters in depressive pathophysiology is known as the biogenic monoamine hypothesis (Fedaku, Shibeshi & Engidawork, 2017). This hypothesis postulates that a functional deficiency of monoamines, specifically, 5HT, NA and DA, is a cause of depressive disorders (Krishnan & Nestler, 2008; Potter & Manji, 1994). Serotonergic neurons project mainly from the dorsal raphe nucleus to multiple regions of the brain, including the amygdala, hypothalamus, striatum, cortex, and cerebellum (Berton & Nestler, 2006; Fuchs & Flugge, 2004). As a result, 5HT plays important roles in mood, pain response, sexual activity, circadian rhythm, and appetite (Nestler, 2006; Blows, 2000). The noradrenergic neurons project from locus coeruleus to similar areas as 5HT neurons including the hypothalamus, cortex, and cerebellum (Fuchs & Flugge, 2004; Blows, 2000). Consequently, NA serves as an essential neurotransmitter regulating arousal and adaptation to internal and external stressors (Goddard *et al.*, 2010). Low levels of NA mediate a broad spectrum of depressive symptoms including decreased sex drive, low appetite, poor concentration, and increased aggression (Brigitta, 2002). Dopaminergic neurons project from the ventral tegmental area of the brain stem to the frontal cortex and nucleus accumbens of the limbic system (Blows, 2000). Additionally, there are also dopaminergic pathways from the substantia nigra to the corpus striatum. Depressive symptoms, such as: psychomotor disturbance, low mood, and poor motivation are attributed to low DA levels in the nucleus accumbens (Nutt *et al.*, 2007; Chinta & Andersen, 2005).

There are several suggested reasons for this monoamine deficiency: disrupted monoamine synthesis, decreased level of cell transporter function, or an abnormality of postsynaptic receptors (Kharade, Gumate & Naikwade, 2010; Brigitta, 2002). Deficiency of these monoamines due to disrupted synthesis could involve a shortage of the essential amino acids that are the precursors to these neurotransmitters, tryptophan for 5HT and either indirectly from phenylalanine or directly from tyrosine for NA and DA (Kvetnansky *et al.*, 2009; Fernstrom, 1983). Issues with enzyme activity along the biochemical synthesis pathway for neurotransmitters may also result in disrupted synthesis (van Donkelaar *et al.*, 2011; Eisenhpfer, Kopin & Goldstein, 2004). Cell transporter proteins play a crucial role in nerve-nerve communication, facilitating re-uptake of neurotransmitters from the synaptic cleft to the pre-synaptic neurons, allowing these molecules to be available for continuous neurotransmission if required (Brigitta, 2002). This reuptake also minimises degradation of neurotransmitters by monoamine oxidase enzymes (Holtzheimer & Nemeroff, 2008; Brigitta, 2002). The final potential pathway to monoamine deficiency in depressive disorders is an abnormality in receptor function, resulting in impaired transmission. In depressed patients, a reduction in the number and binding affinity of the serotonin receptors, 5-HT1A and 5-HT2A, in the PFC was reported and suggested to be linked to depressive symptoms (Wang *et al.*, 2016; Muller *et al.*, 2015). Similarly, increased sensitivity of presynaptic α-adrenoceptors, which modulate NA release, has been reported in the brains of individuals with depressive disorders (Chandley & Ordway, 2012; Ribas, Miralles, Busquets & García-Sevilla, 2001).

The biogenic monoamine hypothesis is the most widely researched hypothesis of depressive disorders, dominating pharmacological approaches to depressive disorder treatment (Massart, Mongeau & Lanfumey, 2012; Kharade *et al.*, 2010). The most commonly used pharmacological treatments for depressive disorders alleviate depressive symptoms via the modification of monoaminergic systems (Figure 1.6) (Hamon & Blier, 2013; Holtzheimer & Nemeroff, 2008; Schechter *et al.*, 2005). These include: monoamine oxidase inhibitors (MAOIs), tricyclic and tetracyclic antidepressants (TCAs), and the reuptake inhibitors.

Figure 1.6: Schematic representation of synapse and the location of antidepressant action in relation to monoaminergic transmission. Monoamine precursors are transported from blood (A). Precursors are converted into monoamine neurotransmitters and stored in vesicles (B). Monoamine neurotransmitters released into the synaptic cleft (C). These transmitters bind with presynaptic autoreceptors, postsynaptic receptors to induce activity downstream, or reuptake into the presynapse and stored in vesicles or degraded by monoamine oxidase (MAO) (D). MAO inhibitors (MAOIs) inhibit the action of MAO to limit degradation of monoaminergic neurotransmitters. Reuptake inhibitors (SSRIs and SNRIs) and TCAs inhibit reuptake of neurotransmitters to the presynapse (adapted from Brigitta, 2002).

Antidepressants that limit monoamine oxidase (MAO) activity are non-specific, increasing the levels of 5HT, NA, and DA by binding to MAO enzymes (Finberg & Rabey, 2016; Fiedorowicz & Swartz, 2004). The TCAs and reuptake inhibitors both inhibit transporter proteins for monoamine neurotransmitters (Hamon & Blier, 2013). The TCAs are a closely related drug class, with each compound containing three or four cyclic hydrocarbon rings (Peveler *et al.*, 2005). The mode of action for TCAs is inhibition of reuptake of both 5HT and NA from the synaptic cleft (Hamon & Blier, 2013). By contrast, reuptake inhibitors such as: selective serotonin reuptake inhibitors (SSRIs), noradrenaline reuptake inhibitors (NRIs), and combined serotonin and noradrenaline reuptake inhibitors (SNRIs) target the neurotransmitter specific transporter proteins, increasing availability in the synaptic cleft (Fekadu, *et al.*, 2017; Hamon & Blier, 2013).

Reuptake inhibitors are the most commonly prescribed antidepressant treatments, largely due to having fewer side effects (Morley, 2017; Coupland *et al.*, 2011; Jain, 2004). A main issue with the use of any pharmacological antidepressants is the long therapeutic delay and the low remission rates, even in first-line treatments like SSRIs (Stewart *et al.*, 2014; Tiller, 2012). In addition to this, the experimental depletion of monoamines in healthy individuals has no impact on mood and produces only a mild mood reduction in unmedicated depressed patients (Ruhe *et al.*, 2007). This suggests that monoamines alone are unlikely to play a central role in the development of depressive disorders. However, the development of pharmacological treatments for depressive and anxiety disorders is beyond the scope of this dissertation and will not be further discussed.

1.2.4.4 Endocrine Factors in Depression and Anxiety

A variety of hormonal abnormalities has also been identified as possible contributors to the aetiology of depressive and anxiety disorders. In addition to HPA axis dysregulation and hypercortisolaemia previously discussed, some hormonal changes connected to depressive disorders include: altered levels of growth hormone (GH) and abnormality in thyroid hormone secretion and function (Fekadu, *et al.*, 2017; Brigitta, 2002). However, there is limited information about the possible role of these hormones in depressive and anxiety disorders.

The role of pituitary hormones, like GH, in the development of depressive disorders has been investigated using noradrenergic stimulated secretion (Brigitta, 2002). Specifically, GH release in depressed patients is significantly lower than GH responses in healthy controls (Ansseau *et al.*, 1988; Matussek *et al.*, 1980). Dopamine receptor sensitivity or decreased α_2-adrenoceptor sensitivity was theorised as the reason behind the blunted GH response when subjects received GH challenge tests with apomorphine and clonidine respectively (Ansseau *et al.*, 1988). However, when challenged with an alternative α_2-adrenoceptor agonist, depressed patients demonstrated normal GH responses (Charney, Heninger & Sternberg, 1984). This blunted GH response to the α_2-adrenoceptor agonist, clonidine, is not specific to just depressive disorders as it

has also been observed in generalised anxiety disorder, panic disorder and social phobia (Tancer, Stein & Uhde, 1993; Abelson *et al.*, 1991; Uhde *et al.*, 1989). This suggests that an abnormality in the GH system was responsible for the reduced GH response (Charney *et al.*, 1984). However, the mechanism of GH response dysfunction occurring in depressive and anxiety disorders remains largely unknown.

Changes in thyroid function have been linked repeatedly to depressive disorders, demonstrating elevated tetra-iodothyronine (T_4) and low tri-iodothyronine (T_3) (Hage & Azar, 2012; Kirkegaard & Faber, 1998). The administration of T_3 acts as an effective adjunctive treatment for some depressed patients (Duval *et al.*, 2005; Altshuler *et al.*, 2001). Additionally, hypothyroid patients also appear to possess reduced serotonin responsiveness that is reversible with thyroid hormone therapy (Bauer, Heinz & Whybrow, 2002). Furthermore, some depressive symptoms such as: cognitive dysfunction, apathy, and psychomotor slowing are seen in patients with hypothyroid activity, though not the main symptoms of sadness or depressed mood (Hage & Azar, 2012). Another study found that thyroid hormone might function as a co-transmitter with NA, though the mechanism is not apparent (Gordon, Kaminski, Rozanov & Dratman, 1999). The precise mechanism by which thyroid hormone abnormalities contribute to depressive disorders remains to be fully elucidated, though thyroid hormone is believed to possibly act indirectly through noradrenergic and/or serotonergic systems (Bauer *et al.*, 2002; Brigitta, 2002).

1.2.4.5 Inflammatory Markers in Depression and Anxiety

The inflammatory hypothesis postulates that depressive disorders are a result of behavioural and biochemical alterations due to increased secretion of pro-inflammatory cytokines (Anisman, 2011; Miller, Maletic & Raison, 2009; Danzter *et al.*, 2008). Cytokines are small intracellular proteins secreted by lymphoid cells (mainly white blood cells) in response to stimuli, such as foreign or invasive pathogenic antigens (Wood, 2011). As a result, cytokines function as regulators of all other immune cells that are involved in the process of inflammation in the body

(Male, Brostoff, Roth & Roitt, 2006; Schiepers, Wichers & Maes, 2005). Under normal conditions, cytokines are synthesised and secreted accordingly on the level of immune activation. In depressed individuals, the levels of pro-inflammatory cytokines (tumour necrosis factor alpha [TNF-α], Interleukin [IL]-1β and IL-6) are reported to be elevated in peripheral blood samples (Blume, Douglas & Evans, 2011; O'Brien, Scott & Dinan, 2004). Additionally, administration of inflammatory cytokines or their inducers (e.g. endotoxin) to otherwise healthy, non-depressed individuals triggers depressive-like behaviours such as: fatigue, poor concentration, and social withdrawal (Engler *et al.*, 2016; Lasselin *et al.*, 2016; Capuron *et al.*, 2002; Reichenberg *et al.*, 2001).

The depressive-like behaviours seen in healthy individuals are jointly termed "sickness behaviour", due to the similarity to the behaviour demonstrated during immune system activation while fighting an illness or infection (Hayley, 2011; Muller, Myint & Schwarz, 2011; Sharpley & Agnew, 2011). Sickness behaviours include: hyperthermia, loss of appetite, sleep disturbances, anhedonia, fatigue, hyperalgesia, and loss of interest in social and physical environments (Dantzer, O'Connor, Freund, Johnson & Kelley, 2008). These behaviours result in social withdrawal by an individual as an energy conservation method to cope better with illness.

Treatment with pro-inflammatory cytokines induces symptoms that are not part of depressive or anxiety disorders such as hyperthermia and hyperalgesia (Miller & Raison, 2016; Kent, Bluthé, Kelley & Dantzer, 1992). Furthermore, the use of anti-inflammatory agents only attenuates depressive symptoms in a sub-group of patients that have increased peripheral inflammation (Kohler *et al.*, 2014; Raison *et al.*, 2013). In patients without elevated peripheral inflammation, anti-inflammatory treatments appear to impair placebo responses that contribute to the effectiveness of SSRIs (Warner-Schmidt, Vanover, Chen, Marshall & Greengard, 2011). However, the peripheral anti-inflammatory action does not necessarily imply anti-inflammatory action in the brain (Dantzer *et al.*, 2008; Marques, Cizza & Starnberg, 2007). A study that examined the central anti-inflammatory processes via microglial response found that SSRIs,

specifically fluoxetine, sertraline, paroxetine, fluvoxamine, and citalopram, potently inhibited both TNF-α and nitric oxide production (Tynan *et al.*, 2012). This indicates that, while inflammatory cytokines may play an important role in the development of depressive disorders, they are likely not solely responsible for the development of depression (Dinan, 2008; Dunn, 2008).

1.2.5 Importance of Depressive and Anxiety Disorder Research

Collectively, the preceding sections presented evidence suggesting that the development of clinical depressive and anxiety disorders may have significant genetic and environmental components, involving complex phenomena potentially with multiple aetiologies. The pathophysiological markers associated with depressive and anxiety disorders collectively, with more research into mechanisms and relationships, could be utilised to further the stratification of the diagnosis for depressive and anxiety disorders (Haapakoski, Ebmeier, Alenius & Kivimäki, 2016). The research into such mechanisms and relationships can be carried out using animal models, which allow more detailed examination of central systems. Furthermore, the basic research conducted using animal models informs translational/clinical research for depressive and anxiety disorders in humans conducted under the research domain criteria framework put forward by the National Instiutes of Mental Health (Simmons & Quinn, 2014).

Comparative physiological and biochemical studies, using relevant animal models have been successfully used in medical research to glean information regarding the mechanism(s) that underpin disease states in humans (e.g. cancer research). Indeed, relevant animal models defined objective physiological and genetic markers as well as elucidating potential mechanisms of depressive and anxiety disorders in mammals. These inferences may be used to shed light on physiological markers of human depression or anxiety. However, the caveats when using animal models in comparative depression and anxiety studies are first, stressors used to induce analogous depressive and anxiety behaviours must be comparable between humans and the model organism.

Second, the interpretation of any observed behaviours should consider the perceptual biases of the animal used as the model organism. Therefore, the correlation between depressive- or anxiety-like behaviours in an animal model and the physiological responses of those animals must be established and validated before any animal model is useful for modelling human depressive or anxiety disorders. The next sections will discuss potential model organisms that can be used for research into depressive and anxiety disorders, the requirements for animal model validity, the common types of animal models and the behavioural tests used to assess depressive- and anxiety-like behaviour in animals.

1.3 Use of Model Organisms for Depressive and Anxiety Disorder Research

Animal models hold an important place in biomedical and neurological research, providing valuable insights into the definition of the neurobiological mechanisms underlying depressive and anxiety disorders. In preclinical studies, such animal models have examined novel pharmacological treatments and new therapeutic targets for treatment (Hanell & Murklund, 2014; Abelaira, Reus & Quevedo, 2013). When designing an animal model to investigate human diseases or disorders, the first consideration is the suitability of the model organism. Specifically, will the model organism accurately mimic the human condition as much as possible; and what are the logistical or ethical considerations are present regarding use as a model organism (Justice & Dhillon, 2016; Vallender & Miller, 2013; Hunter, 2008). The main organisms used for depressive and anxiety disorder research include: non-human primates, tree shrews, rats, and mice (Pryce & Fuchs, 2017; Krishnan & Nestler, 2011). The primary issues surrounding the use of model organisms involves first, the generation time for breeding, the lifespan, and the number of offspring. Second, the accessibility or the availability of the animal intended for research use. Third, the cost and time required for husbandry, housing, and feeding. Fourth, the amount of genetic similarity between humans and the selected animal, and fifth, the comparative behaviour between humans and animals *i.e.* similar response to stimuli between human and animal (Czeh,

Fuchs, Wiborg & Simon, 2016; Fuchs, 2015; Prescott, 2010). These issues for the major behavioural model animals used in depressive and anxiety disorder research are summarised in Table 1.2.

Table 1.2: Pros and cons of model organisms for depressive and anxiety disorder research.

Model organism	Advantages	Disadvantages	References
Non-Human Primates	Close phylogenetic relationship, similar perceptual bias, similar developmental timeframe	Ethical concern due to close relation, long lifespan, low offspring number, higher husbandry associated costs	Prescott, 2010; Pryce *et al.*, 2005; Shively & Willard, 2012
Tree Shrews	Phylogenetically closer to humans than rodents, moderate breeding cycles, similar behavioural and physiological changes to social stress as humans	Small litter size, literature clustered in psychosocial stress models, cannot be bred large scale	Pryce & Fuchs, 2017; Fuchs 2015; Cao *et al.*, 2003
Rats	Litter size, lifespan, breeding cycles, simple husbandry requirements, socially reliant, extensive previous literature	Different perceptual bias, difficult to translate results to the analogous human situation	Pryce & Fuchs, 2017; Czeh *et al.*, 2016; Pryce *et al.*, 2005
Mice	Gene knockout viability, litter size, lifespan, simple husbandry requirements, breeding cycles. Some background literature	Prey exclusive animal, different perceptual bias, less socially reliant, difficult to translate results to the analogous human situation	Pryce & Fuchs, 2017; Czeh *et al.*, 2016

In terms of genetic similarity and ease of translation for results, the optimum model organism to use in depressive and anxiety disorder research are non-human primates (NHP). The advantage of NHP use is their close phylogenetic relationship with humans. This allows for easier result interpretation because of similar biochemical and neurobiological mechanisms and behavioural responses in NHPs when compared to humans (Shively & Willard, 2012; VandeBerg & Williams-Blangero, 1997). However, there are limitations regarding the use of NHP due to the

long lifespan and low offspring numbers extending study duration, resulting in a long time under experimental conditions, which represents an ethical concern as well (Prescott, 2010). Another limitation is the much higher associated cost of acquisition and husbandry (Nelson & Winslow, 2009; VandeBerg & Williams-Blangero, 1997).

Tree shrews represent an intermediate model organism between NHP and rodents for depressive and anxiety disorder research, due to the close phylogenetic placement with primates (Pryce & Fuchs, 2017). Additionally, under stressful stimuli, such as social defeat from a more dominant animal, the subordinate tree shrews demonstrate behavioural and physiological changes similar to human patients with depressive disorders (Fuchs, 2015; Cao, Yang, Su, Li & Chow, 2003). Specifically, subordinate male tree shrews lose body weight, demonstrate reduced locomotor activity and have increased concentrations of cortisol and increased NA concentrations (Pryce & Fuchs, 2017; Meng, Shen, Li, Li & Wang, 2016). These behavioural and physiological changes in the tree shrews were reversible with chronic antidepressant treatment, which has contributed to the view that the chronic psychosocial stress model in tree shrews is a practical model organism for depressive disorder research (Czeh *et al.*, 2016). A major limitation for the use of tree shrews as model organisms is the difficulty of housing and breeding, which are respectively space and time-consuming, resulting in a limited number of available experimental animals (Fuchs, 2015). As a result, the number of laboratories that can afford to house and maintain tree shrew colonies for experimental use is quite small.

Given the difficulty of housing and maintaining NHP and tree shrews, it is unsurprising that many studies use rodents for depressive and anxiety disorder research (Pryce & Fuchs, 2017). Rodents are cheaper to maintain and house, they are relatively quick to breed and possess short lifespans, reaching maturity quickly which allows for high throughput in antidepressant screening studies (Czeh *et al.*, 2016; Slattery & Cryan, 2014; Abelaira *et al.*, 2013). In the study of depressive and anxiety disorders, rats are often preferred over mice since many of the rodent behavioural tests were developed and validated in rats (Hanell & Marklund, 2014; Cryan &

Mombereau, 2004). However, genetic knockout studies have used mice due to the ready availability of genetically modified mice (Hanell & Marklund, 2014).

1.3.1 Animal Model Validity

There are three criteria to be satisfied when validating animal models of depressive or anxiety disorders (Kedzierska & Wach, 2016). These criteria are face, construct, and pharmacological validity as first proposed by McKinney and Bunney (1969) in relation to animal models of psychiatric disorders. Face validity requires that the model mimic alterations found in humans as closely as possible, for any anatomical, biochemical, neurobiological, or behavioural features (Belzung & Lemoine, 2011; Krishnan & Nestler, 2011). Construct validity determines the relevance of the methods used to generate the model (Willner, 2017). In depression and anxiety research, construct validity is often determined using stress as a behavioural trigger and the response to known antidepressants (Willner, 2017; Belzung & Lemoine, 2011). Finally, pharmacological validity establishes whether the induced symptoms are reversible by current pharmacological treatments (Slattery & Cryan, 2017; Willner, 2017; Valvassori, Budni, Varela & Quevedo, 2013).

Notably, there is no perfect animal model that exhibits all behavioural, biochemical, and neurological abnormalities associated with depressive or anxiety disorders (Abelaira *et al.*, 2013). An ideal animal model is unattainable for two reasons; first, it is very difficult to mimic some symptoms of these disorders such as: suicidal thoughts, guilty feelings, or persistent worry in rodents. Second, the biological mechanisms underpinning depressive and anxiety disorder development have not been fully elucidated and therefore cannot be modelled perfectly (Slattery & Cryan, 2017; Hasler, 2010). However, no single patient demonstrates all potential behavioural or biological symptoms of depressive or anxiety disorders (APA, 2013; Ostergaard, Jensen and Bech, 2011; Tarantino, Sullivan & Meltzer-Brody, 2011). This heterogeneity of depressive and anxiety disorders in humans complicates the examination of developmental mechanisms in animal

models. As such, endophenotypes for depressive disorders separate behavioural and biological symptoms into more stable phenotypes, reproducible and evaluated in animal models (Hasler, Drevets, Manji & Charney, 2004). In the research of depressive and anxiety disorders, endophenotypes occupy an intermediate position between genotype and phenotype because of the complexity of pathophysiological development (Goldstein & Klein, 2014). Some proposed endophenotypes in animal models of depression include: anxiety-related behaviour, anhedonia, metabolic changes (such as weight gain or appetite change), neuroanatomical modifications, and neuroendocrine disturbances (Slattery & Cryan, 2017; Goldstein & Klein, 2014; Hasler *et al.*, 2004). These endophenotypes have been expanded on and incorporated into the research domain criteria framework for human clinical studies (Simmons & Quinn, 2014). However, preclinical animal models allow an investigation into how environmental factors can influence disorder, in a way that is not viable in humans given the heterogeneity of depressive and anxiety disorder development (APA, 2013; Abelaira *et al.*, 2013). While depressive and anxiety disorders in animals would be similarly heterogeneous, factors that are uncontrolled in humans can be limited using animals, for example, standardising the exposure to environmental factors (stress exposure, neonatal manipulation, or enforced social encounters) to examine the effect of the environment on genetic susceptibility or resilience (Halldorsdottir & Binder, 2017; Miller & Hen, 2015). The use of animals does not completely limit the potential heterogeneity of depressive or anxiety disorder symptoms. However, it does allow the triggers for such symptoms to be consistent across all experimental subjects, allowing similar environmental experiences until exposure to the triggering stimuli, so that different phenotypes and the associated triggers are theoretically clearer (Abelaira *et al.*, 2013; Bystritsky, Khalsa, Cameron & Schiffman, 2013; Nestler & Hyman, 2010).

A major caveat in animal models of psychiatric disorders, concerning the model validity, is the incomplete understanding of depressive and anxiety disorder development, which limits the methods used to generate depressive- and anxiety-like behaviours in animals (Czeh *et al.*, 2016; Abelaira *et al.*, 2013). The development of current models of depressive and anxiety disorders have a basis in the responses to stress, or the known actions of current antidepressant treatments

(Kedzierska & Wach, 2016). The efficacy of antidepressant treatments in humans is inconsistent with 50% of depressed patients non-responsive to first-line treatment (Dale, Bang-Andersen & Sanchez, 2015; Berton *et al.*, 2012). The Sequenced Treatment Alternatives to Relieve Depression (STAR*D) trial noted that only 35% of depressed patients achieved remission using a first-line treatment of the SSRI, citalopram (Sinyor, Schaffer & Levitt, 2010; Warden, Rush, Trivedi, Fava & Wisniewski, 2007). Furthermore, the therapeutic effects of current first-line antidepressants are similar to the originally identified anti-depressant agents via the targeting of monoaminergic mechanisms (Dale *et al.*, 2015; Hanell & Marklund, 2014; Berton *et al.*, 2012). This limits the development of novel pharmacological treatments using animal models since it hinders the use of reference drugs to test novel compounds (Berton *et al.*, 2012). Furthermore, this results in a catch-22 situation, whereby novel animal model development may only be responsive to the current pharmacological treatments (Slattery & Cryan, 2017).

1.3.2 Rodent Models of Depressive- and Anxiety-like Disorders

As discussed, the model organisms frequently used in depressive and anxiety disorder research are rodent models. Many behavioural models used today, were developed and validated using rats as the model organism (Hanell & Marklund, 2014; Cryan & Mombereau, 2004). Many rodent models of depressive and anxiety disorders use stress to trigger abnormal behavioural and physiological responses (Slattery & Cryan, 2017). Stress models can use either physical restraint stress, exposure to external stimuli, or social stress, which involves influencing normal social interactions of the rat (Grigor'yan & Gulyaeva, 2017; Beery & Kaufer, 2015). These stress models can apply to adult rats with no prior experimental conditioning, or during the rat developmental period to examine the impact of early life stress (Trujillo, Durando & Suarez, 2016; Boersma & Tamashiro, 2015; Ruedi-Bettschen *et al.*, 2006). In context of rodent models, stress events are defined as adversive events designed to trigger behavioural or physiological disturbance that deviates from normal animal responses, such as; environmental disruption or

instability, inflicted physical damage, and loss or limitation of social support (Grigor'yan & Gulyaeva, 2017; Menard *et al.*, 2016; Young & Dietrich, 2015). Common types include: chronic mild stress (CMS), learned helplessness (LH), social defeat, and maternal deprivation/separation (MD/S) (Menard *et al.*, 2016; Krishnan & Nestler, 2011). Additionally, decades of selective breeding have developed inbred lines of laboratory rats such as Flinders sensitive line and the Kyoto-Wistar lines, which are well suited to behavioural studies due to their depressive- or anxiety-like phenotypes, which modify the rat's response to stressful stimuli making them more stress susceptible (Ellenbroek *et al.*, 2016; Fischer, Liebenberg, Elfving, Lund & Wegener, 2012; Pajer *et al.*, 2012; Neumann *et al.* 2011). Finally, a few rodent models rely on damage to brain regions, such as the olfactory bulbs, to induce depressive- or anxiety-like behaviours or prompt depressive-like behaviours following chemical manipulation, such as injection of lipopolysaccharide (LPS) which triggers a similar increase in inflammatory cytokines observed in depressed patients (Kedzierska & Wach, 2016; Koo, Russo, Ferguson, Nestler & Duman, 2010; Carobrez, Gasparotto, Buwalda & Bohus, 2002). The reversibility of manifested behavioural and physiological symptoms in rodent models, which is a validity requirement, is assessed using the response to antidepressant treatment (Kedzierska & Wach, 2016). An overview of rodent models used to induce depressive- and anxiety-like behaviours is shown in Table 1.3. Three rat behavioural models namely, CMS, MD/S and the olfactory bulbectomy (OB) will be discussed in more detail.

Table 1.3: Overview of rat models used to induce depressive- and anxiety-like behaviours.

Model Used	**Method of conducting**	**References**
Chronic Mild Stress	Rats subjected to mild stressors on a rotating schedule. Stressor for 1-2 d. Model for 3-4 wks. Common stressors include: sleep interference, soiled bedding, limited bedding, loud noises	Herrera-Perez *et al.*, 2017; Menard *et al.*, 2016; Rana *et al.*, 2016; You *et al.*, 2011; Nestler & Hyman, 2010; Willner, 1997; Katz *et al.*, 1981
Learned Helplessness	Rats exposed to inescapable stress. Subsequent exposure to escapable stress shows greater escape latency than controls	Menard *et al.*, 2016; Krishnan & Nestler, 2011; Dalla *et al.*, 2009; Vollmayr *et al.*, 2007; Cryan & Mombereau, 2004

LPS-induced Sickness Behaviour	Rats injected with LPS, triggers infection-like immune response. Causes 'sickness behaviour' similar to depression, physiological parameters like elevated biomarkers also observed	Schmidt *et al.*, 2011; Dalla *et al.*, 2009; Dunn *et al.*, 2005
Maternal Separation/ Deprivation	Mothers separated from offspring for a set duration. Common forms: repeated or single separation, pups separated from mother and littermates	Ellenbroek *et al.*, 2016; Menard *et al.*, 2016; Trujillo *et al.*, 2016; Volodina *et al.*, 2012; Schmidt *et al.*, 2011; Ivy *et al.*, 2008; El Khoury *et al.*, 2006
Social Defeat	Rats subjected to repeated bouts of social subordination. Subordinate rats show behavioural alterations similar to depression. Common forms are: resident-intruder and sensory stress	Menard *et al.*, 2016; Krishnan & Nestler, 2011; Yu *et al.*, 2011; Nestler & Hyman, 2010; Tsankova *et al.*, 2006
Social Isolation	Rats reared in isolation from others after weaning. Behaviour is often compared to group-raised subjects	Evans *et al.*, 2012; Fischer *et al.*, 2012; Lukkes *et al.*, 2009
Olfactory Bulbectomy	Rats subjected to bilateral lesions of olfactory bulbs. Triggers behavioural changes	Menard *et al.*, 2016; Skelin *et al.*, 2011; Cryan & Mombereau, 2004; Cryan *et al.*, 2002
Flinders Sensitive Line (FSL)/ Flinders Resistance Line (FRL)	Sensitive Line shows phenotypic traits similar to depressed humans; ↑ behavioural despair, ↓ sucrose preference, ↓ body weight and HPA axis dysregulation. Behaviours normalised w/ chronic antidepressant treatment. FRL is control	Fischer *et al.*, 2012; Hascup *et al.*, 2011; Dalla *et al.*, 2009; El Khoury *et al.*, 2006
High Anxiety-like Behaviour (HAB)	The HAB strain demonstrates ↑ behavioural despair, ↓ sucrose preference and altered social behaviour. These alterations can be normalised w/ antidepressant treatment	Carnevali *et al.*, 2016; Neumann *et al.* 2011
Wistar Kyoto sub-strains (WMI & WLI)	Strain demonstrates behavioural, hormonal and physiological parameters similar to symptom-presenting depressives. Bred specifically for "depressive" behaviour	Shetty & Sadananda, 2017; Pajer *et al.*, 2012; Andrus *et al.*, 2010

1.3.2.1 The Chronic Mild Stress Model

Chronic stress exposure is a well-established risk factor for the development of depressive and anxiety disorders in humans (Moscati, Flint & Kendler, 2016; APA, 2013). As a result, CMS studies in rats use various mild stressors applied daily over a prolonged period, usually between one to seven weeks with three weeks being most common (Kedzierska & Wach, 2016; Abelaira *et al.*, 2013; Mao, Xian, Ip, Tsai & Che, 2010). The CMS model, first described by Katz, Roth and Carroll (1981), involved exposing animals to severe uncontrollable stress to trigger an anhedonic phenotype in rats with increased plasma corticosteroid levels, and reduced sucrose preference assumed to be evidence of anhedonia. Willner (1984) adapted the chronic stress model into its current method of CMS by reducing the stressor severity. The milder stress exposure was sufficient to trigger the same reduced sucrose preference as severe stress (Slattery & Cryan, 2017). Some stressors used in CMS include; overnight illumination, limited bedding material, mild food or water deprivation (1-2 days), cage tilt, white noise, isolation, and crowding, applied in an unpredictable manner to increase the impact (Slattery & Cryan, 2017; You *et al.*, 2011; Mao *et al.*, 2010).

As a model, CMS demonstrates strong face validity with behavioural and biochemical manifestations similar to symptoms of depressed human patients, strong construct validity with the use of stress to trigger similar symptoms, and good pharmacological validity with chronic antidepressant treatment reversing the effect of the chronic stress exposure (Willner, 2017; Kedzierska & Wach, 2016; Stepanichev, Dygalo, Grigoryan, Shishkina & Gulyaeva, 2014). The CMS model does have certain disadvantages. It is labour intensive and requires large amounts of space and time (Kedzierska & Wach, 2016). These requirements make the CMS method expensive and difficult to use and have led to suggestions that this model has poor inter-laboratory reliability (Menard *et al.*, 2016; Stepanichev *et al.*, 2014; Willner, 2005). Despite these disadvantages, a recent survey of laboratories that used the CMS model conducted by Willner (2017) found that 75% of respondents said that CMS provided robust data when used in their

laboratories. This suggests that despite the extensive time and cost requirements, the CMS model is a good model for researching depressive disorders (Czeh *et al.*, 2016; Kedzierska & Wach, 2016; Willner 2017).

1.3.2.2 The Maternal Deprivation/Separation (MD/S) Model

Early life stress represents a major risk factor for the development of depressive and anxiety disorders in humans (Menard *et al.*, 2016; APA, 2013). As a result, MD/S models are also well regarded in depressive and anxiety disorder research (Menard *et al.*, 2016; Trujillo *et al.*, 2016; Marais, van Rensburg, van Zyl, Stein & Daniels, 2008). These MD/S studies are examples of developmental models that use an imposition of early life stress on rats via the disruption of parental care (Molet, Maras, Avishai-Eliner & Baram, 2014; Abelaira *et al.*, 2013). In studies using MD/S, rat pups are separated from the mother, and in some cases isolated from the littermates, during the first two weeks of life between postnatal day 2-14, either as a single or repeated stress (Réus *et al.*, 2011; Schmidt, Wang & Meijer, 2011). The main advantage of MD/S models is that they allow an investigation into how early life stress can trigger vulnerability to developing depressive or anxiety disorders during adulthood (Czeh *et al.*, 2016; Valvassori *et al.*, 2013). This vulnerability is suggested to be a consequence of the manipulation of the early postnatal environment influencing adult behaviour (Abelaira *et al.*, 2013; Heim, Plotsy & Nemeroff, 2004). As with the CMS model, the MD/S design also has strong validity in the three criteria, with similar behavioural and biochemical symptoms triggered by developmental stress that is reversed by chronic antidepressant treatment (Grigor'yan & Gulyaeva, 2017; Ellenbroek *et al.*, 2016; Molet *et al.*, 2014). However, the disadvantages are also like the CMS model. The MD/S model is both time-consuming and requires significant space for the duration of stress exposure (Czeh *et al.*, 2016; Slattery & Cryan, 2014). Despite these limitations, the MD/S model is one of the most widely used preclinical models that examine early life vulnerability to depressive and anxiety disorders (Menard *et al.*, 2016; Valvassori *et al.*, 2013).

1.3.2.3 The Olfactory Bulbectomy Model

Another model of depressive disorders is the olfactory bulbectomy (OB) model, which is conducted by surgically removing or inflicting bilateral lesions on the olfactory bulbs of rats (Hendriksen, Mechiel Korte, Olivier & Oosting, 2015; Stepanichev *et al.*, 2014; Song & Leonard, 2005). The OB model triggers behavioural, neurochemical, neuroendocrine, and immune alterations in rats similar to the symptoms observed in depressed humans (Czeh *et al.*, 2016; Harkin, Kelly & Leonard, 2003). The symptoms observed in OB rats are theorised to arise from dysfunction or compensatory mechanisms in the limbic system since the peripherally induced loss of olfaction does not trigger the same symptoms (Czeh *et al.*, 2016; Song & Leonard, 2005). Additionally, the modifications induced by the OB model are reversible with chronic, but not acute, antidepressant treatment, which has deemed the OB model an excellent tool for screening potential antidepressant compounds (Hendriksen *et al.*, 2015; Slattery & Cryan, 2014). Thus, the OB model has good face and pharmacological validity, but the construct validity is lacking, considering that individuals with depressive or anxiety disorders do not have olfactory bulb lesions (Czeh *et al.*, 2016; Harkin *et al.*, 2003).

1.3.3 Behavioural Tests for Depressive- and Anxiety-like Behaviour

The difference between a model and a test is not always clear, with considerable terminology overlap present in the literature (Slattery & Cryan, 2014; Abelaira *et al.*, 2013; Nestler & Hymen, 2010). A model requires both a manipulation, such as CMS or MD/S, and a readout, which assesses the impact of the manipulation, while a test involves just a read-out such as sucrose preference test or open field test (Slattery & Cryan, 2017; Kedzierska & Wach, 2016). Some common examples of animal tests designed to assess depressive- and anxiety-like behaviour include: forced swim test (FST), sucrose preference (SP), open field test (OFT), and elevated plus maze (EPM) (Wood *et al.*, 2015; Andrus *et al.*, 2010). Therefore, rodent models must include a readout, which is often provided by behavioural tests, to assess the models outcome(s) but

behavioural tests do not need the manipulation from a model to be used for assessment. An example of this would be a rodent model that uses social isolation in adulthood as a manipulation to induce anxiety behaviour, and the elevated plus maze to assess the change in rodent behaviour from rats that were not isolated. Table 1.4 summarises the symptoms of depressive and anxiety disorders in humans, the comparative behaviours identified in rats and the preclinical test(s) used to assess those behaviours.

Table 1.4: Human depressive and anxiety disorder symptoms, analogous behaviour in rats and the preclinical test(s) used for assessment (Adapted from Czeh et al., 2016; Castagne, Moser & Porsolt, 2009).

Human Symptom	Analogous Behaviour in Rats	Example Preclinical Test(s)
Depressed mood	Resignation	Forced swim test Tail suspension test
Loss of interest or pleasure	Anhedonia	Sucrose preference Intracranial self-stimulation Female urine sniffing Novelty seeking
Irritability	Aggressiveness	Social behaviour Muricidal behaviour
Changes in weight	Body weight	Body weight Food and water intake
Sleep disturbance	Altered diurnal rhythm	Sleep EEG Circadian rhythms
Psychomotor disturbance	Locomotor activity	Open field test Home cage activity Treadmill running
Anxiety or restlessness	Exploratory behaviour changes	Open field test Elevated plus maze Elevated zero maze Light/dark box
Feelings of guilt	No analogous behaviour	Not applicable
Poor concentration	No analogous behaviour	Not applicable
Suicidal ideation	No analogous behaviour	Not Applicable

Rat behavioural tests assess the effect of an environmental, genetic, or pharmacological manipulation used in rat models (Czeh *et al.*, 2016; Abelaira *et al.*, 2013). In this way, rat models are used to examine gene-environment interactions, controlling environmental factors to investigate their effects on any quantifiable parameter such as behaviour, biochemistry, neurobiology, or pharmacological response (Czeh *et al.*, 2016; Dick, 2011). Table 1.5 summarises preclinical tests of depressive- and anxiety-like behaviour in rats, as well as the advantages and disadvantages of these tests. Two behavioural tests will be discussed in more detail regarding the assumptions that are made with these tests in addition to the advantages and disadvantages, these tests are: the FST and the OFT.

Table 1.5: Summary of rat behavioural tests, advantages and disadvantages related to the use of these tests.

Behavioural test	Behaviour(s) measured	Advantages	Disadvantages	References
Elevated plus maze	Thigmotaxis, exploration, anxiety	Simple, high throughput, no conditioning or reward influencing behaviour	Inconsistent result with different antidepressants, sensitive to handling prior to testing	Karson *et al.*, 2013; Chiba *et al.*, 2012; Fischer *et al.*, 2012; Andrus *et al.*, 2010
Forced swim test	Behavioural despair	Ease of use, high throughput, low cost	Sensitive to acute antidepressant treatment only, can give false positives with compounds that increase general activity, strain differences	Kedzierska & Wach, 2016; Liu *et al.*, 2013; Suo *et al.*, 2013; Chen *et al.*, 2012; Will *et al.*, 2003
Light/dark box	Behavioural withdrawal, exploratory behaviour	Uses innate fear of brightly lit areas, no conditioning or reward influencing behaviour	Not sensitive to all antidepressants, responses sensitive to rat age	Steimer, 2011; Ennaceur *et al.*, 2006; Mineur *et al.*, 2006
Morris water maze	Spatial learning,	No motivation issues, no	Can have large variability, requires	First *et al.*, 2011; Ferraz *et al.*, 2011;

	recognition memory	olfactory cues	training days, time-consuming, large space requirement	Schaar *et al.*, 2010
Novel object test	Recognition memory	Simple, ease of use, low cost	Size of objects can affect rat response, requires training	Toth *et al.*, 2013; Larsen *et al.*, 2010; Schrijver *et al.*, 2002
Open field test	Locomotor activity, anxiety	Uses innate fear of brightly lit areas, simple	Difficult to discriminate between anxiety and novelty seeking behaviours, not sensitive to all antidepressants	Reus *et al.*, 2012; Liu *et al.*, 2011; Qi *et al.*, 2008; Zhao *et al.*, 2008
Sleep EEG	Circadian rhythms	Reliable, consistent, no interpretation bias	Cost of use, invasive, requires technical expertise	Wang *et al.*, 2014; Mrdalj *et al.*, 2013
Social interaction test	Interaction with an unknown animal	Simple, ease of use	Dependant on the gender of novel and subject rats	Rana *et al.*, 2016; Beery & Kaufer, 2015; Lukkes *et al.*, 2009
Sucrose preference	Preference for a sucrose solution compared to water. Anhedonia	Easy, monitors anhedonia (core depressive symptom)	Requires single housing for accurate results, can be time-consuming	Wood *et al.*, 2015; Mao *et al.*, 2010; Luo *et al.*, 2008; Muscat *et al.*, 1990
Tail suspension test	Behavioural despair	Same as the forced swim test	Same as the forced swim test	Kedzierska & Wach, 2016; Liu *et al.*, 2013; Suo *et al.*, 2013; Chen *et al.*, 2012; Will *et al.*, 2003

1.3.3.1 The Forced Swim Test

Porsolt, Le Pichon and Jalfre originally developed the FST, also known as the Porsolt Swim Test (PST), in 1977 as a screening test for potential antidepressant agents (Kedzierska & Wach,

2016; Porsolt, Bertin & Jalfre, 1977). In recent years, the FST is commonly described in the literature as a test to assess what is defined as rat behavioural despair following environmental manipulations (de Kloet & Molendijk, 2016; Kedzierska & Wach, 2016; Suo *et al.*, 2013). A rat is placed in an inescapable cylinder of water for 15 minutes as a pre-test, after which it is dried and returned to the home cage. Twenty-four hours later the rat undergoes a second swim experience for five minutes, with most rats demonstrating passive behaviour, just enough movement to keep the head above water, soon after the second test starts (Castagne, Moser, Roux & Porsolt, 2010; Porsolt, Bertin & Jalfre, 1977). Immobility, defined as passive floating in the water, is interpreted as behavioural despair due to inability to escape and antidepressant administration causes the escape-oriented behaviour to persist for longer than controls (Suo *et al.*, 2013; Porsolt *et al.*, 1977). The main benefits of the FST are that it is a straightforward simple test to use, with high throughput and low costs, resulting in widespread use in behavioural studies (Kedzierska & Wach, 2016; Abelaira *et al.*, 2013). The main limitations of the FST are that the reduced mobility may demonstrate learning or habituation rather than behavioural despair and that the test does not reflect the clinical situation due to use of acute antidepressant treatment (de Kloet & Molendijk, 2016; Slattery & Cryan, 2014; Valvassori *et al.*, 2013; Armario, Escorihuela & Nadal, 2008). The potential for habituation and adaptation to the FST suggests that this test may be more effectively used to investigate the mechanisms of stress coping and adaptation rather than an assessment of behavioural despair (de Kloet & Molendijk, 2016).

1.3.3.2 The Open Field Test

The OFT is primarily used to assess anxiety-like behaviour due to the ease of assessment for locomotion and stereotypic behaviours (Rabasa *et al.*, 2016). This test involves placing the rat in an arena 100cm x 100cm for the rat to move around freely, with the general activity of the rat monitored, specifically horizontal and vertical movement (Mao *et al.*, 2010). High locomotion and time spent in the central areas indicate low anxiety-like behaviour, while less locomotion and a

preference for the peripheral areas demonstrates higher anxiety-like behaviour (Ennaceur, 2014; Will, Aird & Redei, 2003). A predominant criticism of the OFT is that just the open field offers no incentive for continued exploration after initial exposure, resulting in initial high levels of activity but rapid habituation and corresponding low activity (Clemens, Jansson, Portal, Riess & Nguyen, 2014; Ennaceur, 2014; Ramos, 2008). Thus, a modified OFT, with other objects to induce exploratory behaviour, is preferred (Ramos, 2008). This rapid habitation in the OFT is identified as a lack of exploratory behaviour, rather than the behavioural despair due to the inability to escape that is observed in the FST (Clemens *et al.*, 2014; Suo *et al.*, 2013). Such habituation leads to reduced exploratory behaviours that are not interpreted as depressive-like but can be used as reference behaviours to determine the impact of experimental manipulations (Bailey & Crawley, 2009; Nosek *et al.*, 2008).

1.3.4 Why Use Rodent Models?

As mentioned in section 1.2.4 Pathophysiology of Depression and Anxiety in Humans, similar comparative pathophysiology has also been reported from rat studies of depressive and anxiety disorders. The main reasons for the use of rat models in depressive and anxiety disorder research include, first, the requirement for tissue use (such as brain tissues) where alternative methods are not available or cannot provide the same level of detail. Second, rat models allow the investigation into the mechanism(s) of development such as examining neurogenesis or protein localisation after a set period during development rather than at an end-point after death in humans. Third, rat models allow more rapid development of novel treatment options during preclinical research (Hanell & Murklund, 2014; Krishnan & Nestler, 2011; Nestler *et al.*, 2002). As a result, there is additional information regarding neurological changes in depressive and anxiety disorders obtainable from rat models using more invasive techniques than what is viable in live human subjects. This is an important reason rat models are used to investigate neurological changes because of stress or development of depressive or anxiety disorders (Hanell & Murklund,

2014). These changes include neurogenesis, protein localisation in specific brain regions of interest, and the correlation between central and peripheral concentrations of biomarkers (Klein *et al.*, 2011; Sierra, Encinas & Maletic-Savatic, 2011). It is possible to examine some of these neurological changes using post-mortem studies in subjects that suffered from depression while alive, such as protein localisation and to a limited degree, neurogenesis (Ho, Hooker, Sahay, Holt & Roffman, 2013; Matthews & Harrison, 2012).

However, a caveat with the usage of post-mortem tissue is that there is no way to control or minimise the heterogeneity of depressive and anxiety disorder development, or any confounding factors relating to the post-mortem tissue use (Sierra *et al.*, 2011). Confounding factors against brain tissue use in human post-mortem studies can include: age at death, general health in life, cause of death, duration before tissue acquisition, possible medications, and state of the depressive disorder at time of death (e.g. during a depressive episode or while in remission) (Muller *et al.*, 2015; Chandley & Ordway, 2012; Sierra *et al.*, 2011). In rat models, the trigger for the development of defined depressive- or anxiety-like behaviours is consistent for all rats in the experiment, reducing the heterogeneity of the induced behaviours, when compared to humans with depressive or anxiety disorders (Gass *et al.*, 2016). There is the potential that individual rodents will respond differently to the same stimulus, such as using coping mechanisms, like grooming, differently when stressed (Franklin, Saab & Mansuy, 2012; Koolhaas, de Boer, Coppens & Buwalda, 2010). However, it is easier to compare the observed behaviours in a rodent model due to similar stimulus exposure, as opposed to differences in prior stress and in human depressive or anxiety disorder symptomatology (Gass *et al.*, 2016). Therefore, behavioural and physiological differences between experimental rats can be limited to individual variation between animals or genetic differences when not using a specifically bred rat strain like the Flinders Sensitive Line or Wistar-Kyoto rats, since the stimulus used for triggering depressive- or anxiety-like behaviours is consistent for all animals (Burke *et al.*, 2016; Ellenbroek *et al.*, 2016).

In the investigation of depressive and anxiety disorders, an area of active investigation is the role of neurogenesis and neurotrophins in development of these disorders, particularly given the evidence of reduced neurotrophin levels in rats demonstrating depressive- and anxiety-like behaviour as well as in humans with depressive disorders (Stepanichev *et al.*, 2014; Chiba *et al.*, 2012; Sen, Duman & Sanacora, 2008). Neurogenesis refers to the process of generating new neurons from neural stem cells and neurotrophins are growth factors that promote survival, differentiation and growth of neurons, thereby stimulating and controlling neurogenesis (Kim, Na, Myint & Leonard, 2016; Petrik, Lagace & Eisch, 2012; Neto, Borges, Torres-Sanchez, Mico & Berrocoso, 2011). The main benefit of rat models in the examination of neurogenesis is that the potential growth or atrophy of neuronal tissue is assessable at any predetermined period following induction of depressive- or anxiety-like behaviour (Yazir, Utkan, Gacar & Aricioglu, 2015; Liu *et al.*, 2014; Marco *et al.*, 2012). Neurogenesis is commonly measured by injecting bromodeoxyuridine (BrdU) to quantify cell proliferation or lack thereof, that results from experimental manipulation (Sailor, Schinder & Lledo, 2017; Evans, Sun, McGregor & Connor, 2012; Malberg & Duman, 2003). Measurement of neurogenesis in humans is possible but is restricted to resected or post-mortem brain samples, which, as mentioned, can be easily confounded by factors other than depressive or anxiety disorder status (Sierra *et al.*, 2011).

Together with the same parameters that are effectively measurable in humans, these stresses induced pathophysiological markers and associated behavioural measures can better define phenotypes of depressive- and anxiety-like symptoms in rats. These phenotypes in rats can be an indication of the physiological parameters to examine in humans with depressive or anxiety disorders. This can outline potential physiological markers to be examined in human post-mortem and neuroimaging studies of depressive and anxiety disorders, after which, the associated genotypes can be examined to further elucidate specific mechanisms of action in depressive and anxiety disorders (Grigor'yan & Gulyaeva, 2017; Mehta-Raghavan *et al.*, 2016). Consequently, this information could also be used to attempt to better stratify the diagnosis of depressive and anxiety disorders using additional markers.

1.4 Study Overview

As discussed, exposure to stress often precedes the development of depressive and anxiety disorders. Thus, stress was the mechanism used to alter the behaviour of the rats in this study. The current study uses a factorial design (2 x 2) to subject rats to combinations of early life stress (maternal deprivation) and isolation, followed by recent life stress to investigate how behavioural, hormonal, and cellular measures changed after these different levels of environmental stress. Early life stress and adult isolation are often forms of stress used that can be used alone to induce depressive- or anxiety-like symptoms in animal models. Furthermore, such environmental factors can impact the development of depressive or anxiety disorders in humans (Section 1.2.3). The use of recent life stress in this study, in the form of CMS, is representative of stressful events that frequently occur during adulthood in humans.

The original design incorporated a blood sampling time course to be examined in conjunction with longitudinal behavioural changes. This would have allowed the change to physiological parameters to be analysed and correlated behavioural modifications. However due to the failure of the longitudinal blood sampling only post mortem blood and tissue samples were obtained. The longitudinal behavioural response to stress was assessed as depressive- or anxiety-like using a modified OFT termed the behavioural observation open field (BOOF) test. The post-mortem hormonal and cellular changes associated with the stress exposure were assessed using liquid column mass spectrometry, flow cytometry, and histological structural staining. Finally, the stress-induced behaviours were examined for association with the hormonal and cellular markers that were altered because of the same stress exposure.

1.5 Rationale and Limitations of the Study Design

The combination of stressors used in the current study (MD, isolation, and CMS) was selected based on the available literature indicating that these types of stress (early life trauma,

social isolation, and chronic stress) often precede the development of depressive or anxiety disorders in humans. First, children exposed to early life trauma have a higher risk for development of future mental disorders, particularly following recent life stress in adulthood (Menard *et al.,* 2016; APA, 2013; Kaufman *et al.,* 2000). Second, social isolation or a lack of social support in humans can have a negative effect on the development of depressive and anxiety disorders during chronic isolation. However, as mentioned in Section 1.2.3, the availability of social support has been noted to confer a protective effect against the development of these mental disorders. Finally, exposure to stressful life events during adulthood is frequently observed prior to the development of depressive or anxiety disorders (Kendler & Aggen, 2017; Taporoski *et al.*, 2015; Koolhaas *et al.*, 2011). Stressful life events can include, but are not limited to; spousal death, marital discord, abuse, loss of job, or major accidents. It is important to note that significant literature has observed similar types of stress (early life stress, social isolation, and chronic stress) having the potential to induce depressive- or anxiety-like behaviours in rats as well. In Section 1.3.2, Table 1.3 outlines common rodent models of depressive- and anxiety-like disorders, with MD, social isolation and CMS noted as commonly used methods to research depressive- and anxiety-like disorders in rodents.

Early life stress and social isolation are environmental variables in humans that may or may not be present prior to the development of depressive and anxiety disorders. Chronic stress, or exposure to multiple small stresses over time in everyday life, is considered a normal part of adult life (Willner, 2005; Muscat, Papp & Willner, 1992). Furthermore, there are both negative and positive stressful life events; though identifying such events as negative or positive is highly dependent on individual perception as discussed in Section 1.2.3. Given that stress exposure, and cumulative small stresses over time, is a normal part of adult life, the decision was made to subject all animals to CMS exposure. As a result, the current study design uses a factorial design with combinations of social instability (maternal deprivation and adult isolation), followed by environmental disruption (chronic mild stress) for all animals in adulthood to investigate how

these environmental stress combinations impact behavioural, hormonal, and cellular measures in rats.

Thus, the current study can be best described as an examination of the response to combinations of environmental stress across a lifetime. However, there were significant limitations in the current study due to the lack of a control condition for the CMS exposure that must be noted. As mentioned in Section 1.4 Study Overview the original experimental design called for longitudinal blood sampling to control for the change before and after CMS exposure. This would have allowed the longitudinal behavioural parameters to be compared to longitudinal physiological measures. The lack of biological time course data resulted in no way to determine if the observed differences in the physiological parameters were significantly altered from pre-stress values. This deviation from the original experimental design, due to the inability to obtain blood samples prior to CMS exposure confounded the study, limiting the conclusions that could be drawn from the correlations between end point behavioural and physiological data.

There was also a massive limitation in the lack of a facility reared control group, even when considering the original study design. In the original study design the time course of biological samples would only allow the physiological changes within the same cohort to be assessed. Further, comparisons could be made between the cohorts due to the same treatment exposure, but the lack of facility reared rodents subjected to normal husbandry practices, meant there was no way to determine if the rats that received only CMS were significantly different in behaviour, hormonal or cellular measures from facility reared rats. This lack of a facility reared control group means no negative control to compare the behavioural and physiological measures against, which in turn, limits the comparisons that can be made against similarly designed studies. More importantly, it was not possible to determine how different the stress combination of the MD, isolation, and CMS may be from normal facility reared control rats, and whether the observed changes were significantly different from such control animals. Thus, the size of the effect of the stress manipulation when compared to rodents that experience no manipulation as all.

As a result of this drawback in the current study design, there were limits to what could reasonably be concluded from the data analyses. Specifically, conclusions were made that compared the rats subjected to combinations of MD and isolation followed by CMS to the rats only subjected to CMS if any statistical difference was observed. This prevents any categorical statements that imply the rats in the current study were more or less stressed than rats subjected to no stress. Hypotheses were made regarding why these combinations had the observed results and how this could be applied to future studies based on the results of the current study and other similar studies. However, the scope of these hypotheses was restricted due to the lack of a control group for CMS. Several hypotheses were put forward with potential explanations for the observed results based on what was concluded from the literature. The observed differences between the experimental cohorts in the current study were attributed to the combination of MD and isolation manipulations, not the CMS manipulation since all cohorts received the same CMS exposure. However, the combinations of stressors used were hypothesised to be more analogous to how depressive and anxiety disorders develop in humans, where symptoms often manifest following cumulative stress, rather than being attributed to a single life event.

1.6 Thesis Hypothesis and Aims

The manifestation of clinical depressive and anxiety disorders have significant genetic and environmental components, involving complex phenomena with possibly multiple aetiologies. Stress is a common environmental trigger that precedes the development of depressive or anxiety disorders. Rodent models can be effectively used to define phenotypes of comparative behaviour and physiological modifications in response to stress to elucidate the potential mechanisms of depressive and anxiety disorders. These induced phenotypes can be used to further shed light on physiological markers of human depressive or anxiety disorders, effectively narrowing down potential examinable areas for use in human post-mortem and neuroimaging studies.

The hypothesis of this dissertation is that combinations of early life stress and isolation in addition to adult life stress in rats, will manifest physiological (hormone levels) and anatomical (hypertrophy and hypotrophy in the brain and adrenal regions) changes associated with stress-induced depressive- or anxiety-like behaviour in these animals.

The objectives of this dissertation were to:

1) Examine whether exposure to maternal deprivation and isolation altered rat behaviour following chronic mild stress.
2) Determine rat hormonal levels following maternal deprivation, isolation, and chronic mild stress.
3) Assess cellular and morphological changes in relation to volume in the adrenal gland, hypothalamus and hippocampus following maternal deprivation, isolation and chronic mild stress.
4) Identify associations between behavioural and physiological measures separately determined as significantly different following maternal deprivation, isolation, and chronic mild stress.

Chapter 2 – Materials and Methods

2.1 Materials

2.1.1 Reagents and consumables

All chemicals used were of analytical grade or higher. These chemicals were obtained from the manufacturers listed below.

Armidale Pet Shop & Aquarium (Armidale, Australia): Cane finch nests.

Ajax Chemicals (Sydney, Australia): Absolute Ethanol, Eosin, 30% w/w Formaldehyde, Glacial Acetic Acid, Haematoxylin, Potassium Alum, Potassium Chloride, Sodium Carbonate, Sodium Dihydrogen Orthophosphate, Xylene HP, Sodium Iodate.

Becton Dickinson Biosciences (Sydney, Australia): 25G needles, K2EDTA blood tubes.

BHD/Merck Chemicals (Melbourne, Australia): Disodium Hydrogen Orthophosphate, Hydrochloric acid, Potassium Dihydrogen Orthophosphate, Sodium Chloride, Sodium Hydroxide.

Bunnings Armidale (Armidale, Australia): Insulation tape, 10mm Aviary Wire Mesh.

Chem-Supply (Adelaide, Australia): Water B&J Brand for gradient HPLC/ spectrometry, Methanol ACS/HPLC Certified.

Dollars & Sense (Armidale, Australia): Clothes pegs, Aquarium figures, Cat toys.

eBioscience (Waltham, USA): Permeabilisation Buffer (10X) 100 ml, 10x RBC Lysis Buffer (Multi-species) 50 ml.

Heidolph Instruments (Schwabach, Germany): Heidolph DIAX-900 homogeniser.

Leica Microsystems (Wetzlar, Germany): Paraplast™ Pelletized Paraffin Wax (MP: 56°C).

Livingston International Pty. Ltd. (Rosebery, Australia): Liv-wipe alcohol swabs, latex gloves.

Phenomenex (Torrance, USA): Kinetex 2.6 µm EVO C18, LC column – 50 x 2.1 mm, Kinetex 2.6 µm EVO F5, LC column – 100 x 2.1 mm.

ProSciTech (Townsville, Australia): Feather R35 Microtome Blades, Slide boxes.

Ridley Agriproducts (Melbourne, Australia): Rat and Mouse Chow.

Sarstedt (Ingle Farm, Australia): 50 ml centrifuge tubes, 15 ml centrifuge tubes, 1 ml microfuge tubes, sterile transfer pipettes, pipette tips, plate sealers, 96-well v-bottomed plates, 2 ml Cryopure tube.

Sigma-Aldrich Incorporated (Sydney, Australia): DPX Mounting Media.

SDR Scientific (Sydney, Australia): Rodent Restraint Cones.

Thermo Fisher Scientific (Waltham, USA): Menzel-Glaser Superfrost Plus Microscope slides, Menzel-Glaser Coverslips, Micro Vial PP Snap Ring 0.3 ml Transparent, 11 mm combination seal PE Snap Ring Cap, AbC Total Antibody Compensation Bead Kit, BCA protein assay kit.

United Biosciences (Brisbane, Australia): Solv21C.

UNE Science Workshop (Armidale, Australia): Behavioural Arena, Glass Cylinder.

2.1.2 Antibodies

Bioss Inc. (Woburn, USA): Rabbit Anti-CD19 Polyclonal Antibody, PE-Cy5 Conjugated, Rabbit Anti-CD56 Polyclonal Antibody PE-Cy7 Conjugated, Rabbit Anti-Glucocorticoid Receptor (GR) Polyclonal Antibody, ALEXA FLUOR 647 Conjugated, Rabbit IgG Isotype Control A647 Conjugated.

Invitrogen Life Technologies (Mulgrave, Australia): Rat Anti-CD8 FITC Conjugated, Rat Anti-CD4 PE-conjugated.

2.1.3 Software Packages

GraphPad Software Inc. (California, USA): GraphPad Prism 6.

International Business Machines Corporation (New York, USA): IBM SPSS Statistics Ver. 22.

IDEAS® Software (Seattle, USA): Analysis software specific to Amnis FlowSight flow cytometer.

Hamamatsu Photonics (Hamamatsu City, Japan): NDP view 2 Viewing software.

2.2 Methods

2.2.1 General Information

All solutions were prepared with glass-distilled water, further purified by the Millipore Milli-Q filtration system (Millipore, Sydney, NSW). All solutions requiring sterilisation were autoclaved at 121°C for 15 minutes. The pH of solutions was measured and adjusted as necessary using a pH meter (Activon model 209).

2.2.2 Animals

Sixty male Wistar rats were bred in-house, from the UNE rat colony, at the University of New England (UNE) Animal House. This decision was made to prevent transport stress to females prior to the study from compromising maternal behaviours and resulting in additional stress on the offspring. To increase the statistical power and prevent additional complication, due to estrous cycle, during analysis of behavioural and physiological markers only male offspring were used in the study. Ten breeding females from the UNE rat colony contributed litters, providing the required numbers (n = 60; four cohorts with n = 15 per cohort) for the study. After mating, females were checked daily for litter birth. The presence of litter was designated postnatal day 0 (PND0). Pups were sexed and the female offspring from all litters were culled on PND4 at the start of the maternal deprivation protocol, control litters were also sexed at this time. On PND30, rats were weaned as per normal operating procedure in the UNE Animal House. Weaned rats were separated four per cage until the start of isolation on PND45. After PND45, the rats were single-housed in opaque polypropylene cages (64 × 41 × 25 cm) with stainless steel lids. Cages were lined with dust-free wood shavings and shredded paper. Cages were maintained with a 12-hour light and dark cycle (lights on at 0700, off at 1900), and constant temperature (23 ± 2°C). Tap water and standard rat chow (Ridley Agriproducts, NSW) were provided *ad libitum* unless stated otherwise. The University of New England Animal Ethics Committee (AEC13-050) approved all procedures (Appendix A).

2.2.3 Maternal Deprivation Design

Early life stress (ELS) is a common method of altering rodent behaviour and has been used extensively to induce depressive- and anxious-like behaviour in rats (Molet *et al.,* 2014; Ruedi-Bettschen *et al.,* 2006). Maternal deprivation (MD) represents a common form of postnatal ELS used in rodent models, with prolonged separation considered to be a laboratory model for childhood neglect due to the overall lack of maternal care (de Kloet *et al.,* 2005). The MD protocol began on PND4 and continued for ten days until PND13. Litters were randomly assigned to MD protocol or control. Each day the pups were removed from the home cage and isolated in separate containers on a fresh bed of clean wood shavings. Maternal deprivation was conducted for 180 minutes per day. Deprivation start time alternated between morning and afternoon for ten consecutive days, beginning between 0700 – 1130 hours for the morning or 1200 – 1530 hours for the afternoon. The variable start time prevented an established routine and limited the pups' adaptation to the stress (Gagliano, Fuentes, Nadal & Armario, 2008; Eklund & Arborelius, 2006). Control litters were maintained together under normal husbandry conditions. Animals weaned on PND30 as per normal UNE Animal House procedures and group housed (four rats/cage) until the start of the chronic stress protocol.

2.2.4 Chronic Mild Stress Exposure Design

The animal study was designed as a 2×2 factorial design to maximise the statistical power for the behavioural and physiological analysis (Table 2.1). The rats from the two ELS groups (Control and Maternally Deprived) were randomly assigned to visual or non-visual isolation when placed in single housing on PND45. These groups were designated Visual and Non-Visual isolation. Social isolation has been noted in the literature (Section 1.2.3 and Section 1.3.2) to influence the development of depressive- or anxiety-like behaviours. However, there is also evidence that the presence of social support can provide a protective effect against the

development of depressive and anxiety disorders (Section 1.2.3). Rats in the non-visual group had a piece of stiff cardboard placed between the visible portions of adjacent cages to preventing any face-to-face interaction. Rats in the visual group had limited interaction due to the lack of this cardboard barrier. All cohorts received the same chronic mild stress exposure through environmental disruption during adulthood, similar to how humans experience different stressful events across a lifetime. Figure 2.1 provides the timeline for the experimental protocols experienced by the rats.

Table 2.1: Description of treatments for the experimental cohorts in the factorial study design.

Treatments	**Experimental cohorts**	
	Visual (Control)	**Non-Visual (Treatment)**
Not Maternally Deprived (Control)	No MD and visual social support after weaning	No MD and no visual social support after weaning
Maternally Deprived (Treatment)	MD received and visual social support after weaning	MD received and no visual social support after weaning

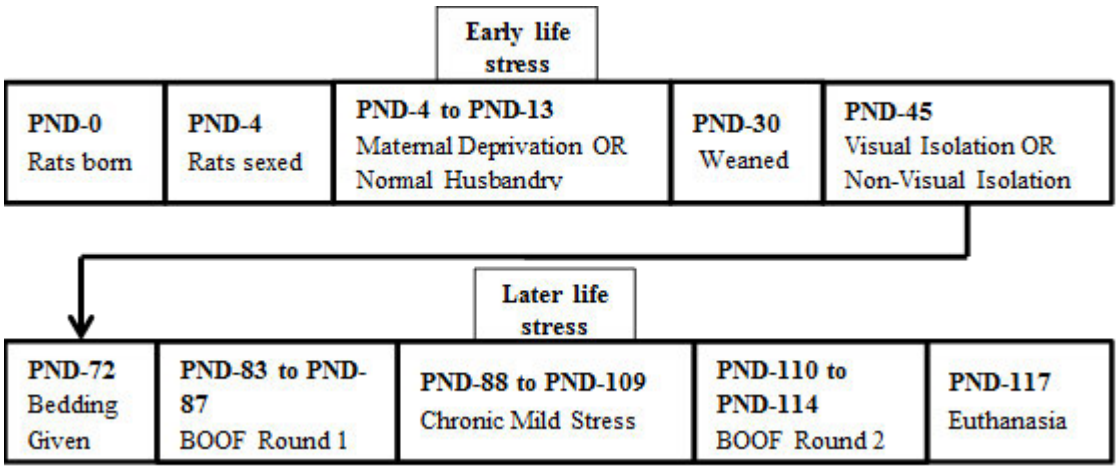

Figure 2.1: Experimental timeline used for behavioural study based on factorial study design.

On PND72, all rats were provided with additional bedding items in the form of a wicker finch nest, five cotton balls and five strips of cotton as materials to construct a nesting area involving the finch nest, in addition to the normal cage bedding (Figure 2.2-A). This nest area was used as a landmark for application of chronic mild stress, and thus nest construction was not analysed.

The chronic mild stress protocol started on PND88 and lasted for 21 consecutive days with all rats experiencing chronic mild stress exposure. The stressor was an environmental disruption, applied by removing access to the established nest area (Gurfein *et al.*, 2012; Wurbel, 2001). During stress, all bedding except the nest area was removed from the cage and a wire barrier was fitted to prevent access to the nest by the rat (Figure 2.2-B). The stress exposure duration was cycled each day lasting between 90, 120 or 150 minutes for an average of 120 minutes of stress each day. The start of stress exposure each day fell between 0700 to 1300 hours. During chronic stress exposure, food and water access were removed. The rats were video monitored for 15 minutes at the beginning and end of stress exposure. Video monitoring occurred over the first five days and every fifth day thereafter, to monitor the overall health of the rats.

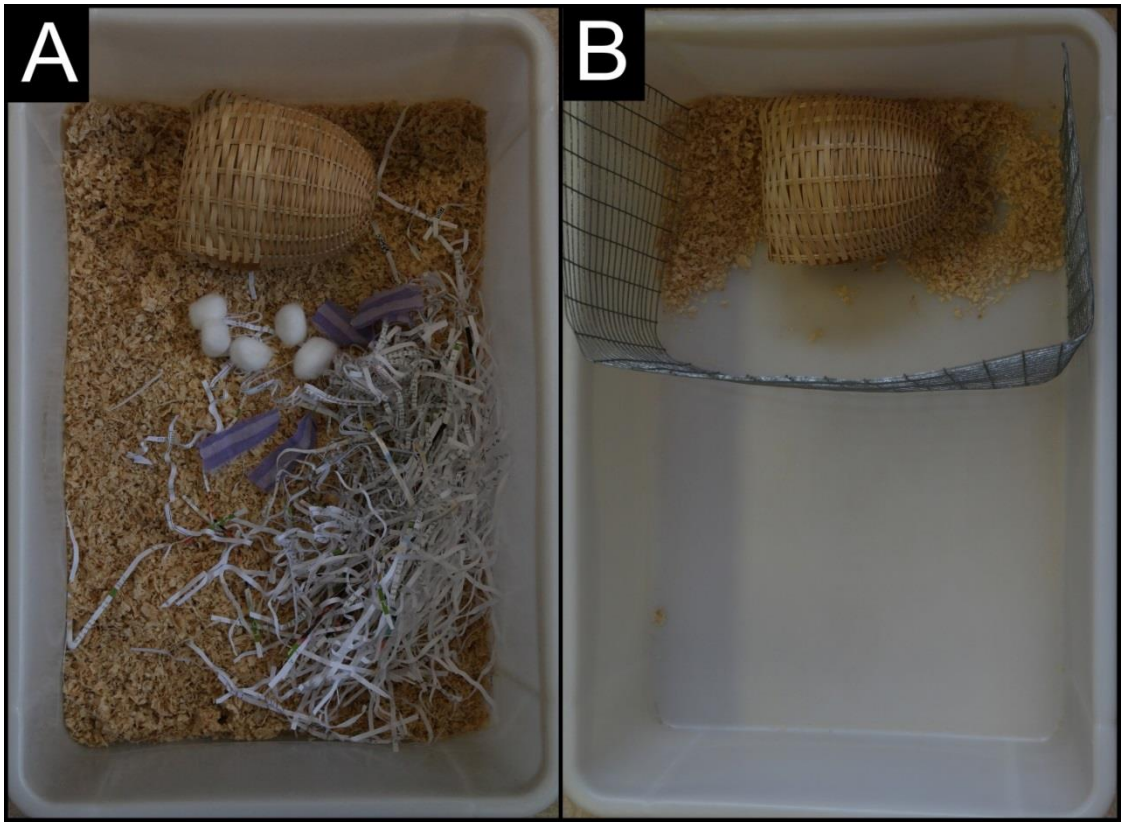

Figure 2.2: Set up of rodent cages before chronic stress exposure (A) and during chronic stress exposure (B).

2.2.5 Behavioural Observation Open Field (BOOF) Test Design

All rats underwent behavioural observation for five days before (PND83 – 87) and five days after (PND110 – 114) stress exposure, allowing the behaviour and habituation to the testing area before stress to be monitored and compared to the behaviour and habituation post stress. The behavioural observation open field (BOOF) test was a modified open field test, utilising novel objects to motivate exploration (Ennaceur, 2014; Ramos, 2008). The BOOF test was conducted in a 100 × 100 × 50 cm arena made of blue melamine, the base of which was divided into 25 squares with green electrical tape (20 × 20 cm) (Figure 2.3). A sheet of clear plastic (100 × 100 cm) covered the base of the arena for easy cleaning between tests. Within the arena, there were four objects to examine exploratory behaviour; a glass tunnel, a cat toy, an aquarium figure, and a clothes peg. One object changed each day of testing for another object of the same type but different size or colour, except for the glass tunnel, which remained consistent.

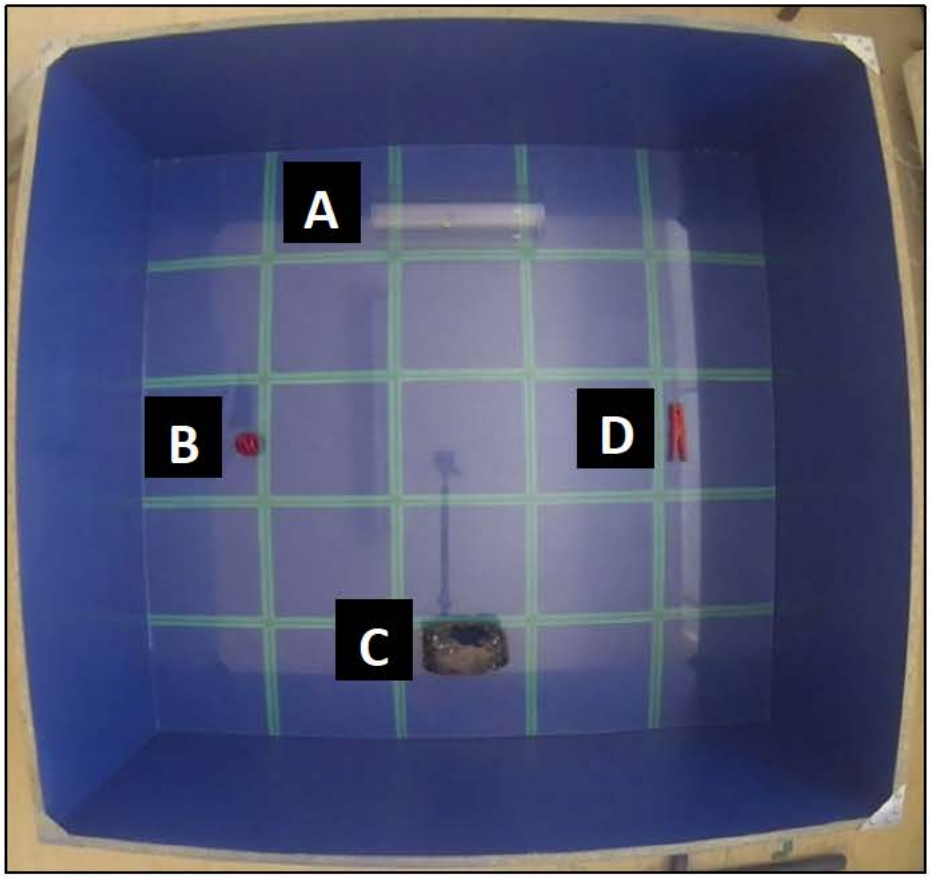

Figure 2.3: Behavioural observation open field arena. Objects are (A) Object 1 the glass tunnel; (B) Object 2 the cat toy; (C) Object 3 the aquarium figure; and (D) Object 4 the clothes peg.

Table 2.2 details, which object was changed on which day of behavioural testing. This same pattern was used for all rats during testing before and after chronic stress exposure. The behavioural tests were video recorded using a GoPro camera for high-resolution, positioned 140 cm above the centre of the arena. All behavioural testing conducted under a light intensity of 25 Lux.

Table 2.2: Object changes during behavioural observation open field-testing by test day.

Behavioural Testing Day	**Objects used**	**Object changed**
1	Glass tunnel, spherical cat toy, barrel aquarium figure, red peg	N/A
2	Glass tunnel, spherical cat toy, skull figure, red peg	Aquarium Figure
3	Glass tunnel, cylindrical cat toy, skull figure, red peg	Cat Toy
4	Glass tunnel, cylindrical cat toy, bridge figure, red peg	Aquarium Figure
5	Glass tunnel, cylindrical cat toy, bridge figure, yellow peg	Peg

Rat cages were covered and transported in less than a minute to the testing room. Each rat was placed in the central square of the arena to limit animal bias for any specific corner over another and given 10 minutes to explore the testing arena (Lambas-Senas *et al.* 2009). This length of time allowed each rat time to adjust and begin to investigate the testing arena (Antunes & Biala, 2012). Behaviours of interest such as locomotion, grooming, immobility, and object investigation activities were hand scored from the video recording for frequency and duration to determine if there were any differences between experimental groups due to stress combinations (Appendix B; BOOF checklist and behaviour definitions). After observation was completed, the arena cleaned thoroughly with 70% ethanol to remove rat olfactory trails between subjects.

2.2.6 Euthanasia, Animal Dissection and Tissue Preservation

On PND116, rats were fasted overnight and moved to the kill room two hours before euthanasia. Each rat was given an intraperitoneal injection of sodium pentobarbitone (70mg/kg) and returned to the home cage to wait for the anaesthetic to take effect. At post-mortem blood was

collected via a cardiac puncture using a 20G needle into a 2 ml syringe coated with 20 µl of 0.5M EDTA (pH 8.0). Following blood collection, animals were perfused through the left ventricle with ice-cold phosphate buffered saline (PBS; 137 mM NaCl, 2.7 mM KCl, 10 mM Na_2HPO_4, 1.8 mM KH_2PO_4). The right atrium was cut to allow blood to drain from the body. After perfusion, the head was removed, and the brain quickly dissected from the skull and hemisected. The right hemisphere was snap frozen in liquid nitrogen and stored at -80ºC and the left hemisphere was stored at room temperature in 10% neutral buffered formalin for histology. Additionally, the adrenal glands were excised. The right adrenal gland was snap frozen in liquid nitrogen and stored at -80ºC and the left adrenal gland was stored at room temperature in 10% neutral buffered formalin for histology.

2.2.7 Liquid Column Mass Spectrometry (LC-MS)

2.2.7.1 Sample Preparation for LC-MS

Adrenal glands were weighed (10 – 61 mg) and homogenised using a Heidolph DIAX-900 homogeniser (Heidolph, Germany) in 500 µL of ice-cold PBS. The hypothalamus (8 – 28 mg), hippocampus (7 – 39 mg), and prefrontal cortex regions (8 – 33 mg) were dissected from the right hemisphere of the brain. The brain region samples (hypothalamus, hippocampus, and prefrontal cortex) were homogenised in volumes of PBS to give a final concentration of 100mg/ml. Any remaining tissue homogenate not used for LC-MS sample preparation was snap frozen and stored at -80 ºC. The protein concentrations of the tissue homogenates were determined using the BCA protein assay kit (Thermo Scientific, USA).

Samples for catecholamine analysis were prepared as follows. For all samples (plasma, adrenal gland, brain region [hypothalamus, hippocampus, and prefrontal cortex]) 50 µL plasma, 7 mg/ml of the adrenal gland or 100 mg/ml of brain homogenates were added to 200 µL 2 µg/µL MES in methanol (100% LC-MS grade). Samples were vortexed for 1 minute to mix and microfuged 13000 × g for 4 minutes. The supernatant was removed, the pellet discarded, and the

supernatant was vacuum dried. The sample was resuspended in 50 µL H_2O (100% LC-MS grade) and vortexed in a 0.3 ml snap ring micro-vial.

Samples for steroid hormone analysis were prepared as follows. For all samples (plasma, adrenal gland, and brain region) 50 µL plasma, adrenal gland (7 mg/ml) or brain homogenates (100 mg/ml) was added to 200 µL 2 µg/µL MES in methanol (100% LCMS grade). Incubated on ice for 30 minutes and microfuged at 13000 × g for 4 minutes, 150 µL of supernatant was diluted with an equivalent volume of LCMS grade water in a 0.3 ml snap ring micro-vial.

2.2.7.2 Liquid Column Separation of Metabolites

Chromatography was performed using a Shimadzu Nexera X2 HPLC [LC-30AD] (Shimadzu Scientific Instruments, Japan) coupled to the mass spectrometer. Catecholamine separation was run on a Kinetex F5 column (100 mm × 2.1 mm, 2.6 µm, Phenomenex, USA), with a total run time of 8 minutes. Solvent A was 0.1% formic acid in LC-MS grade water, and solvent B was 0.1% formic acid in LC-MS grade acetonitrile. An isocratic solvent gradient was used to separate the catecholamine metabolites (noradrenaline, adrenaline, and dopamine) as shown in Figure 2.4, with a flow rate of 0.25 ml/minute. All separations were performed at 40ºC.

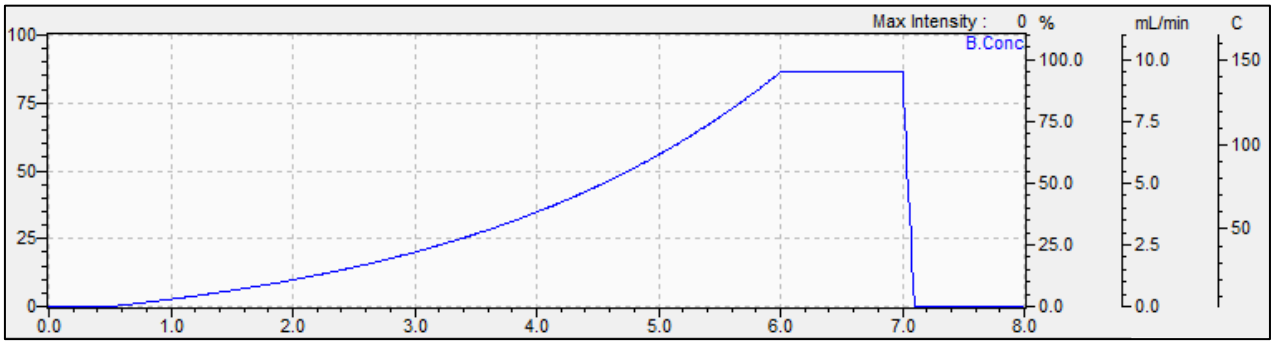

Figure 2.4: Solvent gradient used for Catecholamine LCMS protocol.

Steroid hormones were run on a Kinetex C18 column (50 mm × 2.1 mm, 2.6 µm, Phenomenex, USA), with a total run time of 3.75 minutes. Solvent A was 0.2µmol ammonium fluoride in LC-MS grade water, and solvent B was 0.2µmol ammonium fluoride in LC-MS grade methanol. An isocratic solvent gradient was used to separate the steroid hormone metabolites

(corticosterone, 11-deoxycorticosterone, testosterone, and progesterone) as shown in Figure 2.5, with a flow rate of 0.25 ml/minute. All separations were performed at 40ºC.

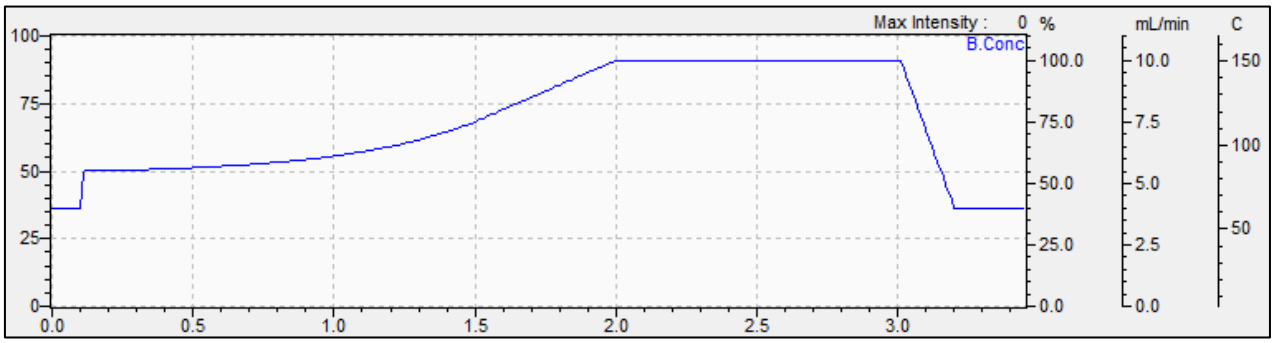

Figure 2.5: Solvent gradient used for Steroid hormone LCMS protocol.

2.2.7.3 Mass Spectrometry of Metabolites

The separated metabolites were detected on a Shimadzu LCMS-8050 triple quadrupole mass spectrometer (Shimadzu Scientific Instruments, Japan). Multiple-reaction monitoring (MRM) was applied for detection and the parameters for data acquisition, such as precursor-to-product mass to charge ratio, retention time, and collision energy, are available for all metabolites in Table 2.3.

Table 2.3: Parameters of LC-MS metabolites. Mass to charge ratio of precursor and product-ions for MRM, column retention time and collision energy of MRMs.

Catecholamine metabolite	**MRM**		**Retention Time (minute)**	**Collision Energy**
	Precursor (m/z)	**Products (m/z)**		
Noradrenaline	170.10	152.15, 107.1	2.0	-10, -21
Adrenaline	184.10	166.1, 77.00	2.1	-12, -44
Dopamine	154.10	91.05, 137.05	2.58	-27, -15
Steroid metabolite	**Precursor (m/z)**	**Products (m/z)**	**Retention Time (minute)**	**Collision Energy**
Corticosterone	347.20	329.20, 121.10, 311.20	1.53	-16, -25, -16
11-Deoxycorticosterone	331.50	97.10, 109.10, 79.15	1.87	-22, -25, -53
Testosterone	289.40	97.10, 109.10	1.95	-25, -26
Progesterone	315.10	97.10, 109.10	2.6	-23, -26

2.2.8 Flow Cytometry of Peripheral Blood Lymphocyte Subpopulations

2.2.8.1 Flow Cytometer Compensation

The output from the flow cytometer was standardised using AbC total antibody Compensation Bead Kit (ThermoFisher Scientific, USA). AbC capture beads were incubated on ice in the dark with each of the antibodies for 15 minutes. To each tube, 2 ml of PBS was added. Samples were centrifuged for five minutes at 250 × g. The supernatant was discarded, and the samples were resuspended in 0.5 ml of PBS. AbC negative beads were added to the tubes and thoroughly mixed. Each sample was run through the flow cytometer and automatic compensation was performed for each antibody. Figure 2.6 shows the theoretical spectra of the fluorophores conjugated to the antibodies used.

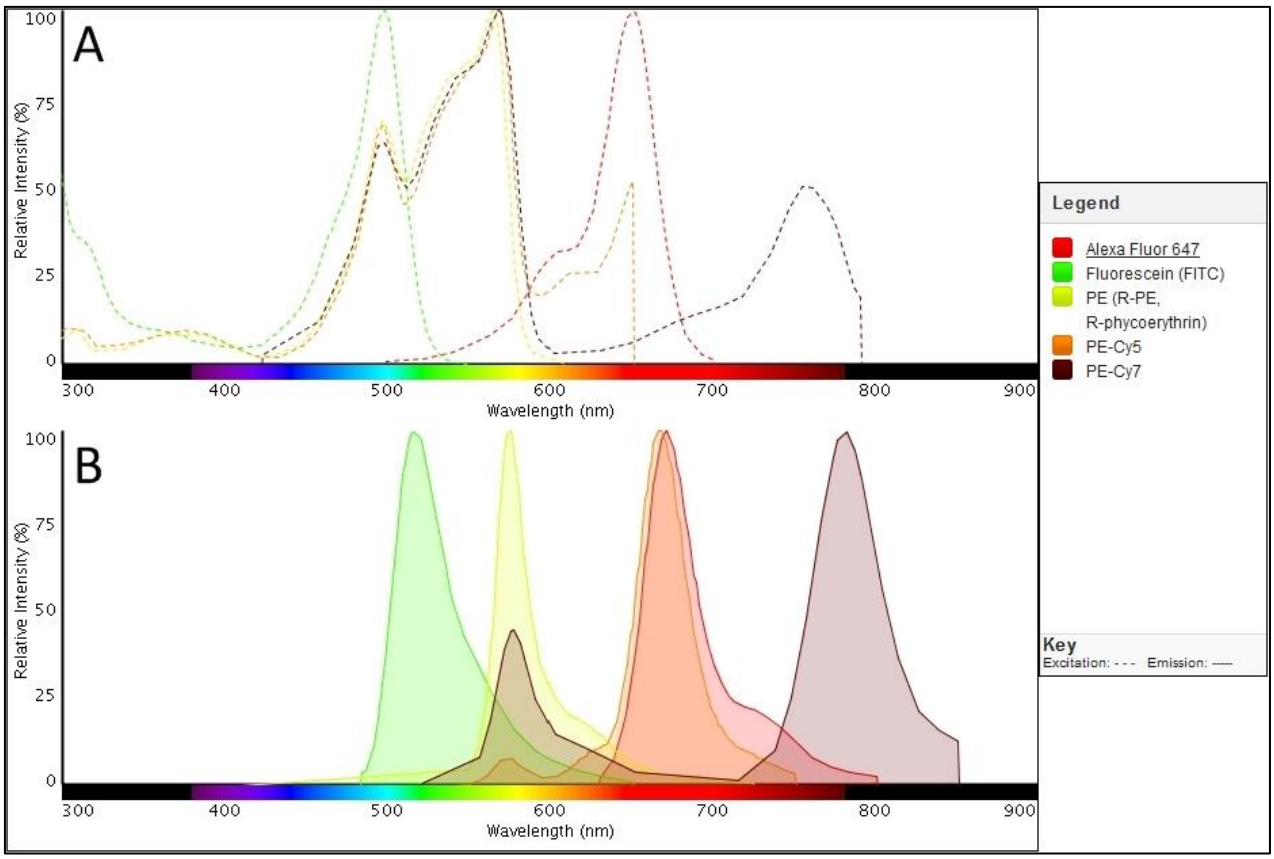

Figure 2.6: Five colour fluorophores theoretical spectra. (A) Percentage excitation spectra. (B) Percentage fluorescence emission.

2.2.8.2 Labelling of Lymphocytes with Antibodies

Whole blood samples (100 µl) for flow cytometric analysis were lysed using 1 ml RBC Lysis Buffer (eBioscience, USA). Samples were incubated in the dark at room temperature for 10 minutes. After lysis was completed, the samples were centrifuged at 400 × g for 5 minutes and the supernatant was aspirated. Cells were washed with cold PBS with 0.1% BSA (300 µl) and centrifuged at 400 × g for 5 minutes twice. The pellet was resuspended with the appropriate amounts and combinations of extracellular markers as shown in Table 2.4. These extracellular markers identified the lymphocyte sub-type as follows: $CD19^+$ B-lymphocytes, $CD4^+$ T-helper lymphocytes, $CD8^+$ cytotoxic T lymphocytes, and $CD56^+$ Natural Killer (NK) cells.

Table 2.4: Antibodies used to label extracellular markers for flow cytometry procedure.

Extracellular Antibodies				
Sample Tube	PECy5 – CD19 1 µg/µl	PECy7 – CD56 1 µg/µl	PE – CD4 0.5 1 µg/µl	FITC – CD8 0.5 1 µg/µl
Control Tube	N/A	N/A	N/A	N/A

*Note**Fluorochrome key: PECy5: Phycoerythrin-Cy5, PECy7: Phycoerythrin-Cy7, PE: Phycoerythrin, FITC: Fluorescein.

The tubes were gently vortexed and incubated on ice in the dark for one hour. Tubes were centrifuged at 400 x g for five minutes. Cells were washed with ice-cold PBS (300 µl) and centrifuged twice more. The pellet was resuspended in permeabilisation buffer (eBioscience, USA) and incubated in the dark for two minutes. Cells were washed with cold PBS, centrifuged at 400 × g and the supernatant was aspirated. An intracellular antibody (Rabbit anti-Glucocorticoid Receptor) or isotype control (Rabbit IgG isotype control) were added to the tubes (Table 2.5), vortexed gently and incubated on ice and in the dark for 30 minutes.

Table 2.5: Antibodies used to label intracellular markers for flow cytometry procedure.

Intracellular Antibodies	
Sample Tube	Rabbit Anti-Glucocorticoid Receptor AlexaFluor 647 (1 µg/µl)
Control Tube	Rabbit IgG Isotype Control AlexaFluor 647 (1 µg/µl)

Cells were washed twice and centrifuged at 400 x g for five minutes. Pellet was resuspended in 200 µl of ice-cold PBS with 0.5% paraformaldehyde (PFA). The tubes were stored at 4ºC until flow cytometric analysis was performed using compensation settings as described above. All flow cytometric data were acquired within 24 hours of sample collection. Data acquisition and analysis were performed on a flow cytometer (Amnis FlowSight, Amnis Corporation) using 488 nm, 658 nm, and 785 nm lasers. A total of 10,000 cells were collected and gated to isolate single cells to analyse the percentage of lymphocyte subpopulation (B-lymphocytes, T-helper lymphocytes, cytotoxic T-lymphocytes, and NK cells) with positive co-expression of CD identifier and glucocorticoid receptor (GR) markers.

2.2.9 Histology Procedures

Adrenal gland and brain region (hypothalamus and hippocampus) samples were taken from all rats. A randomly selected subset (n = five per factorial group; total n = 20) were used for histological examination of morphological changes in adrenal gland and brain (hypothalamus and hippocampus) tissues. The same randomly selected subjects were used for all cellular parameters assessed.

2.2.9.1 Adrenal Gland

All tissue processing procedures were performed on a Leica TP 1020 Processor (Leica Biosystems, Germany). Adrenal gland samples were dehydrated for two hours each in four increasing concentrations of alcohol (30%, 50%, 70% and 80%) followed by one change of 95% alcohol and two changes of absolute alcohol for one hour each. Samples were then cleared through a change of 50:50% absolute alcohol: xylene, followed by two changes of xylene for one hour each. Samples were then immersed in two changes of paraplast (paraffin) for two hours each. After this procedure, the samples were then vacuum-embedded for 20 minutes. Samples were embedded in paraffin blocks on a Leica EG1150H Embedding Station (Leica Biosystems,

Germany) and stored at room temperature until required. These embedded samples were then serially sectioned on a microtome (Leitz model 1516) at 6 µm. These sections were transferred to superfrost plus glass slides (Thermo Fisher Scientific, USA) and dried overnight at room temperature. Slides were kept at 4 °C until required.

2.2.9.2 Brain Regions

All tissue processing procedures were performed on a Leica TP 1020 Processor (Leica Biosystems, Germany). The fixed left-brain hemisphere samples for each rat were trisected, using the optic chiasma and mid-brain as markers for separation, into three sample cassettes. These samples were dehydrated for 12 hours each in two increasing concentrations of alcohol (50% and 70%). This was followed by three increasing concentrations of alcohol (80%, 90% and 95%) for two hours each and two changes of absolute alcohol for one hour each. Samples were then cleared through a change of 50:50% absolute alcohol: xylene, followed by two changes of xylene for 1.5 hours each. Samples were then immersed in two changes of paraplast (paraffin) for 1.5 hours each. After this procedure, the samples were then vacuum-embedded for 20 minutes. Brain samples were then embedded coronally in paraffin blocks on a Leica EG1150H Embedding Station (Leica Biosystems, Germany). Tissue blocks were stored at room temperature until required. These embedded samples were then serially sectioned on a microtome (Leitz model 1516) at 5 µm. These sections were transferred to superfrost plus glass slides (Thermo Fisher Scientific, USA) and dried overnight at room temperature. These slides were kept at 4 °C until required.

2.2.9.3 Histological Staining

2.2.9.3.1 H&E Structural Staining

Four slides with sections containing the central medulla of the adrenal gland were stained with Harris' Haematoxylin and Eosin (Harris, 1900) to examine morphological changes. Slides were deparaffinised in three solutions of Solv21C for 1-2 minutes each, then rehydrated in two solutions of absolute alcohol, one solution of 80% alcohol and one solution of 50% alcohol for two minutes each. Slides were rinsed with distilled water and placed in a solution of Harris' haematoxylin for 12 minutes and were differentiated in Acid-Alcohol and then rinsed under running tap water for 15 minutes. The slides were placed in a solution of eosin for five minutes. Following this, the slides were further rinsed with tap water and then dehydrated with immersion for one minute in 50% alcohol, two minutes in 80% alcohol and two minutes each in two solutions of absolute alcohol. The slides were then cleared through a further three solutions of Solv21C for 1-2 minutes each before they were mounted with DPX Mounting Media (Distyrene, Plasticiser (Tricresyl Phosphate), and Xylene) and dried for 24 hours.

2.2.9.3.2 Nissl Structural Staining

Every second slide of brain tissue was stained with cresyl violet (Paxinos & Watson, 2007) to examine morphological changes specific to neurological tissue. Briefly, slides were deparaffinised in three solutions of Solv21C for 1-3 minutes each. Slides were rehydrated in two solutions of absolute alcohol for 2-3 minutes, a solution of 95% alcohol for two minutes and a solution of 70% alcohol for three minutes. Slides were rinsed with distilled water and placed in a solution of distilled water with acetic acid for two minutes. Next, the slides were stained with 0.1 % cresyl violet for 10 minutes and were rinsed in an additional solution of distilled water and acetic acid for 15 seconds, differentiated and dehydrated in solutions of 70% alcohol and 80% alcohol for 30 seconds each and a solution of 95% alcohol and acetic acid. Slides were rinsed in two solutions of absolute alcohol, to ensure complete differentiation. The slides were then cleared

through three more solutions of Solv21C for 1-3 minutes each before they were mounted with DPX Mounting Media (Distyrene, Plasticiser (Tricresyl Phosphate), and Xylene) and dried for 24 hours.

2.2.10 Slide Image Analysis

Light micrographs were acquired using a Hamamatsu NanoZoomer 2.0-RS Digital Pathology Slide Scanner (Hamamatsu Photonics K.K., Japan) to collect the entire slide field. Histological slides were analysed using Hamamatsu NDP Viewer 2 (Hamamatsu Photonics K.K., Japan) viewing software to annotate and measure areas of interest for each slide.

The adrenal glands were measured for the width of the adrenal regions, which was averaged over eight randomly chosen sections containing a full cross-section of the central medulla. The size of the cortex and medulla region was used to determine hypo- or hypertrophy of any adrenal regions for the experimental cohorts.

The size of the hippocampus and paraventricular nucleus (PVN) of the hypothalamus were analysed on the brain sections using image analysis. The area of the hippocampus measured from the formation of the dentate gyrus (Bregma -1.92 mm; Interaural 7.08 mm) through the next 10 sections. The area of the PVN of the hypothalamus (Bregma -1.92 mm; Interaural 7.08 mm) was measured over five randomly selected sections. The average of these measurements for the hippocampus and hypothalamus were used to determine the presence of any hypo- or hypertrophy.

2.3 Statistical Analysis

All statistical analysis was conducted with IBM SPSS Statistics 22 and any graphing used GraphPad Prism 6. The behavioural parameters were analysed using MANOVA to determine the

main effect of stress on rat behaviour. A Bonferroni correction ($p < 0.002$) was applied to the univariate effects from MANOVA analysis.

Individual behavioural parameters were also analysed separately with repeated measure ANOVA, to determine the degree of behavioural habituation to BOOF test before and after stress. These analyses were grouped based on the type of behaviours analysed (locomotion, immobility, grooming, and object investigation). A Bonferroni correction was applied to the repeated measure ANOVA behavioural groupings based on the number of behaviours in each sub-grouping (locomotion, immobility, and grooming correction $p < 0.017$; object investigation correction $p < 0.01$).

The results from physiological assays were analysed with one-way ANOVA to investigate the statistical difference in the catecholamine (noradrenaline, adrenaline, and dopamine) concentrations and steroid hormone (corticosterone, 11-deoxycorticosterone, testosterone, and progesterone) concentrations in adrenal glands, brain tissue (hypothalamus, hippocampus, and prefrontal cortex), and plasma. A Bonferroni correction was applied to the catecholamine ($p < 0.017$) and steroid hormone ($p < 0.013$) concentrations, since in each case the metabolites are in the same biosynthetic pathway. One-way ANOVA was used to determine any significant differences in the cellular morphology of adrenal gland and brain (hypothalamus and hippocampus) tissues between the experimental cohorts. A two-way ANOVA was used to analyse the difference in glucocorticoid receptor expression in leukocyte subpopulations. Cellular measures and the level of glucocorticoid receptor expression with a $p < 0.05$ were deemed statistically significant.

Spearman's coefficient correlation analysis was conducted to determine the correlation coefficient between behavioural and physiological parameters. Only parameters deemed statistically significant from previous analyses were used in the correlation coefficient analysis between the behavioural and physiological measures.

Chapter 3 – Behavioural Changes in Response to Early and Recent Life Stress in Rats

3.1 The Behavioural Response to Stress in Rats

It was hypothesised that imposition of chronic stress can trigger deviations in normal animal behaviour (Chapter 1, Section 1.4). The previous chapter (specifically Section 2.2.3 and Section 2.2.4) detailed a factorial designed study using maternal deprivation (MD), isolation, and chronic mild stress (CMS) exposure that was designed to test this hypothesis in a rodent model. The outcome of the MD, isolation, and CMS exposure on the rats was monitored using the behavioural observation open field (BOOF) test (Chapter 2, Section 2.2.5). During the BOOF test, the frequency and duration of the rats' behaviours in the BOOF arena were scored from the five, 10-minute BOOF sessions before CMS exposure and five sessions, of the same duration, after CMS exposure.

The monitored behaviours were *post-hoc* grouped into locomotion, grooming, immobility, and object investigation (Appendix B, BOOF Checklist and Behaviour Definitions). The behavioural data generated from the BOOF test were used to examine the change in behaviour from MD and isolation before and after CMS exposure (Table 3.1). The behaviours before CMS exposure formed a baseline of the impact of MD and isolation prior to CMS exposure for the experimental cohorts. The second round of BOOF testing after CMS exposure examined the differences in the behavioural responses of the experimental cohorts due to recent life stress following early life stress (MD and isolation) experiences.

Table 3.1: Description of the experimental cohorts in the current study.

Treatments	**Experimental cohorts**	
	Visual (Control)	**Non-Visual (Treatment)**
Not Maternally Deprived (Control)	No MD and visual social support after weaning	No MD and no visual social support after weaning
Maternally Deprived (Treatment)	MD received and visual social support after weaning	MD received and no visual social support after weaning

The aim of this chapter was to examine whether exposure to maternal deprivation and isolation, altered rat behaviour following chronic mild stress exposure. Therefore, the research questions for this chapter were:

1) Did maternal deprivation, isolation, and chronic mild stress trigger a depressive- or anxiety-like behavioural profile in the rats, in terms of their observed behaviour during the BOOF test?
2) Did habituation (reduction in scored behaviours due to loss of novelty) occur during the BOOF test before or after chronic mild stress?
3) Did the experimental cohorts have significantly different behaviours after maternal deprivation, isolation, and chronic mild stress?

The data were analysed by Multivariate Analysis of Variance (MANOVA) and repeated measures ANOVA. A Bonferroni correction ($p < 0.002$) was applied to the univariate effects from MANOVA analysis. The repeated measure ANOVAs also had Bonferroni corrections applied based on the type of grouped behaviours (locomotion, immobility, and grooming corrections $p < 0.017$; object investigation correction $p < 0.01$). Tukey's *post-hoc* analysis was used to determine the difference in behaviours between the experimental cohorts due to MD and isolation. Due to the size of the data set, the data were not normalised for these tests as the sample size was deemed sufficiently large based on the central limit theorem. Specifically, the central limit theorem states that if samples that possess $n > 30$, then sampled distributions are assumed as normally distributed, regardless of the actual distributions of the variables (Tabachnick & Fidell, 2013). The current study possessed $n = 60$ and was deemed to be sufficiently large to not require normalisation for behaviours that did not have an observed normal distribution.

3.2 Behaviours Observed in the BOOF Test Resulting from Stress Exposure

3.2.1 The Frequency of Behaviours in the BOOF Test

3.2.1.1 Overall Impact of Stress on the Behaviour Frequency During the BOOF Test

The frequencies of behaviours (locomotion, grooming, immobility, and object investigation) were examined to assess behaviours performed during the BOOF test. To determine whether there was an immediate significant difference between the start and end of CMS exposure, the frequency for all scored behaviours from day five (last day of BOOF before CMS) and day six (the first day of BOOF after CMS) were analysed using MANOVA. Table 3.2 shows the frequency scores for each of the scored behaviours for the experimental cohorts, plus univariate results for each variable. However, there was no significant difference in the combined behaviours of the experimental cohorts for the immediate impact of CMS [F (66, 105.4) = 1.42, p = 0.054; *Wilks'* λ = 0.15, partial η^2 = 0.47]. The partial η^2 indicated a large effect (Pallant, 2013) of stress with 47% of the experimental variation in the dependent variables accounted for by CMS exposure across the experimental cohorts.

Table 3.2: Means (SD) and univariate results for frequency of scored behaviours from pre-stress day 5 and post-stress day1. Bonferroni adjustment for significant value ($p \leq 0.002$).

Behaviour	Not Deprived Visual M (SD)	Not Deprived Non-Visual M (SD)	Deprived Visual M (SD)	Deprived Non-Visual M (SD)	F	*p*	Partial η^2
Vertical Motor Activity (VMA) Pre-Stress Day 5	14.2 (12.78)	20.93 (17.86)	14.07 (8.92)	23.53 (18.74)	1.51	0.22	0.08
Vertical Motor Activity (VMA) Post-Stress Day 1	17.27 (12.80)	24.13 (17.28)	16.87 (11.31)	22.87 (17.76)	0.93	0.43	0.05
Horizontal Motor Activity (HMA) Pre-Stress Day 5	78.27 (54.93)	106.20 (81.81)	95.00 (48.32)	122.07 (87.88)	1.03	0.39	0.05
Horizontal Motor Activity (HMA) Post-Stress Day 1	84.40 (57.48)	116.53 (74.02)	126.00 (45.14)	131.07 (73.94)	1.62	0.20	0.08
Centre Entries (CE) Pre-Stress Day 5	3.87 (3.66)	6.27 (6.53)	3.87 (3.56)	5.60 (6.29)	0.83	0.48	0.04
Centre Entries (CE) Post-Stress Day 1	5.07 (6.98)	7.20 (6.94)	5.67 (4.52)	5.60 (5.26)	0.35	0.79	0.02
Total Grooming (TG) Pre-Stress Day 5	3.40 (2.20)	2.47 (1.81)	2.93 (2.58)	2.53 (2.36)	0.55	0.65	0.03
Total Grooming (TG) Post-Stress Day 1	3.07 (1.34)	2.87 (2.75)	1.93 (2.28)	1.80 (2.08)	1.31	0.28	0.07
Normal Grooming (NG) Pre-Stress Day 5	1.47 (0.99)	1.60 (1.06)	1.60 (0.99)	1.53 (0.92)	0.06	0.98	0.003
Normal Grooming (NG) Post-Stress Day 1	1.53 (0.99)	1.33 (0.82)	0.87 (0.52)	1.13 (1.13)	1.53	0.22	0.08
Stereotypic Grooming (SG) Pre-Stress Day 5	1.93 (1.62)	0.87 (1.46)	1.33 (2.06)	1.00 (2.00)	1.04	0.38	0.05
Stereotypic Grooming (SG) Post-Stress Day 1	1.53 (1.46)	1.53 (2.48)	1.07 (2.15)	0.67 (1.40)	0.71	0.55	0.04
Total Immobility (TI) Pre-Stress Day 5	16.20 (9.99)	12.07 (8.12)	22.20 (11.26)	15.33 (9.17)	2.85	0.05	0.13
Total Immobility (TI) Post-Stress Day 1	19.67 (9.66)	13.27 (9.24)	25.40 (14.54)	19.67 (9.48)	3.07	0.04	0.14
Corner Immobility (CI) Pre-Stress Day 5	15.53 (10.23)	11.33 (8.49)	19.80 (10.59)	14.93 (9.43)	1.91	0.14	0.09
Corner Immobility (CI) Post-Stress Day 1	18.73 (10.17)	12.60 (9.58)	23.80 (14.62)	18.33 (10.45)	2.43	0.08	0.12
Square Immobility (SI) Pre-Stress Day 5	0.67 (0.98)	0.73 (1.39)	2.40 (3.89)	0.40 (0.63)	2.71	0.05	0.13

Square Immobility (SI) Post-Stress Day 1	0.93 (1.67)	0.67 (1.11)	1.60 (1.92)	1.33 (1.50)	1.04	0.38	0.05
Total Object Investigation (TOI) Pre-Stress Day 5	10.07 (9.33)	15.20 (14.27)	12.87 (7.60)	15.87 (13.93)	0.76	0.52	0.04
Total Object Investigation (TOI) Post-Stress Day 1	12.07 (9.02)	19.13 (12.84)	18.40 (10.57)	16.80 (11.72)	1.22	0.31	0.06
Object 1 Investigation (O1) Pre-Stress Day 5	3.60 (3.81)	6.33 (5.53)	4.73 (3.28)	6.53 (6.33)	1.21	0.32	0.06
Object 1 Investigation (O1) Post-Stress Day 1	4.00 (3.93)	6.87 (5.34)	7.73 (4.62)	8.60 (6.48)	2.23	0.10	0.11
Object 2 Investigation (O2) Pre-Stress Day 5	1.13 (1.73)	1.60 (1.55)	2.07 (1.58)	2.27 (1.87)	1.35	0.27	0.07
Object 2 Investigation (O2) Post-Stress Day 1	1.60 (1.30)	3.47 (3.20)	2.00 (1.96)	1.47 (1.36)	2.86	0.05	0.13
Object 3 Investigation (O3) Pre-Stress Day 5	3.40 (2.92)	4.33 (5.56)	3.73 (2.05)	4.80 (4.83)	0.35	0.79	0.02
Object 3 Investigation (O3) Post-Stress Day 1	3.87 (3.50)	5.20 (3.75)	5.40 (3.83)	5.20 (4.68)	0.48	0.70	0.03
Object 4 Investigation (O4) Pre-Stress Day 5	1.93 (1.71)	2.93 (2.96)	2.33 (2.13)	2.27 (2.02)	0.51	0.68	0.03
Object 4 Investigation (O4) Post-Stress Day 1	2.60 (1.68)	3.60 (3.16)	3.27 (2.15)	1.53 (1.46)	2.55	0.07	0.12

A second MANOVA was conducted using the mean of the scored behaviours (locomotion, grooming, immobility, and object investigation behaviours) over the five days of BOOF testing before and after CMS. This was done to determine if a significant difference was present between MD and isolation exposure after accounting for day-to-day variability in the frequency of the behaviour during BOOF. This assessed whether there was any change in the mean scored behaviours of the experimental cohorts due to exposure to CMS. The MANOVA revealed a significant difference between the experimental cohorts for the combined dependent variables, F (69, 102.4) = 1.68, $p = 0.012$; *Wilks'* $\lambda = 0.11$, partial $\eta^2 = 0.52$. The partial η^2 indicated a large effect (Pallant, 2013) of stress with 52% of the experimental variation in the dependent variables accounted for by CMS exposure across the experimental cohorts. Table 3.3 shows the frequency scores for each of the scored behaviours for the experimental cohorts, plus univariate results for each variable. Univariate analysis indicated no significant effect of CMS exposure on any of the individual behavioural frequencies before or after CMS exposure following Bonferroni adjustment for a significance value of $p < 0.002$ (Table 3.3).

Table 3.3: Means (SD) and univariate results for the mean frequency of scored behaviours before and after CMS exposure. Bonferroni adjustment for significant value ($p \leq 0.002$).

Behaviour	Not Deprived Visual M (SD)	Not Deprived Non-Visual M (SD)	Deprived Visual M (SD)	Deprived Non-Visual M (SD)	F	*p*	Partial η^2
Vertical Motor Activity (VMA) Pre-Stress Mean	16.52 (10.74)	19.28 (10.07)	17.21 (6.26)	23.15 (12.28)	1.31	0.28	0.07
Vertical Motor Activity (VMA) Post-Stress Mean	20.52 (10.67)	23.77 (10.41)	19.25 (9.57)	26.21 (14.89)	1.12	0.35	0.06
Horizontal Motor Activity (HMA) Pre-Stress Mean	96.19 (56.94)	112.33 (64.65)	128.60 (41.32)	139.79 (63.76)	1.65	0.19	0.08
Horizontal Motor Activity (HMA) Post-Stress Mean	86.72 (44.85)	107.08 (52.05)	127.25 (40.98)	142.65 (57.31)	3.66	0.02	0.16
Centre Entries (CE) Pre-Stress Mean	4.93 (3.80)	5.35 (4.02)	5.29 (2.99)	5.65 (4.73)	0.08	0.97	0.01
Centre Entries (CE) Post-Stress Mean	4.83 (3.44)	6.49 (4.64)	6.59 (3.86)	7.81 (4.58)	1.30	0.28	0.07
Total Grooming (TG) Pre-Stress Mean	2.80 (0.99)	2.11 (1.39)	2.67 (1.97)	2.36 (1.43)	0.66	0.58	0.03
Total Grooming (TG) Post-Stress Mean	3.05 (1.15)	2.69 (1.26)	2.55 (1.70)	1.88 (1.03)	2.12	0.11	0.10
Normal Grooming (NG) Pre-Stress Mean	1.35 (0.56)	1.21 (0.66)	1.36 (0.66)	1.52 (0.78)	0.53	0.67	0.03
Normal Grooming (NG) Post-Stress Mean	1.56 (0.29)	1.67 (0.68)	1.40 (0.43)	1.25 (0.48)	2.06	0.12	0.10
Stereotypic Grooming (SG) Pre-Stress Mean	1.45 (0.81)	0.89 (1.16)	1.31 (1.62)	0.84 (1.05)	0.96	0.42	0.05
Stereotypic Grooming (SG) Post-Stress Mean	1.49 (1.13)	1.03 (1.09)	1.15 (1.78)	0.63 (0.81)	1.22	0.31	0.06
Total Immobility (TI) Pre-Stress Mean	15.55 (6.42)	12.39 (5.14)	19.43 (8.25)	15.28 (7.42)	2.63	0.06	0.12
Total Immobility (TI) Post-Stress Mean	16.68 (7.30)	12.67 (5.03)	18.53 (8.96)	16.03 (6.34)	1.81	0.16	0.09
Corner Immobility (CI) Pre-Stress Mean	15.00 (6.77)	11.71 (5.43)	17.36 (7.81)	14.37 (7.20)	1.72	0.17	0.08
Corner Immobility (CI) Post-Stress Mean	15.93 (7.59)	11.64 (5.20)	15.63 (7.31)	14.40 (6.29)	1.29	0.29	0.07
Square Immobility (SI) Pre-Stress Mean	0.55 (0.73)	0.68 (0.93)	1.80 (1.93)	0.91 (0.68)	3.43	0.02	0.16

Square Immobility (SI) Post-Stress Mean	0.75 (0.76)	1.03 (0.96)	2.91 (3.69)	1.63 (0.97)	3.44	0.02	0.16
Total Object Investigation (TOI) Pre-Stress Mean	12.35 (7.64)	15.75 (9.51)	16.63 (5.84)	17.21 (8.36)	1.12	0.35	0.06
Total Object Investigation (TOI) Post-Stress Mean	11.88 (6.67)	17.20 (9.47)	19.87 (6.69)	20.61 (9.75)	3.42	0.02	0.16
Object 1 Investigation (O1) Pre-Stress Mean	4.57 (2.85)	6.19 (4.01)	6.73 (2.75)	7.44 (4.98)	1.58	0.21	0.08
Object 1 Investigation (O1) Post-Stress Mean	4.23 (3.13)	6.80 (3.88)	8.49 (3.28)	9.21 (5.63)	4.38	0.01	0.19
Object 2 Investigation (O2) Pre-Stress Mean	2.07 (1.32)	2.51 (1.69)	2.25 (.86)	2.59 (1.16)	0.51	0.68	0.03
Object 2 Investigation (O2) Post-Stress Mean	1.76 (1.22)	2.56 (1.80)	2.65 (1.22)	2.61 (1.0)	1.52	0.22	0.08
Object 3 Investigation (O3) Pre-Stress Mean	3.44 (2.04)	4.29 (2.14)	4.87 (1.87)	4.89 (2.17)	1.64	0.19	0.08
Object 3 Investigation (O3) Post-Stress Mean	3.32 (1.94)	4.75 (2.76)	5.56 (1.83)	6.21 (2.45)	4.51	0.01	0.19
Object 4 Investigation (O4) Pre-Stress Mean	2.27 (2.01)	2.76 (2.09)	2.77 (1.07)	2.29 (1.23)	0.43	0.73	0.02
Object 4 Investigation (O4) Post-Stress Mean	2.57 (1.75)	3.09 (2.22)	3.16 (1.32)	2.57 (1.50)	0.52	0.67	0.03

However, Tukey *post-hoc* analysis indicated the presence of a significant difference between the experimental cohorts after CMS exposure for horizontal motor activity (HMA) and object investigation (Total Object Investigation [TOI], Object 1 Investigation [O1], and Object 3 investigation [O3]) behaviours as shown in Table 3.4. Specifically, rats in the *"deprived non-visual"* cohort, which were subjected to MD and had visual barriers placed between the cages, had significantly higher behaviour frequencies for HMA, TOI, O1, and O3 than the control *"not deprived visual"* rats, which were not subjected to MD and had no visual barrier after weaning. A similar significant increase in the frequency of object exploration was also noted in the *"deprived visual"* cohort of rats subjected to MD with no visual barrier between the cages when compared to the control *"not deprived visual"* cohort. Collectively, these behavioural changes indicate that MD but not isolation had a significant effect on horizontal motor activity and exploration following CMS.

*Table 3.4: Tukey post-hoc analysis from a MANOVA of mean behaviour frequencies for all experimental cohorts after CMS exposure. Limited to dependent variables with a significant difference between experimental cohorts. Please note * indicates significant result ($p < 0.05$).*

Behaviour	Cohort	Comparison Cohorts	Mean Difference	Std. Error	Sig.
Horizontal Motor Activity Post-CMS mean	Not Deprived Visual	Not Deprived Non-Visual	-20.36	17.97	0.67
		Deprived Visual	-40.53	17.97	0.12
		Deprived Non-Visual	-55.93*	17.97	0.02
Total Object Investigation post-CMS mean	Not Deprived Visual	Not Deprived Non-Visual	-5.32	3.02	0.30
		Deprived Visual	-7.99	3.02	0.05
		Deprived Non-Visual	-8.73*	3.02	0.03
Object 1 Investigation post-CMS mean	Not Deprived Visual	Not Deprived Non-Visual	-2.57	1.50	0.32
		Deprived Visual	-4.27*	1.50	0.03
		Deprived Non-Visual	-4.99*	1.50	0.01
Object 3 Investigation post-CMS mean	Not Deprived Visual	Not Deprived Non-Visual	-1.43	0.83	0.33
		Deprived Visual	-2.24*	0.83	0.05
		Deprived Non-Visual	-2.89*	0.83	0.01

3.2.1.2 Habituation of Behaviour Frequency During the BOOF Test

After assessment of the overall impact of MD, isolation, and CMS, additional repeated measure ANOVAs were conducted on each set of behaviours, before and after CMS exposure. These analyses examined whether there was habituation in the frequency of the individual behavioural groupings (locomotion, grooming, immobility, and object investigation) for the experimental cohorts. In each case, a Bonferroni correction was applied based on the number of behaviours in each grouping, with details provided in each section. Tukey's *post-hoc* analysis was conducted to determine if there were any significant differences between the experimental cohorts in their behavioural response in the BOOF test over time following MD and isolation exposure.

3.2.1.2.1 Locomotion Behaviours in the BOOF Test

The impact of CMS exposure on rats' ambulatory movement was examined using the temporal profiles of the locomotion behaviours (vertical motor activity, horizontal motor activity, and centre entries). Table 3.5 presents the mean and standard deviation for locomotion behaviours in the BOOF test before and after CMS. A Bonferroni correction was applied to the p-values of the locomotion behaviours to minimise type 1 errors, resulting in a corrected p-value threshold of $p < 0.017$.

There was a significant decrease in the frequency over the five days of BOOF for vertical ($F = 3.60$; $p < 0.017$) and horizontal ambulatory movement ($F = 16.58$; $p < 0.017$) in all experimental cohorts during the BOOF test before CMS (Figure 3.1-A and –B). However, no similar significant change was observed in the frequency of centre entries ($F = 1.53$; $p > 0.017$) (Figure 3.1-C). Following CMS exposure, there was no significant reduction (habituation) for behaviour frequency over time for vertical motor activity ($F = 1.07$; $p > 0.017$), horizontal motor activity ($F = 2.60$; $p > 0.017$), or centre entries ($F = 1.67$; $p > 0.017$) based on MD and isolation (Appendix C, Table 1).

However, Tukey's *post-hoc* analysis revealed a significant difference between the experimental groups in the frequency of horizontal motor activity following CMS (Appendix C, Table 2). Specifically, the *"deprived non-visual*" rats that had been subjected to MD and had visual barriers placed between the cages after weaning, had a significantly higher ($p < 0.017$) total horizontal motor activity after CMS when compared to the control "*not deprived visual*" rats.

Prior to stress exposure, there were no significant differences between the experimental cohorts for vertical motor activity ($F = 1.31$; $p > 0.017$), horizontal motor activity ($F = 1.65$; $p > 0.017$), or centre entries ($F = 0.08$; $p > 0.017$). Nor were there any significant differences between the experimental cohorts after CMS exposure for vertical motor activity ($F = 1.12$; $p > 0.017$), horizontal motor activity ($F = 3.66$; $p > 0.017$), or centre entries ($F = 1.30$; $p > 0.017$) (Appendix C, Table 1).

Finally, there was no significant interaction between time and experimental cohort treatment before stress for vertical motor activity ($F = 0.58$; $p > 0.017$), horizontal motor activity ($F = 0.93$; $p > 0.017$), or centre entries ($F = 1.39$; $p > 0.017$). After stress exposure, there was also no significant interaction between time and experimental cohort treatment for vertical motor activity ($F = 1.13$; $p > 0.017$), horizontal motor activity ($F = 0.97$; $p > 0.017$), or centre entries ($F = 0.95$; $p > 0.017$) (Appendix C, Table 1).

Collectively, these data suggested a lack of habituation to the BOOF arena following CMS exposure, where no significant reduction in locomotor activity occurred. It also suggested that maternal deprivation had no significant impact on locomotion behaviours in the BOOF test until after exposure to a later life chronic stress.

Table 3.5: Means (SD) for the frequency of locomotion behaviours (vertical motor activity, horizontal motor activity, and centre entries) before and after CMS.

Behaviour	Not Deprived Visual (*n* = 15) M (SD)	Not Deprived Non-Visual (*n* = 15) M (SD)	Deprived Visual (*n* = 15) M (SD)	Deprived Non-Visual (*n* = 15) M (SD)	Total (*n* = 60) M (SD)
Vertical Motor Activity Pre-Stress Day 1	22.60 (9.60)	25.93 (13.02)	22.53 (9.77)	25.67 (12.12)	24.18 (11.06)
Vertical Motor Activity Pre-Stress Day 2	17.93 (15.79)	20.00 (18.81)	16.07 (8.27)	22.33 (14.42)	19.08 (14.64)
Vertical Motor Activity Pre-Stress Day 3	15.80 (14.05)	17.00 (10.68)	18.40 (10.78)	21.27 (15.89)	18.12 (12.87)
Vertical Motor Activity Pre-Stress Day 4	12.07 (15.30)	12.53 (13.24)	15.00 (12.71)	22.93 (18.67)	15.63 (15.41)
Vertical Motor Activity Pre-Stress Day 5	14.20 (12.78)	20.93 (17.86)	14.07 (8.92)	23.53 (18.74)	18.18 (15.30)
Vertical Motor Activity Post-Stress Day 1	17.27 (12.80)	24.13 (17.28)	16.87 (11.31)	22.87 (17.76)	20.28 (15.03)
Vertical Motor Activity Post-Stress Day 2	20.87 (14.91)	24.13 (13.73)	23.47 (11.03)	24.27 (12.31)	23.18 (12.82)
Vertical Motor Activity Post-Stress Day 3	19.13 (13.53)	25.87 (10.97)	20.80 (12.24)	28.33 (21.12)	23.53 (15.08)
Vertical Motor Activity Post-Stress Day 4	23.47 (13.84)	25.93 (16.85)	16.20 (11.66)	26.73 (13.23)	23.08 (14.29)
Vertical Motor Activity Post-Stress Day 5	21.87 (16.74)	18.80 (11.19)	18.93 (14.05)	28.87 (18.37)	22.12 (15.49)
Horizontal Motor Activity Pre-Stress Day 1	153.60 (82.98)	167.20 (87.49)	188.20 (59.50)	195.53 (63.68)	176.13 (74.43)
Horizontal Motor Activity Pre-Stress Day 2	103.80 (79.29)	109.20 (89.25)	119.33 (58.61)	132.27 (71.98)	116.15 (74.47)
Horizontal Motor Activity Pre-Stress Day 3	79.53 (69.25)	104.07 (76.21)	136.73 (70.46)	120.87 (83.91)	110.30 (76.30)
Horizontal Motor Activity Pre-Stress Day 4	65.73 (63.50)	75.00 (74.33)	103.73 (61.12)	128.20 (73.27)	93.17 (71.02)
Horizontal Motor Activity Pre-Stress Day 5	78.27 (54.93)	106.20 (81.81)	95.00 (48.32)	122.07 (87.88)	100.38 (70.36)
Horizontal Motor Activity Post-Stress Day 1	84.40 (57.48)	116.53 (74.02)	126.00 (45.14)	131.07 (73.94)	114.50 (64.80)
Horizontal Motor Activity Post-Stress Day 2	101.40 (63.16)	111.20 (68.33)	154.00 (67.43)	152.53 (62.15)	129.78 (67.99)

Horizontal Motor Activity Post-Stress Day 3	77.13 (52.2)	108.00 (47.29)	125.87 (44.35)	137.60 (65.29)	112.15 (56.43)
Horizontal Motor Activity Post-Stress Day 4	90.80 (52.89)	110.33 (58.63)	108.07 (51.38)	144.67 (52.08)	113.47 (56.01)
Horizontal Motor Activity Post-Stress Day 5	79.87 (51.83)	89.33 (61.77)	122.33 (64.41)	147.40 (68.71)	109.73 (66.19)
Centre Entries Pre-Stress Day 1	7.73 (6.34)	5.40 (4.10)	7.33 (4.17)	5.20 (3.36)	6.42 (4.65)
Centre Entries Pre-Stress Day 2	5.40 (5.84)	5.67 (6.15)	3.53 (3.48)	4.47 (5.53)	4.77 (5.28)
Centre Entries Pre-Stress Day 3	4.53 (5.77)	5.13 (4.88)	6.53 (4.88)	6.47 (7.42)	5.67 (5.75)
Centre Entries Pre-Stress Day 4	3.13 (4.29)	4.27 (6.08)	5.20 (5.14)	6.53 (6.44)	4.78 (5.55)
Centre Entries Pre-Stress Day 5	3.87 (3.66)	6.27 (6.53)	3.87 (3.56)	5.60 (6.29)	4.90 (5.18)
Centre Entries Post-Stress Day 1	5.07 (6.98)	7.20 (6.94)	5.67 (4.51)	5.60 (5.26)	5.88 (5.92)
Centre Entries Post-Stress Day 2	6.33 (5.15)	7.20 (5.66)	7.60 (4.01)	8.73 (5.80)	7.47 (5.14)
Centre Entries Post-Stress Day 3	3.93 (4.25)	6.53 (5.68)	6.67 (6.31)	7.20 (5.17)	6.08 (5.42)
Centre Entries Post-Stress Day 4	4.40 (3.98)	6.27 (4.70)	5.53 (4.76)	9.67 (5.98)	6.47 (5.18)
Centre Entries Post-Stress Day 5	4.40 (4.21)	5.27 (5.15)	7.47 (6.73)	7.87 (4.87)	6.25 (5.39)

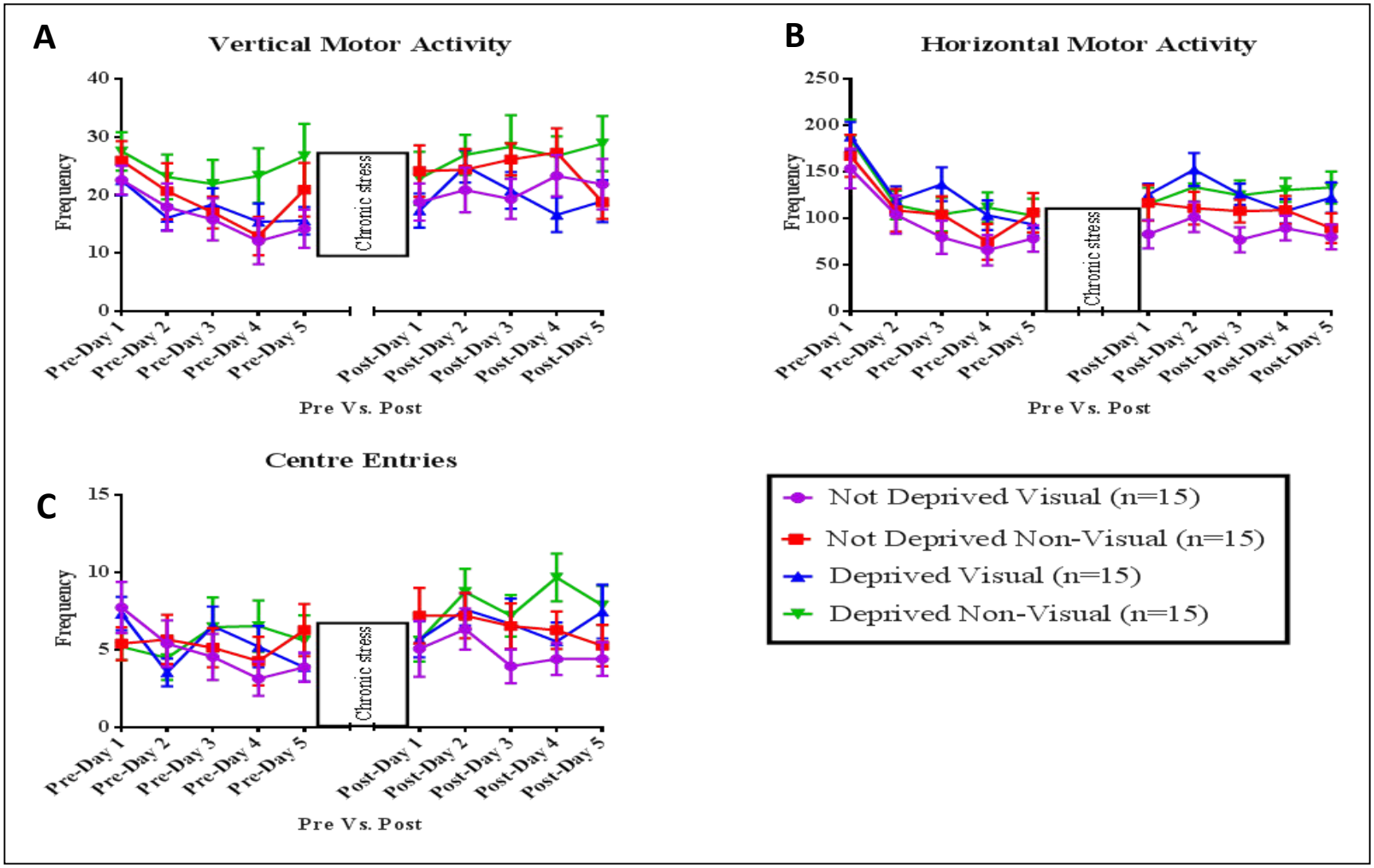

Figure 3.1: Frequency of behavioural parameters measured during BOOF. (A) Vertical Motor Activity (VMA); (B) Horizontal Motor Activity (HMA); (C) Centre Entries (CE) by experimental cohort on each day of BOOF before and after stress. n = 15. Data are mean ± SEM.

3.2.1.2.2 Grooming Behaviours in the BOOF Test

The temporal profiles of grooming behaviours (total grooming, normal grooming, and stereotypic grooming) were examined to investigate the impact of CMS exposure on the behavioural frequency between the experimental cohorts. Table 3.6 presents the mean and standard deviation for the grooming behaviours in the BOOF test before and after CMS. A Bonferroni correction was applied to the p-values of the grooming behaviours to minimise type 1 errors, resulting in a corrected p-value threshold of $p < 0.017$.

There was no significant difference in total ($F = 1.30$; $p > 0.017$), normal ($F = 2.28$; $p > 0.017$), or stereotypic ($F = 0.57$; $p > 0.017$) grooming behaviours over time before CMS exposure (Figure 3.2-A, -B and -C). Additionally, there was no significant difference in the total ($F = 1.72$; $p > 0.017$) or stereotypic ($F = 1.85$; $p > 0.017$) grooming frequencies after CMS exposure. There was a significant increase in normal grooming incidents ($F = 3.90$; $p < 0.017$) during the five BOOF test sessions, after CMS exposure (Appendix C, Table 3).

Before CMS exposure, there were no significant differences between experimental cohorts in total ($F = 0.66$; $p > 0.017$), normal ($F = 0.53$; $p > 0.017$), or stereotypic ($F = 0.96$; $p > 0.017$) grooming behaviours. There were also no significant differences between the experimental cohorts after stress exposure for total ($F = 2.12$; $p > 0.017$), normal ($F = 2.06$; $p > 0.017$), or stereotypic ($F = 1.22$; $p > 0.017$) grooming behaviours (Appendix C, Table 3).

Finally, there was no interaction between time and the experimental cohorts before stress for total ($F = 1.10$; $p > 0.017$), normal ($F = 0.68$; $p > 0.017$), or stereotypic ($F = 1.67$; $p > 0.017$) grooming behaviours. After stress exposure, there was also no significant interactions between time and the experimental cohorts for total ($F = 0.87$; $p > 0.017$), normal ($F = 1.40$; $p > 0.017$) or stereotypic ($F = 0.85$; $p > 0.017$) grooming behaviours (Appendix C, Table 3).

However, Tukey's *post-hoc* analysis revealed no significant differences between the experimental cohorts (not deprived visual, not deprived non-visual, deprived visual, or deprived

non-visual) for any grooming behaviours, before or after CMS exposure (Appendix C, Table 4). Collectively, this suggested that MD and isolation had no effect on the grooming behaviours observed in the BOOF test following CMS.

Table 3.6: Means (SD) for the frequency of grooming behaviours (total, normal, and stereotypic grooming) before and after CMS.

Behaviour	Not Deprived Visual (*n* = 15) M (SD)	Not Deprived Non-Visual (*n* = 15) M (SD)	Deprived Visual (*n* = 15) M (SD)	Deprived Non-Visual (*n* = 15) M (SD)	Total (*n* = 60) M (SD)
Total Grooming Pre-Stress Day 1	3.20 (1.42)	2.20 (1.82)	2.13 (1.88)	2.87 (2.00)	2.60 (1.81)
Total Grooming Pre-Stress Day 2	2.20 (2.01)	1.73 (1.28)	3.27 (2.87)	2.53 (2.17)	2.43 (2.17)
Total Grooming Pre-Stress Day 3	2.73 (1.75)	2.20 (2.60)	2.47 (2.72)	2.13 (1.88)	2.38 (2.23)
Total Grooming Pre-Stress Day 4	2.47 (2.36)	1.93 (1.94)	2.53 (2.29)	1.73 (1.16)	2.17 (1.98)
Total Grooming Pre-Stress Day 5	3.40 (2.20)	2.47 (1.81)	2.93 (2.58)	2.53 (2.36)	2.83 (2.23)
Total Grooming Post-Stress Day 1	3.07 (1.33)	2.87 (2.75)	1.93 (2.28)	1.80 (2.08)	2.42 (2.19)
Total Grooming Post-Stress Day 2	3.13 (1.77)	1.87 (1.46)	2.67 (1.54)	1.67 (1.29)	2.33 (1.60)
Total Grooming Post-Stress Day 3	3.13 (2.00)	2.87 (1.68)	2.33 (2.32)	1.53 (1.19)	2.47 (1.90)
Total Grooming Post-Stress Day 4	3.00 (1.85)	3.33 (2.29)	3.47 (2.92)	2.47 (1.41)	3.07 (2.17)
Total Grooming Post-Stress Day 5	2.93 (2.22)	2.53 (1.81)	2.33 (1.84)	1.93 (1.49)	2.43 (1.84)
Normal Grooming Pre-Stress Day 1	1.33 (0.49)	1.33 (1.11)	1.33 (1.05)	1.93 (1.10)	1.48 (0.98)
Normal Grooming Pre-Stress Day 2	1.27 (1.10)	1.13 (0.83)	1.33 (1.05)	1.13 (0.92)	1.22 (0.96)
Normal Grooming Pre-Stress Day 3	1.53 (1.06)	1.00 (1.20)	1.20 (1.15)	1.60 (1.35)	1.33 (1.19)
Normal Grooming Pre-Stress Day 4	1.13 (0.83)	1.00 (1.00)	1.33 (0.90)	1.40 (1.12)	1.22 (0.96)
Normal Grooming Pre-Stress Day 5	1.47 (0.99)	1.60 (1.06)	1.60 (0.99)	1.53 (0.92)	1.55 (0.96)
Normal Grooming Post-Stress Day 1	1.53 (0.99)	1.33 (0.82)	0.87 (0.52)	1.13 (1.13)	1.22 (0.90)
Normal Grooming Post-Stress Day 2	2.07 (0.96)	1.40 (0.99)	1.73 (1.03)	1.07 (0.70)	1.57 (0.98)

Normal Grooming Post-Stress Day 3	1.40 (0.99)	1.93 (1.10)	1.47 (1.06)	1.07 (0.96)	1.47 (1.05)
Normal Grooming Post-Stress Day 4	1.73 (0.88)	2.20 (1.78)	1.60 (1.06)	1.80 (1.21)	1.83 (1.26)
Normal Grooming Post-Stress Day 5	1.07 (0.59)	1.47 (0.92)	1.33 (0.90)	1.20 (0.68)	1.27 (0.78)
Stereotypic Grooming Pre-Stress Day 1	1.87 (1.41)	0.87 (1.30)	0.80 (1.66)	0.93 (1.49)	1.12 (1.50)
Stereotypic Grooming Pre-Stress Day 2	0.93 (1.39)	0.60 (0.91)	1.93 (2.46)	1.40 (1.88)	1.22 (1.79)
Stereotypic Grooming Pre-Stress Day 3	1.20 (1.37)	1.20 (2.31)	1.27 (2.02)	0.53 (0.99)	1.05 (1.73)
Stereotypic Grooming Pre-Stress Day 4	1.33 (2.23)	0.93 (1.39)	1.20 (1.90)	0.33 (0.62)	0.95 (1.65)
Stereotypic Grooming Pre-Stress Day 5	1.93 (1.62)	0.87 (1.46)	1.33 (2.06)	1.00 (2.00)	1.28 (1.80)
Stereotypic Grooming Post-Stress Day 1	1.53 (1.46)	1.53 (2.47)	1.07 (2.15)	0.67 (1.40)	1.20 (1.91)
Stereotypic Grooming Post-Stress Day 2	1.07 (1.16)	0.47 (0.92)	0.93 (1.44)	0.60 (0.99)	0.77 (1.14)
Stereotypic Grooming Post-Stress Day 3	1.73 (1.87)	0.93 (1.58)	0.87 (2.07)	0.47 (1.06)	1.00 (1.71)
Stereotypic Grooming Post-Stress Day 4	1.27 (1.79)	1.13 (1.46)	1.87 (3.18)	0.67 (0.90)	1.23 (2.01)
Stereotypic Grooming Post-Stress Day 5	1.87 (2.36)	1.07 (1.58)	1.00 (1.89)	0.73 (1.28)	1.17 (1.82)

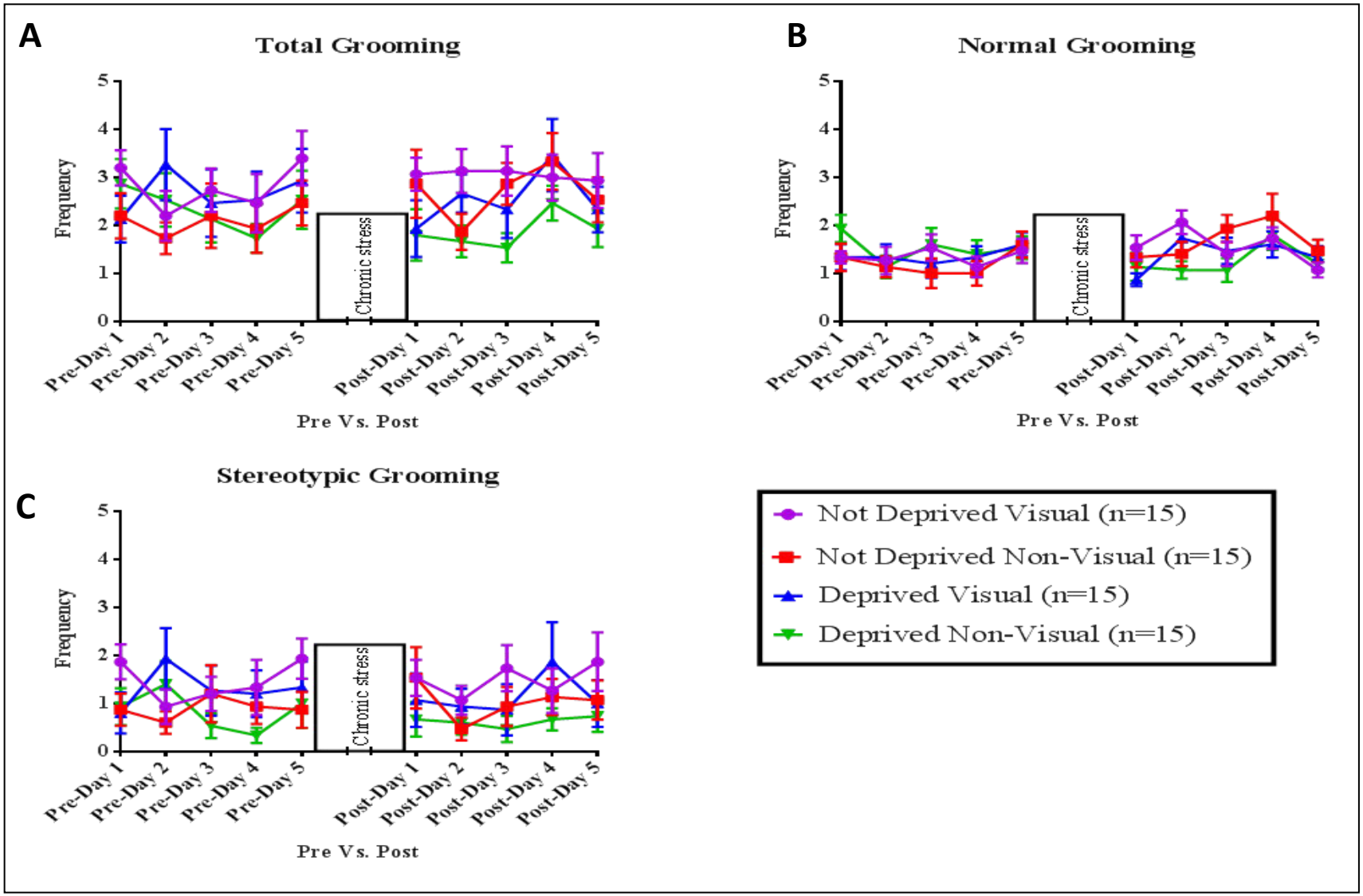

Figure 3.2: Frequency of behavioural parameters measured during BOOF. (A) Total Grooming incidents (TG); (B) Normal Grooming incidents (NG); (C) Stereotypic Grooming incidents (SG) by experimental cohort on each day of BOOF before and after stress. n = 15. Data are mean ± SEM.

3.2.1.2.3 Immobility Behaviours in the BOOF Test

The frequency of immobility behaviours (total, in the corner and in any square) was assessed to investigate the impact of CMS exposure between the experimental cohorts. Table 3.7 presents the mean and standard deviation for the immobility behaviours in the BOOF test before and after CMS. A Bonferroni correction was applied to the p-values of the immobility behaviours to minimise type 1 errors, resulting in a corrected p-value threshold of $p < 0.017$.

There was a significant increase over time in total immobility incidents ($F = 12.31$; $p < 0.017$) and corner immobility incidents ($F = 13.89$; $p < 0.017$) for all experimental cohorts during BOOF before CMS exposure (Figure 3.3-A and –B). However, there was no significant increase over time for incidents of immobility in any other marked square ($F = 1.44$; $p > 0.017$) (Appendix C, Table 5). After CMS exposure, there was a significant decrease over time in total ($F = 6.17$; $p < 0.017$), and corner immobility ($F = 6.66$; $p < 0.017$) incidents for all experimental cohorts, but not for square immobility incidents ($F = 1.91$; $p < 0.017$). This suggested that the rats in all experimental cohorts habituated to the BOOF test arena.

Prior to CMS exposure, there were no significant differences between the experimental cohorts for total ($F = 2.62$; $p > 0.017$), corner ($F = 1.72$; $p > 0.017$), or square ($F = 3.43$; $p > 0.017$) immobility incidents. Furthermore, there were also no significant differences between the experimental cohorts after CMS exposure for total ($F = 1.81$; $p > 0.017$), corner ($F = 1.30$; $p > 0.017$), or square ($F = 3.44$; $p > 0.017$) immobility incidents (Appendix C, Table 5).

However, there was a significant interaction between experimental cohorts and time before CMS exposure for both total ($F = 2.62$; $p < 0.017$) and corner ($F = 2.78$; $p < 0.017$) immobility incidents. However, there was no significant interaction between time and the experimental cohort after stress CMS in total ($F = 1.79$; $p > 0.017$) immobility incidents or corner ($F = 1.88$; $p > 0.017$) immobility incidents. There was no significant interaction between time and experimental cohort treatment for square immobility incidents before ($F = 0.48$; $p > 0.017$) or after ($F = 0.89$; $p > 0.017$) stress exposure (Appendix C, Table 5).

Tukey's *post-hoc* analysis revealed no significant difference between the experimental groups prior to or after CMS exposure (Appendix C, Table 6) for total and corner immobility incidents. However, from Tukey's *post-hoc* analysis the *"deprived visual"* cohort was found to have a significantly higher frequency ($p < 0.05$) of square immobility incidents after stress exposure when compared to the control *"not deprived visual"* rats.

Collectively, these immobility data illustrated the opposite pattern to the ambulatory movement behaviours. Before CMS exposure, there was an increase in the frequency of immobility during the BOOF test. Following CMS exposure, there was an overall decrease in immobility incidents. While there was a significant difference between the *"deprived visual"* and *"not deprived visual"* rats for square immobility incident this appeared to be due to variation between the "*deprived visual*" rats for square immobility behaviours, with several of the rats being significant outliers from the mean square immobility frequency. This suggested that MD or isolation on male Wistar rats did not significantly alter the immobility behaviour in the BOOF test, but CMS exposure did decrease the incidence of immobility behaviours.

Table 3.7: Means (SD) for the frequency of immobility behaviours (total, corner, and square immobility) before and after CMS.

Behaviour	Not Deprived Visual (*n* = 15) M (SD)	Not Deprived Non-Visual (*n* = 15) M (SD)	Deprived Visual (*n* = 15) M (SD)	Deprived Non-Visual (*n* = 15) M (SD)	Total (*n* = 60) M (SD)
Total Immobility Pre-Stress Day 1	9.13 (6.76)	8.07 (7.51)	14.87 (7.74)	10.47 (6.09)	10.63 (7.35)
Total Immobility Pre-Stress Day 2	16.00 (6.81)	12.73 (7.17)	23.67 (8.91)	17.13 (9.08)	17.38 (8.81)
Total Immobility Pre-Stress Day 3	16.33 (7.61)	12.93 (6.03)	18.67 (10.8)	17.93 (9.17)	16.47 (8.66)
Total Immobility Pre-Stress Day 4	20.07 (10.8)	16.13 (6.88)	17.73 (10.53)	15.53 (9.33)	17.37 (9.43)
Total Immobility Pre-Stress Day 5	16.20 (9.99)	12.07 (8.12)	22.20 (11.26)	15.33 (9.17)	16.45 (10.15)
Total Immobility Post-Stress Day 1	19.67 (9.66)	13.27 (9.24)	25.40 (14.53)	19.67 (9.48)	19.50 (11.52)
Total Immobility Post-Stress Day 2	18.53 (10.24)	14.07 (6.78)	15.27 (9.31)	15.53 (6.57)	15.85 (8.33)
Total Immobility Post-Stress Day 3	14.87 (10.18)	10.93 (6.13)	19.40 (11.48)	17.73 (8.34)	15.73 (9.58)
Total Immobility Post-Stress Day 4	13.80 (7.61)	10.73 (6.98)	15.93 (10.28)	13.93 (7.93)	13.60 (8.30)
Total Immobility Post-Stress Day 5	16.53 (7.42)	14.33 (6.60)	16.67 (9.18)	13.27 (6.76)	15.20 (7.51)
Corner Immobility Pre-Stress Day 1	8.33 (7.02)	7.27 (7.23)	13.00 (7.50)	9.27 (5.02)	9.47 (6.94)
Corner Immobility Pre-Stress Day 2	15.47 (7.02)	12.20 (7.47)	22.2 (8.36)	16.13 (8.63)	16.50 (8.51)
Corner Immobility Pre-Stress Day 3	16.00 (7.87)	12.67 (6.15)	15.93 (11.04)	17.07 (9.87)	15.42 (8.86)
Corner Immobility Pre-Stress Day 4	19.67 (11.06)	15.07 (7.60)	15.87 (10.31)	14.47 (8.80)	16.27 (9.51)
Corner Immobility Pre-Stress Day 5	15.53 (10.23)	11.33 (8.49)	19.80 (10.59)	14.93 (9.43)	15.40 (9.94)
Corner Immobility Post-Stress Day 1	18.73 (10.17)	12.60 (9.58)	23.80 (14.62)	18.33 (10.45)	18.37 (11.79)
Corner Immobility Post-Stress Day 2	17.53 (10.88)	12.47 (7.59)	11.53 (7.69)	13.67 (7.10)	13.80 (8.54)

Corner Immobility Post-Stress Day 3	14.67 (10.08)	9.67 (5.91)	17.47 (10.04)	16.27 (8.15)	14.52 (9.0)
Corner Immobility Post-Stress Day 4	12.67 (8.12)	9.60 (7.08)	11.47 (8.15)	12.13 (6.21)	11.47 (7.34)
Corner Immobility Post-Stress Day 5	16.07 (7.73)	13.87 (7.02)	13.87 (9.66)	11.60 (6.65)	13.85 (7.81)
Square Immobility Pre-Stress Day 1	0.80 (1.37)	0.80 (1.08)	1.87 (1.77)	1.20 (1.66)	1.17 (1.52)
Square Immobility Pre-Stress Day 2	0.53 (1.25)	0.53 (1.06)	1.47 (1.88)	1.00 (1.60)	0.88 (1.50)
Square Immobility Pre-Stress Day 3	0.33 (1.29)	0.27 (0.46)	1.40 (1.92)	0.87 (1.13)	0.72 (1.35)
Square Immobility Pre-Stress Day 4	0.40 (1.06)	1.07 (1.98)	1.87 (3.09)	1.07 (1.44)	1.10 (2.06)
Square Immobility Pre-Stress Day 5	0.67 (0.98)	0.73 (1.39)	2.40 (3.89)	0.40 (0.63)	1.05 (2.24)
Square Immobility Post-Stress Day 1	0.93 (1.67)	0.67 (1.11)	1.60 (1.92)	1.33 (1.5)	1.13 (1.58)
Square Immobility Post-Stress Day 2	1.00 (1.00)	1.60 (2.20)	3.73 (5.65)	1.87 (2.33)	2.05 (3.36)
Square Immobility Post-Stress Day 3	0.20 (0.41)	1.27 (1.91)	1.93 (2.37)	1.47 (1.30)	1.22 (1.75)
Square Immobility Post-Stress Day 4	1.13 (1.51)	1.13 (1.25)	4.47 (7.47)	1.80 (2.46)	2.13 (4.18)
Square Immobility Post-Stress Day 5	0.47 (0.83)	0.47 (0.74)	2.80 (4.04)	1.67 (1.54)	1.35 (2.39)

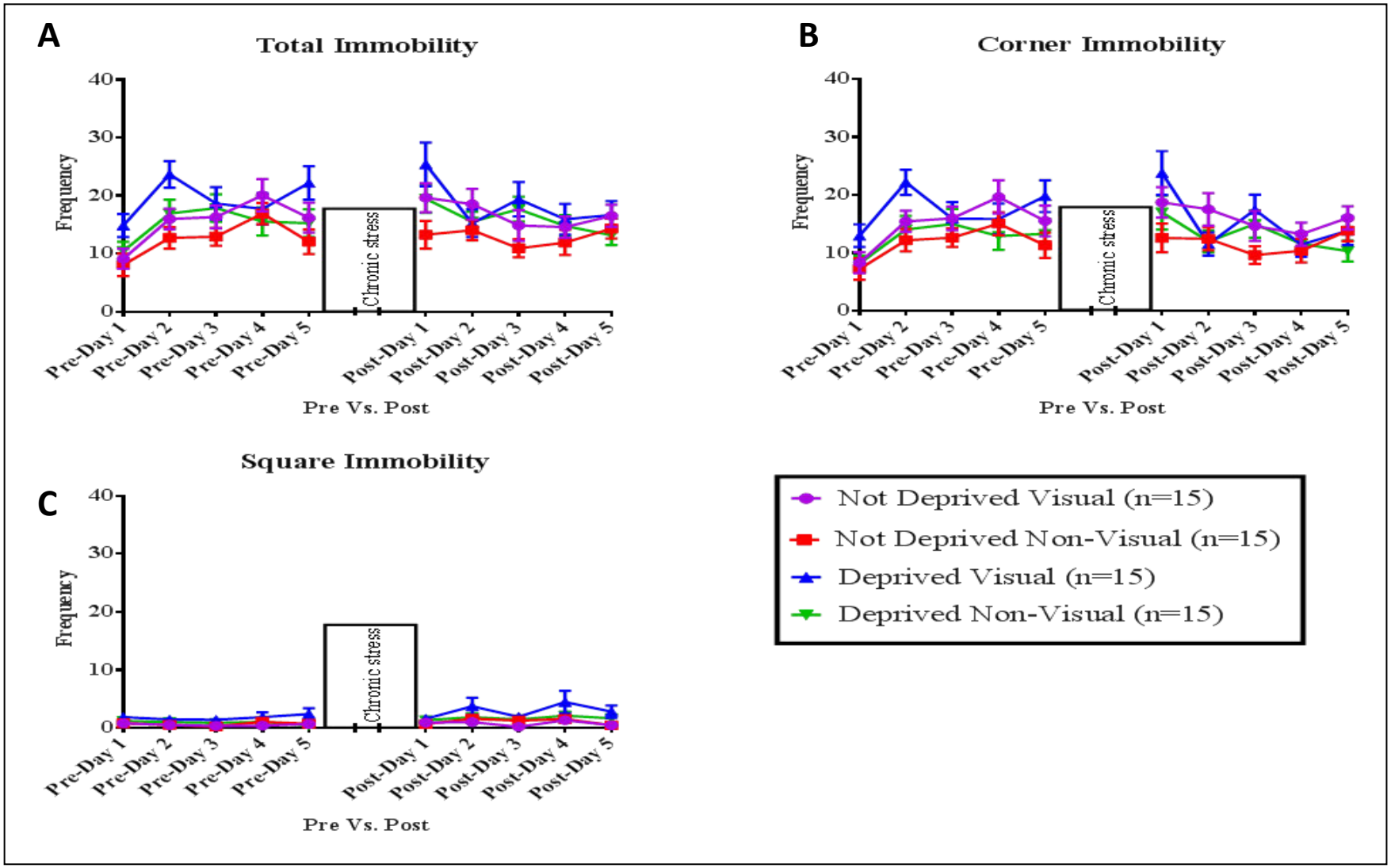

Figure 3.3: Frequency of behavioural parameters measured during BOOF. (A) Total Immobility incidents (TI); (B) Corner Immobility incidents (CI); (C) Square Immobility incidents (SI) by experimental cohort on each day of BOOF before and after stress. n = 15. Data are mean ± SEM.

3.2.1.2.4 Object Investigation Behaviours in the BOOF Test

Finally, the frequency of exploratory behaviours, using object investigation, was analysed to determine the impact of CMS exposure following MD and isolation. Table 3.8 presents the mean and standard deviation for the grooming behaviours in the BOOF test before and after CMS. A Bonferroni correction was applied to the p-values of the object investigation behaviours to minimise type 1 errors, resulting in a corrected p-value threshold of $p < 0.01$.

There was a significant decrease over time in total object ($F = 11.79$; $p < 0.01$), object 1 exploration ($F = 13.14$; $p < 0.01$), object 2 ($F = 5.58$; $p < 0.01$), object 3 ($F = 9.38$; $p < 0.01$), and object 4 ($F = 3.03$; $p < 0.01$) exploration incidents for all experimental groups during the BOOF test prior to CMS (Figure 3.4). A similar pattern was noted after CMS, with a decrease for total object ($F = 6.45$; $p < 0.01$), object 2 ($F = 4.01$; $p < 0.01$), and object 3 ($F = 7.40$; $p < 0.01$) investigation incidents over the five days of the BOOF test. This indicated that the rats were habituating normally to these objects both before and after stress. However, there was no significant change over time in object 1 investigation or object 4 investigation after CMS (Appendix C, Table 7).

There was no significant difference based on the MD and isolation manipulations used for the experimental cohorts prior to CMS exposure for total object investigation ($F = 1.12$; $p > 0.01$), object 1 investigation ($F = 1.58$; $p > 0.01$), object 2 investigation ($F = 0.51$; $p > 0.01$), object 3 investigation ($F = 1.64$; $p > 0.01$), or object 4 investigation ($F = 0.43$; $p > 0.01$). After CMS exposure, there were no significant differences in the interaction between the time and the experimental cohorts for total object investigation ($F = 3.42$; $p > 0.01$), object 2 investigation ($F = 1.52$; $p > 0.01$), or object 4 investigation ($F = 0.52$; $p > 0.01$). However, there was a significant difference between the experimental cohorts after stress in object 1 investigation frequency ($F = 4.38$; $p < 0.01$) and object 3 investigation frequency ($F = 4.51$; $p < 0.01$) (Appendix C, Table 7).

After stress exposure, Tukey's *post-hoc* analysis revealed there was a significant difference between the experimental cohorts for object 1 investigation (Figure 3.4-B). The *"deprived visual"*

($p < 0.05$) cohort and *"deprived non-visual"* ($p < 0.05$) cohort demonstrated a significantly higher frequency of investigation incidents than the control *"not deprived visual"* cohort (Appendix C, Table 8). A similar significant difference between the groups was noted in object 3 investigation after stress (Figure 3.4-D), with the control *"not-deprived visual"* rats demonstrating significantly less investigation than rats in the *"deprived visual"* ($p < 0.05$) and the *"deprived non-visual"* ($p < 0.05$) groups (Appendix C, Table 8).

There was no significant difference in the interaction between time and the experimental cohorts for total object investigation ($F = 1.00$; $p > 0.01$), object 1 investigation ($F = 0.63$; $p > 0.01$), object 2 investigation ($F = 1.28$; $p > 0.01$), object 3 investigation ($F = 1.62$; $p > 0.01$), or object 4 investigation ($F = 1.02$; $p > 0.01$) before CMS exposure. After CMS exposure, there were no significant differences in the interaction between the time and the experimental cohorts for total object investigation ($F = 1.29$; $p > 0.01$), object 1 investigation ($F = 0.78$; $p > 0.01$), object 2 investigation ($F = 1.58$; $p > 0.01$), object 3 investigation ($F = 1.29$; $p > 0.01$), or object 4 investigation ($F = 1.16$; $p > 0.01$) (Appendix C, Table 7).

These data indicated that there was a significant impact of MD status on the object investigation but no impact of isolation status after CMS exposure. Furthermore, increased investigation of objects 1 and 3 suggested a lack of habituation in the rats following CMS exposure. There was no impact of MD or isolation before CMS exposure, only after stress exposure. This suggested that early life manipulation in male Wistar rats did not significantly alter their behaviours in the BOOF test, but later life exposure to a chronic stressor resulted in significantly modified behaviour based on MD and isolation.

Table 3.8: Means (SD) for the frequency of object investigation behaviours (total, object 1, object 2, object 3, and object 4 investigation) before and after CMS.

Behaviour	Not Deprived Visual (*n* = 15) M (SD)	Not Deprived Non-Visual (*n* = 15) M (SD)	Deprived Visual (*n* = 15) M (SD)	Deprived Non-Visual (*n* = 15) M (SD)	Total (*n* = 60) M (SD)
Total Object Investigation Pre-Stress Day 1	21.27 (12.40)	22.00 (10.33)	24.20 (10.30)	22.80 (6.27)	22.57 (9.88)
Total Object Investigation Pre-Stress Day 2	12.53 (10.15)	15.53 (13.01)	14.40 (11.11)	15.67 (11.31)	14.53 (11.22)
Total Object Investigation Pre-Stress Day 3	10.20 (9.21)	15.27 (12.24)	17.87 (9.64)	14.00 (10.09)	14.33 (10.47)
Total Object Investigation Pre-Stress Day 4	7.67 (8.68)	10.73 (12.94)	13.80 (9.89)	17.73 (10.05)	12.48 (10.90)
Total Object Investigation Pre-Stress Day 5	10.07 (9.33)	15.20 (14.27)	12.87 (7.60)	15.87 (13.93)	13.50 (11.58)
Total Object Investigation Post-Stress Day 1	12.07 (9.02)	19.13 (12.84)	18.40 (10.57)	16.80 (11.72)	16.60 (11.19)
Total Object Investigation Post-Stress Day 2	15.4 (11.31)	19.00 (12.41)	24.60 (10.94)	24.60 (11.04)	20.90 (11.82)
Total Object Investigation Post-Stress Day 3	10.33 (8.03)	17.00 (8.68)	17.13 (7.51)	15.93 (9.03)	15.10 (8.59)
Total Object Investigation Post-Stress Day 4	11.13 (7.09)	17.40 (9.62)	18.93 (11.38)	24.00 (12.14)	17.87 (11.00)
Total Object Investigation Post-Stress Day 5	10.47 (8.38)	13.47 (11.59)	20.27 (12.12)	21.73 (11.92)	16.48 (11.80)
Object 1 Investigation Pre-Stress Day 1	8.20 (5.14)	8.33 (5.14)	10.20 (5.23)	10.87 (5.22)	9.40 (5.18)
Object 1 Investigation Pre-Stress Day 2	4.67 (3.66)	6.00 (5.92)	6.27 (5.28)	7.20 (6.89)	6.03 (5.50)
Object 1 Investigation Pre-Stress Day 3	3.47 (3.85)	6.20 (5.63)	7.00 (4.07)	6.13 (5.34)	5.70 (4.85)
Object 1 Investigation Pre-Stress Day 4	2.93 (2.94)	4.07 (5.19)	5.47 (4.69)	6.47 (5.19)	4.73 (4.68)
Object 1 Investigation Pre-Stress Day 5	3.60 (3.81)	6.33 (5.52)	4.73 (3.28)	6.53 (6.33)	5.30 (4.92)
Object 1 Investigation Post-Stress Day 1	4.00 (3.93)	6.87 (5.34)	7.73 (4.62)	8.60 (6.48)	6.80 (5.34)
Object 1 Investigation Post-Stress Day 2	4.60 (4.66)	6.87 (4.79)	9.93 (4.88)	10.60 (6.30)	8.00 (5.62)

Object 1 Investigation Post-Stress Day 3	3.67 (3.37)	7.33 (4.01)	8.07 (4.22)	7.53 (5.95)	6.65 (4.72)
Object 1 Investigation Post-Stress Day 4	4.60 (4.05)	7.47 (5.77)	8.47 (4.91)	10.40 (6.09)	7.73 (5.55)
Object 1 Investigation Post-Stress Day 5	4.27 (3.77)	5.47 (5.50)	8.27 (6.13)	8.93 (6.70)	6.73 (5.82)
Object 2 Investigation Pre-Stress Day 1	3.73 (2.37)	3.67 (3.33)	2.67 (1.59)	3.4 (1.59)	3.37 (2.31)
Object 2 Investigation Pre-Stress Day 2	2.07 (1.91)	2.33 (2.23)	1.53 (1.68)	2.47 (1.92)	2.10 (1.93)
Object 2 Investigation Pre-Stress Day 3	2.13 (2.03)	3.20 (3.10)	2.87 (1.96)	2.00 (2.07)	2.55 (2.33)
Object 2 Investigation Pre-Stress Day 4	1.27 (1.49)	1.73 (2.31)	2.13 (1.88)	2.80 (1.70)	1.98 (1.91)
Object 2 Investigation Pre-Stress Day 5	1.13 (1.73)	1.60 (1.55)	2.07 (1.58)	2.27 (1.87)	1.77 (1.70)
Object 2 Investigation Post-Stress Day 1	1.60 (1.30)	3.47 (3.20)	2.00 (1.96)	1.47 (1.36)	2.13 (2.20)
Object 2 Investigation Post-Stress Day 2	2.40 (2.67)	2.93 (2.84)	3.47 (1.96)	3.53 (1.96)	3.08 (2.37)
Object 2 Investigation Post-Stress Day 3	1.67 (2.13)	2.47 (1.85)	2.33 (1.59)	1.87 (1.25)	2.08 (1.72)
Object 2 Investigation Post-Stress Day 4	1.67 (1.18)	2.00 (1.25)	2.67 (1.59)	2.93 (1.39)	2.32 (1.42)
Object 2 Investigation Post-Stress Day 5	1.47 (1.46)	1.93 (2.02)	2.80 (1.86)	3.27 (2.19)	2.37 (1.98)
Object 3 Investigation Pre-Stress Day 1	6.00 (4.11)	6.73 (3.37)	7.60 (2.77)	5.47 (2.95)	6.45 (3.35)
Object 3 Investigation Pre-Stress Day 2	3.13 (2.95)	4.40 (3.87)	4.47 (3.56)	4.67 (3.46)	4.17 (3.44)
Object 3 Investigation Pre-Stress Day 3	2.47 (2.26)	3.13 (2.36)	5.20 (3.93)	3.40 (2.64)	3.55 (2.98)
Object 3 Investigation Pre-Stress Day 4	2.20 (3.45)	2.87 (2.83)	3.33 (2.72)	6.13 (3.74)	3.63 (3.47)
Object 3 Investigation Pre-Stress Day 5	3.40 (2.92)	4.33 (5.56)	3.73 (2.05)	4.80 (4.83)	4.07 (4.02)
Object 3 Investigation Post-Stress Day 1	3.87 (3.50)	5.20 (3.75)	5.40 (3.83)	5.20 (4.68)	4.92 (3.91)
Object 3 Investigation Post-Stress Day 2	4.93 (4.17)	5.40 (3.42)	7.27 (3.63)	7.00 (3.72)	6.15 (3.79)
Object 3 Investigation Post-Stress Day 3	2.67 (2.29)	3.93 (2.05)	4.27 (2.31)	4.40 (1.80)	3.82 (2.18)
Object 3 Investigation Post-Stress Day 4	2.73 (2.31)	5.07 (3.61)	4.73 (3.88)	7.80 (4.13)	5.08 (3.91)

Object 3 Investigation Post-Stress Day 5	2.40 (2.16)	4.13 (4.14)	6.13 (3.85)	6.67 (3.56)	4.83 (3.82)
Object 4 Investigation Pre-Stress Day 1	3.33 (2.82)	3.27 (1.91)	3.73 (2.60)	3.07 (1.79)	3.35 (2.28)
Object 4 Investigation Pre-Stress Day 2	2.67 (2.89)	2.80 (2.81)	2.13 (1.73)	1.33 (1.23)	2.23 (2.29)
Object 4 Investigation Pre-Stress Day 3	2.13 (3.20)	2.73 (2.81)	2.80 (1.52)	2.47 (2.13)	2.53 (2.45)
Object 4 Investigation Pre-Stress Day 4	1.27 (2.09)	2.07 (3.24)	2.87 (2.13)	2.33 (1.72)	2.13 (2.38)
Object 4 Investigation Pre-Stress Day 5	1.93 (1.71)	2.93 (2.96)	2.33 (2.13)	2.27 (2.02)	2.37 (2.22)
Object 4 Investigation Post-Stress Day 1	2.60 (1.68)	3.60 (3.16)	3.27 (2.15)	1.53 (1.46)	2.75 (2.30)
Object 4 Investigation Post-Stress Day 2	3.47 (2.90)	3.80 (2.73)	3.93 (3.26)	3.47 (2.17)	3.67 (2.73)
Object 4 Investigation Post-Stress Day 3	2.33 (2.64)	3.27 (3.24)	2.47 (0.92)	2.13 (1.60)	2.55 (2.27)
Object 4 Investigation Post-Stress Day 4	2.13 (1.85)	2.87 (2.23)	3.07 (2.09)	2.87 (2.61)	2.73 (2.19)
Object 4 Investigation Post-Stress Day 5	2.33 (2.19)	1.93 (1.91)	3.07 (2.28)	2.87 (2.07)	2.55 (2.11)

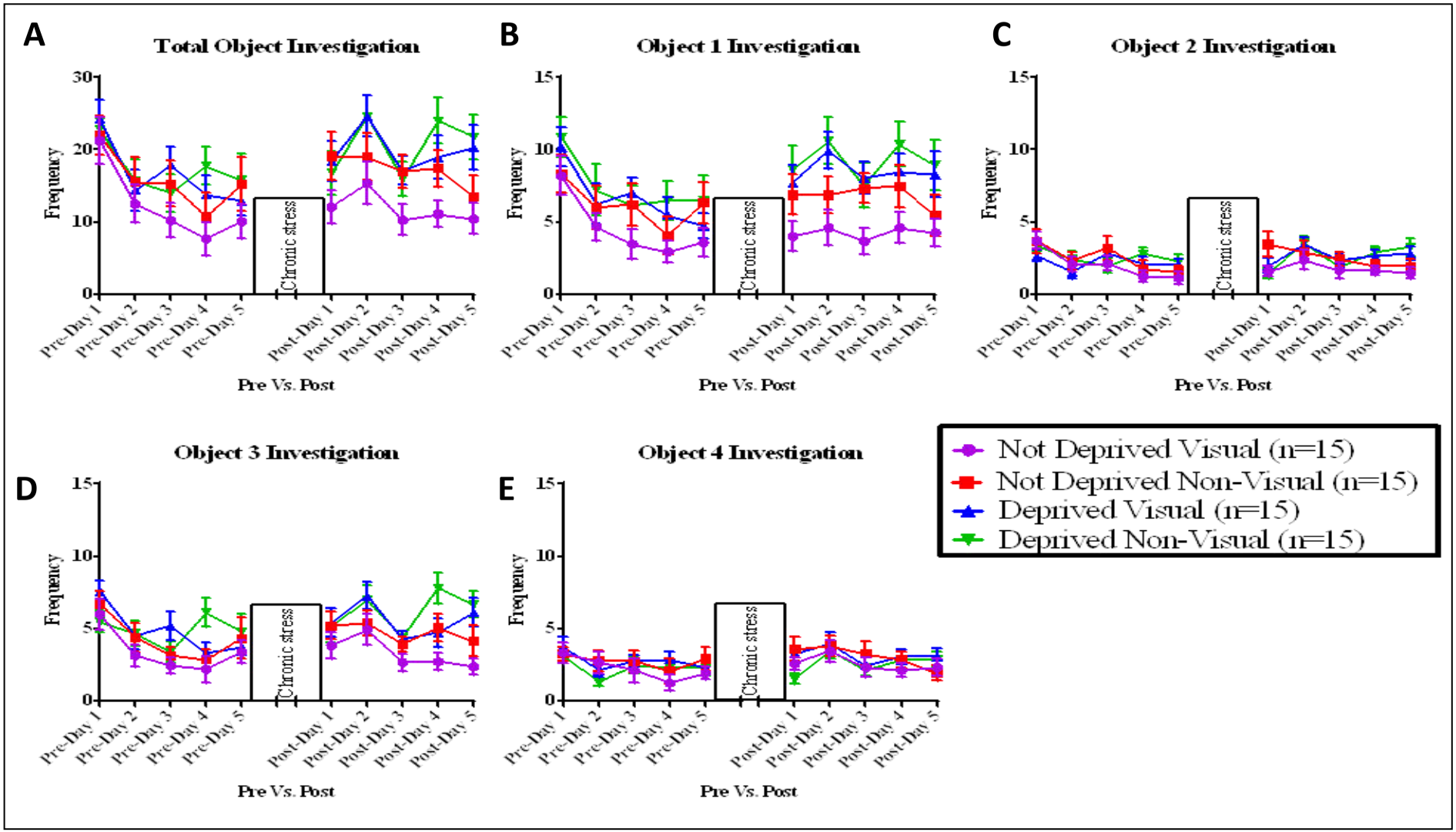

Figure 3.4: Frequency of behavioural parameters measured during BOOF. (A) Total Object Investigation (TOI); (B) Object 1 Investigation O1; (C) Object 2 Investigation (O2); (D) Object 3 Investigation (O3); (E) Object 4 Investigation (O4) by experimental cohort on each day of BOOF before and after stress. n = 15. Data are mean ± SEM.

3.2.2 The Duration of Behaviours in the BOOF Test

3.2.2.1 Overall Impact of Stress on the Behaviour Duration During the BOOF Test

In addition to the frequencies of the behaviours under scrutiny, the duration of those behaviours (locomotion, grooming, immobility, and object investigation) was also examined. The length of time spent on each of the behaviours was examined from day five (the last day of BOOF before CMS exposure) and day six (the first day of BOOF after CMS exposure). A MANOVA was conducted to determine whether there was an immediate effect of CMS exposure on the time spent on any of the behaviours. There was a significant difference between the experimental cohorts for the combined dependent variables [F (72, 99.5) = 1.47, p = 0.037; *Wilks'* λ = 0.11, partial η^2 = 0.52]. The η^2 indicated a large main effect (Pallant, 2013) of stress with 52% of the experimental variation in the dependent variables accounted for by CMS exposure across the experimental cohorts. Table 3.9 shows the duration of the scored behaviours for the experimental cohorts, plus univariate results for each variable. Univariate analysis indicated there was no significant effect of CMS exposure on the duration of the individual behaviours before or after stress, following Bonferroni adjustment for a significance value of $p < 0.002$.

Table 3.9: Means (SD) and univariate results for the duration of scored behaviours from pre-stress day 5 and post-stress day 1. Bonferroni adjustment for significant value ($p \leq 0.002$).

Behaviour	Not Deprived Visual M (SD)	Not Deprived Non-Visual M (SD)	Deprived Visual M (SD)	Deprived Non-Visual M (SD)	F	*p*	partial η^2
Vertical Motor Activity (VMA) Pre-Stress Day 5	37.73 (34.87)	57.60 (49.63)	38.40 (30.16)	70.20 (56.83)	1.91	0.14	0.09
Vertical Motor Activity (VMA) Post-Stress Day 1	43.47 (36.69)	68.67 (49.78)	43.53 (33.39)	61.53 (56.21)	1.22	0.31	0.06
Horizontal Motor Activity (HMA) Pre-Stress Day 5	51.68 (41.83)	66.01 (56.63)	53.07 (28.12)	67.47 (54.10)	0.48	0.70	0.03
Horizontal Motor Activity (HMA) Post-Stress Day 1	56.39 (38.84)	74.56 (56.07)	71.51 (32.02)	71.67 (45.34)	0.52	0.67	0.03
Central Time (CT) Pre-Stress Day 5	15.13 (14.26)	34.67 (39.70)	20.73 (23.09)	21.47 (21.99)	1.47	0.23	0.07
Central Time (CT) Post-Stress Day 1	21.13 (26.24)	38.47 (30.79)	30.80 (27.77)	24.40 (20.85)	1.24	0.31	0.06
Total Grooming (TG) Pre-Stress Day 5	44.20 (28.96)	23.80 (20.51)	33.73 (33.54)	23.67 (22.17)	2.00	0.13	0.10
Total Grooming (TG) Post-Stress Day 1	32.27 (20.44)	30.07 (36.06)	36.80 (59.14)	19.73 (22.81)	0.55	0.65	0.03
Normal Grooming (NG) Pre-Stress Day 5	12.93 (5.84)	12.60 (9.16)	14.60 (6.39)	12.27 (9.51)	0.26	0.86	0.01
Normal Grooming (NG) Post-Stress Day 1	11.13 (7.82)	11.07 (7.10)	11.33 (7.24)	10.33 (10.913)	0.04	0.99	0.002
Stereotypic Grooming (SG) Pre-Stress Day 5	31.27 (31.62)	11.20 (18.61)	19.13 (29.97)	11.40 (21.18)	1.98	0.13	0.10
Stereotypic Grooming (SG) Post-Stress Day 1	21.13 (18.84)	19.00 (33.31)	25.47 (57.13)	9.40 (18.29)	0.55	0.65	0.03
Total Immobility (TI) Pre-Stress Day 5	122.67 (97.58)	142.27 (146.30)	159.00 (87.79)	143.33 (129.46)	0.24	0.87	0.01
Total Immobility (TI) Post-Stress Day 1	139.60 (104.45)	151.53 (151.76)	169.60 (97.98)	167.13 (137.25)	0.19	0.90	0.01
Corner Immobility (CI) Pre-Stress Day 5	119.87 (98.76)	138.47 (148.01)	147.47 (89.10)	142.00 (130.57)	0.15	0.93	0.01
Corner Immobility (CI) Post-Stress Day 1	135.13 (106.90)	148.93 (153.62)	160.93 (96.71)	162.93 (139.43)	0.16	0.93	0.01
Square Immobility (SI) Pre-Stress Day 5	2.80 (4.60)	3.80 (9.28)	11.53 (18.02)	1.33 (2.06)	2.86	0.05	0.13

Square Immobility (SI) Post-Stress Day 1	4.47 (9.82)	2.60 (6.61)	8.67 (12.48)	4.20 (5.12)	1.25	0.30	0.06
Total Object Investigation (TOI) Pre-Stress Day 5	24.13 (25.76)	35.87 (32.72)	30.47 (17.83)	36.40 (34.59)	0.61	0.62	0.03
Total Object Investigation (TOI) Post-Stress Day 1	27.13 (23.72)	43.13 (32.71)	44.80 (29.69)	45.13 (37.79)	1.14	0.34	0.06
Object 1 Investigation (O1) Pre-Stress Day 5	9.27 (13.66)	16.00 (15.96)	13.27 (9.15)	17.40 (17.05)	0.94	0.43	0.05
Object 1 Investigation (O1) Post-Stress Day 1	10.40 (11.93)	18.93 (16.38)	22.27 (15.95)	28.93 (25.76)	2.68	0.06	0.13
Object 2 Investigation (O2) Pre-Stress Day 5	2.07 (3.52)	2.60 (2.44)	3.67 (2.82)	3.13 (2.45)	0.88	0.46	0.05
Object 2 Investigation (O2) Post-Stress Day 1	2.27 (2.52)	6.27 (6.46)	2.87 (2.90)	1.93 (1.75)	4.00	0.01	0.18
Object 3 Investigation (O3) Pre-Stress Day 5	9.33 (10.57)	11.33 (13.62)	10.27 (8.79)	11.60 (13.67)	0.12	0.95	0.01
Object 3 Investigation (O3) Post-Stress Day 1	9.47 (12.22)	11.27 (10.17)	14.20 (11.83)	12.27 (13.40)	0.41	0.75	0.02
Object 4 Investigation (O4) Pre-Stress Day 5	3.47 (3.02)	5.93 (6.10)	3.27 (2.66)	4.27 (3.65)	1.32	0.28	0.07
Object 4 Investigation (O4) Post-Stress Day 1	5.00 (3.14)	6.67 (6.60)	5.47 (4.66)	2.00 (2.10)	2.97	0.04	0.14

However, Tukey *post-hoc* analysis revealed significant differences before and after CMS exposure between the experimental cohorts for square immobility and object investigation as shown in Table 3.10. There was a significant increase ($p = 0.047$) in the duration of square immobility in the *"deprived visual"* cohort when compared to the "*deprived non-visual"* cohort prior to CMS stress. However, there was no significant difference between these cohorts for square immobility immediately after CMS exposure. There were also significant differences noted from Tukey's *post-hoc* analysis between the experimental cohorts for the duration of object 1, object 2 and object 4 investigation behaviours after stress. The control "*not deprived visual*" cohort spent significantly ($p = 0.036$) less time in object 1 investigation when compared to the *"deprived non-visual"* cohort. There was significantly more time spent on object 2 investigation by the *"not deprived non-visual"* cohort when compared to *"not deprived visual"* ($p = 0.031$) and *"deprived non-visual"* ($p = 0.017$) cohorts.

*Table 3.10: Tukey post-hoc analysis from a MANOVA of behavioural durations of experimental groups for pre-day 5 before & post-day 1 after CMS. Limited to dependent variables with a significant difference between experimental cohorts. Please note * indicates significant result (p < 0.05).*

Dependent Variable	Cohort	Comparison cohort	Mean Difference	Std. Error	Sig.
Square Immobility Pre-Stress Day 5	Deprived Visual	Not Deprived Visual	8.73	3.81	0.11
		Not Deprived Non-Visual	7.73	3.81	0.19
		Deprived Non-Visual	10.20*	3.81	0.05
Object 1 Investigation Post-Stress Day 1	Not Deprived Visual	Not Deprived Non-Visual	-8.53	6.66	0.58
		Deprived Visual	-11.87	6.66	0.29
		Deprived Non-Visual	-18.53*	6.66	0.04
Object 2 Investigation Post-Stress Day 1	Not Deprived Non-Visual	Not Deprived Visual	4.00*	1.41	0.03
		Deprived Visual	3.40	1.41	0.09
		Deprived Non-Visual	4.33*	1.41	0.02
Object 4 Investigation Post-Stress Day 1	Not Deprived Non-Visual	Not Deprived Visual	1.67	1.63	0.74
		Deprived Visual	1.20	1.63	0.88
		Deprived Non-Visual	4.67*	1.63	0.03

To elucidate the overall impact of CMS exposure on the experimental cohorts, a second MANOVA was performed on the mean behavioural duration, over five days, before and after CMS exposure to account for day-to-day variability in the duration of behaviours during BOOF. There were no significant differences found between the experimental cohorts following CMS exposure on the combined dependent variables, however, a trend toward significance was present, F (81, 90.6) = 1.40, p = 0.058; *Wilks'* λ = 0.088, partial η^2 = 0.56. The partial η^2 indicated a large effect (Pallant, 2013) of stress, with 56% of the experimental variation in the dependent variables accounted for by the CMS exposure across the experimental cohorts. Table 3.11 shows the frequency scores for each of the scored behaviours for the experimental cohorts, plus univariate results for each dependent variable. However, though there was a trend to significance in the main effect of the MANOVA, none of the univariate effects reached the adjusted level of significance.

The reversal in analytical significance between the frequency and duration may be due to the short duration spent on these behaviours during the BOOF test. Since the total duration spent on any assessed behaviour was short (< 150 seconds) when compared to the total time spent within the BOOF test arena (600 seconds). Overall, the duration of the behaviours when analysed using the mean before or after CMS exposure was not significant, while the frequency of the same behaviours was significantly different. The variation in result significance was due to each behaviour incidence (the frequency) potentially lasting for several seconds or only a single second (the duration). This discrepancy suggested greater day-to-day variation in the duration of the behaviours than the frequency of the behaviours.

Table 3.11: Means (SD) and univariate results for the mean duration of scored behaviours before and after CMS exposure. Bonferroni adjustment for significant value ($p \leq 0.002$).

Behaviour	Not Deprived Visual M (SD)	Not Deprived Non-Visual M (SD)	Deprived Visual M (SD)	Deprived Non-Visual M (SD)	F	*p*	partial η^2
Vertical Motor Activity (VMA) Pre-Stress Mean	41.81 (30.98)	48.67 (25.71)	39.88 (14.49)	60.91 (33.35)	1.84	0.15	0.09
Vertical Motor Activity (VMA) Post-Stress Mean	56.56 (33.28)	69.00 (31.00)	50.88 (27.67)	72.81 (47.02)	1.26	0.30	0.06
Horizontal Motor Activity (HMA) Pre-Stress Mean	62.57 (39.62)	72.14 (48.81)	71.30 (21.73)	77.29 (37.99)	0.38	0.77	0.02
Horizontal Motor Activity (HMA) Post-Stress Mean	59.95 (38.44)	67.40 (39.19)	72.94 (35.00)	79.35 (36.71)	0.73	0.54	0.04
Central Time (CT) Pre-Stress Mean	23.99 (16.31)	25.80 (18.24)	26.13 (21.19)	24.88 (15.99)	0.04	0.99	0.002
Central Time (CT) Post-Stress Mean	26.11 (19.09)	35.48 (21.11)	41.93 (29.99)	38.52 (18.33)	1.36	0.27	0.07
Total Grooming (TG) Pre-Stress Mean	36.32 (20.62)	23.57 (21.72)	39.13 (40.25)	25.59 (17.44)	1.27	0.29	0.06
Total Grooming (TG) Post-Stress Mean	37.39 (24.36)	27.76 (22.14)	38.07 (48.18)	19.21 (13.94)	1.33	0.27	0.07
Normal Grooming (NG) Pre-Stress Mean	12.28 (3.00)	10.05 (4.97)	12.32 (4.54)	11.92 (9.52)	0.48	0.70	0.03
Normal Grooming (NG) Post-Stress Mean	13.56 (4.060)	12.59 (5.11)	13.04 (5.03)	10.71 (5.12)	0.99	0.41	0.05
Stereotypic Grooming (SG) Pre-Stress Mean	24.04 (20.14)	13.52 (19.23)	26.81 (38.22)	13.67 (15.59)	1.16	0.33	0.06
Stereotypic Grooming (SG) Post-Stress Mean	23.83 (23.09)	15.17 (19.56)	25.03 (46.23)	8.51 (12.25)	1.13	0.34	0.06
Total Immobility (TI) Pre-Stress Mean	127.85 (76.95)	150.80 (111.94)	137.49 (67.22)	117.20 (92.76)	0.39	0.76	0.02
Total Immobility (TI) Post-Stress Mean	120.65 (76.40)	113.56 (67.86)	129.72 (61.11)	115.43 (73.25)	0.16	0.92	0.01
Corner Immobility (CI) Pre-Stress Mean	125.77 (78.07)	146.96 (114.05)	129.48 (66.89)	109.32 (85.48)	0.46	0.71	0.02
Corner Immobility (CI) Post-Stress Mean	117.87 (77.82)	107.71 (70.02)	115.15 (54.83)	109.27 (73.58)	0.07	0.98	0.004
Square Immobility (SI) Pre-Stress Mean	2.08 (2.55)	3.84 (5.73)	8.01 (9.42)	3.23 (2.33)	3.01	0.04	0.14

Square Immobility (SI) Post-Stress Mean	2.79 (3.01)	5.85 (6.04)	14.57 (20.22)	6.16 (3.61)	3.28	0.03	0.15
Total Object Investigation (TOI) Pre-Stress Mean	27.84 (18.09)	36.43 (24.92)	38.59 (13.93)	39.43 (22.08)	1.04	0.38	0.05
Total Object Investigation (TOI) Post-Stress Mean	28.05 (16.87)	42.51 (25.86)	52.28 (22.76)	52.28 (22.76)	3.46	0.02	0.16
Object 1 Investigation (O1) Pre-Stress Mean	11.19 (7.26)	16.16 (12.03)	17.24 (8.03)	19.45 (15.62)	1.45	0.24	0.07
Object 1 Investigation (O1) Post-Stress Mean	12.07 (9.62)	19.75 (13.42)	27.45 (14.09)	26.32 (17.53)	3.87	0.01	0.17
Object 2 Investigation (O2) Pre-Stress Mean	3.65 (2.55)	5.29 (4.52)	4.15 (2.40)	4.25 (1.86)	0.79	0.51	0.04
Object 2 Investigation (O2) Post-Stress Mean	3.27 (2.67)	4.79 (3.81)	4.64 (2.45)	4.63 (2.45)	0.90	0.45	0.05
Object 3 Investigation (O3) Pre-Stress Mean	8.23 (6.01)	9.13 (5.19)	11.85 (5.19)	11.25 (5.14)	1.52	0.22	0.08
Object 3 Investigation (O3) Post-Stress Mean	7.84 (5.82)	11.83 (8.80)	14.19 (5.82)	15.55 (7.16)	3.49	0.02	0.16
Object 4 Investigation (O4) Pre-Stress Mean	4.77 (4.44)	5.84 (4.70)	5.37 (1.83)	4.40 (2.42)	0.48	0.70	0.03
Object 4 Investigation (O4) Post-Stress Mean	4.99 (3.32)	6.15 (4.51)	6.00 (3.02)	4.85 (2.56)	0.57	0.64	0.03

3.2.2.2 Habituation of Behaviour Duration During the BOOF Test

After assessment of the overall impact of stress on the duration of the behaviours, additional repeated measure ANOVAs were conducted on each set of behaviours, before and after CMS exposure. These analyses examined whether habituation occurred in the duration of the individual behaviours for the experimental cohorts. In each case, a Bonferroni correction was applied based on the number of behaviours in each grouping, with details provided in each section. Tukey's *post-hoc* analysis was conducted to determine if there were any significant differences in the behavioural durations following MD and isolation exposures.

3.2.2.2.1 Locomotion Behaviours in the BOOF Test

The impact of CMS exposure on the rats' duration of ambulatory movement was examined using locomotion behaviours (vertical motor activity, horizontal motor activity, and centre entries) to determine the level of habituation to the BOOF arena before and after stress and if there were any significant differences between the experimental cohorts. Table 3.12 presents the mean and standard deviation for the grooming behaviours in the BOOF test before and after CMS. A Bonferroni correction was applied to the p-values of the locomotion behaviours to minimise type 1 errors, resulting in a corrected p-value threshold of $p < 0.017$.

There was a significant increase ($F = 4.23$; $p < 0.017$) in the duration of vertical motor activity and a significant decrease ($F = 14.67$; $p < 0.017$) in the duration of horizontal motor activity before CMS exposure (Figure 3.5-A and –B). However, there were no significant differences in the duration of vertical ($F = 1.93$; $p > 0.017$) or horizontal motor activity ($F = 2.34$; $p > 0.017$) over the course of the BOOF test after CMS exposure (Appendix C, Table 9). Analysis of the time spent in the centre of the arena (Figure 3.5-C) reveals the opposite pattern, no significant change in the central area time prior to stress exposure (Appendix C, Table 9). However, there was a significant increase ($F = 4.35$; $p < 0.017$) in central duration over the five

days of the BOOF test after CMS exposure. This suggested that, while there was no change in the duration of ambulatory movement, a greater time was spent in the centre of the BOOF arena after CMS exposure, which indicated reduced anxiety.

There were no significant differences between the experimental cohorts on the time spent on vertical motor activity ($F = 1.84$; $p > 0.017$), horizontal motor activity ($F = 0.38$; $p > 0.017$), or time spent in the centre area ($F = 0.04$; $p > 0.017$) before stress exposure. After stress exposure, there were also no significant differences between the experimental cohorts for vertical motor activity duration ($F = 1.26$; $p > 0.017$), horizontal motor activity duration ($F = 0.73$; $p > 0.017$), or time spent in centre area ($F = 1.36$; $p > 0.017$) (Appendix C, Table 9).

Finally, there were no significant interactions between time and experimental cohort before stress exposure for vertical motor activity duration ($F = 0.68$; $p > 0.017$), horizontal motor activity duration ($F = 1.01$; $p > 0.017$), and time spent in the centre area ($F = 1.87$; $p > 0.017$). The same lack of significant interaction between time and experimental cohorts was noted after stress exposure for vertical motor activity duration ($F = 1.19$; $p > 0.017$), horizontal motor activity duration ($F = 0.93$; $p > 0.017$), and time spent in the centre area ($F = 0.65$; $p > 0.017$) (Appendix C, Table 9). Tukey's *post-hoc* analysis revealed no significant difference due to MD or isolation exposure, before or after CMS, for vertical or horizontal motor activity durations (Appendix C, Table 10).

Thus, analysis of the behaviour durations demonstrated no change in the duration of vertical and horizontal motor activities in the BOOF arena following CMS exposure, regardless of MD or isolation exposures.

Table 3.12: Means (SD) for the duration of locomotion behaviours (vertical motor activity, horizontal motor activity, and central time) before and after CMS.

Behaviour	Not Deprived Visual (*n* = 15) M (SD)	Not Deprived Non-Visual (*n* = 15) M (SD)	Deprived Visual (*n* = 15) M (SD)	Deprived Non-Visual (*n* = 15) M (SD)	Total (*n* = 60) M (SD)
Vertical Motor Activity Pre-Stress Day 1	52.47 (23.06)	62.47 (31.00)	50.67 (25.15)	58.33 (28.02)	55.98 (26.70)
Vertical Motor Activity Pre-Stress Day 2	50.00 (53.97)	50.80 (50.07)	38.80 (21.20)	64.93 (39.02)	51.13 (42.91)
Vertical Motor Activity Pre-Stress Day 3	39.07 (40.29)	40.33 (27.91)	41.07 (22.67)	53.53 (42.03)	43.50 (33.85)
Vertical Motor Activity Pre-Stress Day 4	29.80 (39.87)	32.13 (30.55)	30.47 (26.12)	57.53 (43.54)	37.48 (36.71)
Vertical Motor Activity Pre-Stress Day 5	37.73 (34.87)	57.60 (49.63)	38.40 (30.16)	70.20 (56.83)	50.98 (45.22)
Vertical Motor Activity Post-Stress Day 1	43.47 (36.69)	68.67 (49.77)	43.53 (33.39)	61.53 (56.21)	54.30 (45.24)
Vertical Motor Activity Post-Stress Day 2	54.07 (44.32)	72.00 (41.05)	63.33 (36.98)	67.60 (51.06)	64.25 (43.06)
Vertical Motor Activity Post-Stress Day 3	53.93 (39.29)	81.40 (40.07)	59.40 (46.60)	82.93 (62.76)	69.42 (48.65)
Vertical Motor Activity Post-Stress Day 4	65.27 (40.68)	72.67 (56.99)	39.47 (29.26)	75.20 (40.46)	63.15 (44.25)
Vertical Motor Activity Post-Stress Day 5	66.07 (53.90)	50.27 (25.52)	48.67 (36.28)	76.80 (53.88)	60.45 (44.52)
Horizontal Motor Activity Pre-Stress Day 1	96.36 (51.81)	107.43 (60.79)	101.87 (21.95)	109.77 (40.06)	103.86 (45.13)
Horizontal Motor Activity Pre-Stress Day 2	72.31 (58.86)	69.92 (58.74)	67.89 (36.39)	73.41 (44.40)	70.88 (49.27)
Horizontal Motor Activity Pre-Stress Day 3	48.83 (41.57)	67.89 (55.40)	75.85 (40.36)	66.07 (46.52)	64.66 (46.23)
Horizontal Motor Activity Pre-Stress Day 4	43.68 (41.88)	49.45 (58.22)	57.80 (35.12)	69.71 (38.97)	55.16 (44.39)
Horizontal Motor Activity Pre-Stress Day 5	51.68 (41.83)	66.01 (56.63)	53.07 (28.12)	67.47 (54.09)	59.56 (45.95)
Horizontal Motor Activity Post-Stress Day 1	56.39 (38.84)	74.56 (56.07)	71.51 (32.02)	71.67 (45.34)	68.53 (43.43)
Horizontal Motor Activity Post-Stress Day 2	67.55 (48.34)	71.35 (55.97)	88.63 (47.18)	84.52 (38.8)	78.01 (47.56)

Horizontal Motor Activity Post-Stress Day 3	52.87 (40.95)	68.79 (38.92)	72.51 (32.83)	78.17 (42.12)	68.08 (39.03)
Horizontal Motor Activity Post-Stress Day 4	64.52 (45.27)	67.37 (35.14)	62.89 (42.49)	81.56 (35.03)	69.09 (39.42)
Horizontal Motor Activity Post-Stress Day 5	58.41 (44.58)	54.93 (39.68)	69.19 (46.11)	80.83 (40.65)	65.84 (42.96)
Central Time Pre-Stress Day 1	35.60 (30.63)	22.00 (16.02)	31.27 (21.78)	20.27 (12.64)	27.28 (21.80)
Central Time Pre-Stress Day 2	30.47 (26.16)	24.47 (22.11)	16.93 (19.48)	21.07 (20.14)	23.23 (22.13)
Central Time Pre-Stress Day 3	22.80 (28.96)	24.67 (27.15)	35.67 (38.54)	29.33 (29.03)	28.12 (30.85)
Central Time Pre-Stress Day 4	15.93 (18.74)	23.20 (24.46)	26.07 (30.31)	32.27 (28.74)	24.37 (25.97)
Central Time Pre-Stress Day 5	15.13 (14.26)	34.67 (39.70)	20.73 (23.09)	21.47 (21.99)	23.00 (26.75)
Central Time Post-Stress Day 1	21.13 (26.23)	38.47 (30.79)	30.80 (27.77)	24.40 (20.85)	28.70 (26.82)
Central Time Post-Stress Day 2	33.40 (29.59)	38.87 (32.06)	51.13 (34.20)	44.40 (26.67)	41.95 (30.69)
Central Time Post-Stress Day 3	23.33 (27.76)	33.27 (25.27)	35.60 (32.93)	33.87 (26.69)	31.52 (28.00)
Central Time Post-Stress Day 4	24.00 (20.73)	40.07 (27.18)	45.40 (50.24)	48.60 (30.20)	39.52 (34.40)
Central Time Post-Stress Day 5	28.67 (28.61)	26.73 (25.07)	46.73 (49.01)	41.33 (30.97)	35.87 (34.83)

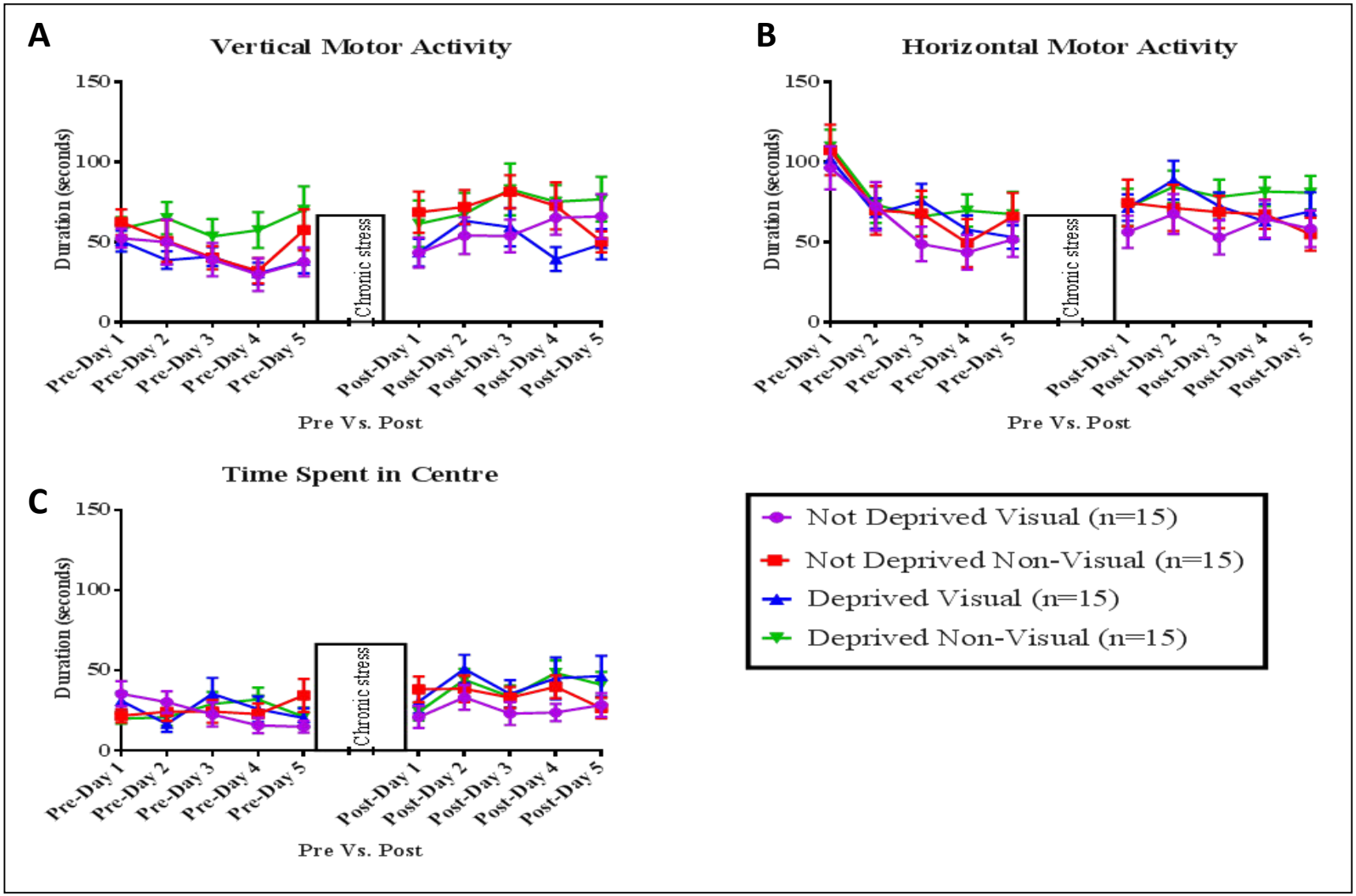

Figure 3.5: Duration of behavioural parameters measured during BOOF. (A) Vertical Motor Activity (VMA); (B) Horizontal Motor Activity (HMA); (C) Time Spent in Centre (CT) by experimental cohort on each day of BOOF before and after stress. n = 15. Data are mean ± SEM.

3.2.2.2.2 Grooming Behaviours in the BOOF Test

The temporal profiles of the duration of grooming behaviours (total grooming, normal grooming, and stereotypic grooming) were examined to investigate the impact of CMS exposure on the experimental cohorts. Table 3.13 presents the mean and standard deviation for the grooming behaviours in the BOOF test before and after CMS. A Bonferroni correction was applied to the p-values of the grooming behaviours to minimise type 1 errors, resulting in a corrected p-value threshold of $p < 0.017$.

There were no significant differences in the duration of total grooming ($F = 0.25$; $p > 0.017$), normal grooming ($F = 1.74$; $p > 0.017$), or stereotypic grooming ($F = 0.31$; $p > 0.017$) behaviours over the five days of the BOOF test before CMS exposure. There were also no significant differences over time in the duration of total grooming ($F = 1.29$; $p > 0.017$), normal grooming ($F = 1.61$; $p > 0.017$), or stereotypic grooming ($F = 1.44$; $p > 0.017$) behaviours after CMS exposure (Figure 3.6-A, -B and –C) (Appendix C, Table 11).

There were no significant differences between the experimental cohorts in the duration of total grooming ($F = 1.27$; $p > 0.017$), normal grooming ($F = 0.48$; $p > 0.017$), or stereotypic grooming ($F = 1.16$; $p > 0.017$) behaviours before CMS exposure. There were also no significant differences between the experimental cohorts for total grooming ($F = 1.33$; $p > 0.017$), normal grooming ($F = 0.99$; $p > 0.017$), or stereotypic grooming ($F = 1.13$; $p > 0.017$) behaviours after CMS exposure (Appendix C, Table 11).

Finally, there were no signification interactions between time and the experimental cohorts for total grooming ($F = 0.99$; $p > 0.017$), normal grooming ($F = 0.32$; $p > 0.017$), or stereotypic grooming ($F = 1.13$; $p > 0.017$) behaviour durations before CMS exposure. Nor were there any significant interactions between time and the experimental cohorts after stress exposure for total grooming ($F = 1.16$; $p > 0.017$), normal grooming ($F = 1.61$; $p > 0.017$), or stereotypic grooming ($F = 0.62$; $p > 0.017$) (Appendix C, Table 11). Furthermore, from Tukey's *post-hoc* analysis, there

were no significant differences between the experimental groups in the duration of any grooming behaviours (Appendix C, Table 12). The high degree of variation in grooming behaviour duration was present both prior to and after CMS exposure.

Table 3.13: Means (SD) for the duration of grooming behaviours (total, normal and stereotypic grooming) before and after CMS.

Behaviour	Not Deprived Visual (*n* = 15) M (SD)	Not Deprived Non-Visual (*n* = 15) M (SD)	Deprived Visual (*n* = 15) M (SD)	Deprived Non-Visual (*n* = 15) M (SD)	Total (*n* = 60) M (SD)
Total Grooming Pre-Stress Day 1	40.47 (28.44)	22.27 (22.96)	32.93 (34.98)	30.73 (25.29)	31.60 (28.32)
Total Grooming Pre-Stress Day 2	29.87 (28.61)	20.47 (25.33)	44.40 (51.50)	31.27 (25.91)	31.50 (34.77)
Total Grooming Pre-Stress Day 3	37.80 (37.13)	30.07 (41.69)	43.00 (59.11)	20.80 (25.92)	32.92 (42.42)
Total Grooming Pre-Stress Day 4	29.27 (19.85)	21.27 (22.80)	41.60 (50.54)	21.47 (20.09)	28.40 (31.44)
Total Grooming Pre-Stress Day 5	44.20 (28.96)	23.80 (20.51)	33.73 (33.54)	23.67 (22.17)	31.35 (27.48)
Total Grooming Post-Stress Day 1	32.27 (20.44)	30.07 (36.06)	36.80 (59.14)	19.73 (22.81)	29.72 (37.43)
Total Grooming Post-Stress Day 2	30.33 (21.17)	16.47 (24.15)	37.07 (48.35)	18.27 (16.14)	25.53 (30.58)
Total Grooming Post-Stress Day 3	38.00 (21.38)	32.80 (35.89)	29.87 (40.98)	14.47 (12.35)	28.78 (30.44)
Total Grooming Post-Stress Day 4	43.73 (48.52)	31.00 (28.45)	40.87 (47.81)	24.40 (17.28)	35.00 (37.75)
Total Grooming Post-Stress Day 5	42.60 (40.09)	28.47 (22.48)	45.73 (60.93)	19.20 (18.13)	34.00 (39.72)
Normal Grooming Pre-Stress Day 1	13.20 (5.66)	10.47 (7.08)	12.40 (8.54)	14.40 (8.75)	12.62 (7.55)
Normal Grooming Pre-Stress Day 2	11.47 (7.32)	7.33 (5.91)	11.73 (9.46)	10.67 (12.09)	10.30 (8.95)
Normal Grooming Pre-Stress Day 3	12.87 (8.82)	10.27 (8.41)	10.73 (10.85)	11.07 (14.54)	11.23 (10.69)
Normal Grooming Pre-Stress Day 4	10.93 (7.95)	9.60 (10.3)	12.13 (7.56)	11.20 (11.33)	10.97 (9.22)
Normal Grooming Pre-Stress Day 5	12.93 (5.84)	12.60 (9.16)	14.60 (6.39)	12.27 (9.51)	13.10 (7.75)
Normal Grooming Post-Stress Day 1	11.13 (7.82)	11.07 (7.1)	11.33 (7.24)	10.33 (10.91)	10.97 (8.20)
Normal Grooming Post-Stress Day 2	16.80 (4.31)	7.80 (6.84)	12.67 (9.75)	10.13 (7.07)	11.85 (7.83)

Normal Grooming Post-Stress Day 3	14.00 (6.94)	16.80 (7.87)	11.27 (9.08)	9.47 (7.93)	12.88 (8.27)
Normal Grooming Post-Stress Day 4	14.53 (6.97)	14.67 (12.29)	15.33 (9.17)	13.93 (9.22)	14.62 (9.37)
Normal Grooming Post-Stress Day 5	11.33 (7.93)	12.60 (8.83)	14.60 (9.87)	9.67 (5.94)	12.05 (8.26)
Stereotypic Grooming Pre-Stress Day 1	27.27 (24.80)	11.80 (21.55)	20.53 (33.80)	16.33 (22.71)	18.98 (26.13)
Stereotypic Grooming Pre-Stress Day 2	18.40 (27.92)	13.13 (24.41)	32.67 (49.35)	20.60 (24.89)	21.20 (33.22)
Stereotypic Grooming Pre-Stress Day 3	24.93 (31.50)	19.80 (40.63)	32.27 (56.82)	9.73 (21.19)	21.68 (39.60)
Stereotypic Grooming Pre-Stress Day 4	18.33 (17.36)	11.67 (17.35)	29.47 (46.99)	10.27 (18.19)	17.43 (28.35)
Stereotypic Grooming Pre-Stress Day 5	31.27 (31.61)	11.20 (18.61)	19.13 (29.97)	11.40 (21.18)	18.25 (26.59)
Stereotypic Grooming Post-Stress Day 1	21.13 (18.84)	19.00 (33.31)	25.47 (57.13)	9.40 (18.29)	18.75 (35.16)
Stereotypic Grooming Post-Stress Day 2	13.53 (18.66)	8.67 (18.45)	24.40 (44.93)	8.13 (13.38)	13.68 (26.98)
Stereotypic Grooming Post-Stress Day 3	24.00 (23.86)	16.00 (32.58)	18.60 (40.50)	5.00 (13.21)	15.90 (29.43)
Stereotypic Grooming Post-Stress Day 4	29.20 (48.78)	16.33 (24.09)	25.53 (47.79)	10.47 (14.35)	20.38 (36.72)
Stereotypic Grooming Post-Stress Day 5	31.27 (39.61)	15.87 (23.70)	31.13 (57.72)	9.53 (15.78)	21.95 (38.04)

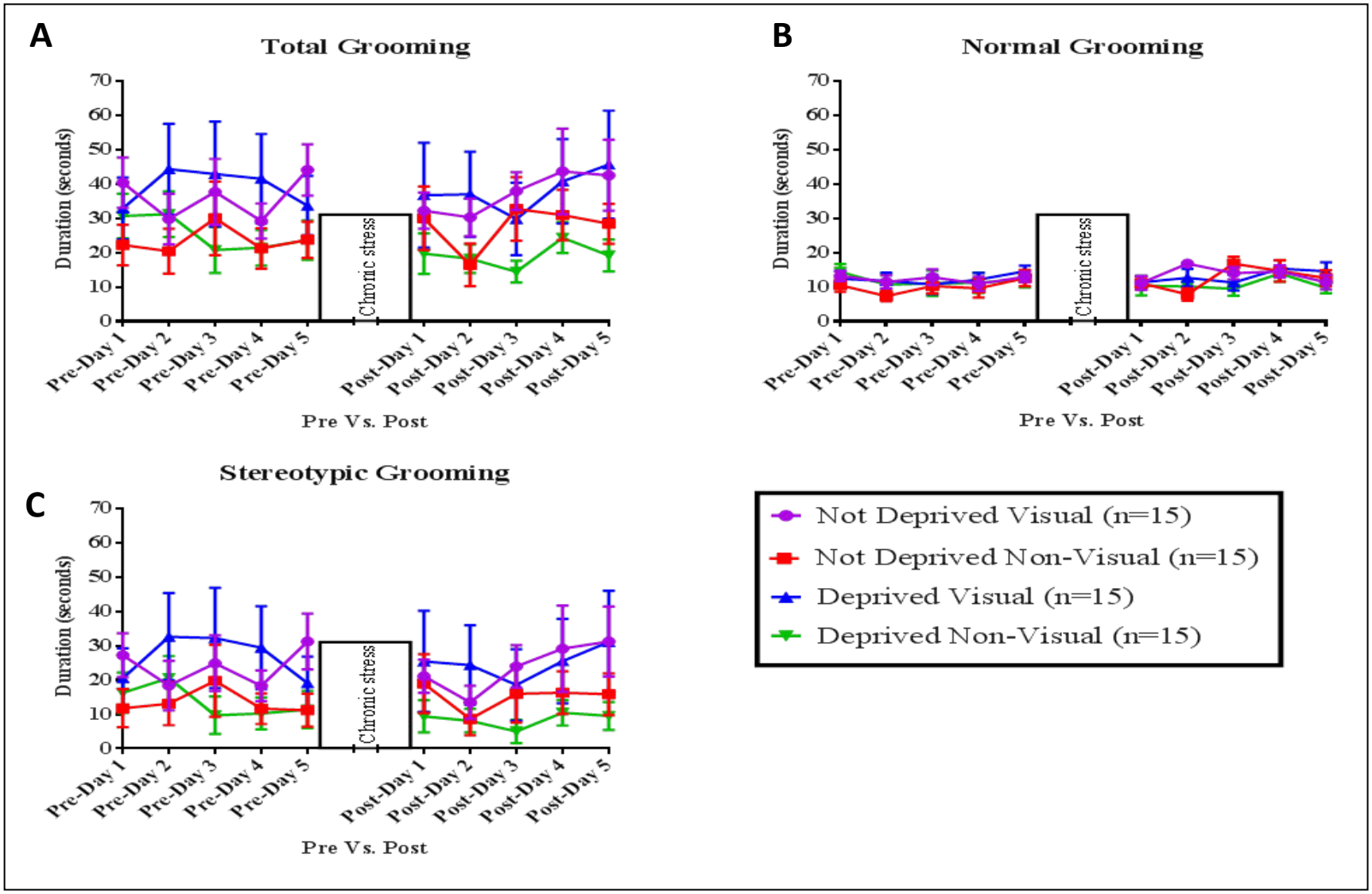

Figure 3.6: Duration of behavioural parameters measured during BOOF. (A) Total Grooming incidents (TG); (B) Normal Grooming incidents (NG); (C) Stereotypic Grooming incidents (SG) by experimental cohort on each day of BOOF before and after stress. n = 15. Data are mean ± SEM.

3.2.2.2.3 Immobility Behaviours in the BOOF Test

The duration of immobility behaviours (total, in the corner, in any square) were assessed separately using ANOVA to investigate the impact of CMS exposure following MD and isolation. Table 3.14 presents the mean and standard deviation for the grooming behaviours in the BOOF test before and after CMS. A Bonferroni correction was applied to the p-values of the immobility behaviours to minimise type 1 errors, resulting in a corrected p-value threshold of $p < 0.017$.

Prior to CMS exposure, there was a significant increase over time in total ($F = 10.14$; $p < 0.017$) and corner immobility duration ($F = 10.23$; $p < 0.017$) for all experimental groups during BOOF (Figure 3.7-A and –B). There were no significant differences over time in the duration of square immobility, prior to ($F = 2.48$; $p > 0.017$) or after ($F = 0.89$; $p > 0.017$) CMS exposure (Appendix C, Table 13). After stress exposure, there was also a significant decrease over time for the total ($F = 8.23$; $p < 0.017$) and corner immobility ($F = 8.42$; $p < 0.017$) duration, for all experimental groups.

There were no significant differences between the experimental cohorts before stress for the durations of total immobility ($F = 0.39$; $p > 0.017$), corner immobility ($F = 0.46$; $p > 0.017$), or square immobility ($F = 3.01$; $p > 0.017$). There were also no significant differences between the experimental cohorts for durations of total immobility ($F = 0.16$; $p > 0.017$), corner immobility ($F = 0.07$; $p > 0.017$), or square immobility ($F = 3.28$; $p > 0.017$) after CMS exposure (Appendix C, Table 13).

Finally, there were no significant interactions between time and experimental cohort prior to stress exposure for total immobility duration ($F = 1.05$; $p > 0.017$), corner immobility duration ($F = 1.43$; $p > 0.017$), or square immobility duration ($F = 0.81$; $p > 0.017$). Nor were there significant interactions between time and experimental cohort after stress exposure for total immobility ($F = 1.56$; $p > 0.017$), corner immobility ($F = 1.48$; $p > 0.017$), or square immobility ($F = 1.13$; $p > 0.017$) durations (Appendix C, Table 13).

There was a noted significant change over time for immobility duration. However, Tukey's *post-hoc* analysis indicated that there was no significant difference between the experimental groups before or after CMS exposure for total or corner immobility (Appendix C, Table 14). There was however, a significant difference between experimental groups for time spent immobile in any arena square, with *"deprived visual"* cohort rats spending a significantly longer time in square immobility when compared to the *"not deprived non-visual"* cohort, both before ($p < 0.05$) and after ($p < 0.05$) CMS exposure.

Collectively, these results suggest that all the experimental groups spent a similar amount of time immobile and demonstrated similar habituation profiles. However, the "*deprived visual*" cohort was more prone to immobility anywhere in the BOOF arena, as opposed to one of the corners due to a significant difference in square immobility time but no significant difference in corner immobility duration. Hence, the "*deprived visual*" cohort spent a greater time away from the corners of the BOOF arena during the test.

Table 3.14: Means (SD) for the duration of immobility behaviours (total, corner, and square immobility) before and after CMS.

Behaviour	Not Deprived Visual (*n* = 15) M (SD)	Not Deprived Non-Visual (*n* = 15) M (SD)	Deprived Visual (*n* = 15) M (SD)	Deprived Non-Visual (*n* = 15) M (SD)	Total (*n* = 60) M (SD)
Total Immobility Pre-Stress Day 1	56.93 (65.88)	73.53 (99.81)	88.67 (70.06)	51.67 (32.85)	67.70 (70.92)
Total Grooming Pre-Stress Day 2	143.53 (94.62)	183.20 (164.08)	161.80 (103.43)	120.33 (97.25)	152.22 (117.65)
Total Immobility Pre-Stress Day 3	148.00 (93.97)	152.87 (141.83)	121.20 (99.41)	155.00 (148.64)	144.27 (121.01)
Total Immobility Pre-Stress Day 4	168.13 (128.25)	202.13 (141.62)	156.80 (119.33)	115.67 (114.84)	160.68 (127.05)
Total Immobility Pre-Stress Day 5	122.67 (97.58)	142.27 (146.29)	159.00 (87.79)	143.33 (129.46)	141.82 (115.38)
Total Immobility Post-Stress Day 1	139.60 (104.45)	151.53 (151.76)	169.60 (97.98)	167.13 (137.25)	156.97 (122.28)
Total Immobility Post-Stress Day 2	134.00 (103.80)	118.13 (83.54)	110.67 (88.97)	109.47 (104.32)	118.07 (93.65)
Total Immobility Post-Stress Day 3	114.67 (101.33)	67.80 (44.11)	143.80 (100.84)	126.60 (77.41)	113.22 (86.84)
Total Immobility Post-Stress Day 4	79.80 (58.85)	89.80 (101.82)	120.67 (83.37)	77.53 (49.58)	91.95 (76.26)
Total Immobility Post-Stress Day 5	135.20 (107.17)	140.53 (104.38)	103.87 (58.63)	96.40 (85.11)	119.00 (90.65)
Corner Immobility Pre-Stress Day 1	54.20 (66.07)	67.93 (97.42)	81.33 (70.21)	47.40 (30.68)	62.72 (69.67)
Corner Immobility Pre-Stress Day 2	140.67 (97.18)	181.27 (165.67)	156.33 (104.03)	117.27 (96.32)	148.88 (118.64)
Corner Immobility Pre-Stress Day 3	147.07 (95.10)	150.47 (143.43)	115.73 (99.99)	152.07 (150.59)	141.33 (122.5)
Corner Immobility Pre-Stress Day 4	167.07 (129.25)	196.67 (145.68)	146.53 (118.85)	87.87 (79.76)	149.53 (124.39)
Corner Immobility Pre-Stress Day 5	119.87 (98.76)	138.47 (148.01)	147.47 (89.10)	142.00 (130.57)	136.95 (116.41)
Corner Immobility Post-Stress Day 1	135.13 (106.90)	148.93 (153.62)	160.93 (96.71)	162.93 (139.43)	151.98 (123.57)
Corner Immobility Post-Stress Day 2	130.40 (106.08)	109.33 (84.77)	95.93 (93.27)	103.60 (106.05)	109.82 (96.31)

Corner Immobility Post-Stress Day 3	114.00 (101.32)	61.93 (43.05)	136.60 (96.16)	118.53 (78.56)	107.77 (85.55)
Corner Immobility Post-Stress Day 4	76.13 (60.62)	84.13 (103.4)	94.60 (76.65)	71.60 (45.91)	81.62 (73.36)
Corner Immobility Post-Stress Day 5	133.67 (108.41)	134.2 (96.42)	87.67 (63.28)	89.67 (86.24)	111.30 (90.72)
Square Immobility Pre-Stress Day 1	2.73 (4.46)	5.60 (8.41)	7.33 (9.51)	4.27 (4.85)	4.98 (7.17)
Square Immobility Pre-Stress Day 2	2.87 (7.47)	1.93 (4.76)	5.47 (5.77)	3.07 (5.39)	3.33 (5.93)
Square Immobility Pre-Stress Day 3	0.93 (3.61)	2.40 (4.24)	5.47 (8.31)	2.93 (4.18)	2.93 (5.54)
Square Immobility Pre-Stress Day 4	1.07 (3.10)	5.47 (13.89)	10.27 (17.62)	4.53 (9.43)	5.33 (12.40)
Square Immobility Pre-Stress Day 5	2.80 (4.60)	3.80 (9.28)	11.53 (18.02)	1.33 (2.06)	4.87 (10.92)
Square Immobility Post-Stress Day 1	4.47 (9.82)	2.60 (6.61)	8.67 (12.48)	4.20 (5.12)	4.98 (9.03)
Square Immobility Post-Stress Day 2	3.60 (4.12)	8.80 (14.80)	14.73 (22.16)	5.87 (6.44)	8.25 (14.14)
Square Immobility Post-Stress Day 3	0.67 (1.40)	5.87 (9.15)	7.20 (8.68)	8.07 (11.05)	5.45 (8.69)
Square Immobility Post-Stress Day 4	3.67 (6.06)	5.67 (6.84)	26.07 (48.00)	5.93 (8.12)	10.33 (25.83)
Square Immobility Post-Stress Day 5	1.53 (2.95)	6.33 (18.39)	16.20 (27.13)	6.73 (7.10)	7.70 (17.25)

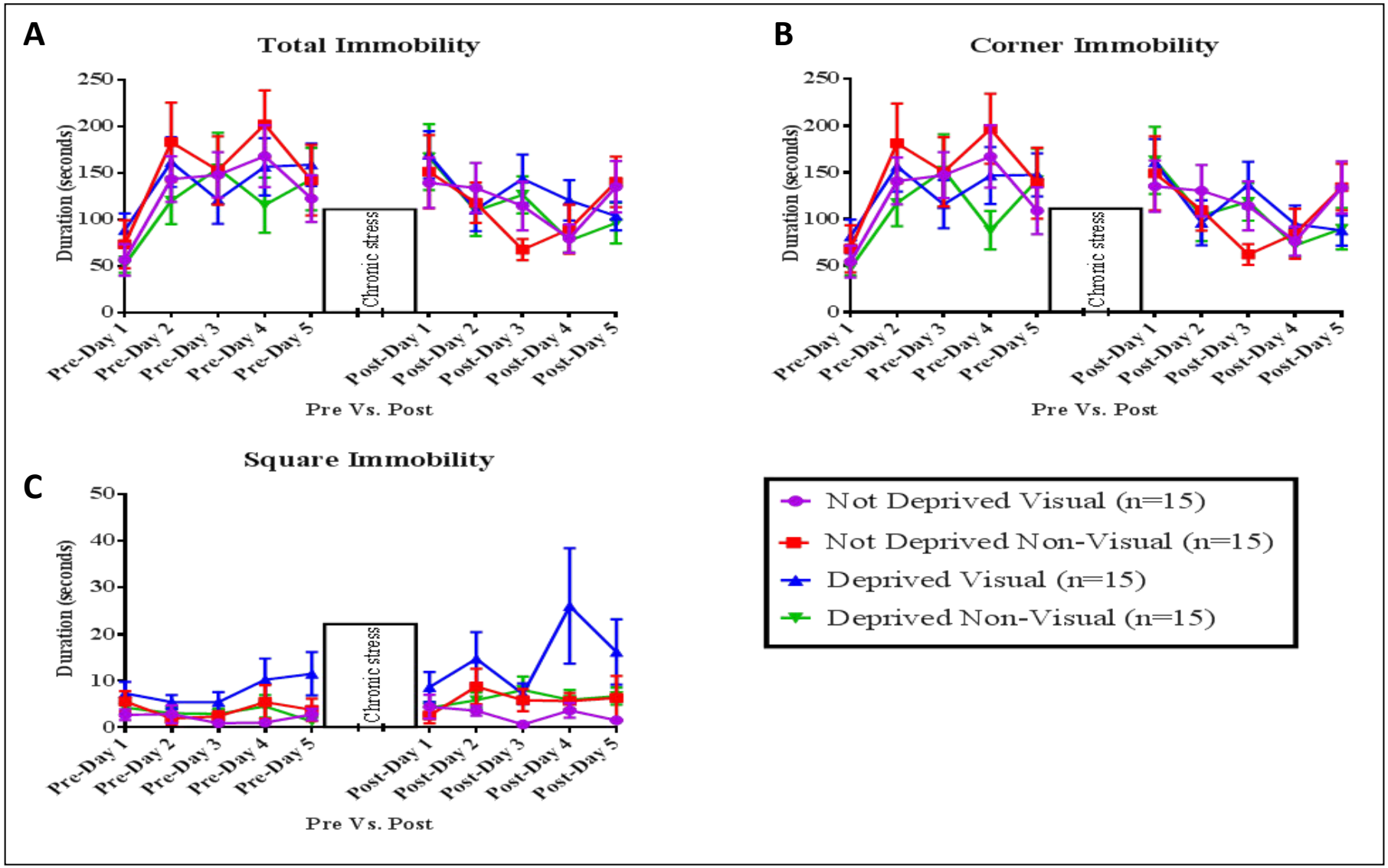

Figure 3.7: Duration of behavioural parameters measured during BOOF. (A) Total Immobility incidents (TI); (B) Corner Immobility incidents (CI); (C) Square Immobility incidents (SI) by experimental cohort on each day of BOOF before and after stress. n = 15. Data are mean ± SEM.

3.2.2.2.4 Object Investigation Behaviours in the BOOF Test

To investigate the impact of CMS exposure following MD and isolation, the exploratory behaviours involving object investigation were examined separately using repeated measure ANOVA. This was done to determine if there was any habituation, as measured by reduced time spent investigating an object, in the investigation of any objects. Table 3.15 presents the mean and standard deviation for the grooming behaviours in the BOOF test before and after CMS. A Bonferroni correction was applied to the p-values of the object investigation behaviours to minimise type 1 errors, resulting in a corrected p-value threshold of $p < 0.01$.

There were significant decreases over time in the duration of total object ($F = 11.74$; $p < 0.01$), object 1 exploration ($F = 9.76$; $p < 0.01$), object 2 ($F = 7.06$; $p < 0.01$), object 3 ($F = 3.92$; $p < 0.01$), and object 4 ($F = 3.84$; $p < 0.01$) exploration for all experimental groups during the BOOF test prior to CMS exposure (Figure 3.8). There was also a significant decrease over time in the duration of object exploration over the five days of the BOOF test, after CMS exposure for object 2 ($F = 3.49$; $p < 0.01$), and object 3 ($F = 3.36$; $p < 0.01$) investigation duration. This indicated that the rats were habituating normally to these objects before and after stress. However, there was no significant change in the duration of total object ($F = 3.39$; $p > 0.01$), object 1 ($F = 0.73$; $p > 0.01$), or object 4 ($F = 2.13$; $p > 0.01$) investigations over time after CMS, indicating a lack of normal habituation to the familiar environment and objects (Appendix C, Table 15). Additionally, there were no significant differences between the experimental groups for any object investigation duration before CMS exposure.

Before stress exposure there were no significant differences between the experimental cohorts for duration of total object ($F = 1.04$; $p > 0.01$), object 1 ($F = 1.45$; $p > 0.01$), object 2 ($F = 0.79$; $p > 0.01$), object 3 ($F = 1.52$; $p > 0.01$), or object 4 ($F = 0.48$; $p > 0.01$) investigations. There were also no significant differences between the experimental cohorts after CMS exposure for the duration of total object ($F = 3.46$; $p > 0.01$), object 2 ($F = 0.90$; $p > 0.01$), object 3 ($F = 3.49$; $p > 0.01$), or object 4 ($F = 0.57$; $p > 0.01$) investigation behaviours. However, there was a

significant difference ($F = 3.87$; $p < 0.01$) in object 1 investigation (Figure 3.8-B) between the experimental cohorts after CMS exposure (Appendix C, Table 15).

Finally, there were no significant interactions between time and experimental cohort before stress for time spent on total object ($F = 1.35$; $p > 0.01$), object 1 ($F = 0.91$; $p > 0.01$), object 2 ($F = 0.98$; $p > 0.01$), object 3 ($F = 1.59$; $p > 0.01$), or object 4 ($F = 1.18$; $p > 0.01$) investigation behaviours. After CMS exposure there were also no significant interactions between time and experimental cohort for the duration of total object ($F = 1.25$; $p > 0.01$), object 1 ($F = 0.95$; $p > 0.01$), object 3 ($F = 1.29$; $p > 0.01$), or object 4 ($F = 1.33$; $p > 0.01$) investigation behaviours. However, there was a significant interaction between time and experimental cohort for the duration of object 2 ($F = 2.47$; $p < 0.01$) investigation (Appendix C, Table 15).

However, Tukey's *post-hoc* analysis revealed that rats from the *"deprived visual"* ($p < 0.05$) and *"deprived non-visual"* ($p < 0.05$) cohorts spent significantly more time investigating object 1 when compared to the *"control not-deprived visual"* cohort (Appendix C, Table 16). A similar significant difference ($p < 0.05$) between the groups was noted in object 3 investigation (Figure 3.8-D), with the control *"not-deprived visual"* cohort spending significantly less time investigating object 3 than rats in the *"deprived non-visual"* ($p < 0.05$) cohort (Appendix C, Table 16). No differences were found between the experimental cohorts for the duration of object 2 or object 4 investigations.

Collectively, these data indicated that there was a significant impact of MD on the duration of object investigation but no impact of isolation status. Furthermore, the increased duration of investigation for objects 1 and 3 suggested a lack of habituation following CMS exposure. This is like what was observed in the behavioural frequency.

Table 3.15: Means (SD) for the duration of object investigation behaviours (total, object 1, object 2, object 3, and object 4 investigation) before and after CMS.

Behaviour	Not Deprived Visual (*n* = 15) M (SD)	Not Deprived Non-Visual (*n* = 15) M (SD)	Deprived Visual (*n* = 15) M (SD)	Deprived Non-Visual (*n* = 15) M (SD)	Total (*n* = 60) M (SD)
Total Object Investigation Pre-Stress Day 1	48.67 (31.81)	47.53 (22.36)	54.07 (20.03)	54.87 (17.52)	51.28 (23.18)
Total Object Investigation Pre-Stress Day 2	28.87 (23.03)	34.60 (28.14)	32.20 (24.02)	34.40 (28.89)	32.52 (25.58)
Total Object Investigation Pre-Stress Day 3	21.40 (19.06)	39.27 (38.32)	42.67 (25.05)	30.47 (25.14)	33.45 (28.33)
Total Object Investigation Pre-Stress Day 4	16.13 (20.85)	24.87 (33.00)	33.53 (25.70)	41.00 (28.40)	28.88 (28.25)
Total Object Investigation Pre-Stress Day 5	24.13 (25.76)	35.87 (32.72)	30.47 (17.83)	36.40 (34.59)	31.72 (28.21)
Total Object Investigation Post-Stress Day 1	27.13 (23.72)	43.13 (32.71)	44.80 (29.68)	45.13 (37.79)	40.05 (31.50)
Total Object Investigation Post-Stress Day 2	32.40 (20.51)	47.20 (31.39)	59.00 (31.91)	58.27 (27.67)	49.22 (29.58)
Total Object Investigation Post-Stress Day 3	30.93 (26.24)	43.13 (25.98)	47.07 (27.38)	37.07 (25.99)	39.55 (26.45)
Total Object Investigation Post-Stress Day 4	25.53 (18.93)	43.40 (27.98)	51.87 (31.84)	61.07 (36.31)	45.47 (31.60)
Total Object Investigation Post-Stress Day 5	24.27 (20.01)	35.67 (35.34)	58.67 (42.44)	55.33 (30.01)	43.48 (35.16)
Object 1 Investigation Pre-Stress Day 1	19.87 (14.55)	19.87 (11.94)	25.60 (13.35)	30.93 (17.06)	24.07 (14.73)
Object 1 Investigation Pre-Stress Day 2	12.67 (10.57)	16.13 (13.44)	15.47 (13.51)	17.13 (20.81)	15.35 (14.77)
Object 1 Investigation Pre-Stress Day 3	7.47 (8.70)	18.13 (21.07)	16.13 (10.03)	15.00 (17.21)	14.18 (15.30)
Object 1 Investigation Pre-Stress Day 4	6.67 (7.69)	10.67 (16.27)	15.73 (15.66)	16.80 (16.46)	12.47 (14.71)
Object 1 Investigation Pre-Stress Day 5	9.27 (13.66)	16.00 (15.96)	13.27 (9.15)	17.40 (17.05)	13.98 (14.26)
Object 1 Investigation Post-Stress Day 1	10.40 (11.93)	18.93 (16.38)	22.27 (15.95)	28.93 (25.76)	20.13 (18.99)
Object 1 Investigation Post-Stress Day 2	10.60 (9.22)	20.47 (15.30)	32.07 (19.50)	28.87 (17.85)	23.00 (17.66)

Object 1 Investigation Post-Stress Day 3	13.80 (14.71)	21.93 (16.29)	26.53 (19.51)	19.67 (18.28)	20.48 (17.47)
Object 1 Investigation Post-Stress Day 4	13.00 (11.15)	19.60 (15.43)	27.47 (18.05)	29.47 (20.48)	22.38 (17.51)
Object 1 Investigation Post-Stress Day 5	12.53 (11.91)	17.80 (23.11)	28.93 (25.15)	24.67 (17.19)	20.98 (20.51)
Object 2 Investigation Pre-Stress Day 1	6.07 (3.49)	7.73 (9.69)	4.33 (2.32)	5.73 (2.87)	5.97 (5.47)
Object 2 Investigation Pre-Stress Day 2	3.40 (4.05)	3.93 (4.08)	2.80 (2.93)	4.00 (2.78)	3.53 (3.46)
Object 2 Investigation Pre-Stress Day 3	4.87 (5.28)	9.73 (14.84)	6.73 (10.35)	4.53 (5.77)	6.47 (9.83)
Object 2 Investigation Pre-Stress Day 4	1.87 (2.00)	2.47 (3.87)	3.20 (3.19)	3.87 (2.26)	2.85 (2.95)
Object 2 Investigation Pre-Stress Day 5	2.07 (3.51)	2.60 (2.44)	3.67 (2.82)	3.13 (2.45)	2.87 (2.83)
Object 2 Investigation Post-Stress Day 1	2.27 (2.52)	6.27 (6.46)	2.87 (2.90)	1.93 (1.75)	3.33 (4.14)
Object 2 Investigation Post-Stress Day 2	4.60 (4.63)	5.33 (6.21)	5.40 (2.26)	6.27 (4.88)	5.40 (4.63)
Object 2 Investigation Post-Stress Day 3	4.40 (8.60)	5.73 (4.37)	3.53 (2.80)	2.67 (2.26)	4.08 (5.14)
Object 2 Investigation Post-Stress Day 4	2.73 (2.02)	2.93 (2.71)	5.80 (4.83)	5.73 (4.27)	4.30 (3.84)
Object 2 Investigation Post-Stress Day 5	2.33 (2.29)	3.67 (4.27)	5.60 (5.29)	6.53 (5.15)	4.53 (4.61)
Object 3 Investigation Pre-Stress Day 1	14.33 (16.19)	13.07 (6.71)	16.53 (6.84)	12.33 (6.15)	14.07 (9.78)
Object 3 Investigation Pre-Stress Day 2	7.27 (6.87)	9.67 (9.71)	9.00 (6.86)	11.00 (9.24)	9.23 (8.17)
Object 3 Investigation Pre-Stress Day 3	4.93 (5.09)	5.13 (4.34)	14.00 (13.46)	6.80 (7.59)	7.72 (9.01)
Object 3 Investigation Pre-Stress Day 4	5.27 (10.24)	6.47 (7.29)	9.47 (7.86)	14.53 (9.22)	8.93 (9.24)
Object 3 Investigation Pre-Stress Day 5	9.33 (10.57)	11.33 (13.62)	10.27 (8.79)	11.60 (13.67)	10.63 (11.58)
Object 3 Investigation Post-Stress Day 1	9.47 (12.22)	11.27 (10.17)	14.20 (11.83)	12.27 (13.40)	11.80 (11.78)
Object 3 Investigation Post-Stress Day 2	10.80 (10.11)	13.87 (9.10)	15.80 (9.14)	16.00 (9.74)	14.12 (9.52)
Object 3 Investigation Post-Stress Day 3	7.07 (6.71)	8.33 (6.14)	11.60 (7.48)	10.40 (5.82)	9.35 (6.64)
Object 3 Investigation Post-Stress Day 4	6.67 (9.41)	15.33 (19.52)	12.20 (9.03)	21.27 (16.52)	13.87 (14.96)

Object 3 Investigation Post-Stress Day 5	5.20 (5.20)	10.33 (10.47)	17.13 (11.76)	17.80 (11.33)	12.62 (11.09)
Object 4 Investigation Pre-Stress Day 1	8.40 (8.58)	6.87 (3.98)	7.73 (5.09)	5.87 (3.54)	7.22 (5.59)
Object 4 Investigation Pre-Stress Day 2	5.53 (6.16)	4.87 (4.34)	4.93 (4.46)	2.27 (2.22)	4.40 (4.58)
Object 4 Investigation Pre-Stress Day 3	4.13 (6.51)	6.27 (7.84)	5.80 (4.36)	4.13 (4.37)	5.08 (5.89)
Object 4 Investigation Pre-Stress Day 4	2.33 (4.06)	5.27 (8.06)	5.13 (4.07)	5.47 (5.33)	4.55 (5.63)
Object 4 Investigation Pre-Stress Day 5	3.47 (3.02)	5.93 (6.10)	3.27 (2.66)	4.27 (3.65)	4.23 (4.12)
Object 4 Investigation Post-Stress Day 1	5.00 (3.14)	6.67 (6.60)	5.47 (4.66)	2.00 (2.10)	4.78 (4.68)
Object 4 Investigation Post-Stress Day 2	6.40 (5.45)	7.53 (5.54)	5.73 (5.82)	7.00 (5.10)	6.67 (5.39)
Object 4 Investigation Post-Stress Day 3	5.67 (7.15)	7.13 (7.22)	5.40 (2.61)	4.33 (3.94)	5.63 (5.55)
Object 4 Investigation Post-Stress Day 4	3.60 (3.18)	5.53 (4.66)	6.40 (5.58)	4.60 (3.56)	5.03 (4.36)
Object 4 Investigation Post-Stress Day 5	4.27 (3.81)	3.87 (4.02)	7.00 (6.52)	6.33 (6.70)	5.37 (5.46)

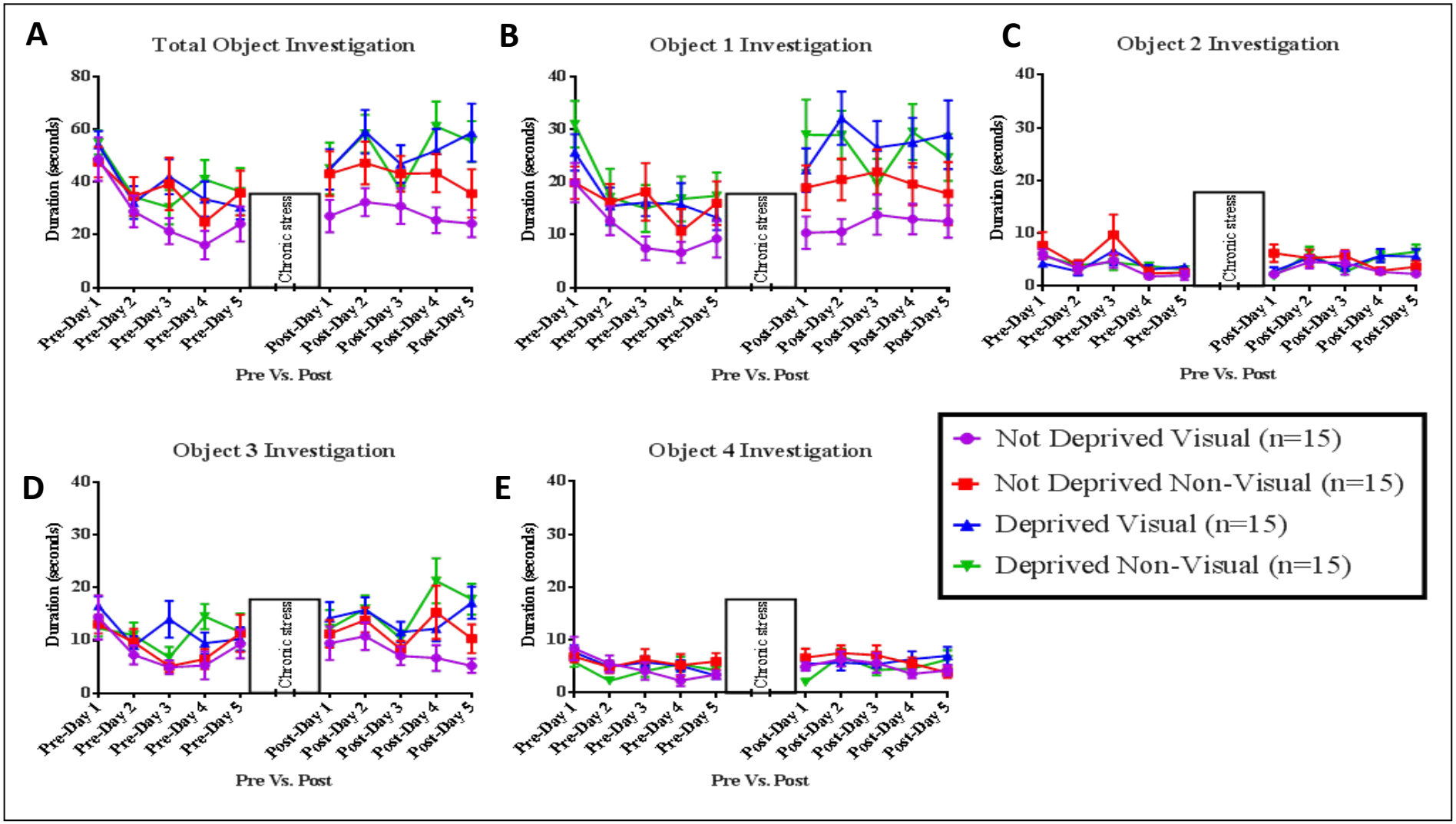

Figure 3.8: Duration of Object investigation behaviours measured during BOOF. (A) Total Object Investigation (TOI); (B) Object 1 Investigation O1; (C) Object 2 Investigation (O2); (D) Object 3 Investigation (O3); (E) Object 4 Investigation (O4) by experimental cohort on each day of BOOF before and after stress. n = 15. Data are mean ± SEM.

3.3 The Behaviours Changed due to Stress Exposure in Stress-Resistant Rats

Following this factorial study, the frequency and duration of the scored rat behaviours (locomotion, immobility, grooming, and object exploration) were significantly different after later life chronic mild stress (CMS), based on the combinations of maternal deprivation (MD) and isolation exposures. The behavioural alterations suggest that anxiety-like behaviours were triggered due to the observed change in behaviour following stress. This finding is a plausible outcome for the study given the high comorbidity between anxiety and depression in humans, and the high overlap of depressive- and anxiety-like behaviours triggered by stress exposure described in the literature for rat studies (Garcia, Acosta & Osman, 2016; Cummings *et al.*, 2014; Tiller, 2012; Berton & Nestler, 2006).

A significant difference was found between the rats that were only subjected to CMS and rats subjected to MD, isolation, and CMS for both frequency and duration of several behaviours. This indicated an effect of MD and degree of social support on behaviour following CMS. The impact of both early life stress and available social support on overall mental state and stress management during current life stress in humans is well known (Kendler & Aggen, 2017; Taporoski *et al.*, 2015; APA, 2013). Individuals who experience early life stress or trauma have an increased likelihood of developing depressive or anxiety disorders in response to recent life stresses (Syed & Nemeroff, 2017; Shapero *et al.*, 2014; Tofoli *et al.*, 2011). Individuals with limited access to a social support network were also more likely to experience more severe depressive or anxiety symptomology, less effective treatment, and lower remission rates when compared to individuals with a strong social support network (Ghorbani Saeedian *et al.*, 2014; Roohafza *et al.*, 2014; Fiske, Wetherell & Gatz, 2009).

Some studies of the impact of early life stress, social support, and recent life stress in rats, has been undertaken. Early life stress, specifically maternal deprivation (MD), has been demonstrated to trigger depressive-like behaviours (Reus *et al.*, 2011; El Khoury, Gruber, Mork & Mathe, 2006). There is also evidence of anxious-like behaviour due to MD exposure (Menard *et*

al., 2016; Marais *et al.*, 2008). A study by Trujillo *et al.* (2016) found, using a combined MD and chronic mild stress (CMS) protocol, that the anxious-like behaviour of the rats was reversible using treatment with the antidepressant tianeptine. Similarly, CMS triggers depressive-like behaviours and is known to also cause ambiguous anxious behaviour as well (Rana *et al.*, 2016; Trujillo *et al.*, 2016; Neumann *et al.*, 2011). A study by Kompagne *et al.* (2008) found CMS generated clear depressive-like behaviour but ambiguous anxiety-like behaviour in Wistar rats. Another study by Chiba *et al.* (2012) found that following chronic restraint stress, adult male Wistar rats demonstrated increased immobility in the forced swim test (FST) (depression-like behaviour) and a decrease in open arm entries in the elevated plus maze (EPM) (anxiety-like behaviour). Additionally, social isolation often triggers anxious-like and depressive-like behaviours in rats, due to their social nature (Evans *et al.*, 2012). However, there has been a limited examination of the interaction of these factors in rodent models (Rana *et al.*, 2016; Trujillo *et al.*, 2016; Zalosnik, Pollano, Trujillo, Suárez & Durando, 2014). The current study aimed to examine the interaction of these factors on behavioural outcome measures in a rodent model using a stress-resistant rat strain to represent a general population.

The data presented in this chapter showed behavioural alterations determined more consistent with anxious behaviour rather than depressive-like behaviour. In rodent models, depressive-like behaviours are usually characterised by general hypo-activity and anhedonia in behavioural screening and assessment tests, particularly decreased mobility in the FST, decreased grooming, and a decrease in preference for sweet solutions (Krishnan & Nestler, 2011). Anxiety, on the other hand, is characterised by hyperactivity and increased stereotypic behaviours such as grooming (Burn, 2008; Ramos, Berton, Mormede & Chaouloff, 1997). The expected result of the current study was increased immobility behaviours and a significant lack of exploratory behaviours during behavioural observation and assessment. However, the opposite of these behaviours (i.e. decreased immobility and increased exploratory behaviours that are characterised as anxious behaviours), were observable at the end of the behavioural manipulation.

The rats demonstrated the expected habituation to the behavioural arena and the novel objects prior to stress. The reduction in frequency and duration of ambulatory movement and object investigation behaviours indicated the expected habituation. Additional support for normal environmental habituation prior to CMS exposure was the increased immobility, in both frequency and duration. Furthermore, the lack of grooming prior to CMS solidifies the general behaviour seen before stress as normal habituation expected from rats. Behaviours of all the subjects primarily occurred in the peripheral quadrants of the arena. This thigmotaxis underlies many tests of anxiety since rats use vertical surfaces such as walls as a method of orientation due to their poor visual ability (Burn, 2008). There were no significant differences between experimental groups for any of the behaviours examined, prior to CMS exposure.

After stress, there was a lack of habituation for locomotion and object 1 (the glass tunnel) and object 3 (the aquarium figure) investigation behaviours during the BOOF test. Specifically, there was an average of nine object 1 investigations taking an average of 24 seconds on the first day of BOOF testing prior to CMS. The frequency dropped to an average of five object 1 investigation behaviours, which took an average of 14 seconds by day 5 of the BOOF test. In contrast, during the BOOF test after CMS, there was an average of seven object 1 investigations, which took 20 seconds, made by the rodents on the first day of the BOOF test. By the final day of the BOOF test after CMS exposure, the average frequency of object 1 investigation behaviours was still seven incidents and took 21 seconds. Similar patterns were present in the frequency of horizontal motor activity, and the frequency and duration of object 3 investigation behaviours. Together, this lack of habituation in the behaviours involving movement and environmental exploration, indicate hyperactivity. This hyperactivity resulted in a repeated investigation of these two objects, as well as the increase horizontal motor activity needed to explore those objects, implying an anxious behavioural phenotype (Neumann *et al.*, 2011; Zorner *et al.*, 2003). There was also a significant difference between the experimental cohorts for the frequency of horizontal motor activity and both the frequency and duration of objects 1 and 3 investigation behaviours. Specifically, the rats that received MD, isolation, and CMS were observed to have significantly

higher frequency and duration of behavioural measures after CMS than the rats that were only subjected to CMS, though there was no significant difference between these cohorts prior to CMS. The other two experimental cohorts fell within the range of these cohorts, though there was no significant difference in the incidence of these behaviours. This indicated that the combination of MD and isolation increased the hyperactive behaviour observed following CMS exposure.

There was a significant difference in the degree of interest given to the objects by rats across the experimental cohorts after stress. The cohort subjected to MD, isolation, and CMS, demonstrated the highest degree of interest in these objects following CMS exposure. There are three hypothesised reasons that object 1 and object 3 were of such interest following CMS. Firstly, the size of these objects was equivalent to (object 3) or slightly larger (object 1) than the rats. As determined by the significant increases in frequency and duration, these larger objects were of more interest to the rats both before and after CMS exposure, possibly because the size of the object may have represented an unknown threat. Secondly, a cognitive or memory deficit in the rats, brought on by CMS exposure may have resulted in the rats' inability to recall these larger objects (Bhagya, Srikumar, Veena & Shankaranarayana Rao, 2017; Jett, Bulin, Hatherall, McCartney & Morilak, 2017; Bondi, Rodriguez, Gould, Frazer & Morilak, 2008). Additionally, exposure to MD, isolation, and CMS resulted in a higher frequency and duration of object 1 and object 3 investigation behaviours in comparison to the cohort was only subjected to CMS. This suggested that if a memory or cognitive deficit was the reason for the observed behaviours than additional stress exposure to MD or isolation potentially exacerbated the inability to remember objects 1 and 3. However, additional behavioural tests would be required to elucidate if the observed behaviour was due to a memory or cognitive deficit, as well as determine why objects 2 and 4 did not trigger the same type of repeated interaction.

The final hypothesised reason was that the visibility of the objects may have also contributed to the increased attention the rats gave these larger objects. Specifically, the rats' inability to see the objects based on the visual ability of albino rats (see Figure 3.9). For example,

there was a greater interest in object 1 over object 3, which was unexpected since object 1 was consistent across the five days of behavioural observation. The expected behaviour would be a reduction in interest in object 1 due to familiarity, while the other objects would have varied levels of the investigation behaviours since they were rotated. Adaptation to all objects occurred prior to CMS exposure for all experimental cohorts. However, after stress exposure, object 1 had the highest degree of investigation, with object 3 receiving the second highest and minimal interest in the other two objects.

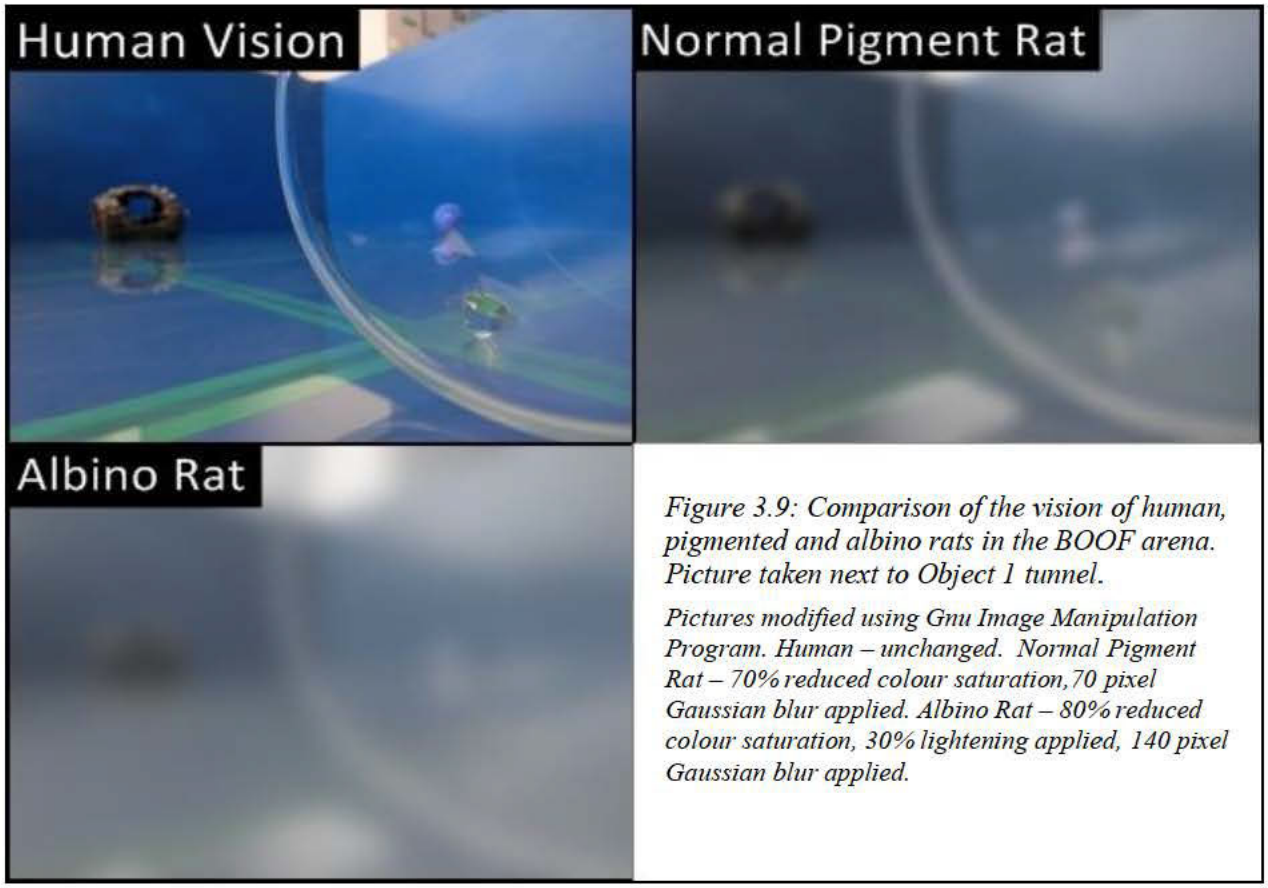

Figure 3.9: Comparison of the vision of human, pigmented and albino rats in the BOOF arena. Picture taken next to Object 1 tunnel.

Pictures modified using Gnu Image Manipulation Program. Human – unchanged. Normal Pigment Rat – 70% reduced colour saturation, 70 pixel Gaussian blur applied. Albino Rat – 80% reduced colour saturation, 30% lightening applied, 140 pixel Gaussian blur applied.

The tunnel, object 1, was made of glass, rendering it almost invisible to a rat with its' poor vision; the other objects are opaque, which would make them more visible to the rat (Ilia & Jeffery, 2000; Curcio, Sloan, Kalina & Hendrickson, 1990; LaVail, 1976). The Wistar rat appears to rely predominantly on its olfactory and auditory senses, in addition to using the vibrissae for close environmental exploration, as opposed to visual senses (Brennan, 2001; Holy, Dulac & Meister, 2000; Heffner & Heffner, 1992; Kelly & Masterton, 1977 [Appendix D]). However, the

objects and BOOF arena were cleaned with 70% ethanol between each animal to remove any olfactory trails, and the two objects of interest, object 1 and 3, made no noise. This limited the usefulness of the rats' primary senses for environmental exploration. Object 1, which was slightly larger than the rodents to allow them to pass through it, was not as visible to the rats compared to the other objects, even when only a short distance away (Figure 3.10).

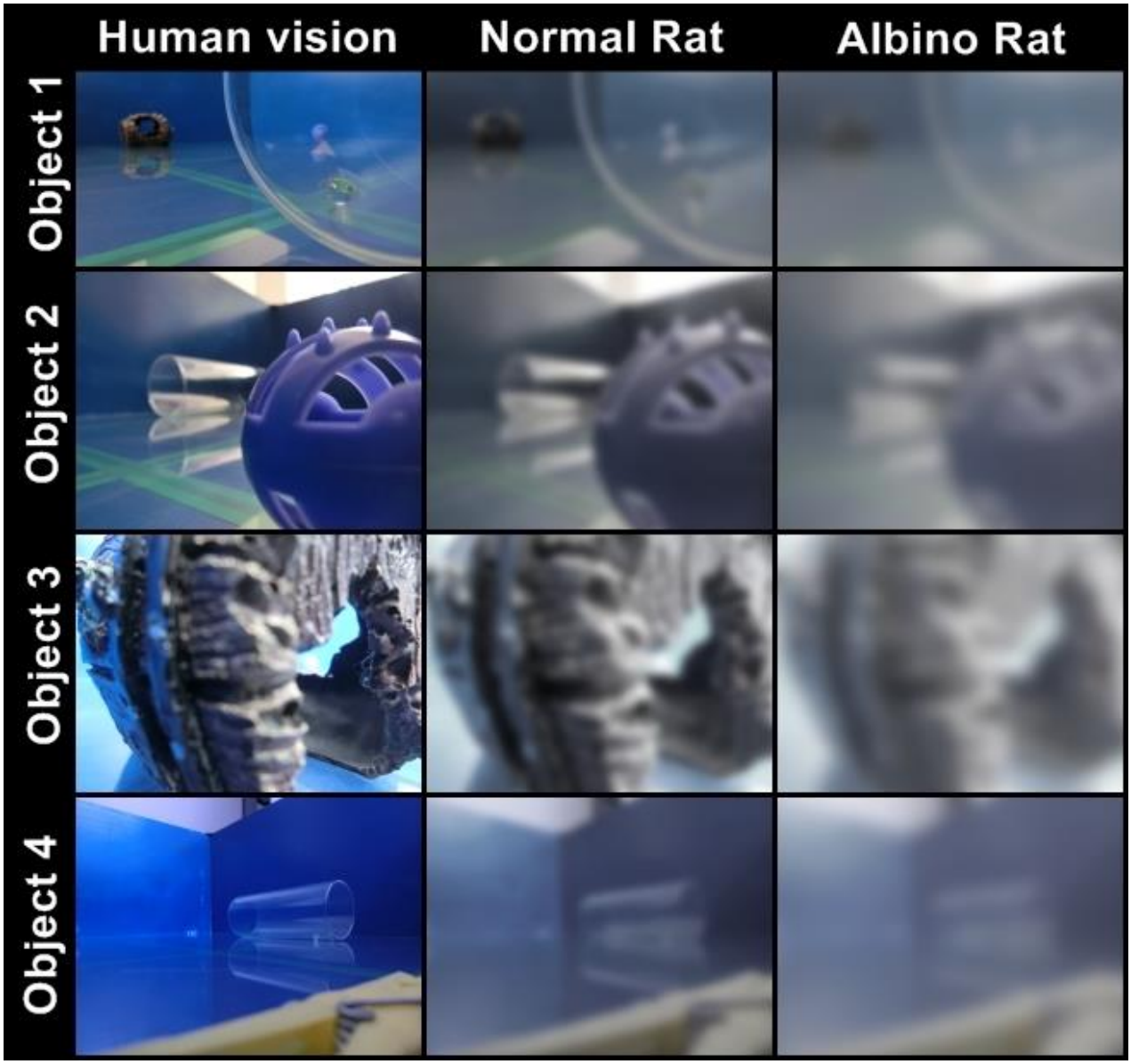

Figure 3.10: Comparison of the vision of human, pigmented and albino rats in the BOOF arena. Pictures were taken 1 cm away from the objects present in the BOOF arena during testing.

Pictures modified using Gnu Image Manipulation Program. Human – unchanged. Normal Pigment Rat – 70% reduced colour saturation, 70-pixel Gaussian blur applied. Albino Rat – 80% reduced colour saturation, 30% lightening applied, 140-pixel Gaussian blur applied.

As a result, following CMS, object 1 was subjected to greater investigation due to its size and the impaired ability to perceive it. The use of environmental instability, bedding removal, as

the stressor in CMS resulted in the rats increased activity in behavioural observation. The rats repeatedly investigated the larger items within the arena, without the reduction expected with a lack of novelty, thereby suggesting that these rats were more anxious about these larger objects. A possible explanation may be that the CMS exposure, therefore, increased the level of anxiety of these Wistar rats.

Additionally, there was an increase over time in the incidence of grooming behaviour for all experimental groups. Grooming can be a normal behaviour, indicative that the rats have fully habituated to the environment (Song, Berridge & Kalueff, 2016; Veloso, Filgueiras, Lorenzo & Estanislau, 2016; Sousa, Almeida & Wotjak, 2006). Rats also use stereotypic behaviours, such as repetitive grooming, as a coping method for stress (Song *et al.*, 2016; Koolhaas *et al.*, 2010; Colorado, Shumake, Conejo, Gonzalez-Pardo & Gonzalez-Lima, 2006; Koolhaas *et al.*, 1999). This increase in grooming, when considered with the increased horizontal motor activity and object investigation, suggested that these rats were under significant stress. However, there is high variability between the experimental subject in grooming behaviours due to the use of such behaviours as a stress coping mechanisms (Franklin *et al.*, 2012; Steimer, 2011). Additionally, such coping mechanisms can vary widely in the execution and intensity depending on the stress exposure (Hofmann, Ellard & Siegle, 2012; Abelson, Khan, Liberzon, Erickson & Young, 2008).

In this behavioural study, there were several limitations, first, the cohort sizes were limited to 15 rats. This is a relatively low number of animals for behavioural examination due to the high variability of stress coping behaviours such as grooming (Boersma & Tamashiro, 2015; Koolhaas *et al.*, 2010; Nosek *et al.*, 2008). However, since this study investigated stress triggers associated with behavioural changes, the number of animals was sufficient given that the total number of subjects was greater than 30 (Tabachnick & Fidell, 2013). Second, the current study used Wistar rats because this strain of rat has been shown to be stress-resistant (Ghadhanfar, Al-Bader & Turcani, 2014; Yang *et al.*, 2012; Roman, Gustafsson, Berg & Nylander, 2006). Thus, if these animals displayed significant behavioural modification in response to the stress imposed upon

them, then this stress is significant since it manifested behavioural changes in subjects resilient to stress. If the same stress was imposed on stress-susceptible rat strains such as Wistar-Kyoto, Flinders Sensitive, and August (Shetty & Sadananda, 2017; Carnevali, Andrews, Neumann, Nalivaiko & Sgoifo, 2016; Fischer *et al.*, 2012; Sudakov, 1999) it is expected that these stress-susceptible strains may manifest statistically significant behavioural changes that only approached significance in the Wistar rats. Thus, the use of Wistar rats facilitated examination of how different environmental stressors can contribute to the development of depressive- and anxiety-like behaviours in a population that is normally less prone to stress. The results also demonstrated the increased vulnerability to recent life stress in subjects that underwent early life MD and had limited access to social support. However, additional research needs to be conducted through a second study that directly compares a stress-susceptible rat breed to the Wistar rats using identical experimental protocols before general transferable conclusions about rat behavioural responses to stress can be reached and objective measures of behaviour established. The final limitation was the use of only one behavioural assessment tool, which limited the conclusions that could be made regarding the observed rodent behaviours. In most behavioural studies, two to three behavioural tests are utilised to quantify and compare any behavioural modifications triggered by environmental manipulation (Ramos, Pereira, Martins, Wehrmeister & Izidio, 2008; Ramos, 2008). However, the focus of the current study was to investigate both behavioural and biological parameters and the association between them, which limited the degree of investigation into either the behavioural or the physiological parameters.

The behavioural manipulations (MD, social isolation, and CMS) were hypothesised to trigger depressive-like behaviour. However, the data suggested the rats possessed an anxious behavioural phenotype. This provides valuable insight into the behavioural changes of rats in response to different MD and isolation exposures, in combinations with CMS. The central aim of this chapter was to determine whether rat behaviour changes in response to early and later life stress. In the current study, rat behaviour did change in response to the combinations of stressors used. The behavioural changes observed were associated with anxiety. These anxious behaviours

(hyperactivity in locomotion and object exploration) reflect the environmental instability experienced by the rats, resulting in circuits of the arena walls and repetitive checking of the objects large enough to be a potential threat. Given that exposure to stress often triggers physiological changes in addition to the behavioural changes observed, the next chapter will explore if the cumulative stress triggered physiological modifications, particularly endocrine and cellular measure, consistent with acute or chronic stress exposures.

Chapter 4 – Physiological Alterations in Response to Early and Later Life Stress in Rats

4.1 The Physiological Response to Stress in Rats

The rats in the current study experienced significant stress that modified the observed behaviour (Chapter 3, Section 3.3). The rats that received maternal deprivation (MD), isolation, and chronic mild stress (CMS) and the rats that only received MD and CMS, with no isolation had behaviour that was significantly different from the rats that only experienced CMS. Specifically, there were significant increases in the frequency and duration of horizontal motor activity, total object investigation, object 1 investigation (glass tunnel), and object 3 investigation (aquarium figurine) in the rats subjected to MD, isolation, and CMS. While rats that received MD, no isolation and CMS had significant increases in object 1 (glass tunnel) investigation and object 3 (aquarium figurine) investigation frequency and object 1 investigation duration when compared to rats that were only subjected to CMS. The results indicated an anxious behavioural phenotype in the rats.

As outlined in Chapter 1 (Section 1.3.2), other rat studies have observed similar anxiety behaviours following stress exposure using individual MD, isolation, or CMS manipulations using the open field test (OFT) (Shetty & Sadananda, 2017; Lambas-Senas *et al.*, 2009) and the elevated plus maze (EPM) (Herrera-Perez, Benitez-Coronel, Jimenez-Rubio, Hernandez-Hernandez & Martinez-Mota, 2017; Shetty & Sadananda, 2017; Trujillo *et al.*, 2016). Specifically, Shetty and Sadananda (2017) found early adolescent isolation in rats increased anxiety behaviours in both the EPM and OFT. An older study by Lambas-Senas *et al.* (2009) observed an increase in anxiety with reduced exploration in the OFT and increased depressive-like behaviours with decreased mobility in the forced swim test (FST) following three hours of MD over 14 days. Herrera-Perez *et al.* (2017) observed that CMS exposure resulted in anxiety behaviours with decreased open arm entries in the EPM. Trujillo *et al.* (2016) noted increased anxiety behaviour in the EPM, following three weeks of daily (4.5 hours) maternal separation, where the litter was maintained together during separation.

The next step was to examine whether stress-associated physiological measures were triggered, in addition to the changed behaviours observed in Chapter 3. Specifically, endocrine and cellular parameters associated with acute and chronic stress exposure were examined. As outlined in Chapter 1 (Section 1.2.4 and Section 1.3.4), the physiological consequences in rats subjected to similar stresses to those in the current study involved changes in the concentration of the glucocorticoid corticosterone, usually an increase in the serum or plasma concentrations (Liu *et al.*, 2014; Liu, W. *et al.*, 2013; Liu, X. *et al.*, 2013; Volodina *et al.*, 2012; Ferraz *et al.*, 2011). However, in humans, there are some cases, where the prolonged elevation of glucocorticoids has led to an association of depressive and anxiety symptoms with reduced plasma concentrations of glucocorticoids (Maripuu *et al.*, 2017; Maripuu *et al.*, 2016). The increased systemic concentration of glucocorticoids often results in decreased expression of glucocorticoid receptors, to maintain normal homeostasis during stress exposure (McVicar & Clancy, 2011; de Kloet *et al.*, 2005). Additionally, an increased level of serum progesterone in rats exposed to chronic stress has also been observed (Andersen, Bignotto, Machado & Tufik, 2004). There are also anatomical modifications that occur in the hippocampus and adrenal glands due to stress exposure (Drevets *et al.*, 2008; Koko, Djordjeviae, Cvijiae & Davidoviae, 2004). Specifically, decreases in hippocampal volume have been observed in rats following chronic stress exposure (Vollmayr, Mahlstedt & Henn, 2007; Malberg & Duman, 2003). The changes in rat adrenal glands involve the distribution of the adrenal cortex, with increases in the volume of the zona fasciculata following stress (Ulrich-Lai *et al.*, 2006).

Thus, the endocrine stress measures examined in the current study included quantification of acute and chronic stress associated hormones; specifically, catecholamines and steroid hormones. As discussed in Chapter 1 (Section 1.2.4.3), the catecholamines (dopamine, noradrenaline, and adrenaline) produced in the adrenal medulla and brain act as central neurotransmitters and as hormones in systemic circulation regulating the physiological response to acute stress (Ranabir & Reetu, 2011; Goldstein, 2003). Acute stress measures were included to determine if the acute stress exposure during euthanasia would confound the cumulative stress the rats were subjected to

during the current study. Steroid hormones (corticosterone, 11-deoxycorticosterone, testosterone, and progesterone) produced in the adrenal cortex and to a lesser degree the gonads (Bellavance & Rivest, 2014; Pardridge & Mietus, 1979) play a role in managing glucose metabolism and circadian rhythms, as well as regulating the hypothalamic-pituitary-adrenal (HPA) axis response to stress (McVicar & Clancy, 2011; Andersen *et al.*, 2004; Retana-Marquez, Bonilla-Jaime, Vázquez-Palacios, Martínez-García & Velázquez-Moctezuma, 2003).

These measures were examined in samples from rat adrenal glands, plasma, and brain regions (hypothalamus, hippocampus, and prefrontal cortex [PFC]) that are associated with the assessment and regulation of stress. This series of observations enabled evaluation of the hormone concentration at the tissues producing these hormones (adrenal glands) as well as in the transporter (plasma) and target tissues (hypothalamus, hippocampus, and PFC) of interest for the impact of the stress exposure on the development of depressive- or anxiety-like symptoms. The glucocorticoid receptor expression in leukocyte subpopulations was also assayed in whole blood due to the role of the corticosterone in an organism's response to stress.

The cellular parameters associated with the tissues involved with the endocrine markers were examined using histology to identify the impact of maternal deprivation, isolation, and chronic mild stress exposure. Specifically, cross sections of the adrenal gland, the hypothalamus and the hippocampus were examined for changes in volume. The adrenal gland is the source of catecholamine and steroid hormone production in the peripheral systemic circulation (Ulrich-Lai *et al.*, 2006; Kvetnansky *et al.*, 1995). In the brain, the hypothalamus receives sympathetic stimulation due to internal or external stressors, as well as signals from the amygdala, which triggers secretion of corticotrophin releasing hormone (CRH) (Raglan *et al.*, 2017; McVicar & Clancy, 2011). The hippocampus mediates the development of behavioural responses to stress exposure, via both the role as a negative feedback site for the glucocorticoid pathway and due to the involvement in declarative memory (Arnone *et al.*, 2012; Nestler & Carlezon, 2006). These

tissue regions are of interest due to the involvement in the organism's response to stress via secretion of stress-associated hormones or as sites for feedback regulation of stress.

It was hypothesised that the change in rodent behaviour triggered by stress would also induce physiological changes at an endocrine and cellular level. The aims of this chapter were to determine the rat acute and chronic stress-hormone concentrations and to assess the anatomical changes in relation to the morphology of the adrenal gland, hypothalamus, and hippocampus. These changes were assessed in the experimental cohorts following maternal deprivation (MD), isolation, and chronic mild stress (CMS) (Table 4.1).

Table 4.1: Description of experimental cohorts in the current study.

Treatments	**Experimental cohorts**	
	Visual (Control)	**Non-Visual (Treatment)**
Not Maternally Deprived (Control)	No MD and visual social support after weaning	No MD and no visual social support after weaning
Maternally Deprived (Treatment)	MD received and visual social support after weaning	MD received and no visual social support after weaning

As a result, the research questions for this chapter were:

1) Did maternal deprivation, isolation, and chronic mild stress trigger significant changes in the hormone levels between the experimental cohorts?
2) Did maternal deprivation, isolation, and chronic mild stress change the expression of the glucocorticoid receptor in leukocyte subpopulations?
3) Did maternal deprivation, isolation, and chronic mild stress trigger significant changes in the cellular morphology of the adrenal gland, hypothalamus, or hippocampus between the experimental cohorts?

Comparisons across the experimental cohorts were made to determine the impact of MD and CMS, as opposed to a baseline, due to the difficulty of obtaining sufficient sample volumes prior to CMS without causing excessive distress for the rats. Results from these tests were

analysed using one-way Analysis of Variance (ANOVA). The glucocorticoid receptor expression in leukocyte subpopulations was analysed using two-way ANOVA. A Bonferroni correction was applied to the analysis of catecholamine ($p < 0.017$) and steroid hormones ($p < 0.013$) using liquid-column mass spectrometry since the metabolites are in the same biosynthetic pathways. In all cases, Tukey's *post-hoc* analysis was used to determine the difference in the behaviour between the experimental cohorts due to MD and isolation exposures.

4.2 Post-Mortem Stress-Induced Physiological Results

4.2.1 Post-Mortem Catecholamine Concentration in Rats

Rats were injected with sodium pentobarbitone and the right atrium was cut. A blood sample was collected via cardiac punch prior to perfusion. The post-mortem catecholamine concentrations present in rat adrenal glands, plasma, and brains (hypothalamus, hippocampus, and PFC) were assayed to determine the degree of acute stress response, as determined by catecholamine concentrations, in response to the MD, isolation, and CMS exposures 48 hours after the last stress treatment. This was also done to determine if there the acute stress from the method of euthanasia would confound the examination of the impact of cumulative exposure to MD, isolation, and CMS.

4.2.1.1 Adrenal Gland Catecholamine Concentration

The adrenal medulla secretes the catecholamines, adrenaline, and noradrenaline in response to acute stress (Arnsten, Raskind, Taylor & Connor, 2015; Ranabir & Reetu, 2011; Selye, 1951). The post-mortem concentrations of noradrenaline, adrenaline, and dopamine in the adrenal glands were determined by liquid column mass spectroscopy. The average concentration of the catecholamines (noradrenaline, adrenaline, and dopamine) for the experimental cohorts was shown in Table 4.2. Based on the data, the adrenal glands of the rats produced approximately 40-

fold more adrenaline than noradrenaline (Figure 4.1). However, there were no significant differences in the levels of noradrenaline ($F = 0.84$; $p > 0.017$), adrenaline ($F = 0.60$; $p > 0.017$), and dopamine ($F = 0.52$; $p > 0.017$) between the different experimental cohorts. Physiologically, these data indicated that at post-mortem, adrenal gland acute stress hormone concentrations did not significantly vary between the experimental cohorts.

Table 4.2: Mean (SD) and ANOVA results for catecholamine (noradrenaline, adrenaline, and dopamine) concentrations in the adrenal gland between the experimental cohorts. Bonferroni adjustment for significance value ($p \leq 0.017$).

Measure	**Not Deprived Visual M (SD) (ng/mg)**	**Not Deprived Non-Visual M (SD) (ng/mg)**	**Deprived Visual M (SD) (ng/mg)**	**Deprived Non-Visual M (SD) (ng/mg)**	**F**	***p***
Adrenal Noradrenaline	79.16 (38.68)	84.93 (106.87)	54.69 (49.85)	63.94 (59.20)	0.60	0.62
Adrenal Adrenaline	3221.21 (1282.77)	3122.10 (1876.90)	2366.62 (1645.32)	2574.20 (1604.09)	0.84	0.48
Adrenal Dopamine	0.14 (0.20)	0.09 (0.21)	0.07 (0.15)	0.06 (0.12)	0.52	0.67

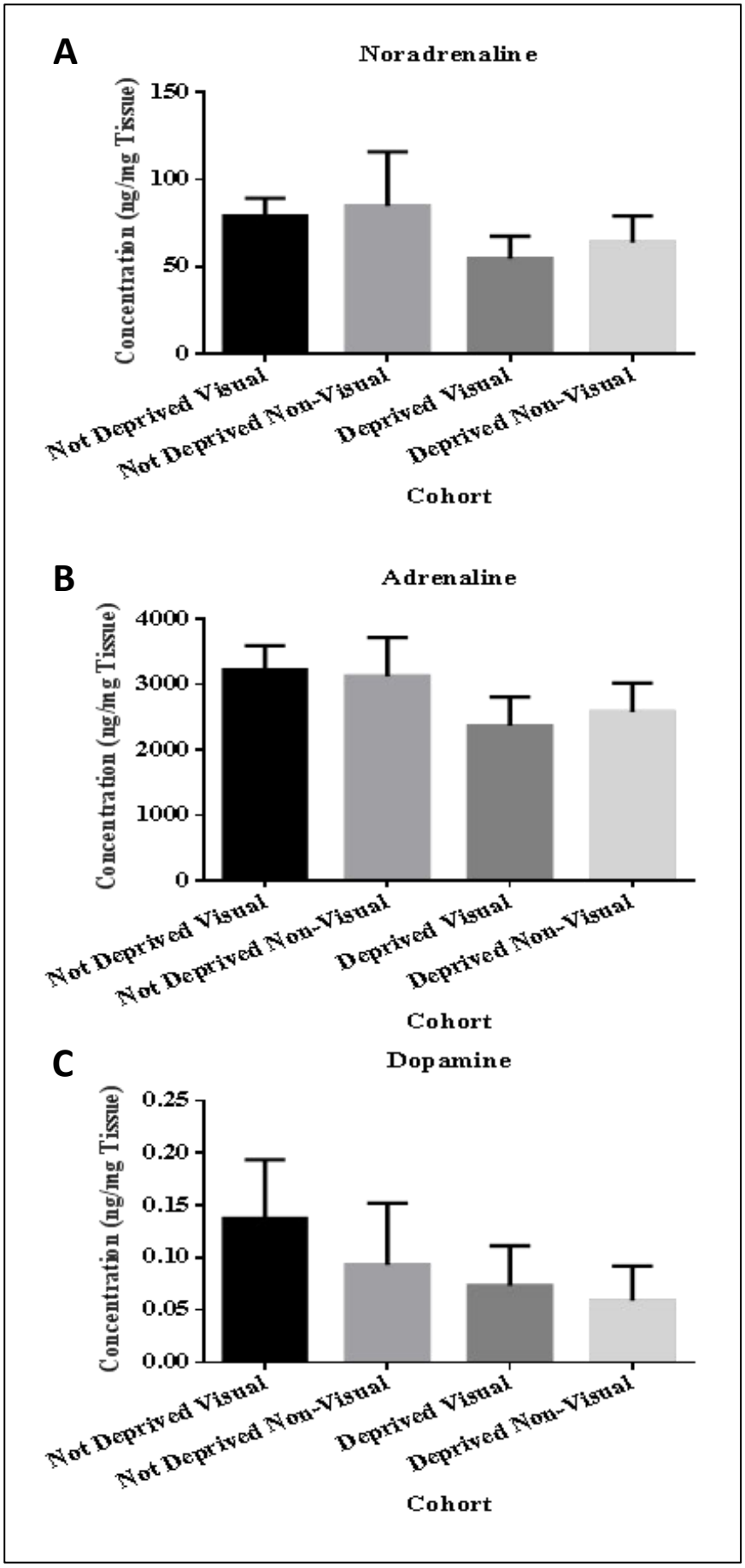

Figure 4.1: Catecholamine levels from the adrenal gland of rats subjected to different stress within their environments. (A) Noradrenaline; (B) Adrenaline; (C) Dopamine levels expressed by the rats in the experimental cohort. n = 15. Data represented as mean ± SEM.

4.2.1.2 Plasma Catecholamine Concentration

The systemic plasma concentrations of noradrenaline, adrenaline, and dopamine were also determined (Figure 4.2). The concentrations in the plasma of noradrenaline and dopamine were below the minimum threshold of noise for the assay (sensitivity = 0.01 ng/ml for both noradrenaline and dopamine) and therefore could not be reliably quantified. The average concentration of adrenaline for the experimental cohorts was shown in Table 4.3. There were no significant differences ($F = 0.22$; $p > 0.017$) in the plasma adrenaline levels between the experimental cohorts.

Table 4.3: Mean (SD) and ANOVA results for adrenaline concentrations in the adrenal gland between the experimental cohorts. Bonferroni adjustment for significance value ($p \leq 0.017$).

Measure	Not Deprived Visual M (SD) (ng/ml)	Not Deprived Non-Visual M (SD) (ng/ml)	Deprived Visual M (SD) (ng/ml)	Deprived Non-Visual M (SD) (ng/ml)	F	*p*
Plasma Adrenaline	15.29 (7.80)	16.6 (12.52)	18.08 (7.36)	18.07 (10.81)	0.22	0.88

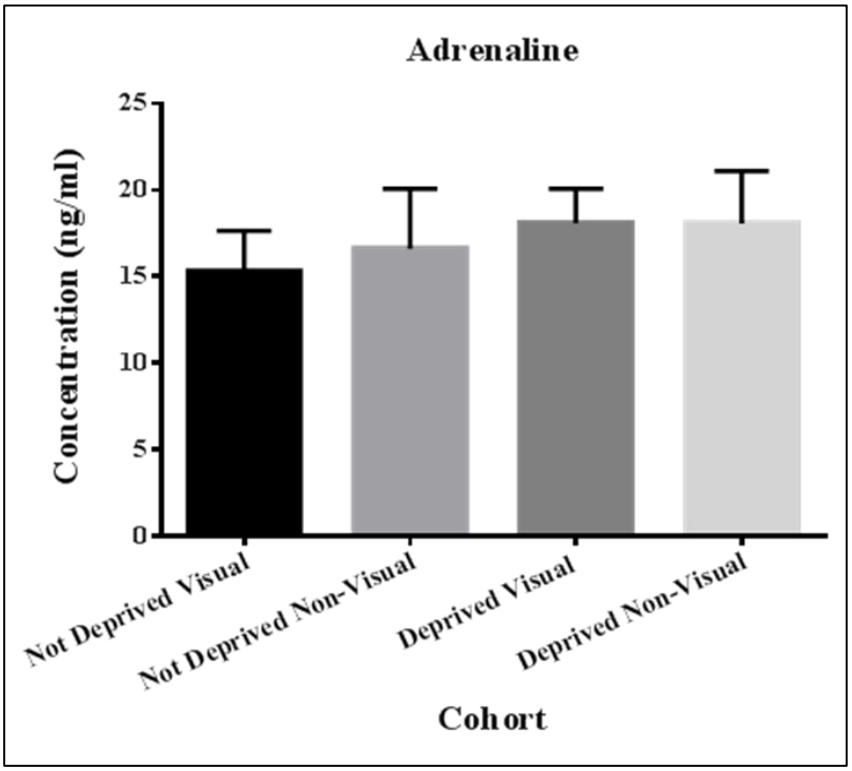

Figure 4.2: Adrenaline levels in the plasma of rats subjected to different stress within their environments. Levels expressed by experimental cohort. n = 15. Data represented as mean ± SEM.

4.2.1.3 Brain Region Catecholamine Concentration

Finally, catecholamine levels were examined separately in the hypothalamus, hippocampus, and PFC regions of the brain at post-mortem in rats that underwent stress testing (Figure 4.3). The concentration of noradrenaline in all assayed brain regions was below the threshold of noise within the assay (sensitivity = 0.01 ng/ml) and therefore could not be reliably quantified. The average concentration of the catecholamines (adrenaline and dopamine) for the experimental cohorts was shown in Table 4.4.

Table 4.4: Mean (SD) and ANOVA results for catecholamine (adrenaline and dopamine) concentrations in the hypothalamus, hippocampus, and PFC between the experimental cohorts. Bonferroni adjustment for significance value ($p \leq 0.017$).

Measure	Not Deprived Visual M (SD) (ng/mg)	Not Deprived Non-Visual M (SD) (ng/mg)	Deprived Visual M (SD) (ng/mg)	Deprived Non-Visual M (SD) (ng/mg)	F	*p*
Hypothalamus Adrenaline	0.34 (0.09)	0.35 (0.08)	0.35 (0.03)	0.29 (0.09)	1.36	0.27
Hypothalamus Dopamine	0.01 (0.01)	0.01 (0.003)	0.01 (0.01)	0.01 (0.01)	0.79	0.51
Hippocampus Adrenaline	0.42 (0.10)	0.47 (0.08)	0.42 (0.15)	0.42 (0.10)	0.62	0.61
Hippocampus Dopamine	0.02 (0.01)	0.01 (0.003)	0.01 (0.01)	0.01 (0.01)	1.28	0.30
PFC Adrenaline	0.57 (0.10)	0.66 (0.13)	0.67 (0.12)	0.60 (0.08)	2.66	0.06
PFC Dopamine	0.02 (0.01)	0.02 (0.002)	0.01 (0.01)	0.02 (0.01)	0.38	0.77

There were no significant differences in the post-mortem levels of hypothalamic adrenaline ($F = 1.36$; $p > 0.017$) or dopamine ($F = 0.79$; $p > 0.017$) following stress (Figure 4.3-A). The post-mortem level of hippocampus adrenaline ($F = 0.62$; $p > 0.017$) and dopamine ($F = 1.28$; $p > 0.017$) after stress also had no significant differences (Figure 4.3-B). Finally, there was no significant difference in the post-mortem PFC dopamine ($F = 0.38$; $p > 0.017$) after CMS (Figure 4.3-C). However, the "*not deprived visual*" control rats had lower post-mortem concentrations of adrenaline in the PFC which approached significance ($F = 2.66$; $p = 0.06$) than the "*deprived visual*" cohort rats. Collectively, these data suggest there were no post-mortem significant differences in systemic, adrenal, or brain catecholamine levels between the cohorts of rats subjected to MD, isolation, and CMS.

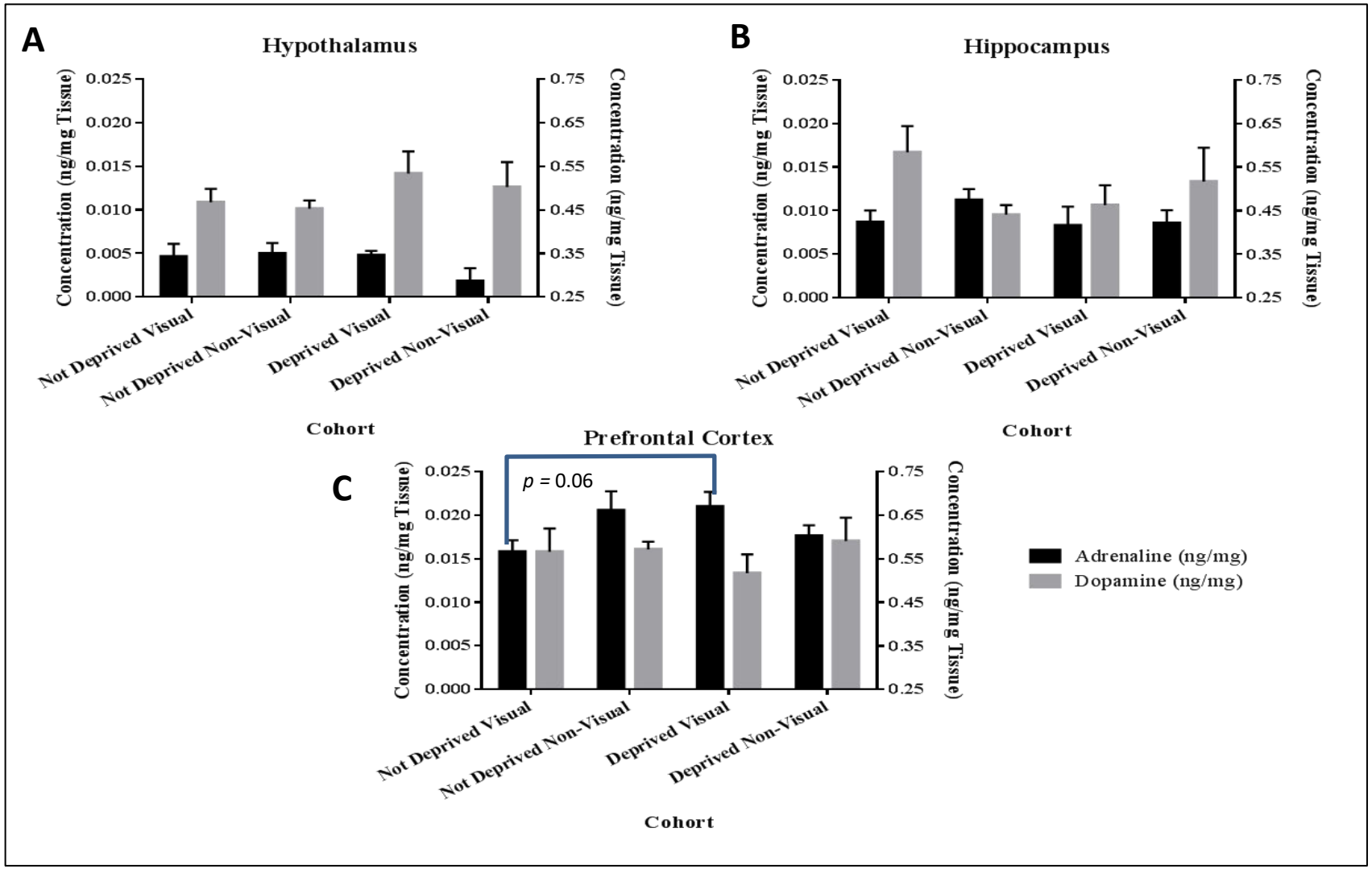

Figure 4.3: Catecholamine levels in the brain regions of rats subjected to different stress within their environments. (A) Hypothalamus; (B) Hippocampus; (C) Prefrontal Cortex levels of adrenaline and dopamine by experimental cohort. n = 15. Data represented as mean ± SEM. Dopamine on left y-axis, Adrenaline on right y-axis.

4.2.2 Post-Mortem Steroid Hormone Concentration in Rats

4.2.2.1 Adrenal Gland Steroid Hormone Concentration

The steroid hormone, corticosterone, is recognised as a marker of chronic stress in mammals (Franklin *et al.*, 2012; Trevino *et al.*, 2012). Therefore, to determine if the behavioural manipulations of MD, isolation, and CMS exposure were associated with post-mortem concentrations of steroid hormones, the rat adrenal glands were assayed for a panel of steroid hormones (corticosterone, 11-deoxycorticosterone, testosterone, or progesterone; Figure 4.4). Table 4.5 shows the average concentration of the steroid hormones (corticosterone, 11-deoxycorticosterone, testosterone, or progesterone) for the experimental cohorts. There were no significant differences in the post-mortem concentrations of corticosterone ($F = 0.36$; $p > 0.013$), 11-deoxycorticosterone ($F = 1.30$; $p > 0.013$), testosterone ($F = 0.23$; $p > 0.013$), or progesterone ($F = 1.12$; $p > 0.013$) between the experimental groups. Physiologically, these data indicated that cohort environmental manipulation did not affect the post-mortem concentrations of the steroid hormones synthesised by the adrenal cortex.

Table 4.5: Mean (SD) and ANOVA results for steroid hormone (corticosterone, 11-deoxycorticosterone, testosterone, and progesterone) concentrations in the adrenal glands between the experimental cohorts. Bonferroni adjustment for significance value ($p \leq 0.017$).

Measure	Not Deprived Visual M (SD) (ng/mg)	Not Deprived Non-Visual M (SD) (ng/mg)	Deprived Visual M (SD) (ng/mg)	Deprived Non-Visual M (SD) (ng/mg)	F	*p*
Adrenal Corticosterone	12.22 (13.68)	11.64 (13.62)	16.22 (10.56)	14.35 (10.82)	0.36	0.78
Adrenal 11-Deoxycorticosterone	7.28 (4.77)	8.80 (5.30)	10.85 (4.91)	9.14 (4.56)	1.30	0.28
Adrenal Testosterone	0.02 (0.02)	0.02 (0.02)	0.03 (0.02)	0.02 (0.02)	0.23	0.88
Adrenal Progesterone	4.10 (4.87)	3.05 (3.91)	5.97 (5.24)	5.41 (4.23)	1.12	0.35

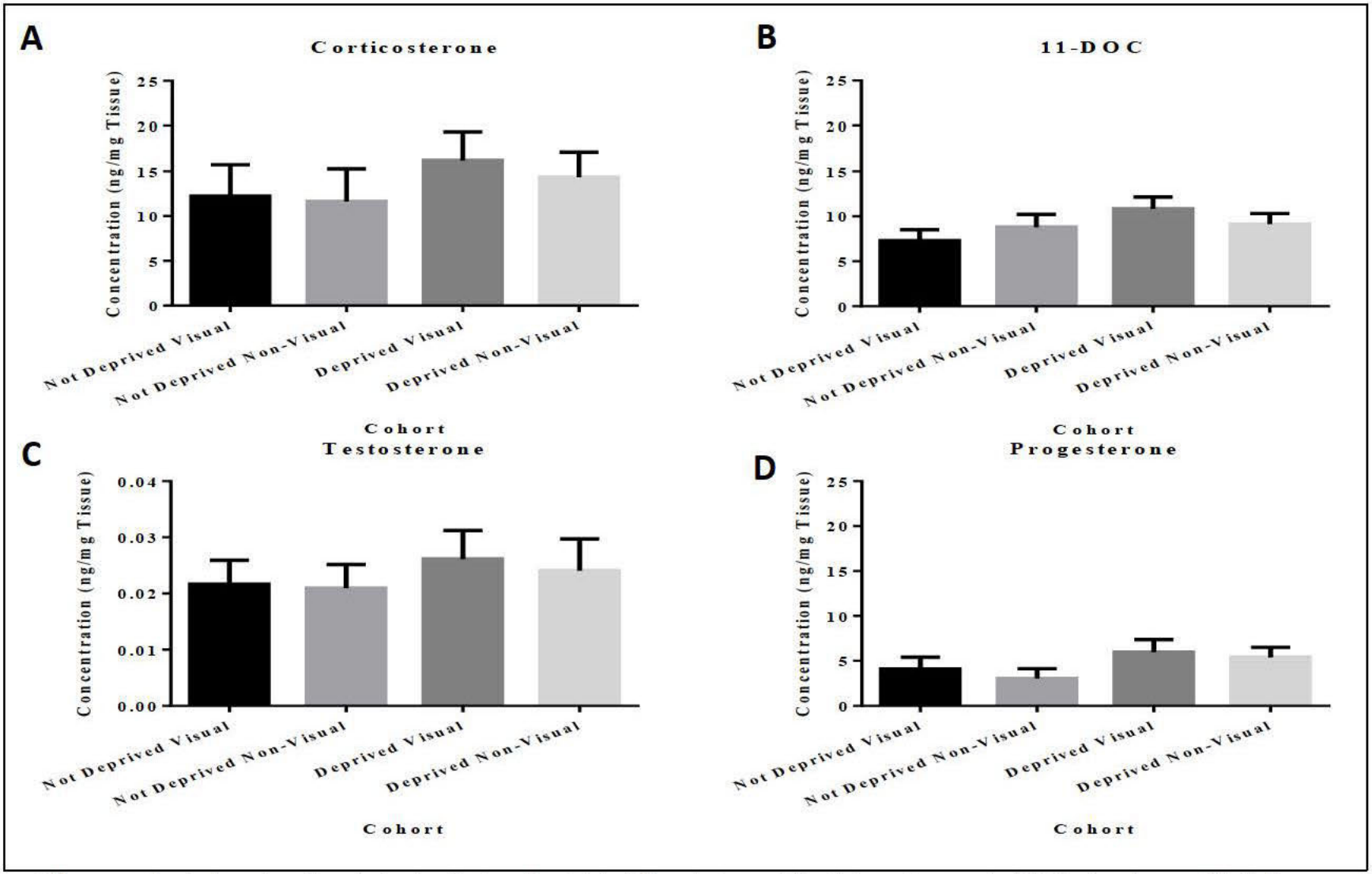

Figure 4.4: Steroid hormone levels from the adrenal glands of rats subjected to different stress within their environments. (A) Corticosterone; (B) 11-Deoxycorticosterone; (C) Testosterone; (D) Progesterone levels expressed by the rats in the experimental cohort. n = 15. Data represented as mean ± SEM.

4.2.2.2 Plasma Steroid Hormone Concentration

The post-mortem plasma concentrations of the steroid hormones (corticosterone, 11-deoxycorticosterone, testosterone, and progesterone) were determined using LC-MS. Table 4.6 shows the average concentration of the steroid hormones (corticosterone, 11-deoxycorticosterone, testosterone, and progesterone) for the experimental cohorts.

Table 4.6: Mean (SD) and ANOVA results for plasma steroid hormone (corticosterone, 11-deoxycorticosterone, testosterone, and progesterone) concentrations between the experimental cohorts. Bonferroni adjustment for significance value ($p \leq 0.017$).

Measure	Not Deprived Visual M (SD) (ng/ml)	Not Deprived Non-Visual M (SD) (ng/ml)	Deprived Visual M (SD) (ng/ml)	Deprived Non-Visual M (SD) (ng/ml)	F	*p*
Plasma Corticosterone	92.98 (33.59)	101.09 (44.04)	82.01 (23.20)	58.94 (29.80)	4.11	0.01
Plasma 11-Deoxycorticosterone	3.24 (1.53)	4.19 (1.96)	2.18 (1.48)	1.70 (1.18)	5.84	0.002
Plasma Testosterone	0.54 (0.20)	0.80 (0.68)	0.97 (0.75)	0.54 (0.53)	1.56	0.21
Plasma Progesterone	1.59 (1.13)	0.82 (0.50)	0.91 (0.64)	0.69 (0.60)	3.23	0.03

There was no significant difference in plasma testosterone ($F = 1.56$; $p > 0.013$) or plasma progesterone ($F = 3.23$; $p > 0.013$) between the experimental cohorts. In contrast, there were significant differences in the post-mortem plasma levels of corticosterone ($F = 4.11$; $p < 0.013$) and 11-deoxycorticosterone ($F = 5.84$; $p < 0.013$) between experimental groups.

Tukey's *post-hoc* analysis revealed that the rats in the *"deprived non-visual"* group possessed significantly lower levels of corticosterone than rats in both the *"not deprived non-visual"* cohort ($p < 0.05$; 1.72-fold decrease) and the *"not deprived visual"* control group ($p = 0.05$; 1.57-fold decrease) (Figure 4.5). In the samples assayed, there was approximately 30-fold more circulating corticosterone (81.3 ng/ml) observed than 11-deoxycorticosterone (2.6 ng/ml) with the distribution between experimental groups having a similar pattern. Rats from the *"not deprived*

non-visual" cohort exhibited plasma 11-deoxycorticosterone levels that were significantly higher than the *"deprived visual"* ($p < 0.05$; 1.9-fold more) and *"deprived non-visual"* ($p < 0.05$; 2.5-fold more) cohorts. Finally, there was a significant ($p < 0.05$) difference in plasma progesterone between rats in the control *"not deprived visual"* cohort and the *"deprived non-visual"* cohort, with the latter 2.3-fold less circulating progesterone than the "*not deprived visual*" control cohort. Physiologically, these data suggest that rats that received both MD and CMS had significantly lower concentrations of selected plasma steroid hormones.

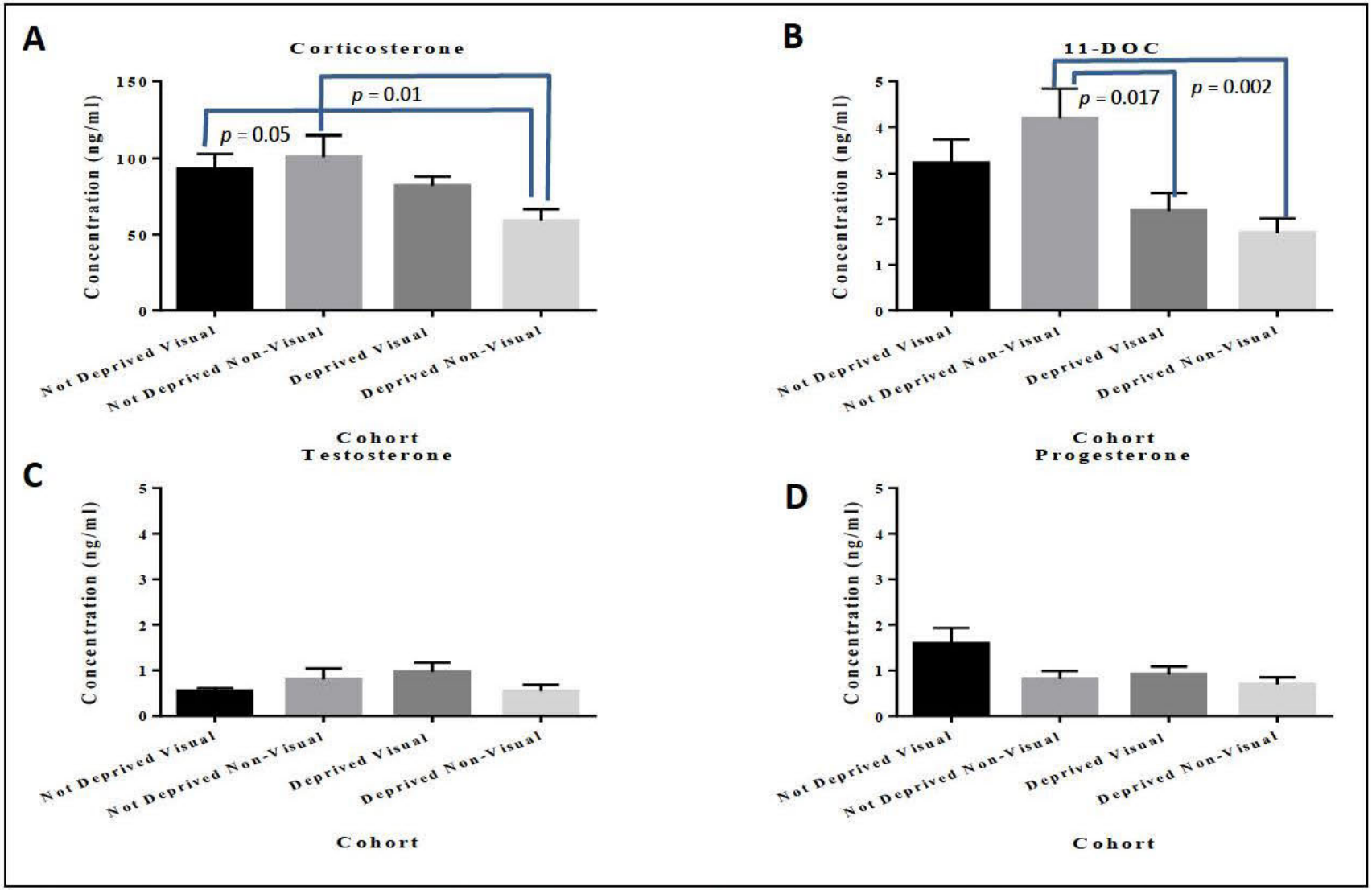

Figure 4.5: Steroid hormone levels from the plasma of rats subjected to different stress within their environments. (A) Corticosterone; (B) 11-Deoxycorticosterone; (C) Testosterone; (D) Progesterone levels expressed by the rats in the experimental cohort. n = 15. Data represented as mean ± SEM.

4.2.2.3 Brain Region Steroid Hormone Concentration

To complete the picture, these steroid hormone levels from the HPA feedback response to the (hypothalamus, hippocampus and PFC) were examined (Figure 4.6). Table 4.7 shows the average concentration of the steroid hormones (corticosterone, 11-deoxycorticosterone, testosterone, and progesterone) for the experimental cohorts.

Table 4.7: Mean (SD) and ANOVA results for steroid hormone (corticosterone, 11-deoxycorticosterone, testosterone, and progesterone) concentrations in the hypothalamus, hippocampus, and PFC between the experimental cohorts. Bonferroni adjustment for significance value ($p \leq 0.017$).

Measure	Not Deprived Visual M (SD) (ng/mg)	Not Deprived Non-Visual M (SD) (ng/mg)	Deprived Visual M (SD) (ng/mg)	Deprived Non-Visual M (SD) (ng/mg)	F	*p*
Hypothalamus Corticosterone	0.015 (0.008)	0.017 (0.012)	0.017 (0.008)	0.013 (0.007)	0.39	0.76
Hypothalamus 11-Deoxycorticosterone	0.005 (0.004)	0.007 (0.004)	0.005 (0.003)	0.006 (0.005)	0.73	0.54
Hypothalamus Testosterone	0.001 (0.001)	0.002 (0.002)	0.001 (0.001)	0.001 (0.001)	2.63	0.06
Hypothalamus Progesterone	0.002 (0.002)	0.003 (0.003)	0.002 (0.002)	0.002 (0.003)	0.29	0.83
Hippocampus Corticosterone	0.007 (0.005)	0.009 (0.010)	0.005 (0.003)	0.009 (0.008)	1.19	0.32
Hippocampus 11-Deoxycorticosterone	0.004 (0.003)	0.006 (0.005)	0.003 (0.002)	0.006 (0.005)	1.96	0.13
Hippocampus Testosterone	0.001 (0.001)	0.002 (0.001)	0.001 (0.001)	0.001 (0.001)	2.01	0.13
Hippocampus Progesterone	0.003 (0.004)	0.003 (0.004)	0.002 (0.002)	0.003 (0.003)	0.56	0.64
PFC Corticosterone	0.002 (0.001)	0.002 (0.001)	0.001 (0.001)	0.001 (0.001)	1.28	0.29
PFC 11-Deoxycorticosterone	0.005 (0.003)	0.007 (0.005)	0.004 (0.003)	0.005 (0.004)	1.16	0.33
PFC Testosterone	0.013 (0.006)	0.014 (0.007)	0.013 (0.007)	0.015 (0.009)	0.17	0.92
PFC Progesterone	0.003 (0.003)	0.003 (0.003)	0.003 (0.003)	0.003 (0.003)	0.09	0.97

In all the hypothalamus there were no significant differences in the post-mortem corticosterone ($F = 0.39$; $p > 0.013$), 11-deoxycorticosterone ($F = 0.73$; $p > 0.013$), testosterone ($F = 2.63$; $p > 0.013$), or progesterone ($F = 0.29$; $p > 0.013$) levels between the experimental cohorts. There were no significant differences in the post-mortem concentrations of corticosterone ($F = 1.19$; $p > 0.013$), 11-deoxycorticosterone ($F = 1.96$; $p > 0.013$), testosterone ($F = 2.01$; $p > 0.013$), or progesterone ($F = 0.56$; $p > 0.013$) in the hippocampus between the experimental cohorts. Nor were there any significant differences in the post-mortem PFC corticosterone ($F = 1.28$; $p > 0.013$), 11-deoxycorticosterone ($F = 1.16$; $p > 0.013$), testosterone ($F = 0.17$; $p > 0.013$), or progesterone ($F = 0.09$; $p > 0.013$) concentrations between the experimental cohorts. Physiologically, this suggested that the exposure to MD and isolation manipulations, in addition to CMS exposure, had similar effects on the level of steroid hormones present in the hypothalamus, hippocampus, and PFC.

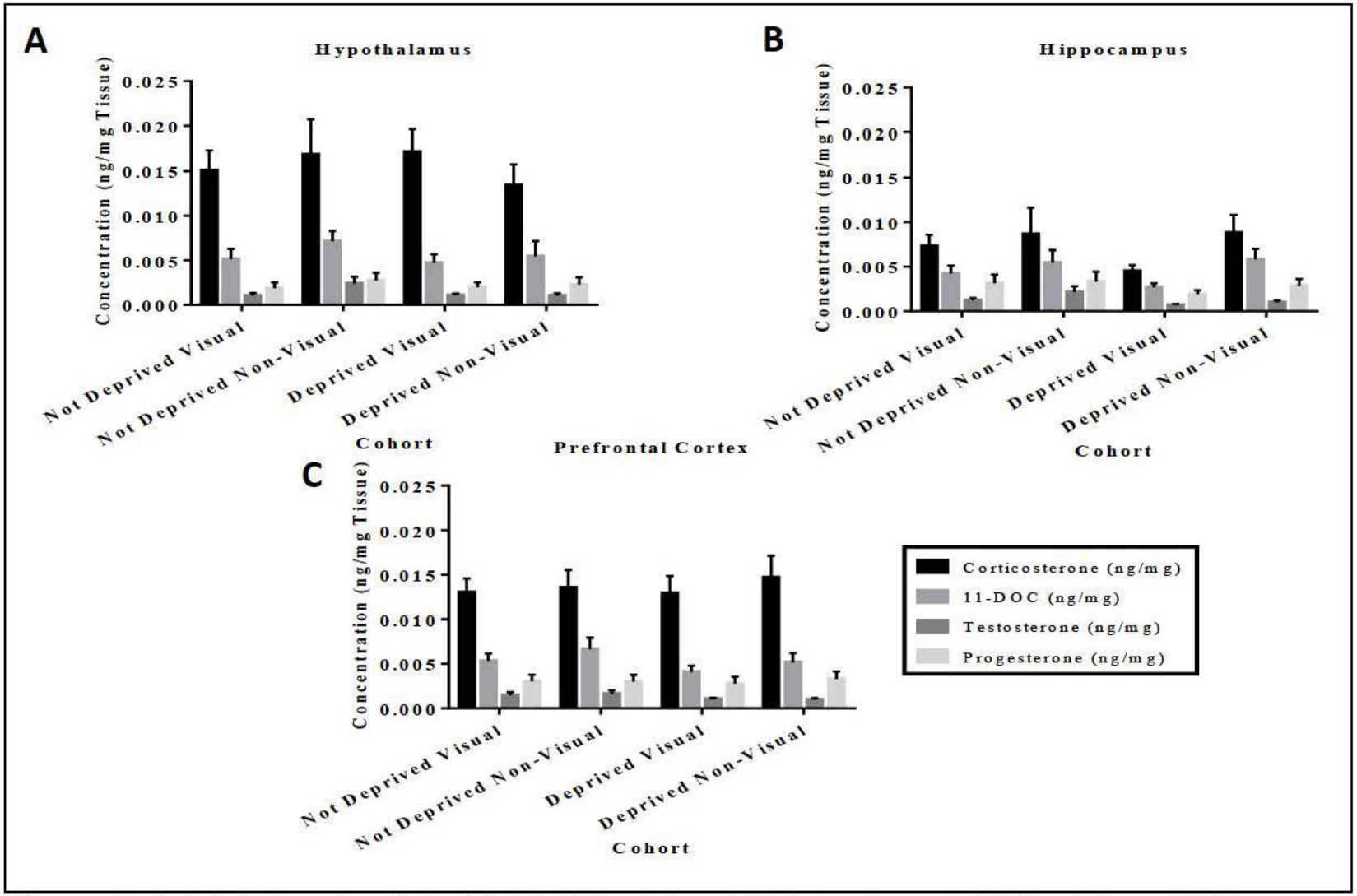

Figure 4.6: Steroid hormone levels in the brain regions of rats subjected to different stress within their environments. (A) Hypothalamus; (B) Hippocampus; (C) Prefrontal Cortex levels of corticosterone, 11-deoxycorticosterone, testosterone, and progesterone by the experimental cohort. n = 15. Data represented as mean ± SEM.

4.2.3 Flow Cytometry of Glucocorticoid Receptor Expression in Peripheral Blood Lymphocyte Subpopulations

Since there were significant differences in the post-mortem plasma concentrations of corticosterone between the experimental cohorts, the glucocorticoid receptor (GR) expression in the lymphocyte subpopulations was assayed to determine if GR expression was associated with stress responses in these animals (Figure 4.7). The average GR expression percentages separated by leukocyte subtype for each of the experimental cohorts are shown in Table 4.8.

Table 4.8: Mean (SD) and ANOVA results for the percentage of expression for the glucocorticoid receptor for the leukocyte sub-types between the experimental cohorts.

Measure	Not Deprived Visual M (SD) (%)	Not Deprived Non-Visual M (SD) (%)	Deprived Visual M (SD) (%)	Deprived Non-Visual M (SD) (%)	F	*p*
T-helper (CD4)	12.50 (15.36)	8.07 (4.20)	6.30 (3.65)	9.77 (7.10)	1.23	0.31
Cytotoxic (CD8)	13.33 (13.61)	8.82 (5.81)	8.31 (4.33)	12.43 (7.41)	1.22	0.31
B-Cells (CD19)	13.26 (12.12)	7.56 (6.08)	17.48 (11.10)	20.53 (14.64)	2.89	0.05
NK Cells (CD56)	5.88 (14.02)	2.25 (2.43)	2.37 (1.27)	3.60 (2.50)	0.74	0.54

There was a significant difference in expression of GRs in $CD19^+$ B-lymphocytes between the experimental cohorts ($F = 2.90$; $p = 0.045$), but not $CD4^+$ T-helper lymphocytes ($F = 1.23$; $p > 0.05$), $CD8^+$ cytotoxic T lymphocytes ($F = 1.22$; $p > 0.05$), or $CD56^+$ NK cells ($F = 0.74$; $p > 0.05$). However, Tukey's *post-hoc* analysis revealed that the *"deprived non-visual"* cohort expressed significantly more GR in B-lymphocytes when compared to the *"not deprived non-visual"* cohort ($p < 0.05$) at post-mortem. This indicated that between the rats subjected to isolation and CMS, exposure to MD as well resulted in lower GR expression levels in B-lymphocytes.

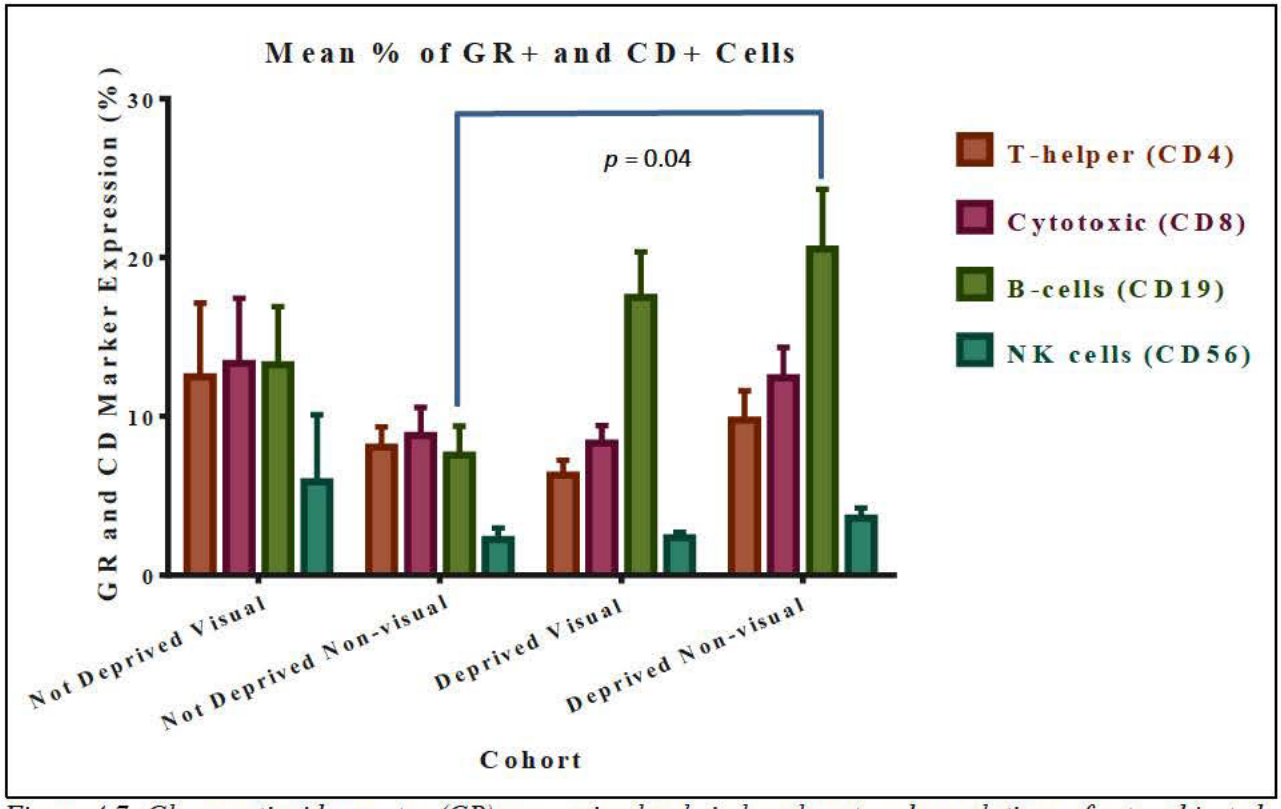

Figure 4.7: Glucocorticoid receptor (GR) expression levels in lymphocyte subpopulations of rats subjected to different environmental stress. n = 15. Data represented as mean ± SEM.

4.2.4 Post-Mortem Histological Analysis in Rats

4.2.4.1 Adrenal Gland Histology

To determine anatomical changes due to stress, the adrenal gland regions (zona reticularis, zona glomerulosa, zona fasciculata, and adrenal medulla) were measured across eight slides at 6 µm thickness. Table 4.9 shows the average area of the adrenal gland regions. Figure 4.8 shows a representative image of the assessed adrenal regions for each of the experimental cohorts.

Table 4.9: Mean (SD) and ANOVA results for the size of the adrenal gland regions (zona glomerulosa, zona fasciculata, zona reticularis, and adrenal medulla).

Measure	Not Deprived Visual M (SD) (μm^2)	Not Deprived Non-Visual M (SD) (μm^2)	Deprived Visual M (SD) (μm^2)	Deprived Non-Visual M (SD) (μm^2)	F	*p*
Zona Glomerulosa	71.33 (6.66)	85.84 (10.26)	75.88 (15.53)	82.90 (10.21)	1.76	0.20
Zona Fasciculata	411.30 (72.45)	410.60 (66.98)	556.00 (91.29)	501.85 (92.72)	3.85	0.03
Zona Reticularis	392.73 (90.63)	367.35 (83.37)	394.48 (80.28)	346.85 (64.08)	0.40	0.76
Adrenal Medulla	1011.50 (146.76)	950.38 (189.25)	1037.23 (279.86)	758.88 (104.59)	2.16	0.13

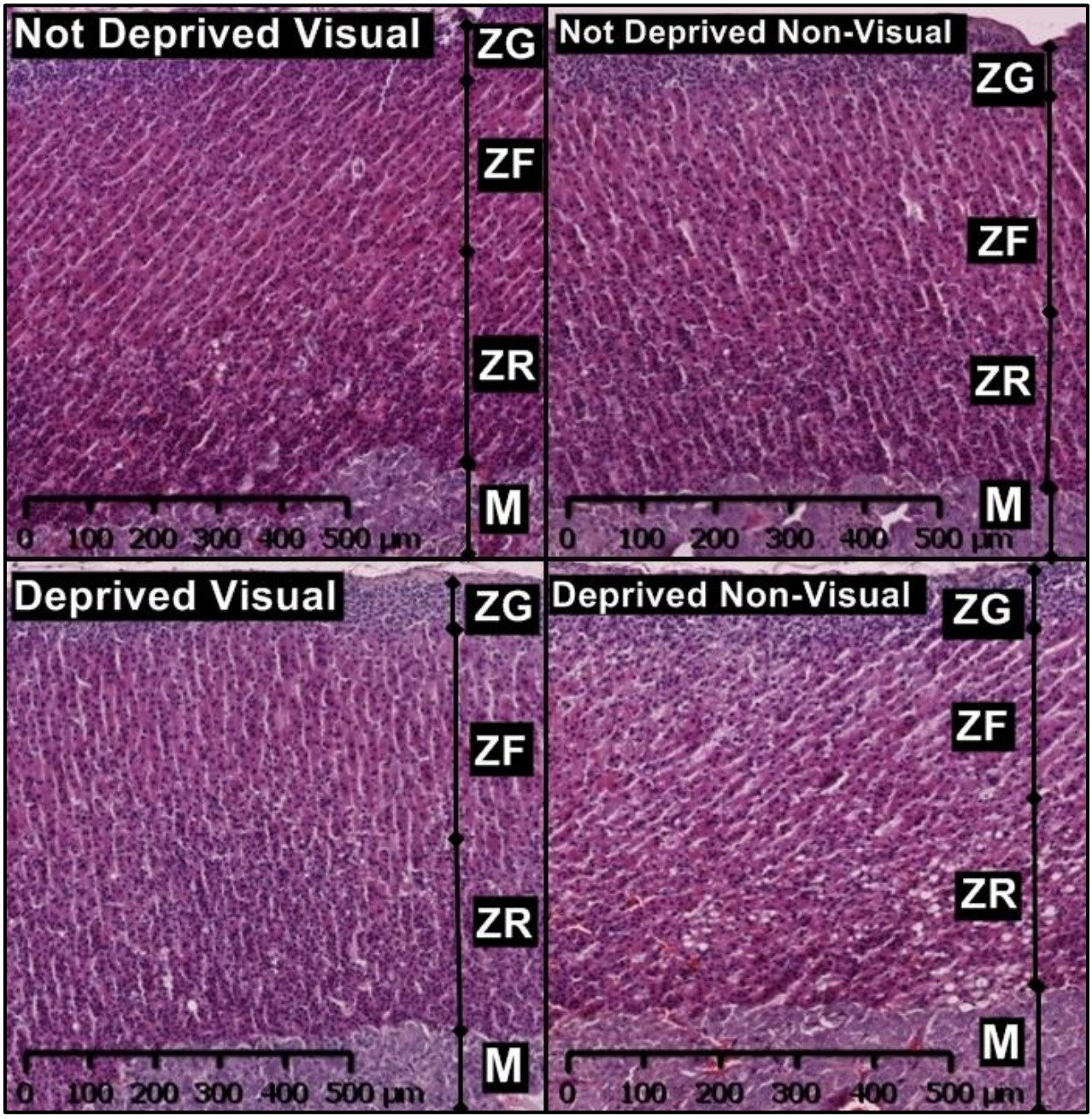

Figure 4.8: Representative micrographs from each experimental cohort showing adrenal gland cortex regions under H & E stain. M: Medulla; ZR: Zona Reticularis; ZF: Zona Fasciculata; ZG: Zona Glomerulosa. Images were taken at 10 x magnification. Scale bars of 500 µm shown in the bottom left.

There was no significant difference between the experimental cohorts in the volume of the medulla ($F = 2.16$; $p > 0.05$), zona reticularis ($F = 0.40$; $p > 0.05$) or zona glomerulosa ($F = 1.76$; $p > 0.05$), as shown in Figure 4.9. There was a significant difference between experimental groups in the volume of the zona fasciculata region ($F = 3.85$; $p < 0.05$).

However, Tukey's *post-hoc* test revealed that the "*deprived visual*" cohort rats were only trending towards ($p = 0.06$) larger zona fasciculata regions when compared to the "*not deprived*

non-visual" cohort and the "*not deprived visual*" control rats. Physiologically, these data indicate no significant hypertrophy of the adrenal gland because of the cumulative effect of stress.

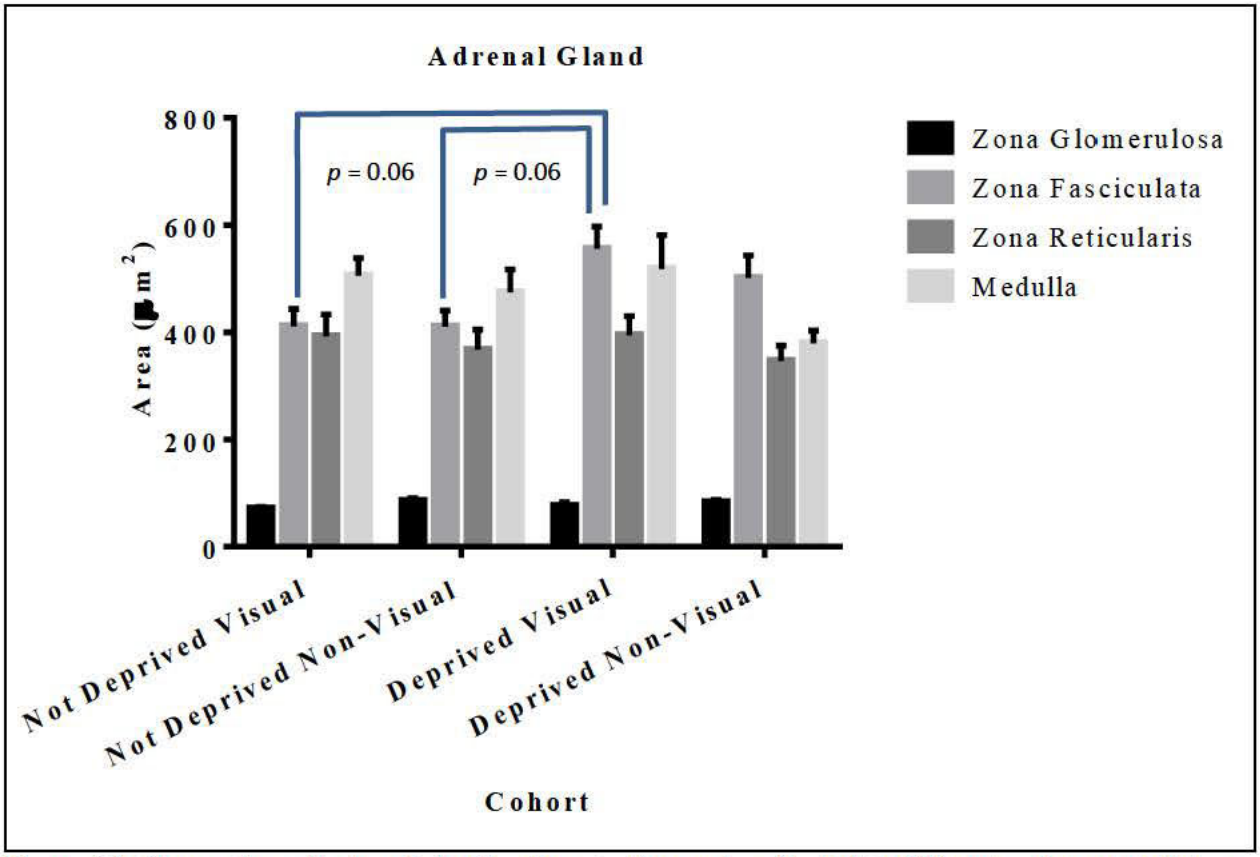

Figure 4.9: Comparison of adrenal gland region size from rats subjected to different environmental stress. Expressed as region size average by rat experimental group. n = 5. Data represented as mean ± SEM.

4.2.4.2 Brain Region Histology

To determine whether there were any anatomical changes in the hypothalamus and hippocampus, due to MD, isolation, and CMS, the total areas for the paraventricular nucleus of the hypothalamus and hippocampus were examined. The area for the PVN of the hypothalamus was measured across five sections at 5 µm thickness and the total area of the hippocampus was measured across 10 sections at 5 µm thickness was examined. Table 4.10 shows the average volume of the hypothalamus and hippocampus for the experimental cohorts.

Table 4.10: Mean (SD) and ANOVA results for the measured volume of the hypothalamus and hippocampus between the experimental cohorts.

Measure	Not Deprived Visual M (SD) (μm^3)	Not Deprived Non-Visual M (SD) (μm^3)	Deprived Visual M (SD) (μm^3)	Deprived Non-Visual M (SD) (μm^3)	F	*p*
Hypothalamus	0.93 (0.06)	0.93 (0.04)	0.86 (0.04)	0.93 (0.07)	1.79	0.20
Hippocampus	38.22 (4.92)	38.34 (6.64)	40.40 (1.79)	38.88 (2.18)	0.26	0.85

Figure 4.10 shows a representative image of the assessed paraventricular nucleus (PVN) of the hypothalamus from each of the experimental cohorts. A similar representation of the hippocampus for the experimental cohorts was generated as shown in Figure 4.11.

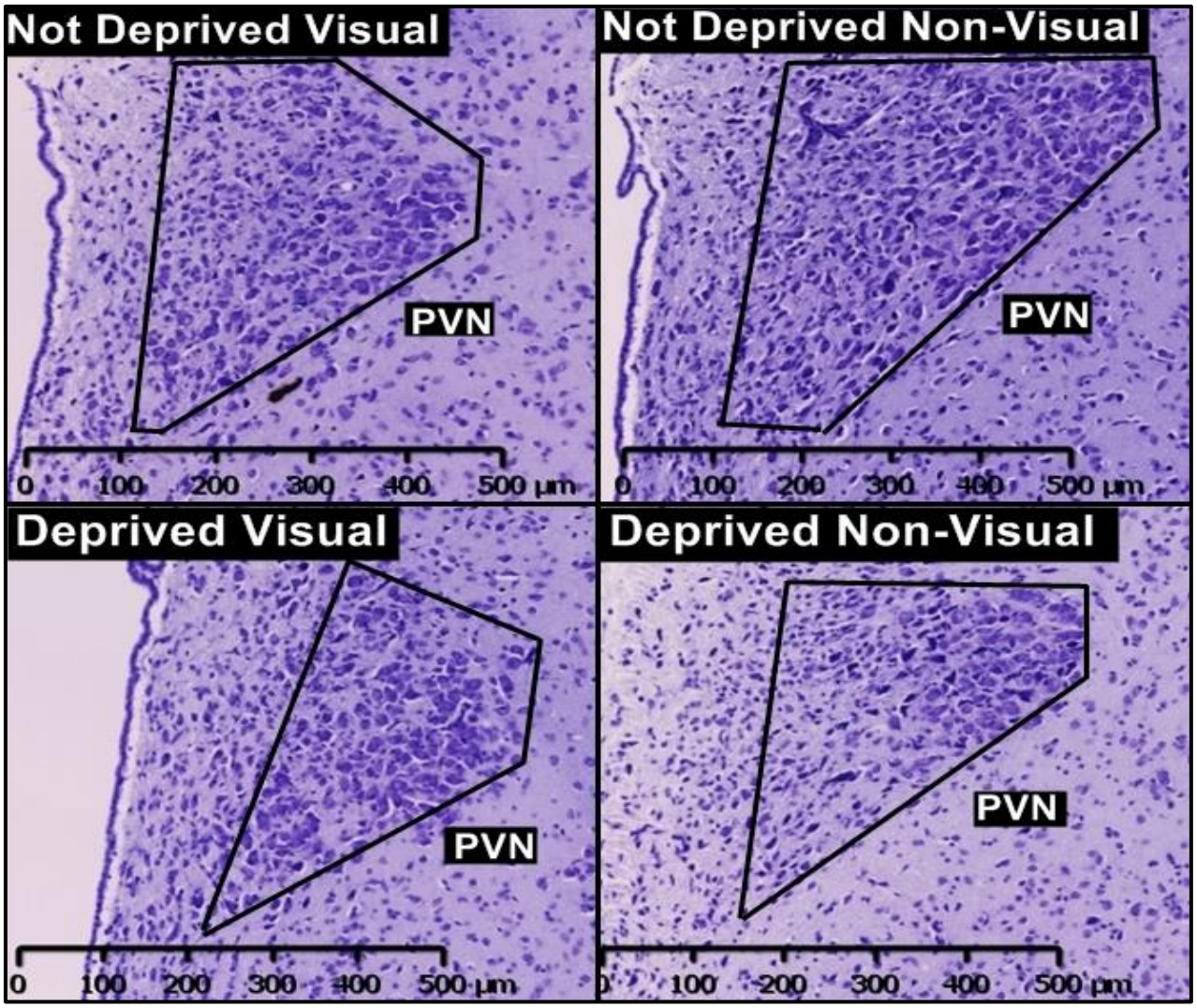

Figure 4.10: Representative micrographs from each experimental cohort showing the PVN of the hypothalamus under the Nissl stain. PVN: Paraventricular Nucleus. Images were taken at 10x magnification. Scale bars of 500 µm shown in the bottom left.

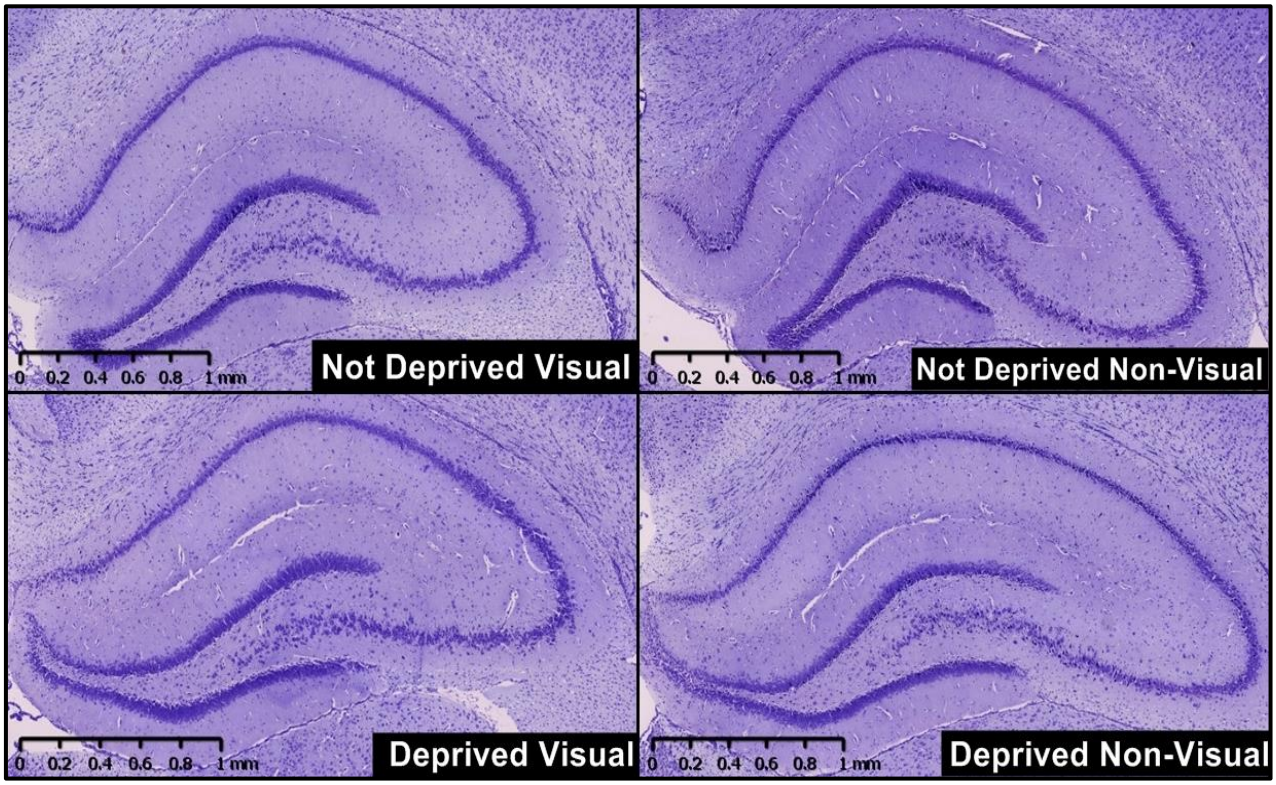

Figure 4.11: Representative micrographs from each experimental cohort showing hippocampus under the Nissl stain. Images were taken at 5x magnification. Scale bars of 1 mm shown in the bottom left.

There was no significant difference between the experimental cohorts in the size of the hypothalamus ($F = 1.79$; $p > 0.05$) or hippocampus ($F = 0.85$; $p > 0.05$), as shown in Figure 4.12. This indicates that environmental stress triggered no significant change in size due to hypertrophy or hypotrophy to the brains of the rats.

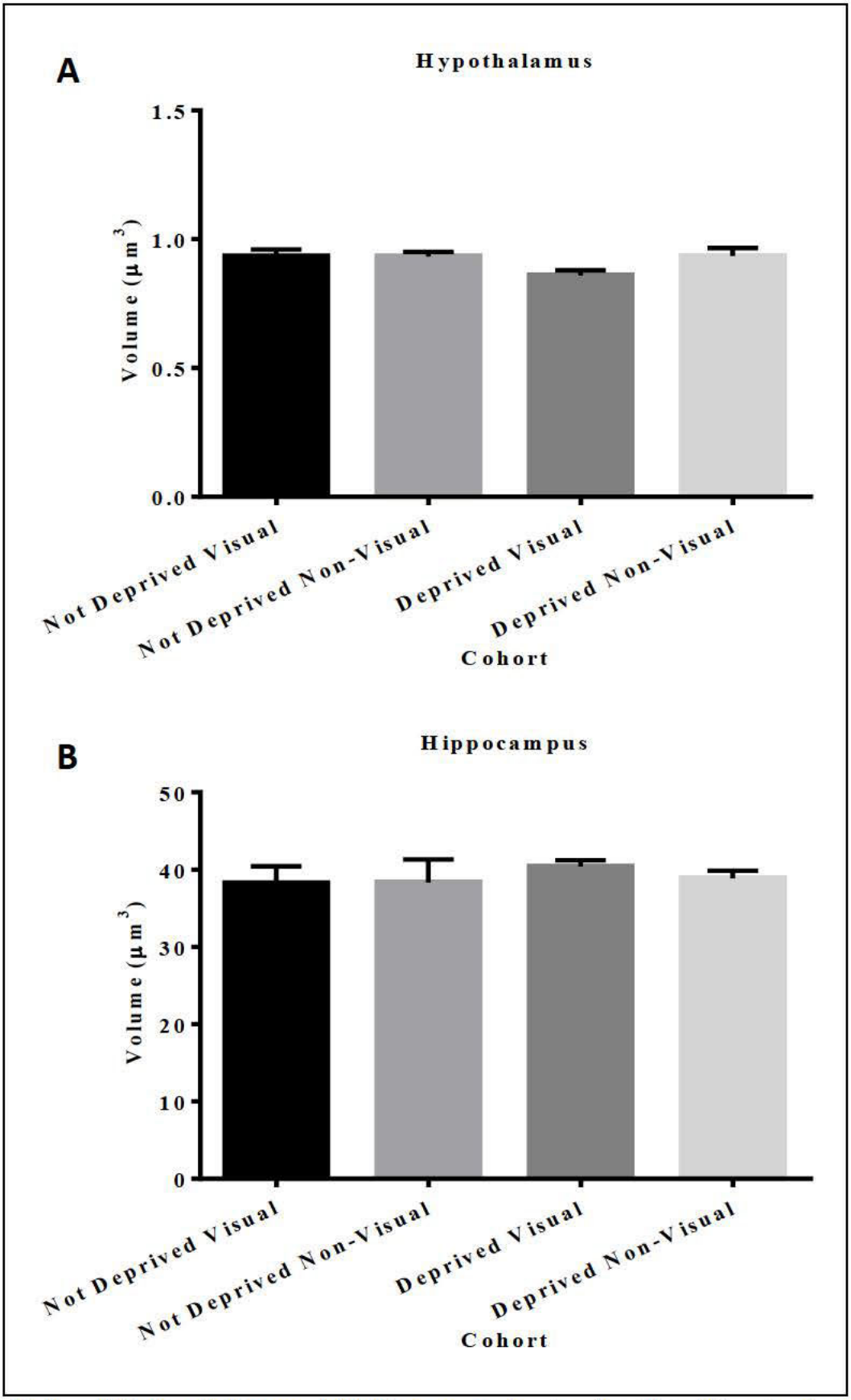

Figure 4.12: Size of (A) Hypothalamus and (B) Hippocampus in rats subjected to different environmental stress. Expressed as average size against rat experimental cohort. n = 5. Data represented as mean ± SEM.

4.3 Physiological Changes in Response to Stress in Stress-Resistant Rats

After the behavioural study, the hormones associated with acute and chronic stress were assayed in post-mortem samples to determine if there was any change from the cumulative stress. Additionally, cellular measures were examined in the adrenal gland, hypothalamus, and hippocampus to determine any stress-induced modifications were present. There were no significant differences in catecholamine concentrations between any of the experimental cohorts, which suggested that none of the stresses induced a detectable acute stress catecholamine response in these animals. In contrast, some of the steroid hormones, specifically corticosterone and 11-deoxycorticosterone, associated with chronic stress were significantly different between the experimental cohorts at post-mortem. However, the cellular measures examined at post-mortem after MD, isolation, and CMS exposure were not significantly different between the experimental cohorts. This suggested that the rats did experience significant chronic stress, but the exposure period was not long enough to result in morphological changes in the adrenal glands or brain regions.

Catecholamines are responsible for mediating the acute stress response in humans and rodents (Ranabir & Reetu, 2011; Goldstein, 2003; Tsigos & Chrousos, 2002). There were no significant differences between the experimental cohorts for catecholamine concentrations in any sample type (adrenal gland, plasma, or brain region). This indicated that the rats did not have significantly different acute stress marker concentrations at the time of death. This suggested that the post-mortem physiological response to 115 days of cumulative stress could be examined in the experimental cohorts without acute stress measures confounding the data. This was hypothesised to be due to all rats receiving 21 days of CMS exposure. A study by Dronjak and Gavrilovic (2006) examined the peripheral and central tissue catecholamine stores in socially isolated rats noted reduced hypothalamic noradrenaline and hippocampal dopamine when compared to non-stressed control rats as measured by radioimmunoassay after decapitation. This further suggested that the lack of significantly different catecholamine concentrations in the current study was due

to all rats undergoing CMS. Another study by Sanchez, Toledo-Pinto, Menezes and Pereira (2003) examined changes in rat adrenal gland post-mortem adrenaline and noradrenaline concentrations following acute immobilisation stress. This study by Sanchez *et al.* (2003) also found that immobilisation did not result in significant changes in rat adrenal gland adrenaline or noradrenaline concentration when compared to control rats after euthanasia. Collectively, in the current study, this indicated that the method of euthanasia did not have significantly different impacts on the acute stress markers of the experimental cohorts. Thus, the post-mortem analysis of the chronic stress endocrine and histological morphology measures could be analysed without acute stress markers confounding the data.

The measurement of the steroid hormones in adrenal glands, brain-regions (hypothalamus, hippocampus, and prefrontal cortex) and plasma, quantified the impact of chronic stress exposure from the behavioural challenge. Specifically, the steroid hormones examined in the current study were all from the same biosynthetic pathway, corticosterone, 11-deoxycorticosterone, testosterone, and progesterone.

There were no significant differences in the steroid hormone concentrations in the adrenal glands. This was expected, since in the current study there was no manipulation of the adrenal glands that would alter the production of steroid hormones or injection of an antagonist compound to interfere with glucocorticoid binding, which are the common reasons in the literature for difference in steroid hormone production (Serova *et al.*, 2008; Raone *et al.*, 2007). In addition, the lack of statistically significant differences in adrenal hormone concentrations indicated that the observed statistical differences in plasma corticosterone and 11-deoxycorticosterone between the experimental cohorts were not due to a difference in the available adrenal hormone concentrations. Additional analysis of the gene expression for upstream regulators of hormone synthesis would confirm if there was any modification to the production of these steroid hormones (Sanderson 2006; Stocco, 2001). However, this was beyond the scope of the current study.

There were no significant differences between the experimental cohorts for steroid hormone concentrations in the examined brain-regions (hypothalamus, hippocampus, prefrontal cortex). This indicated that the feedback of the stress response to the brain after the cumulative stress was not significantly different regardless of MD or isolation exposure (Taves, Ma, Heimovics, Saldanha & Some, 2011). However, the depletion of the plasma steroid hormones suggests different physiological responses to the MD, isolation, and CMS exposures between the experimental cohorts, given the significant difference in the plasma levels of these steroid hormones. The next step would be to examine the expression of the glucocorticoid receptor in the hypothalamus, hippocampus, and PFC (Leonard, 2005). This would determine if a modification in the glucocorticoid receptor expression was mediating the level of available corticosterone in these brain regions (hypothalamus, hippocampus, and PFC). However, examination of the glucocorticoid receptor expression and associated gene expression in these brain regions were beyond the scope of the current study.

There was a significant difference between the experimental cohorts for the plasma concentrations in the chronic stress hormone corticosterone and the precursor hormone 11-deoxycorticosterone. Plasma corticosterone and 11-deoxycorticosterone were significantly higher in rats that only received CMS when compared to rats subjected to MD, isolation, and CMS. This was counter to the expected results of higher glucocorticoid (GC) concentrations in rats that received MD, isolation, and CMS exposure. The physiological response to chronic stress is very complex, with GCs secreted in response to any stress, internal or external that disturbs the maintenance of homeostasis (Leonard, 2005). Acute stress exposure activates the hypothalamic-pituitary-adrenal (HPA) axis resulting in the secretion of GCs from the adrenal cortex (McVicar & Clancy, 2011). The GCs mediate the physiological response to stress, while simultaneously HPA axis activation produces a partial resistance to the feedback inhibition of GC release involving rapid desensitisation of glucocorticoid receptors (GRs) in the brain allowing maintenance of normal signalling responses (Myers, McKlveen & Herman, 2014). However, under chronic stress exposure, HPA axis response can vary based on the development of coping strategies (McVicar &

Clancy, 2011; Koolhaas *et al.*, 2011). Successful coping strategies can result in low plasma GC concentrations (Volodina *et al.*, 2012; Koolhaas *et al.*, 2011; Romero, 2004). This indicated that the rats with lower plasma corticosterone concentrations in the current study may have been able to better cope when exposed to CMS, due to already being subjected to MD and isolation stress. In addition to this, Wistar rats are a stress-resistant strain, which means they do not respond to stressful stimuli the same way as stress-susceptible strains (Ghadhanfar *et al.*, 2014; Yang *et al.*, 2012). Therefore, in the current study, this suggests that the rats exposed to MD, isolation, and CMS were better able to adapt to the stress exposure due to the early life stress period when compared to rats only subjected to CMS.

The early life stress of the loss of the mother in MD can alter GC levels, demonstrated by several studies, though the results are mixed with some reporting higher GC levels (Reus *et al.*, 2011; Kalinichev, Easterling, Plotsky & Holtzman, 2002) and some reporting lower GC levels (Volodina *et al.*, 2012; Ruedi-Bettschen *et al.*, 2006). However, these values were in response to the acute stress exposure during childhood as opposed to additional chronic stress during adulthood after early life stress, as was used in the current study. Furthermore, exposure to CMS or chronic restraint during adulthood with no early life stress manipulations also resulted in increased serum and plasma GCs (Liu *et al.*, 2014; Liu, W. *et al.*, 2013; Liu, X. *et al.*, 2013; Ferraz *et al.*, 2011). In macaques, the long-term consequences of adverse parenting in early infancy resulted in low GC concentrations in later life, even with no additional stress exposures (Kim *et al.*, 2017; Coplan *et al.*, 1996). This was similar to the observable low plasma GC concentrations in the current study. Specifically, this is like the rats in the current study subjected to MD, isolation, and CMS exposure, that possessed the lowest plasma GC concentrations. This suggests that the rats subjected to MD, isolation, and CMS, in addition to adapting to the stress exposure, more efficiently utilised the available plasma steroids to manage the stress response when compared to rats only subjected to CMS.

The cellular examination of the adrenal gland revealed no significant difference between the experimental cohorts in the sizes of the medulla, zona reticularis, or zona glomerulosa regions for the adrenal gland in the current study. There was only a trend towards a significant difference between experimental cohorts for the zona fasciculata. The adrenal gland is the predominant source of catecholamine and steroid hormone synthesis in the periphery, and previous studies have shown that chronic stress exposure can alter the volume and distribution of the adrenal regions (Ulrich-Lai *et al.*, 2006; Koko *et al.*, 2004). In the current study, this suggests that a longer duration of stress exposure may achieve statistical significance. However, since the morphological parameters only used a subset of the total available subjects due to financial constraints it is possible that a greater number of subjects would result in statistical significance.

The other cellular parameters examined were the volume of the hippocampal and hypothalamic regions in the brain. However, there was no observed change in the estimated volume of the hippocampus or hypothalamus between the experimental cohorts. The elevated glucocorticoids from prolonged stress can lead to atrophy of hippocampal neurones. This often results in a reduction of hippocampal volume (Warner-Schmidt & Duman, 2006; Bremner *et al.*, 2000). In the hypothalamus, structural changes may occur because of sustained hypothalamic-pituitary-adrenal (HPA) axis activation (Varghese & Brown, 2001). In the current study, given there was no significant difference in the estimated volume of the hypothalamus or hippocampus at post-mortem it is possible that this was due to the duration or intensity of the stress not being sufficient to result in these changes. Alternatively, as with the adrenal glands, the lack of subject numbers used for assessment of the morphological changes may have played a role in the lack of statistical significance.

Collectively, there are two hypothesised explanations for the observations in the current study. First, the plasma GC levels were lower due to increased GR binding mediating the response to stress. This would require additional testing of the tissues post-mortem to determine potential increase in GR expression, beyond the analyses conducted in lymphocyte subpopulations.

Glucocorticoids exert immunomodulatory effects by acting on all types of immune cells due to the abundant expression of GRs (Bellavance & Rivest, 2014). During acute stress, glucocorticoid binding to GRs enhances immune cell expression of anti-inflammatory cytokines. However, under chronic stress conditions, increasing levels of circulating glucocorticoids suppress immune system function (Petrovsky, 2001). In the current study, there was elevated GR expression in the B-lymphocytes in rats exposed to MD, isolation, and CMS when compared to rats that only received isolation and CMS. This binding of glucocorticoids to the GRs has been noted to inhibit the secretion of antibodies from B-lymphocytes (Zen *et al.*, 2011). Collectively, when considered with the plasma corticosterone data, this implies that the lower corticosterone levels in rats that were subject to MD are due to a higher level of binding. This suggests that in comparison to the rats not subjected to MD, there was a greater impact of the CMS exposure on the rats that were subject to MD. Alternatively, the expression of GR in B-lymphocytes increased to compensate for the significant decrease in plasma corticosterone levels associated with the stress, thus maintaining humoral immune responsiveness.

The second hypothesis is based on the concept that rats subjected to early life stress in the form of MD would respond differently to the recent life stress of CMS because of the early life stress. The rats that experienced MD and isolation had a longer period of cumulative stress that ended with the CMS exposure for a total of 115 days of manipulation. The rats only subjected to CMS spent only the 21-day period of CMS manipulation exposure to stress. This suggests that the rats subjected to MD were able to adapt to stress more effectively and possessed lower GC levels due to this. In the current study, the rats not subjected to MD had higher plasma GC levels possibly due to lack of previous early life stress exposure. This is impossible to determine due to the factorial design of the current study and the lack of facility reared control rats receiving no stress. However, the existing literature notes that rats subjected to MD possessed higher GC concentrations in adulthood than control rats, and similar elevations in GC concentration in rats subjected exposed to CMS when compared to unstressed controls (Liu *et al.*, 2014; Liu, W. *et al.*, 2013; Liu, X. *et al.*, 2013). This indicates that it is possible that the rats that only experienced

CMS had higher plasma GC levels because of no previous exposure to stress. Therefore, the rats only subjected to CMS did not have time to adapt to the stressor.

The behavioural challenge (MD, isolation level, and CMS) did result in different steroid hormone levels between the experimental groups. The plasma corticosterone of rats exposed to MD, isolation, and CMS was significantly lower than that of the other groups. The hypothesis in the current study was that this indicates a greater impact of stress on these rats, compared to the other experimental groups. The lower concentration of corticosterone at the time of death suggests that the available corticosterone had already been utilised by the body to cope with the cumulative stress exposure. The high expression of GRs in B-lymphocytes supports this with either the beginning of immunosuppression from a prolonged elevation of corticosterone or maintenance of humoral immune responsiveness (Blume *et al.*, 2011; Petrovsky, 2001). However, additional testing of GRs expression in the brain and the gene expression of downstream inflammatory markers that glucocorticoids often mediate would be required to elucidate this (Petrovsky, 2001; Russo-Marie, 1992). Collectively these results demonstrate the hormonal and physiological size changes occurred following early and recent life stress exposure in rats, with the significantly lower steroid concentrations found in the rats receiving both early and recent life stress.

There were three main limitations to the physiological component of the current study. Firstly, there were several parameters only trending towards significance, each experimental cohort had 15 rats, so if the number of rats was increased those parameters may reach significance. However, since this was an exploratory study examining the behavioural and physiological response to different stress exposures the number of animals was sufficient. The cellular parameters utilised only a subset of the study population with 20 randomly selected rats (*n* = five per experimental cohort). The histological measures trending towards significance may have been resolved as significant had additional samples been used, however, the financial constraints of the current project limited the number of tissue samples that could be sectioned and stained histologically. As mentioned previously, Wistar rats are a stress-resistant strain

(Ghadhanfar *et al.*, 2014; Yang *et al.*, 2012). Given that, the stress in the current study was sufficient to manifest a measurable change in stress-resilient subjects, exposing stress-susceptible rats (Carnevali *et al.*, 2016; Fischer *et al.*, 2012) to the same stressor combinations used in the current study could result in additional measures that only approached significance being resolved as statistically significant.

Second, the lack of a control group subjected to normal husbandry practices significantly limited the comparison and conclusions that can be drawn from the physiological parameters. As mentioned in Section 1.5 Rationale and Limitations of the Study Design, the original experimental design involved blood samples taken prior to and after the behavioural testing phase. However, due to the failure of the longitudinal blood sampling only post-mortem tissue samples were obtained. This resulted in the results being confound due to the lack of comparison to pre-stress physiological measures.

Third, there was also a limitation in LC-MS analysis since brain region noradrenaline, plasma dopamine, and plasma noradrenaline were unable to be quantified using LC-MS due to a high signal to noise ratio. This limited the comparison of these catecholamine markers. Another method could have been utilised for the detection of these metabolites such as: radioimmunoassay (RIA) or enzyme-linked immunosorbent assay (ELISA). However, LC-MS had the highest and most consistent sensitivity for each of the metabolites (catecholamine sensitivity was 10 pg/ml for all catecholamine metabolites; steroid hormone sensitivity 1.25 pg/ml for all steroid hormone metabolites); particularly, when compared to RIA (catecholamine sensitivity 19pg/ml – 50 pg/ml; steroid hormone sensitivity 0.05 ng/ml – 7.7 ng/ml) (Cisbio, 2018; LDN, 2010) or ELISA (catecholamine sensitivity 10 pg/ml – 49 pg/ml; steroid hormone sensitivity 5.67 pg/ml – 0.35 ng/ml) (Abnova, 2018) with both these methods having different detection limits for each individual metabolite. Overall, LC-MS was deemed the most cost-effective and optimum method given the available sample volume, the equipment availability, and required sensitivity for the metabolites of interest (Maeda *et al.*, 2013; Taves *et al.*, 2011). It was not possible to reanalyse the

metabolites using one of the other methods due to the limited sample volume, the financial constraints, and the lack of available equipment in the case of the RIA method. Therefore, the levels of the markers unable to be quantified (brain region noradrenaline, plasma dopamine, and plasma noradrenaline) should be comparable to the measured marker (adrenaline) in each case, since the catecholamine markers are in the same biosynthetic pathway (Linsell, Lightman, Mullen, Brown & Causon, 1985; Christensen, Vestergaard, Sorensen & Rafaelsen, 1980).

The focus for this chapter was to determine whether the post-mortem hormonal and cellular measures changed in response to early and later life stress, as well as to see if there were differences between the experimental cohorts based on MD and isolation. There was no indication of a significant difference in the impact of acute stress, allowing the analysis of the impact of chronic stress exposure without confound from acute stress due to euthanasia. There was a significant difference in the levels of plasma corticosterone, 11-deoxycorticosterone, and glucocorticoid receptor expression in B-lymphocytes. Overall, this indicated that the cumulative effect of MD, isolation, and CMS that caused a change in rat behaviour also influenced the post-mortem physiological markers of chronic stress for the rats. Given the changes to both the behavioural measures from Chapter 3 and the physiological markers in this chapter were induced in rats exposed to MD, isolation, and CMS when compared to rats that only received CMS. The next chapter explores the associations between these significant stress-induced physiological and behavioural changes.

Chapter 5 – Associations between Behavioural and Physiological Measures

5.1 Behavioural and Physiological Responses to Stress

The rats in the current study were revealed to have significant differences in the physiological measures (Chapter 4, Section 4.3) due to the different maternal deprivation and isolation manipulations that were combined with the chronic mild stress exposures (Table 5.1). This was in addition to the stress-induced behaviour changed observed in Chapter 3 (Section 3.3). The rats that were subjected to maternal deprivation (MD), isolation, and chronic mild stress (CMS) had the lowest concentration of plasma steroid hormones (corticosterone and 11-deoxycorticosterone) when compared to rats that only experienced CMS. This was counter to the expected results of higher glucocorticoid (GC) concentrations in rats that received MD, isolation, and CMS exposure based on the literature (Herrera-Perez *et al.*, 2017; Liu *et al.*, 2014; Liu, W. *et al.*, 2013; Liu, X. *et al.*, 2013; Volodina *et al.*, 2012; Marais *et al.*, 2008; Kalinichev *et al.*, 2002). In addition to this, rats subjected to MD, isolation, and CMS had the highest expression of the glucocorticoid receptor in B-lymphocytes when compared to rats exposed to isolation and CMS.

Based on the results of Chapter 4, the difference in the plasma steroid concentrations was hypothesised to be the rats subjected to MD, isolation, and CMS utilising the available plasma steroid hormones to manage the stress, when compared to rats only subjected to CMS. The difference in the glucocorticoid receptor expression was hypothesised to involve the maintenance of humoral immune responsiveness while the rats were under stress.

Table 5.1: Description of the treatment status for the experimental cohorts in the current study.

Treatments	**Experimental cohorts**	
	Visual (Control)	**Non-Visual (Treatment)**
Not Maternally Deprived (Control)	No MD and visual social support after weaning	No MD and no visual social support after weaning
Maternally Deprived (Treatment)	MD received and visual social support after weaning	MD received and no visual social support after weaning

In the previous chapters, the behavioural and physiological parameters were analysed separately to investigate any changes due to stress exposure, with the results reported in the previous two chapters. In this chapter, the level of association between statistically significant behavioural and physiological variables after MD and isolation manipulation, followed by chronic mild stress exposure was examined. To increase the statistical power the analysis used the pooled results from the experimental groups, on the assumption that any change in behavioural or physiological variables would be consistent within the factorial study design. Only behavioural data obtained post-CMS exposure was used due to only acquiring physiological data post-mortem.

The research question for this chapter was:

1) Are there any associations between the behavioural and physiological measures that significantly changed due to maternal deprivation, isolation, and chronic mild stress?

To address this question the data was analysed using Spearman's Rho correlation coefficient, due to the behavioural data not meeting the criteria for parametric correlation analysis. Measures from the previous chapters identified as significantly ($p < 0.05$) different after CMS exposure were used to calculate the correlation coefficients.

5.2 Spearman's Rho Correlation Analysis Results

The previous two chapters reported the analysis of the behavioural and physiological parameters. The behavioural parameters (Chapter 3) that were found to be statistically significant following stress included: frequency of horizontal motor activity, square immobility incidence, total object investigation, object 1 (glass tunnel) investigation, object 3 (aquarium figurine) investigation and the duration of square immobility incidence, object 1 investigation, and object 3 investigation. These behavioural measures were correlated with physiological markers determined to be statistically significant from Chapter 4 (Table 5.2). The significant markers included: B-

lymphocyte expression of glucocorticoid receptors (GRs), plasma corticosterone, and plasma 11-deoxycorticosterone (11-DOC) concentration.

*Table 5.2: Summary of behavioural measures correlated with physiological measures. Please note * indicates a significant correlation ($p < 0.05$).*

Behavioural Measure	Correlated with Physiological Measure	R^2 Value	*P*-value
Horizontal Motor Frequency Mean Post Stress	B-Lymphocyte Expression of GRs (CD19)	-0.08	0.56
	Plasma Corticosterone Concentration (ng/ml)	-0.14	0.34
	Plasma 11-DOC Concentration (ng/ml)	-0.26	0.08
Total Object Investigation Frequency Mean Post Stress	B-Lymphocyte Expression of GRs (CD19)	-0.08	0.56
	Plasma Corticosterone Concentration (ng/ml)	-0.12	0.41
	Plasma 11-DOC Concentration (ng/ml)	-0.23	0.13
Square Immobility Frequency Mean Post Stress	B-Lymphocyte Expression of GRs (CD19)	0.10	0.49
	Plasma Corticosterone Concentration (ng/ml)	-0.002	0.99
	Plasma 11-DOC Concentration (ng/ml)	-0.05	0.74
Object 1 Investigation Frequency Mean Post Stress	B-Lymphocyte Expression of GRs (CD19)	-0.12	0.41
	Plasma Corticosterone Concentration (ng/ml)	-0.12	0.41
	Plasma 11-DOC Concentration (ng/ml)	-0.29	0.06
Object 3 Investigation Frequency Mean Post Stress	B-Lymphocyte Expression of GRs (CD19)	0.03	0.83
	Plasma Corticosterone Concentration (ng/ml)	-0.31*	0.03
	Plasma 11-DOC Concentration (ng/ml)	-0.25	0.09
Square Immobility Duration Mean Post Stress	B-Lymphocyte Expression of GRs (CD19)	0.08	0.56
	Plasma Corticosterone Concentration (ng/ml)	-0.004	0.98
	Plasma 11-DOC Concentration (ng/ml)	0.05	0.74
Object 1 Investigation Duration Mean Post Stress	B-Lymphocyte Expression of GRs (CD19)	-0.04	0.77
	Plasma Corticosterone Concentration (ng/ml)	-0.08	0.59
	Plasma 11-DOC Concentration (ng/ml)	-0.26	0.08
Object 3 Investigation Duration Mean Post Stress	B-Lymphocyte Expression of GRs (CD19)	-0.06	0.67
	Plasma Corticosterone Concentration (ng/ml)	-0.33*	0.02
	Plasma 11-DOC Concentration (ng/ml)	-0.29*	0.05

There were statistically significant correlations between several behavioural and physiological parameters (Figure 5.1). Object 3 investigation frequency was observed to have a moderate negative correlation with plasma corticosterone concentration ($R^2 = 0.31$; $p = 0.03$). Additionally, the duration measure of object 3 investigation also showed a moderate negative correlation with plasma corticosterone concentration ($R^2 = 0.33$; $p = 0.02$) and a weak negative correlation with plasma 11-DOC concentration ($R^2 = 0.29$; $p = 0.05$).

There were also several correlation coefficients approaching significance between behavioural and physiological measures. There were weak negative correlations that were trending towards significance between the frequencies of horizontal motor activity ($R^2 = 0.26$; $p = 0.08$), object 1 investigation ($R^2 = 0.29$; $p = 0.06$), object 3 investigation ($R^2 = 0.25$; $p = 0.09$) and the plasma 11-DOC concentration. There was also a weak negative correlation trending towards significance between the duration of object 1 investigation and plasma 11-DOC concentration ($R^2 = 0.26$; $p = 0.08$). Collectively, these data added to the statistically significant correlations suggest an association between the concentrations of plasma steroid hormones and object investigation behaviours, specifically, object 1 the glass tunnel and object 3 the aquarium figurine.

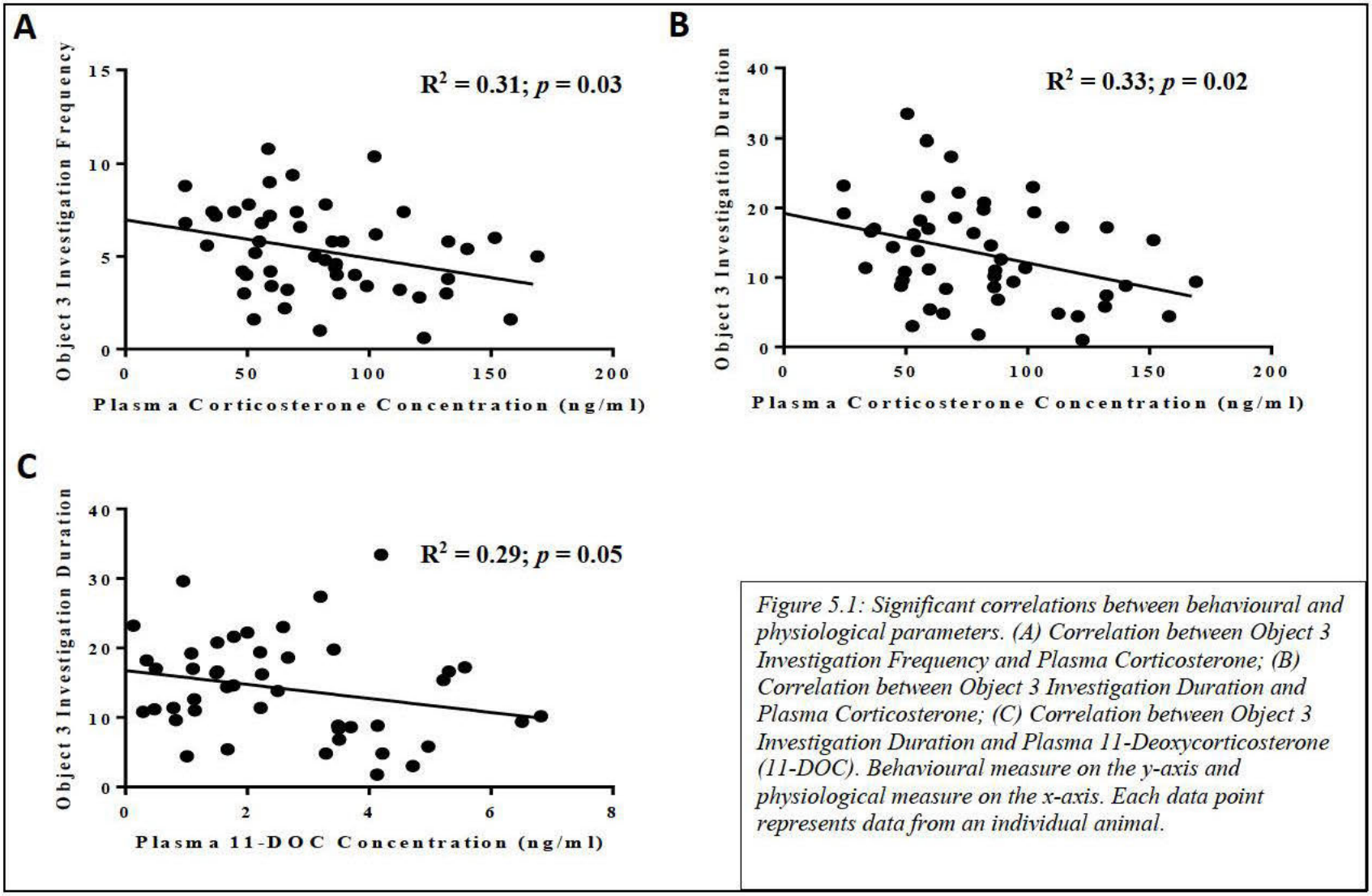

Figure 5.1: Significant correlations between behavioural and physiological parameters. (A) Correlation between Object 3 Investigation Frequency and Plasma Corticosterone; (B) Correlation between Object 3 Investigation Duration and Plasma Corticosterone; (C) Correlation between Object 3 Investigation Duration and Plasma 11-Deoxycorticosterone (11-DOC). Behavioural measure on the y-axis and physiological measure on the x-axis. Each data point represents data from an individual animal.

5.3 Association of Stress-Induced Behavioural and Physiological Changes

The further stratification of the diagnostic methods for depressive and anxiety disorders, particularly those triggered by stress, requires a multidisciplinary approach examining both behavioural phenotypes and potential physiological markers associated with stress. In addition to this, diagnosis of depressive and anxiety disorders currently relies on only behavioural and somatic symptoms (APA, 2013; WHO, 1992). In order to incorporate end-point physiological measures into the diagnosis for these disorders, it must be understood that any observed changes in hormone levels would be a result of the same cumulative stress that initially caused the depressive- or anxiety-like behaviours to manifest. Specifically, observations of changed behaviour or hormone concentrations by a clinician would be the end-point result of cumulative stress experienced by an individual as opposed to a point in time determination that is used as a baseline for any future diagnoses.

The use of animal models is critical in identifying potential physiological parameters for further study and associating those physiological changes with behavioural changes. Animal models have the further benefit of minimising the heterogeneity in how depressive- and anxiety-like behaviours are induced. Additionally, this allows further refinement of what defines animal models of depressive- or anxiety-like behaviours and how those models can respond to new pharmacological treatments. Previous chapters determined the statistically significant changes in longitudinal behavioural (Chapter 3) and end-point post-mortem physiological (Chapter 4) measures associated with stress. In this chapter, the pooled data from the experimental cohorts was used to determine if there was any association between behavioural and physiological parameters after maternal deprivation (MD) and isolation manipulations when followed by chronic mild stress (CMS) exposure.

Separately, MD, isolation, and CMS can each result in increased depressive- and anxiety-like behaviours as discussed in Chapter 3 (Section 3.3). However, there is considerable variability in the duration of behavioural manipulation and the strain of rat used in both MD and CMS

behavioural studies (Appendix E - Table 1 and Table 2 compare of early and later life stress study methodologies respectively). In the current study, correlation analysis used end-point data since the physiological measures were only assessed post-mortem. As a result, the physiological and behavioural data were the result of cumulative manipulations involving MD and isolation combinations, followed by CMS exposure to all rats. Collectively, the data in this chapter revealed an association between the concentrations of plasma steroid hormones (corticosterone and 11-deoxycorticosterone) and object investigation behaviours involving object 1 (the glass tunnel) and object 3 (the aquarium figurine). There was also an association between the frequency of horizontal motor activity and plasma 11-DOC concentration that was trending toward significance. The associations between locomotion and object investigation behaviours (horizontal movement, objects 1 and 3 specifically) and the concentration of post-mortem plasma glucocorticoids (GC) (corticosterone and 11-DOC) were negative associations, with high behavioural frequency or duration associated with lower GC concentration.

There were two hypotheses for this pattern in exploratory behaviour and plasma GC levels. Firstly, the rats subjected to MD and isolation manipulation in addition to CMS had lower plasma GC concentrations than rats that received only CMS. As discussed in Chapter 4 (Section 4.3), the literature indicates that stress exposure should increase the plasma GC concentration (Herrera-Perez *et al.*, 2017; Liu *et al.*, 2014; Liu, W. *et al.*, 2013). Additionally, the plasma GC levels at post-mortem for the rats that received only CMS exposure were not significantly greater than the daily average GC levels in unstressed rats noted in the literature (Romero *et al.*, 2013; Atkinson & Waddell, 1997). This suggested that the rats that experienced MD, isolation, and CMS were more resilient to later life stress as a result, than rats only subjected to CMS exposure. Therefore, the rats subject to MD and isolation manipulation with a lower GC response were hypothesised to have better adaptation to stress from the early life exposure, which minimised the physiological stress response. However, this does not explain the increased anxiety-like behaviour observed in the same rats that had the lowest plasma GC concentrations. Particularly, since a study by Suo *et al.* (2013) found that rats subjected to a predictable mild stress of five minutes of restraint for 28

days reduced anxiety-like behaviour. The increased anxiety-like behaviour indicates the combination of MD, isolation, and CMS did significantly stress the rats and changed the observed behaviour, which counters the assumption that the rats exposed to MD, isolation, and CMS adapted to the later life stress exposure based on the early life stress of the MD.

The other hypothesis for why this pattern of cumulative behavioural and post-mortem physiological measures was observed involved the rats exposed to the highest level of stress (MD, isolation, and CMS) being more advanced along the behavioural and physiological response to cumulative stress. That is, rather than these rats demonstrating adaptation to stress, the high anxiety-like behaviour and low GC concentration reflected the use of energy responding to the stressor (Raglan *et al.*, 2017). Specifically, the lower plasma GCs were due to the GC being utilised in other tissues to regulate the physiological response to stress, based on the observed low plasma GC levels and the increased expression of GR. This hypothesis fits the behavioural and physiological modifications observed in the current study more accurately than if the rats that received MD, isolation, and CMS were more adapted to the later life stress due to the early life stress exposure. Particularly, since early life stress exposure often blunts stress reactivity, reducing the effectiveness of the stress response system (Raglan *et al.*, 2017; Yehuda *et al.*, 2007; Meewisse, Reitsma, de Vries, Gersons & Olff, 2007; Kaufman, Plotsky, Nemeroff & Charney, 2000; Matthews, Robbins, Everitt & Caine, 1999). Collectively, this indicates that all the rats in the current study were responding to the CMS exposure but the rats that also received early life MD manipulation and current isolation were further along in the behavioural and physiological response to the stressor used.

This means that a longer stress period, or additional variable stressors, may result in all rats demonstrating similar behavioural and physiological changes, particularly, if an additional comparison could be made to unstressed control animals. As mentioned in Chapter 1 (Section 1.3.2 and Section 1.3.2.2), studies that examined the impact of early life maternal separation or deprivation observed increases in both depressive- and anxiety-like behaviours in the elevated

plus maze and open field tests (Trujillo *et al.*, 2016; Molet *et al.*, 2014; Marais *et al.*, 2008; Colorado *et al.*, 2006). This was highly dependent on the methodology of early life stress used (Eklund, & Arborelius, 2006; Ruedi-Bettschen, *et al.*, 2006). Studies that used unpredictable chronic stressors, chronic restraint, or isolation during adulthood, also found increased anxiety- and depressive-like behaviours in elevated plus maze, open field, and forced swim tests (Herrera-Perez *et al.*, 2017; Bravo, Torres-Sanchez, Alba-Delgado, Mico & Berrocoso, 2014; Zalosnik *et al.*, 2014; Karson, Demirtas, Bayramgürler, Balcı & Utkan, 2013; Evans *et al.*, 2012). Collectively, this is similar to the increased anxiety-like behaviours observed in the rats that were subjected to MD and isolation, followed by CMS exposure observed in the current study. Interestingly, there is also a varied physiological response to stress in the literature with both increase and decrease in plasma corticosterone concentrations following MD manipulation (Volodina, *et al.*, 2012; Marais, *et al.*, 2008; Kalinichev *et al.*, 2002) and CMS, restraint or isolation stressors separately (Liu *et al.*, 2014; Ferraz *et al.*, 2011; Herzog *et al.*, 2009; Carobrez *et al.*, 2002). No other study has specifically combined MD and isolation manipulations with a later life CMS exposure or used similar methods as the current study. However, a limited number of studies have looked at both behavioural and post-mortem physiological parameters in response to stressors (Gagliano *et al.*, 2008; Belda, Marquez & Armario, 2004) or used combined stress methodology (Trujillo *et al.*, 2016; Zalosnik *et al.*, 2014) like the current study. Furthermore, in other studies, the focus was skewed to either behavioural or physiological outcomes rather than the potential associations between stress-induced behaviours and physiological measures of depressive- or anxiety-like symptoms.

Collectively, this makes comparisons to the current study results difficult, given the combined stress methodology used, but based on what is known from the literature, the results of the current study suggest that longer stress durations could resolve many of the measures only approaching statistical significance. This indicates that the rats only subjected to CMS may take longer to demonstrate the same behavioural and physiological changes observed in the rats that received MD, isolation, and CMS. Furthermore, comparison of the behavioural and physiological

changes observed in the current study to facility-reared control rats would also reveal the extent that the experimental cohorts are different from unstressed rats. A comparison to age-matched unstressed control rats was outside the scope of the current study, but that these changes were evident in Wistar rats is a good indication that comparison to a more stress-susceptible strain, in addition to unstressed rats, would also be beneficial. Particularly, since the development of depressive and anxiety disorders in humans is frequently preceded by a significant stress or a series of stress events, and the response to stress often depends on the given individual and their available support structures. Defining these stress-induced behavioural and physiological measures in a rat model will allow a more in-depth study of depressive and anxiety disorder development, which in turn will improve understanding of how these disorders develop in humans.

The current study investigated the cumulative behavioural and post-mortem physiological changes associated with stress-induced depressive- or anxiety-like symptoms in rats, due to the role stress often plays in the development of depressive and anxiety disorders in humans. The use of stress-resistant Wistar rats in the current study resulted in several of the behavioural and physiological measures examined in the previous chapter only approaching significance. The exclusion of non-significant and approaching significant measures from the correlation analysis limits the insight into the association between the behavioural and physiological measures. However, there would be limited use in including non-significant measures in the analysis given the exploratory nature of the current study into stress-induced behavioural and physiological measures. The development of behavioural indices to be analysed against each physiological metric could help mitigate this, allowing a stronger classification to be applied based on exposure to MD, isolation, and CMS. This would allow even non-significant behavioural metrics to be analysed against the physiological parameters. However, replication of the study using a stress-susceptible rat strain may also resolve some measures as significant and further elucidate potential associations between behavioural and physiological parameters associated with stress. This would also allow further investigation into stress resilience in rodent models of depressive- and anxiety-

like disorder development. Ideally, a comparative study between the stress-resistant Wistar rats and a more stress-responsive rat strain such as Flinders Sensitive or Wistar-Kyoto (Shetty & Sadananda, 2017; Carnevali *et al.*, 2016) may manifest behavioural and physiological changes that only approached significance in Wistar rats. Thus, this would facilitate additional measures to be used to resolve the relationship between cumulative stress-associated behavioural and end-point physiological measures in animal models of depressive- and anxiety-like disorders. Furthermore, this would allow further definition of a combined behavioural and physiological profile for depressive- or anxiety-like symptoms in rodent models that could also be used to further stratify diagnosis of depressive and anxiety disorders in humans.

This chapter examined the associations between cumulative behavioural and post-mortem physiological measures that were determined significant from the previous statistical analysis. There were three statistically significant correlations between different cumulative behavioural and post-mortem physiological measures that were identified in this process, with an additional four correlations approaching significance. However, the significant associations were only moderate strength correlations, while the correlations approaching significance were only weak associations. Additional testing would be required to elucidate the relationship between correlated behavioural and physiological measurements. The aim of this chapter was to determine the associations between the cumulative behavioural and post-mortem physiological measures that were significantly modified as a result of MD, social isolation, and CMS exposure. In the current study, the main associations were between object investigation behaviours and the plasma steroid hormone concentrations, significantly altered due to the behavioural manipulation. However, further investigation would be required into the comparison of significant associations in the stress-resistant strain of Wistar rats to a more stress-susceptible rat strain, to understand the relationship between these associated behaviours and physiological markers.

Chapter 6 – General Conclusions and Future Directions

6.1 Introduction

The fundamental goal of this thesis was to examine the effect of cumulative stress on rodent behaviour and physiology; specifically, to examine potential associations between cumulative behavioural and post-mortem physiological measures in a rodent model of depressive- and anxiety-like disorders. Depressive and anxiety disorders are the most widespread mental disorders worldwide. As discussed in Chapter 1 (Section 1.1 and Section 1.2), these disorders represent an enormous economic burden on society, with both direct and indirect associated costs. There are specific impacts depressive and anxiety disorders have on an individual's ability to maintain occupational, social, and personal commitments. In humans, the manifestation of clinical depressive and anxiety disorders often involves genetic and significant stressful environmental exposures resulting in: sadness, irritable mood, and loss of pleasure in once enjoyed activities. Somatic and cognitive alterations accompany these symptoms, further affecting an individual's ability to function normally. This manifestation of symptoms involves complex phenomena with multiple potential aetiologies.

There is a limitation in the diagnosis of depressive and anxiety disorders in humans due to the large overlap in potential symptoms (Garcia *et al.*, 2016; Cummings *et al.*, 2014; APA, 2013; Tiller, 2012). A shared or causal aetiology has been theorised between depressive and anxiety disorders, though the precise relationship between depressive and anxiety disorders has yet to be fully elucidated (Taporoski *et al.*, 2015; Lohoff, 2010). Investigations into the development of these disorders in humans often use non-invasive investigative methods and post-mortem tissues, or minimally invasive blood tests. These methods do have limitations due to the heterogeneity of depressive and anxiety disorders, inability to examine underlying developmental mechanisms, or confounding factors related to post-mortem tissues, as stated in Chapter 1 (Section 1.3.4). Rodent models can be predictive of human disorders, allowing depressive and anxiety disorders to be studied while minimising of the heterogeneity from genetic and environmental sources. This, in

turn, allows research into the potential underpinning mechanisms and relationships between the pathophysiological markers and behavioural manifestations in depressive and anxiety disorders. This allows further stratification of the diagnosis of depressive and anxiety disorders that can use such physiological markers, with prior information from rodent models effectively limiting the target examinable areas in human post-mortem and neuroimaging studies. The level of association between depressive- and anxiety-like behaviours in a rodent model to the physiological responses needs to be established and validated, so that such models are useful for research into these human disorders.

6.2 Summary of Behavioural and Physiological Response to Stress in Rats

In rodent models, a common method of inducing depressive- and anxiety-like behaviours is exposure to stressful stimuli. The stressful stimulus mimics how stressful life events often precede the development of depressive and anxiety disorders in humans. Previous studies in rats have demonstrated that stress exposure using maternal deprivation (MD), social isolation, and chronic mild stress (CMS) exposure can produce behavioural and physiological changes analogous to depressive and anxiety-like disorders (Herrera-Perez *et al.*, 2017; Willner, 2017; Menard *et al.*, 2016; Trujillo *et al.*, 2016; Evans *et al.*, 2012). In Chapter 2 of the current study, the methods used to trigger stress-associated behavioural and physiological modifications in rats were described. These involved a factorial study design using combinations of MD and isolation, followed up with CMS exposure for all rats. This mimicked combinations of early and recent life stress that often precedes the development of depressive or anxiety disorders in humans. Specifically, assessing stress associated measures, such as: catecholamine and steroid hormone concentrations in plasma, adrenal gland, and brain tissues and cellular changes in the same tissues, with the stress-induced changes to the rat behaviour.

In Chapter 3, the behaviours that significantly changed during behavioural observation open field (BOOF) testing involved increased locomotion and object exploration in the behavioural

testing arena. This hyperactivity in the behavioural arena, when compared to the literature on rat behaviour following stress was determined to indicate anxiety-like behaviour (Herrera-Perez *et al.*, 2017; Menard *et al.*, 2016; Rana *et al.*, 2016; Trujillo *et al.*, 2016; Krishnan & Nestler, 2011; Neumann *et al.*, 2011). This behavioural change was attributed to the different stress combinations of MD and isolation that the experimental cohorts received, since all cohorts were subjected to CMS exposure. In addition to the cumulative behavioural measures, there were also stress associated post-mortem physiological measures that changed following the behavioural challenge.

Chapter 4 involved examination of stress associated post-mortem endocrine and cellular measures after the behavioural study to determine whether there were differences in the stress-associated responses of the experimental cohorts. There was no indication of a statistical difference between the cohorts in post-mortem acute stress associated catecholamines (noradrenaline, adrenaline, and dopamine) nor was there any significant difference between the experimental cohorts for the morphological measures involving the adrenal glands, hippocampus, or paraventricular nucleus of the hypothalamus. There were differences in the post-mortem steroid hormones associated with chronic stress, specifically in plasma samples. The hormones that were significantly different between the experimental cohorts were post-mortem plasma corticosterone and 11-deoxycorticosterone, which are in the same biosynthetic pathway. This indicated that the combination of MD and isolation resulted in different post-mortem physiological measures following CMS exposure. There was also a significant increase in the expression of glucocorticoid receptors (GRs) in a white blood cell subpopulation, specifically B-lymphocytes. The experimental cohorts with the lowest corticosterone concentrations also possessed the highest GR expression levels in B-lymphocytes. This was hypothesised to be due to maintaining humoral immune responsiveness between corticosterone and the glucocorticoid receptor. However, the sample concentrations in the physiological measures were from post-mortem samples only, due to the failure of obtaining time course blood samples prior to stress exposure for additional analysis. This meant that comparisons were only made between experimental cohorts not against baseline

levels prior to CMS. This decision increased statistical power for the study by increasing the number of animals present at the end of the study.

Finally, Chapter 5 examined the results for associations between the cumulative behavioural and post-mortem physiological measures based on the results identified as significant in the previous two chapters. This was based on how depressive and anxiety disorders in humans are diagnosed by cumulative changes in behaviour but there are often physiological changes from the change in behaviour at the time of diagnosis as well (APA, 2013; WHO, 1992). In the current study, there were associations between the stress-induced behavioural and physiological changes. Specifically, associations were observed between the frequency and duration of locomotion and exploratory behaviours (horizontal motor activity, object 1 investigation, and object 3 investigation) with the plasma concentrations of corticosterone and 11-deoxycorticosterone. However, these correlations between the cumulative behaviour and post-mortem physiology were only of moderate and weak strength. It was hypothesised that the different behavioural manipulations (maternal deprivation and isolation combinations, followed by CMS exposure) resulted in the different rat cohorts being at different stages of response to stress. The distinction in the current study, from the literature, was that the stressors applied triggered the cumulative behavioural and end-point physiological changes in Wistar rats. The Wistar strain is considered a stress-resistant rat strain, meaning that they are not as responsive to stressful stimuli compared to other more susceptible strains (Carnevali *et al.*, 2016; Ghadhanfar *et al.*, 2014; Yang *et al.*, 2012). The factorial study design maximised the statistical power for the behavioural analysis in a stress-resistant rat strain.

Collectively, these results reveal that the long-term stress combinations like those used in the current study triggered anxiety-like behaviours and modified physiological markers in stress-resistant rats. The literature notes that both depressive- and anxiety-like behaviours can result from MD, isolation, and CMS as separate stress exposures (Herrera-Perez *et al.*, 2017; Willner, 2017; Menard *et al.*, 2016; Trujillo *et al.*, 2016; Evans *et al.*, 2012). There is also literature that

demonstrates that stress triggers physiological changes (Liu *et al.*, 2014; Liu, W. *et al.*, 2013; Volodina *et al.*, 2012; Reus *et al.*, 2011; Ruedi-Bettschen *et al.*, 2006; Kalinichev *et al.*, 2002). It is likely that the different results in studies from the literature were a result of the various stress methodologies, intensities and durations, as well as different behavioural tests that were used (Chapter 5, Section 5.3 and Appendix E, Table 1 and Table 2). The stress combinations used in the current study did trigger both behavioural and physiological changes in stress-resistant rats and these stress-induced changes can be associated with each other. This suggests that the same stress combinations in stress-susceptible animals may show additional changes in behavioural and physiological markers that could be used to develop physiological profiles for use in conjunction with the behavioural symptoms used to diagnose depressive and anxiety disorders in humans.

The current research represents a key step in determining how physiological markers can be combined with behavioural changes to help stratify depressive and anxiety disorder diagnosis. Even the primary diagnostic guides such as the DSM-5 and ICD-10 note that physiological markers are needed to further improve the diagnosis of depressive and anxiety disorders. However, some of the markers in the current study are difficult to examine effectively in humans with depressive and anxiety disorders due to the heterogeneity of these disorders, as discussed in Chapter 1 Section 1.2.5 and Section 1.3.4. Animal models represent an effective method of examining such stress-induced behavioural and physiological modifications, allowing more effective models to be developed for depressive and anxiety disorder research. Moreover, animal models allow more rapid research to be conducted to screen potential physiological markers for use in further depressive and anxiety disorder research in humans. The current study supports that the behavioural symptoms of depressive- or anxiety-like behaviours in rats can be associated with physiological markers from an external stress trigger. It is possible that when stress precedes the development of depressive and anxiety disorders in humans (Chapter 1, Section 1.2.3) there are physiological markers that may be effectively associated with the behavioural symptoms used for diagnosis. However, further research is still required to expand on potential stress-induced markers.

6.3 Future Research Directions

The results highlight opportunities for future research in a number of areas. Firstly, the methods described in Chapter 2 described the BOOF test (Section 2.2.5) as a method to screen potential behaviours before and after CMS exposure. Additional behavioural tests would expand the potentially changed behaviours examined before and after CMS exposure. This would further develop the behavioural profile of these rats and allow better behavioural classification. Other behavioural tests were described in Chapter 1 (Section 1.3.3), these include: sucrose preference test, social interaction test, or light/dark box. Furthermore, given that the current study used a stress-resistant rat strain, additional insight about stress-induced behaviours could be available by subjecting a stress-susceptible rat strain to the same experimental procedure. This could increase the separation between the experimental cohorts for the behavioural and physiological measures. These assessments could help refine depressive- or anxiety-like behaviour profiles in rats. Behavioural indices from the behaviours (locomotion, immobility, grooming, and object investigation) defined and discussed in Chapter 3, in combination with results from other behavioural tests could be implemented as an association matrix, and would allow a stronger behavioural profile instead of discrete behaviours to be analysed against physiological measures.

Secondly, further investigation into the physiological measures discussed in Chapter 4, would allow the impact of stress on the gene expression levels for noradrenaline, adrenaline, dopamine, corticosterone, 11-deoxycorticosterone, progesterone, and testosterone to be examined. Additionally, the gene expression for the receptors associated with these hormones would allow elucidation of the up- or down-regulation of receptor expression due to stress as well as the location of those markers in tissues involved with depressive or anxiety disorder symptoms. Examination of the upstream regulators and downstream effects of these markers at the rate-limiting steps in the biosynthetic pathways would provide more information about the role of these hormones in depressive or anxiety disorder development. Examination of stress impacted

gene products would allow a greater understanding of the role that environmental stress plays in the development of depressive and anxiety disorders.

Third, additional research should be conducted comparing the results in Wistar rats, to a more stress susceptible rat strains such as the black hooded rat or August rat (Shetty & Sadananda, 2017; Carnevali *et al.*, 2016). This would help to determine additional behavioural and physiological measures that significantly changed due to the stress exposure of MD, isolation, and CMS. Furthermore, additional facility raised rats would help establish baseline reference values to determine whether the change in behavioural and physiological measures in the rats used was significantly different from rodents not exposed to stressful stimuli.

Collectively, this information could help generate corresponding physiological indices that could be analysed in conjunction with a behavioural profile matrix. This would allow better definition of behavioural endophenotypes in rodent models of depressive and anxiety disorders by adding corresponding physiological measures that are present. Furthermore, these matrixes of behavioural and physiological measures can be utilised to elucidate the mechanism(s) of depressive and anxiety-like disorders in rat models. This can expand onwards to stratify the existing diagnostic methods for depressive and anxiety disorders in humans.

6.4 Conclusions

This study has demonstrated that the combination of early life stress, isolation, and later life stress in rats did manifest behavioural and physiological changes associated with stress, but no anatomical changes. The behavioural changes for the rats in the current study were identified as anxiety-like behaviours. There were associations between stress-associated behaviours and post-mortem physiological measures. The research has established that this combination of stress exposure can induce associated cumulative behavioural and end-point physiological changes even in a stress-resistant rat strain. Further research is required to continue to better define depressive- and anxiety-like behaviours in rats, which in turn will further improve the understanding of the development of depressive and anxiety disorders in humans.

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Appendices

Appendix A – Ethics Approval

University of New England

AUTHORITY No.: AEC13-050

ANIMAL ETHICS COMMITTEE

ANIMAL RESEARCH AUTHORITY
And Approval for Animal Experimentation

RESEARCH TEAM:	Ms Linda Agnew, Prof Christopher Sharpley, Ms Nicarla Glyde & Mr Robert Hart
EMERGENCY CONTACT	Ms Linda Agnew 02 67732631, 02 67720114 or 0423505735

Are authorised to conduct the following research:

TITLE:	A rodent model of depression for the development of a panel of biomarkers
LOCATION(S):	UNE Animal House, Ring Road UNE, Armidale NSW 2351

ANIMALS:

Species	Strain	No's Required	Procedure Details
02 - Laboratory Rats	Wistar	70	6

This authority remains in force from **22/05/2013** to **30/12/2014** unless suspended, cancelled or surrendered.

This statement must be read in conjunction with the Conditions for Animal Experimentation at UNE as stated on the reverse.

[redacted]

Jo-Ann Sozou
AEC Secretary and UNE Research

04/06/2014 A13/2270

Appendix B - BOOF Checklist and Behaviour Definitions.

BOOF testing round:

BOOF testing day:

Rat cage number:

Behaviour	Frequency	Duration
Vertical motor activity (Rearing)		
Horizontal motor activity (Line Crossing)		
Time spent in peripheral quadrants		
Time spent in central quadrants		
Middle Quadrant entries		
Grooming Initiated		
Stereotypic behaviour (Grooming)		
Immobility		
Immobility in Corner		
Immobility in Square		
Time Spent in Tunnel (sedentary)		
Time Spent in Tunnel (vigilant)		
Time Spent investigating objects overall		
Object 1 investigation – Tunnel		
Object 2 investigation – Cat Toy		
Object 3 investigation – Aquarium figure		
Object 4 investigation – Peg		

Additional comments:

Behaviour Definitions

Rearing: Defined as the lifting of forepaws from ground and extension of the body in a vertical direction in non-grooming activity. Animals must return to all 4 paws down then rear again for it to be 2 separate rears.

Line crossing: Defined as 2 paws over the gridline

- Peripheral quadrants are the 16 squares that form the outer line against the walls
- Central quadrants are the 9 squares within the middle of the arena
- Central quadrant entries means only the entries into the central quadrants from the peripheral quadrants, not each line cross while in the central area

Grooming initiated: Defined as steady grooming of paws, face and body. Interruption with a different behaviour or object interaction save looking around indicates 2 grooming initiations.

- **Stereotypic Grooming:** Defined as repetitive grooming within a short time frame or frantic repeated grooming

Immobility: Defined as no gross motor movement for 1 sec. Scored using a metronome

- Immobility to have an additional component of in a given square

The objects according to the checklist are as follows:

- Object 1 – Glass tunnel
- Object 2 – Cat toy
- Object 3 – Aquarium figurine (Barrel, Skull or Bridge)
- Object 4 – Coloured peg

Object exploration: Defined as biting, touching the object with nose or paws or sniffing the objects at a <1cm distance. Interruption with a different behaviour must occur for 2 separate investigations to be counted.

The exploration measure is for each item individually as well as a sum of all the exploration

Appendix C – Univariate and Tukey's *post-hoc* test statistical tables from Chapter 3

Table 1: Repeated measure-ANOVA Results for mean frequency of locomotion behaviours during the BOOF test before and after chronic stress exposure.

Measure	Effect	Wilks' Λ Value	F	df	Sig.	Partial η^2
Vertical Motor Activity Pre-Stress	Time	0.786	3.601	4.0	0.011	0.214
	Matsocial	-	1.309	3.0	0.280	0.066
	Time*Matsocial	0.880	0.582	12.0	0.854	0.042
Vertical Motor Activity Post-Stress	Time	0.925	1.068	4.0	0.381	0.075
	Matsocial	-	1.115	3.0	0.351	0.056
	Time*Matsocial	0.784	1.125	12.0	0.345	0.078
Horizontal Motor Activity Pre-Stress	Time	0.444	16.579	4.0	<0.0001	0.556
	Matsocial	-	1.651	3.0	0.188	0.081
	Time*Matsocial	0.818	0.926	12.0	0.523	0.065
Horizontal Motor Activity Post-Stress	Time	0.836	2.593	4.0	0.047	0.164
	Matsocial	-	3.663	3.0	0.018	0.164
	Time*Matsocial	0.810	0.971	12.0	0.480	0.068
Centre Entries Pre-Stress	Time	0.897	1.528	4.0	0.207	0.103
	Matsocial	-	0.084	3.0	0.968	0.005
	Time*Matsocial	0.744	1.388	12.0	0.178	0.094
Centre Entries Post-Stress	Time	0.888	1.669	4.0	0.171	0.112
	Matsocial	-	1.304	3.0	0.282	0.065
	Time*Matsocial	0.813	0.953	12.0	0.496	0.067

Table 2: Tukey *Post-Hoc* analysis from repeated measure ANOVA for mean frequency of locomotion behaviours for all experimental cohorts (Not Deprived Visual, Not Deprived Non-Visual, Deprived Visual, and Deprived Non-Visual) before and after CMS exposure.

Dependent Variable	(I) Maternal Deprivation and Social Support	(J) Maternal Deprivation and Social Support	Mean Difference (I-J)	Std. Error	Sig.
Vertical Motor Activity Pre-Stress	Not Deprived Visual	Not Deprived Non-Visual	-2.76	3.68	0.876
		Deprived Visual	-0.69	3.68	0.998
		Deprived Non-Visual	-6.63	3.68	0.284
	Not Deprived Non-Visual	Deprived Visual	2.07	3.68	0.943
		Deprived Non-Visual	-3.87	3.68	0.721
	Deprived Visual	Deprived Non-Visual	-5.93	3.68	0.380
Vertical Motor Activity Post-Stress	Not Deprived Visual	Not Deprived Non-Visual	-3.25	4.23	0.868
		Deprived Visual	1.27	4.23	0.991
		Deprived Non-Visual	-5.69	4.23	0.537
	Not Deprived Non-Visual	Deprived Visual	4.52	4.23	0.709
		Deprived Non-Visual	-2.44	4.23	0.938
	Deprived Visual	Deprived Non-Visual	-6.96	4.23	0.361
Horizontal Motor Activity Pre-Stress	Not Deprived Visual	Not Deprived Non-Visual	-16.15	20.97	0.868
		Deprived Visual	-32.41	20.97	0.418
		Deprived Non-Visual	-43.60	20.97	0.173
	Not Deprived Non-Visual	Deprived Visual	-16.27	20.97	0.865
		Deprived Non-Visual	-27.45	20.97	0.561
	Deprived Visual	Deprived Non-Visual	-11.19	20.97	0.951
Horizontal Motor Activity Post-Stress	Not Deprived Visual	Not Deprived Non-Visual	-20.36	17.97	0.671
		Deprived Visual	-40.53	17.97	0.121
		Deprived Non-Visual	-55.93*	17.97	0.015
	Not Deprived Non-Visual	Deprived Visual	-20.17	17.97	0.677
		Deprived Non-Visual	-35.57	17.97	0.208
	Deprived Visual	Deprived Non-Visual	-15.40	17.97	0.827
Centre Entries Pre-Stress	Not Deprived Visual	Not Deprived Non-Visual	-0.41	1.44	0.992
		Deprived Visual	-0.36	1.44	0.994
		Deprived Non-Visual	-0.72	1.44	0.958
	Not Deprived Non-Visual	Deprived Visual	0.05	1.44	1.0
		Deprived Non-Visual	-0.31	1.44	0.997
	Deprived Visual	Deprived Non-Visual	-0.36	1.44	0.994
Centre Entries Post-Stress	Not Deprived Visual	Not Deprived Non-Visual	-1.67	1.52	0.693
		Deprived Visual	-1.76	1.52	0.655
		Deprived Non-Visual	-2.99	1.52	0.213
	Not Deprived Non-Visual	Deprived Visual	-0.09	1.52	1.0
		Deprived Non-Visual	-1.32	1.52	0.821
	Deprived Visual	Deprived Non-Visual	-1.23	1.52	0.851

Table 3: Repeated measure-ANOVA Results for mean frequency of grooming behaviours during the BOOF test before and after chronic stress exposure.

Measure	Effect	Wilks' Λ Value	F	df	Sig.	Partial η^2
Total Grooming Pre-Stress	Time	0.911	1.295	4.0	0.284	0.089
	Matsocial	-	0.661	3.0	0.580	0.034
	Time*Matsocial	0.789	1.096	12.0	0.368	0.076
Total Grooming Post-Stress	Time	0.885	1.717	4.0	0.160	0.115
	Matsocial	-	2.115	3.0	0.109	0.102
	Time*Matsocial	0.827	0.874	12.0	0.575	0.061
Normal Grooming Pre-Stress	Time	0.853	2.279	4.0	0.073	0.147
	Matsocial	-	0.526	3.0	0.667	0.027
	Time*Matsocial	0.861	0.679	12.0	0.769	0.049
Normal Grooming Post-Stress	Time	0.773	3.895	4.0	0.008	0.227
	Matsocial	-	2.063	3.0	0.116	0.100
	Time*Matsocial	0.742	1.401	12.0	0.172	0.095
Stereotypic Grooming Pre-Stress	Time	0.959	0.569	4.0	0.686	0.041
	Matsocial	-	0.962	3.0	0.417	0.049
	Time*Matsocial	0.703	1.671	12.0	0.079	0.111
Stereotypic Grooming Post-Stress	Time	0.878	1.847	4.0	0.134	0.122
	Matsocial	-	1.219	3.0	0.311	0.061
	Time*Matsocial	0.831	0.847	12.0	0.603	0.060

Table 4: Tukey *Post-Hoc* analysis from repeated measure ANOVA for mean frequency of grooming behaviours for all experimental cohorts (Not Deprived Visual, Not Deprived Non-Visual, Deprived Visual, and Deprived Non-Visual) before and after CMS exposure.

Dependent Variable	(I) Maternal Deprivation and Social Support	(J) Maternal Deprivation and Social Support	Mean Difference (I-J)	Std. Error	Sig.
Total Grooming Pre-Stress	Not Deprived Visual	Not Deprived Non-Visual	0.69	0.54	0.580
		Deprived Visual	0.13	0.54	0.995
		Deprived Non-Visual	0.44	0.54	0.849
	Not Deprived Non-Visual	Deprived Visual	-0.56	0.54	0.731
		Deprived Non-Visual	-0.25	0.54	0.966
	Deprived Visual	Deprived Non-Visual	0.31	0.54	0.942
Total Grooming Post-Stress	Not Deprived Visual	Not Deprived Non-Visual	0.36	0.48	0.874
		Deprived Visual	0.51	0.48	0.714
		Deprived Non-Visual	1.17	0.48	0.078
	Not Deprived Non-Visual	Deprived Visual	0.15	0.48	0.990
		Deprived Non-Visual	0.81	0.48	0.331
	Deprived Visual	Deprived Non-Visual	0.67	0.48	0.507
Normal Grooming Pre-Stress	Not Deprived Visual	Not Deprived Non-Visual	0.13	0.24	0.948
		Deprived Visual	-0.01	0.24	1.0
		Deprived Non-Visual	-0.17	0.24	0.894
	Not Deprived Non-Visual	Deprived Visual	-0.15	0.24	0.932
		Deprived Non-Visual	-0.31	0.24	0.597
	Deprived Visual	Deprived Non-Visual	-0.16	0.24	0.914
Normal Grooming Post-Stress	Not Deprived Visual	Not Deprived Non-Visual	-0.11	0.18	0.932
		Deprived Visual	0.16	0.18	0.807
		Deprived Non-Visual	0.31	0.18	0.324
	Not Deprived Non-Visual	Deprived Visual	0.27	0.18	0.448
		Deprived Non-Visual	0.41	0.18	0.107
	Deprived Visual	Deprived Non-Visual	0.15	0.18	0.844
Stereotypic Grooming Pre-Stress	Not Deprived Visual	Not Deprived Non-Visual	0.56	0.44	0.579
		Deprived Visual	0.15	0.44	0.987
		Deprived Non-Visual	0.61	0.44	0.503
	Not Deprived Non-Visual	Deprived Visual	-0.41	0.44	0.781
		Deprived Non-Visual	0.05	0.44	0.999
	Deprived Visual	Deprived Non-Visual	0.47	0.44	0.711
Stereotypic Grooming Post-Stress	Not Deprived Visual	Not Deprived Non-Visual	0.47	0.46	0.739
		Deprived Visual	0.35	0.46	0.873
		Deprived Non-Visual	0.87	0.46	0.243
	Not Deprived Non-Visual	Deprived Visual	-0.12	0.46	0.994
		Deprived Non-Visual	0.40	0.46	0.819
	Deprived Visual	Deprived Non-Visual	0.52	0.46	0.669

Table 5: Repeated measure-ANOVA Results for mean frequency of immobility behaviours during the BOOF test before and after chronic stress exposure.

Measure	Effect	Wilks' Λ Value	F	df	Sig.	Partial η^2
Total Immobility Pre-Stress	Time	0.518	12.315	4.0	<0.0001	0.482
	Matsocial	-	2.626	3.0	0.059	0.123
	Time*Matsocial	0.586	2.624	12.0	0.003	0.163
Total Immobility Post-Stress	Time	0.682	6.169	4.0	<0.0001	0.318
	Matsocial	-	1.808	3.0	0.156	0.088
	Time*Matsocial	0.686	1.794	12.0	0.054	0.118
Corner Immobility Pre-Stress	Time	0.488	13.890	4.0	<0.0001	0.512
	Matsocial	-	1.722	3.0	0.173	0.084
	Time*Matsocial	0.569	2.777	12.0	0.002	0.171
Corner Immobility Post-Stress	Time	0.665	6.661	4.0	<0.0001	0.335
	Matsocial	-	1.291	3.0	0.286	0.065
	Time*Matsocial	0.675	1.879	12.0	0.042	0.123
Square Immobility Pre-Stress	Time	0.902	1.441	4.0	0.233	0.098
	Matsocial	-	3.426	3.0	0.023	0.155
	Time*Matsocial	0.899	0.483	12.0	0.922	0.035
Square Immobility Post-Stress	Time	0.874	1.911	4.0	0.122	0.126
	Matsocial	-	3.437	3.0	0.023	0.156
	Time*Matsocial	0.825	0.885	12.0	0.564	0.062

Table 6: Tukey *Post-Hoc* analysis from repeated measure ANOVA for mean frequency of immobility behaviours for all experimental cohorts (Not Deprived Visual, Not Deprived Non-Visual, Deprived Visual, and Deprived Non-Visual) before and after CMS exposure.

Dependent Variable	(I) Maternal Deprivation and Social Support	(J) Maternal Deprivation and Social Support	Mean Difference (I-J)	Std. Error	Sig.
Total Immobility Pre-Stress	Not Deprived Visual	Not Deprived Non-Visual	3.16	2.52	0.596
		Deprived Visual	-3.88	2.52	0.422
		Deprived Non-Visual	0.27	2.52	1.0
	Not Deprived Non-Visual	Deprived Visual	-7.04*	2.52	0.035
		Deprived Non-Visual	-2.89	2.52	0.662
	Deprived Visual	Deprived Non-Visual	4.15	2.52	0.363
Total Immobility Post-Stress	Not Deprived Visual	Not Deprived Non-Visual	4.01	2.58	0.411
		Deprived Visual	-1.85	2.58	0.889
		Deprived Non-Visual	0.65	2.58	0.994
	Not Deprived Non-Visual	Deprived Visual	-5.87	2.58	0.116
		Deprived Non-Visual	-3.36	2.58	0.564
	Deprived Visual	Deprived Non-Visual	2.51	2.58	0.765
Corner Immobility Pre-Stress	Not Deprived Visual	Not Deprived Non-Visual	3.29	2.50	0.557
		Deprived Visual	-2.36	2.50	0.782
		Deprived Non-Visual	0.63	2.50	0.994
	Not Deprived Non-Visual	Deprived Visual	-5.65	2.50	0.121
		Deprived Non-Visual	-2.67	2.50	0.712
	Deprived Visual	Deprived Non-Visual	2.99	2.50	0.634
Corner Immobility Post-Stress	Not Deprived Visual	Not Deprived Non-Visual	4.29	2.43	0.301
		Deprived Visual	0.31	2.43	0.999
		Deprived Non-Visual	1.53	2.43	0.922
	Not Deprived Non-Visual	Deprived Visual	-3.99	2.43	0.366
		Deprived Non-Visual	-2.76	2.43	0.670
	Deprived Visual	Deprived Non-Visual	1.23	2.43	0.958
Square Immobility Pre-Stress	Not Deprived Visual	Not Deprived Non-Visual	-0.13	0.43	0.990
		Deprived Visual	-1.25*	0.43	0.026
		Deprived Non-Visual	-0.36	0.43	0.838
	Not Deprived Non-Visual	Deprived Visual	-1.12	0.43	0.056
		Deprived Non-Visual	-0.23	0.43	0.953
	Deprived Visual	Deprived Non-Visual	0.89	0.43	0.175
Square Immobility Post-Stress	Not Deprived Visual	Not Deprived Non-Visual	-0.28	0.73	0.981
		Deprived Visual	-2.16*	0.73	0.023
		Deprived Non-Visual	-0.88	0.73	0.628
	Not Deprived Non-Visual	Deprived Visual	-1.88	0.73	0.060
		Deprived Non-Visual	-0.60	0.73	0.845
	Deprived Visual	Deprived Non-Visual	1.28	0.73	0.309

Table 7: Repeated measure-ANOVA Results for mean frequency of object investigation behaviours during the BOOF test before and after chronic stress exposure.

Measure	Effect	Wilks' Λ Value	F	df	Sig.	Partial η^2
Total Object Investigation Pre-Stress	Time	0.529	11.793	4.0	<0.0001	0.471
	Matsocial	-	1.124	3.0	0.347	0.057
	Time*Matsocial	0.806	0.996	12.0	0.455	0.069
Total Object Investigation Post-Stress	Time	0.673	6.445	4.0	<0.0001	0.327
	Matsocial	-	3.423	3.0	0.023	0.155
	Time*Matsocial	0.758	1.293	12.0	0.229	0.088
Object 1 Investigation Pre-Stress	Time	0.522	12.135	4.0	<0.0001	0.478
	Matsocial	-	1.578	3.0	0.205	0.078
	Time*Matsocial	0.870	0.632	12.0	0.812	0.045
Object 1 Investigation Post-Stress	Time	0.874	1.905	4.0	0.123	0.126
	Matsocial	-	4.377	3.0	0.008	0.190
	Time*Matsocial	0.844	0.775	12.0	0.676	0.055
Object 2 Investigation Pre-Stress	Time	0.704	5.583	4.0	0.001	0.296
	Matsocial	-	0.509	3.0	0.678	0.027
	Time*Matsocial	0.760	1.281	12.0	0.236	0.087
Object 2 Investigation Post-Stress	Time	0.767	4.014	4.0	0.006	0.233
	Matsocial	-	1.517	3.0	0.220	0.075
	Time*Matsocial	0.715	1.581	12.0	0.104	0.106
Object 3 Investigation Pre-Stress	Time	0.585	9.384	4.0	<0.0001	0.415
	Matsocial	-	1.643	3.0	0.190	0.081
	Time*Matsocial	0.710	1.617	12.0	0.093	0.108
Object 3 Investigation Post-Stress	Time	0.642	7.403	4.0	<0.0001	0.358
	Matsocial	-	4.505	3.0	0.007	0.194
	Time*Matsocial	0.758	1.291	12.0	0.230	0.088
Object 4 Investigation Pre-Stress	Time	0.814	3.029	4.0	0.025	0.186
	Matsocial	-	0.429	3.0	0.733	0.022
	Time*Matsocial	0.801	1.022	12.0	0.432	0.071
Object 4 Investigation Post-Stress	Time	0.783	3.672	4.0	0.010	0.217
	Matsocial	-	0.515	3.0	0.673	0.027
	Time*Matsocial	0.779	1.159	12.0	0.318	0.080

Table 8: Tukey *Post-Hoc* analysis from repeated measure ANOVA for mean frequency of object investigation behaviours for all experimental cohorts (Not Deprived Visual, Not Deprived Non-Visual, Deprived Visual, and Deprived Non-Visual) before and after CMS exposure.

Dependent Variable	(I) Maternal Deprivation and Social Support	(J) Maternal Deprivation and Social Support	Mean Difference (I-J)	Std. Error	Sig.
Total Object Investigation Pre-Stress	Not Deprived Visual	Not Deprived Non-Visual	-3.40	2.90	0.647
		Deprived Visual	-4.28	2.90	0.459
		Deprived Non-Visual	-4.87	2.90	0.345
	Not Deprived Non-Visual	Deprived Visual	-0.88	2.90	0.990
		Deprived Non-Visual	-1.47	2.90	0.957
	Deprived Visual	Deprived Non-Visual	-0.59	2.90	0.997
Total Object Investigation Post-Stress	Not Deprived Visual	Not Deprived Non-Visual	-5.32	3.02	0.303
		Deprived Visual	-7.99	3.02	0.051
		Deprived Non-Visual	-8.73*	3.02	0.027
	Not Deprived Non-Visual	Deprived Visual	-2.67	3.02	0.814
		Deprived Non-Visual	-3.41	3.02	0.673
	Deprived Visual	Deprived Non-Visual	-0.75	3.02	0.995
Object 1 Investigation Pre-Stress	Not Deprived Visual	Not Deprived Non-Visual	-1.61	1.37	0.645
		Deprived Visual	-2.16	1.37	0.402
		Deprived Non-Visual	-2.87	1.37	0.170
	Not Deprived Non-Visual	Deprived Visual	-0.55	1.37	0.978
		Deprived Non-Visual	-1.25	1.37	0.798
	Deprived Visual	Deprived Non-Visual	-0.71	1.37	0.955
Object 1 Investigation Post-Stress	Not Deprived Visual	Not Deprived Non-Visual	-2.57	1.50	0.324
		Deprived Visual	-4.27*	1.50	0.030
		Deprived Non-Visual	-4.99*	1.50	0.008
	Not Deprived Non-Visual	Deprived Visual	-1.69	1.50	0.672
		Deprived Non-Visual	-2.41	1.50	0.381
	Deprived Visual	Deprived Non-Visual	-0.72	1.50	0.963
Object 2 Investigation Pre-Stress	Not Deprived Visual	Not Deprived Non-Visual	-0.44	0.47	0.788
		Deprived Visual	-0.19	0.47	0.979
		Deprived Non-Visual	-0.52	0.47	0.690
	Not Deprived Non-Visual	Deprived Visual	0.25	0.47	0.950
		Deprived Non-Visual	-0.08	0.47	0.998
	Deprived Visual	Deprived Non-Visual	-0.33	0.47	0.894
Object 2 Investigation Post-Stress	Not Deprived Visual	Not Deprived Non-Visual	-0.80	0.49	0.368
		Deprived Visual	-0.89	0.49	0.272
		Deprived Non-Visual	-0.85	0.49	0.311
	Not Deprived Non-Visual	Deprived Visual	-0.09	0.49	0.998
		Deprived Non-Visual	-0.05	0.49	1.0
	Deprived Visual	Deprived Non-Visual	0.04	0.49	1.0
Object 3 Investigation Pre-Stress	Not Deprived Visual	Not Deprived Non-Visual	-0.85	0.75	0.669
		Deprived Visual	-1.43	0.75	0.240
		Deprived Non-Visual	-1.45	0.75	0.226

	Not Deprived Non-Visual	Deprived Visual	-0.57	0.75	0.871
		Deprived Non-Visual	-0.60	0.75	0.855
	Deprived Visual	Deprived Non-Visual	-0.03	0.75	1.0
Object 3 Investigation Post-Stress	Not Deprived Visual	Not Deprived Non-Visual	-1.43	0.83	0.325
		Deprived Visual	-2.24*	0.83	0.045
		Deprived Non-Visual	-2.89*	0.83	0.005
	Not Deprived Non-Visual	Deprived Visual	-0.81	0.83	0.762
		Deprived Non-Visual	-1.47	0.83	0.301
	Deprived Visual	Deprived Non-Visual	-0.65	0.83	0.860
Object 4 Investigation Pre-Stress	Not Deprived Visual	Not Deprived Non-Visual	-0.49	0.61	0.848
		Deprived Visual	-0.51	0.61	0.838
		Deprived Non-Visual	-0.03	0.61	1.0
	Not Deprived Non-Visual	Deprived Visual	-0.01	0.61	1.0
		Deprived Non-Visual	0.47	0.61	0.868
	Deprived Visual	Deprived Non-Visual	0.48	0.61	0.858
Object 4 Investigation Post-Stress	Not Deprived Visual	Not Deprived Non-Visual	-0.52	0.63	0.843
		Deprived Visual	-0.59	0.63	0.790
		Deprived Non-Visual	0.0000	0.63	1.0
	Not Deprived Non-Visual	Deprived Visual	-0.07	0.63	1.0
		Deprived Non-Visual	0.52	0.63	0.843
	Deprived Visual	Deprived Non-Visual	0.59	0.63	0.790

Table 9: Repeated measure-ANOVA results from tests for the mean duration of locomotion behaviours during the BOOF test before and after chronic stress exposure.

Measure	Effect	Wilks' Λ Value	F	df	Sig.	Partial η^2
Vertical Motor Activity Pre-Stress	Time	0.758	4.230	4.0	0.005	0.242
	Matsocial	-	1.843	3.0	0.150	0.090
	Time*Matsocial	0.862	0.676	12.0	0.772	0.048
Vertical Motor Activity Post-Stress	Time	0.873	1.933	4.0	0.118	0.127
	Matsocial	-	1.264	3.0	0.296	0.063
	Time*Matsocial	0.774	1.193	12.0	0.294	0.082
Horizontal Motor Activity Pre-Stress	Time	0.475	14.667	4.0	<0.0001	0.525
	Matsocial	-	0.381	3.0	0.767	0.020
	Time*Matsocial	0.804	1.005	12.0	0.447	0.070
Horizontal Motor Activity Post-Stress	Time	0.850	2.336	4.0	0.498	0.150
	Matsocial	-	0.730	3.0	0.538	0.038
	Time*Matsocial	0.817	0.931	12.0	0.518	0.065
Time in Centre Pre-Stress	Time	0.956	0.606	4.0	0.660	0.044
	Matsocial	-	0.043	3.0	0.988	0.002
	Time*Matsocial	0.676	1.868	12.0	0.043	0.122
Time in Centre Post-Stress	Time	0.753	4.348	4.0	0.004	0.247
	Matsocial	-	1.357	3.0	0.265	0.068
	Time*Matsocial	0.867	0.651	12.0	0.795	0.047

Table 10: Tukey *Post-Hoc* analysis from repeated measure ANOVA for mean duration of locomotion behaviours for all experimental cohorts (Not Deprived Visual, Not Deprived Non-Visual, Deprived Visual, and Deprived Non-Visual) before and after CMS exposure.

Dependent Variable	(I) Maternal Deprivation and Social Support	(J) Maternal Deprivation and Social Support	Mean Difference (I-J)	Std. Error	Sig.
Vertical Motor Activity Pre-Stress	Not Deprived Visual	Not Deprived Non-Visual	-6.85	9.90	0.900
		Deprived Visual	1.93	9.90	0.997
		Deprived Non-Visual	-19.09	9.90	0.228
	Not Deprived Non-Visual	Deprived Visual	8.79	9.90	0.811
		Deprived Non-Visual	-12.24	9.90	0.607
	Deprived Visual	Deprived Non-Visual	-21.03	9.90	0.158
Vertical Motor Activity Post-Stress	Not Deprived Visual	Not Deprived Non-Visual	-12.44	12.97	0.773
		Deprived Visual	5.68	12.97	0.972
		Deprived Non-Visual	-16.25	12.97	0.596
	Not Deprived Non-Visual	Deprived Visual	18.12	12.97	0.506
		Deprived Non-Visual	-3.81	12.97	0.991
	Deprived Visual	Deprived Non-Visual	-21.93	12.97	0.338
Horizontal Motor Activity Pre-Stress	Not Deprived Visual	Not Deprived Non-Visual	-9.57	13.99	0.903
		Deprived Visual	-8.73	13.99	0.924
		Deprived Non-Visual	-14.71	13.99	0.720
	Not Deprived Non-Visual	Deprived Visual	0.85	13.99	1.0
		Deprived Non-Visual	-5.14	13.99	0.983
	Deprived Visual	Deprived Non-Visual	-5.99	13.99	0.973
Horizontal Motor Activity Post-Stress	Not Deprived Visual	Not Deprived Non-Visual	-7.45	13.65	0.947
		Deprived Visual	-13.00	13.65	0.777
		Deprived Non-Visual	-19.40	13.65	0.491
	Not Deprived Non-Visual	Deprived Visual	-5.54	13.65	0.977
		Deprived Non-Visual	-11.95	13.65	0.817
	Deprived Visual	Deprived Non-Visual	-6.41	13.65	0.965
Time in Centre Pre-Stress	Not Deprived Visual	Not Deprived Non-Visual	-1.81	6.59	0.993
		Deprived Visual	-2.15	6.59	0.988
		Deprived Non-Visual	-0.89	6.59	0.999
	Not Deprived Non-Visual	Deprived Visual	-0.33	6.59	1.0
		Deprived Non-Visual	0.92	6.59	0.999
	Deprived Visual	Deprived Non-Visual	1.25	6.59	0.998
Time in Centre Post-Stress	Not Deprived Visual	Not Deprived Non-Visual	-9.37	8.26	0.670
		Deprived Visual	-15.83	8.26	0.233
		Deprived Non-Visual	-12.41	8.26	0.442
	Not Deprived Non-Visual	Deprived Visual	-6.45	8.26	0.862
		Deprived Non-Visual	-3.04	8.26	0.983
	Deprived Visual	Deprived Non-Visual	3.41	8.26	0.976

Table 11: Repeated measure-ANOVA results from tests for the mean duration of grooming behaviours during the BOOF test before and after chronic stress exposure.

Measure	Effect	Wilks' Λ Value	F	df	Sig.	Partial η^2
Total Grooming Pre-Stress	Time	0.981	0.254	4.0	0.906	0.019
	Matsocial	-	1.267	3.0	0.294	0.064
	Time*Matsocial	0.806	0.993	12.0	0.459	0.069
Total Grooming Post-Stress	Time	0.911	1.294	4.0	0.284	0.089
	Matsocial	-	1.331	3.0	0.273	0.067
	Time*Matsocial	0.780	1.156	12.0	0.321	0.080
Normal Grooming Pre-Stress	Time	0.884	1.739	4.0	0.155	0.116
	Matsocial	-	0.479	3.0	0.698	0.025
	Time*Matsocial	0.932	0.315	12.0	0.986	0.023
Normal Grooming Post-Stress	Time	0.892	1.610	4.0	0.185	0.108
	Matsocial	-	0.985	3.0	0.406	0.050
	Time*Matsocial	0.711	1.608	12.0	0.096	0.107
Stereotypic Grooming Pre-Stress	Time	0.977	0.314	4.0	0.867	0.023
	Matsocial	-	1.161	3.0	0.333	0.059
	Time*Matsocial	0.783	1.132	12.0	0.339	0.078
Stereotypic Grooming Post-Stress	Time	0.902	1.442	4.0	0.233	0.098
	Matsocial	-	1.133	3.0	0.344	0.057
	Time*Matsocial	0.872	0.621	12.0	0.822	0.045

Table 12: Tukey *Post-Hoc* analysis from repeated measure ANOVA for mean duration of grooming behaviours for all experimental cohorts (Not Deprived Visual, Not Deprived Non-Visual, Deprived Visual, and Deprived Non-Visual) before and after CMS exposure.

Dependent Variable	(I) Maternal Deprivation and Social Support	(J) Maternal Deprivation and Social Support	Mean Difference (I-J)	Std. Error	Sig.
Total Grooming Pre-Stress	Not Deprived Visual	Not Deprived Non-Visual	12.75	9.70	0.558
		Deprived Visual	-2.81	9.70	0.991
		Deprived Non-Visual	10.73	9.70	0.687
	Not Deprived Non-Visual	Deprived Visual	-15.56	9.70	0.385
		Deprived Non-Visual	-2.01	9.70	0.997
	Deprived Visual	Deprived Non-Visual	13.55	9.70	0.507
Total Grooming Post-Stress	Not Deprived Visual	Not Deprived Non-Visual	9.63	10.95	0.816
		Deprived Visual	-0.68	10.95	1.0
		Deprived Non-Visual	18.17	10.95	0.355
	Not Deprived Non-Visual	Deprived Visual	-10.31	10.95	0.783
		Deprived Non-Visual	8.55	10.95	0.863
	Deprived Visual	Deprived Non-Visual	18.85	10.95	0.323
Normal Grooming Pre-Stress	Not Deprived Visual	Not Deprived Non-Visual	2.23	2.20	0.742
		Deprived Visual	-0.04	2.20	1.0
		Deprived Non-Visual	0.36	2.20	0.998
	Not Deprived Non-Visual	Deprived Visual	-2.27	2.20	0.732
		Deprived Non-Visual	-1.87	2.20	0.831
	Deprived Visual	Deprived Non-Visual	0.40	2.20	0.998
Normal Grooming Post-Stress	Not Deprived Visual	Not Deprived Non-Visual	0.97	1.77	0.946
		Deprived Visual	0.52	1.77	0.991
		Deprived Non-Visual	2.85	1.77	0.381
	Not Deprived Non-Visual	Deprived Visual	-0.45	1.77	0.994
		Deprived Non-Visual	1.88	1.77	0.714
	Deprived Visual	Deprived Non-Visual	2.33	1.77	0.556
Stereotypic Grooming Pre-Stress	Not Deprived Visual	Not Deprived Non-Visual	10.52	9.09	0.656
		Deprived Visual	-2.77	9.09	0.990
		Deprived Non-Visual	10.37	9.09	0.666
	Not Deprived Non-Visual	Deprived Visual	-13.29	9.09	0.467
		Deprived Non-Visual	-0.15	9.09	1.0
	Deprived Visual	Deprived Non-Visual	13.15	9.09	0.477
Stereotypic Grooming Post-Stress	Not Deprived Visual	Not Deprived Non-Visual	8.65	10.33	0.836
		Deprived Visual	-1.20	10.33	0.999
		Deprived Non-Visual	15.32	10.33	0.455
	Not Deprived Non-Visual	Deprived Visual	-9.85	10.33	0.776
		Deprived Non-Visual	6.67	10.33	0.917
	Deprived Visual	Deprived Non-Visual	16.52	10.33	0.388

Table 13: Repeated measure-ANOVA results from tests for the mean duration of immobility behaviours during the BOOF test before and after chronic stress exposure.

Measure	Effect	Wilks' Λ Value	F	df	Sig.	Partial η^2
Total Immobility Pre-Stress	Time	0.566	10.144	4.0	<0.0001	0.434
	Matsocial	-	0.388	3.0	0.762	0.020
	Time*Matsocial	0.797	1.049	12.0	0.408	0.073
Total Immobility Post-Stress	Time	0.617	8.233	4.0	<0.0001	0.383
	Matsocial	-	0.161	3.0	0.922	0.009
	Time*Matsocial	0.719	1.555	12.0	0.112	0.104
Corner Immobility Pre-Stress	Time	0.564	10.232	4.0	<0.0001	0.436
	Matsocial	-	0.463	3.0	0.709	0.024
	Time*Matsocial	0.738	1.428	12.0	0.160	0.096
Corner Immobility Post-Stress	Time	0.612	8.416	4.0	<0.0001	0.388
	Matsocial	-	0.071	3.0	0.975	0.004
	Time*Matsocial	0.730	1.476	12.0	0.140	0.099
Square Immobility Pre-Stress	Time	0.843	2.475	4.0	0.055	0.157
	Matsocial	-	3.009	3.0	0.038	0.139
	Time*Matsocial	0.838	0.808	12.0	0.642	0.057
Square Immobility Post-Stress	Time	0.937	0.887	4.0	0.478	0.063
	Matsocial	-	3.279	3.0	0.027	0.149
	Time*Matsocial	0.783	1.134	12.0	0.338	0.078

Table 14: Tukey *Post-Hoc* analysis from repeated measure ANOVA for mean duration of immobility behaviours for all experimental cohorts (Not Deprived Visual, Not Deprived Non-Visual, Deprived Visual, and Deprived Non-Visual) before and after CMS exposure.

Dependent Variable	(I) Maternal Deprivation and Social Support	(J) Maternal Deprivation and Social Support	Mean Difference (I-J)	Std. Error	Sig.
Total Immobility Pre-Stress	Not Deprived Visual	Not Deprived Non-Visual	-22.95	32.44	0.894
		Deprived Visual	-9.64	32.44	0.991
		Deprived Non-Visual	10.65	32.44	0.988
	Not Deprived Non-Visual	Deprived Visual	13.31	32.44	0.976
		Deprived Non-Visual	33.60	32.44	0.729
	Deprived Visual	Deprived Non-Visual	20.29	32.44	0.923
Total Immobility Post-Stress	Not Deprived Visual	Not Deprived Non-Visual	7.09	25.52	0.992
		Deprived Visual	-9.07	25.52	0.984
		Deprived Non-Visual	5.23	25.52	0.997
	Not Deprived Non-Visual	Deprived Visual	-16.16	25.52	0.921
		Deprived Non-Visual	-1.87	25.52	1.0
	Deprived Visual	Deprived Non-Visual	14.29	25.52	0.943
Corner Immobility Pre-Stress	Not Deprived Visual	Not Deprived Non-Visual	-21.19	32.08	0.911
		Deprived Visual	-3.71	32.08	0.999
		Deprived Non-Visual	16.45	32.08	0.956
	Not Deprived Non-Visual	Deprived Visual	17.48	32.08	0.948
		Deprived Non-Visual	37.64	32.08	0.646
	Deprived Visual	Deprived Non-Visual	20.16	32.08	0.923
Corner Immobility Post-Stress	Not Deprived Visual	Not Deprived Non-Visual	10.16	25.42	0.978
		Deprived Visual	2.72	25.42	1.0
		Deprived Non-Visual	8.60	25.42	0.987
	Not Deprived Non-Visual	Deprived Visual	-7.44	25.42	0.991
		Deprived Non-Visual	-1.56	25.42	1.0
	Deprived Visual	Deprived Non-Visual	5.88	25.42	0.996
Square Immobility Pre-Stress	Not Deprived Visual	Not Deprived Non-Visual	-1.76	2.11	0.838
		Deprived Visual	-5.93*	2.11	0.033
		Deprived Non-Visual	-1.15	2.11	0.948
	Not Deprived Non-Visual	Deprived Visual	-4.17	2.11	0.208
		Deprived Non-Visual	0.61	2.11	0.991
	Deprived Visual	Deprived Non-Visual	4.79	2.11	0.118
Square Immobility Post-Stress	Not Deprived Visual	Not Deprived Non-Visual	-3.08	3.95	0.865
		Deprived Visual	-11.79*	3.95	0.021
		Deprived Non-Visual	-3.37	3.95	0.828
	Not Deprived Non-Visual	Deprived Visual	-8.72	3.95	0.133
		Deprived Non-Visual	-0.31	3.95	1.0
	Deprived Visual	Deprived Non-Visual	8.41	3.95	0.156

Table 15: Repeated measure-ANOVA results from tests for the mean duration of object investigation behaviours during the BOOF test before and after chronic stress exposure.

Measure	Effect	Wilks' Λ Value	F	df	Sig.	Partial η^2
Total Object Investigation Pre-Stress	Time	0.530	11.742	4.0	<0.0001	0.470
	Matsocial	-	1.036	3.0	0.384	0.053
	Time*Matsocial	0.749	1.352	12.0	0.196	0.092
Total Object Investigation Post-Stress	Time	0.796	3.390	4.0	0.015	0.204
	Matsocial	-	3.462	3.0	0.022	0.156
	Time*Matsocial	0.764	1.253	12.0	0.253	0.086
Object 1 Investigation Pre-Stress	Time	0.576	9.761	4.0	<0.0001	0.424
	Matsocial	-	1.450	3.0	0.238	0.072
	Time*Matsocial	0.821	0.908	12.0	0.541	0.064
Object 1 Investigation Post-Stress	Time	0.948	0.728	4.0	0.577	0.052
	Matsocial	-	3.871	3.0	0.014	0.172
	Time*Matsocial	0.813	0.954	12.0	0.496	0.067
Object 2 Investigation Pre-Stress	Time	0.653	7.055	4.0	<0.0001	0.347
	Matsocial	-	0.787	3.0	0.506	0.040
	Time*Matsocial	0.809	0.978	12.0	0.473	0.068
Object 2 Investigation Post-Stress	Time	0.792	3.485	4.0	0.013	0.208
	Matsocial	-	0.904	3.0	0.445	0.046
	Time*Matsocial	0.603	2.470	12.0	0.006	0.155
Object 3 Investigation Pre-Stress	Time	0.772	3.919	4.0	0.007	0.228
	Matsocial	-	1.521	3.0	0.219	0.075
	Time*Matsocial	0.714	1.588	12.0	0.102	0.106
Object 3 Investigation Post-Stress	Time	0.798	3.357	4.0	0.016	0.202
	Matsocial	-	3.488	3.0	0.022	0.157
	Time*Matsocial	0.758	1.292	12.0	0.229	0.088
Object 4 Investigation Pre-Stress	Time	0.776	3.835	4.0	0.008	0.224
	Matsocial	-	0.479	3.0	0.698	0.025
	Time*Matsocial	0.776	1.177	12.0	0.305	0.081
Object 4 Investigation Post-Stress	Time	0.862	2.127	4.0	0.090	0.138
	Matsocial	-	0.573	3.0	0.635	0.030
	Time*Matsocial	0.752	1.333	12.0	0.207	0.091

Table 16: Tukey *Post-Hoc* analysis from repeated measure ANOVA for the mean duration of object investigation behaviours for all experimental cohorts (Not Deprived Visual, Not Deprived Non-Visual, Deprived Visual, and Deprived Non-Visual) before and after CMS exposure.

Dependent Variable	(I) Maternal Deprivation and Social Support	(J) Maternal Deprivation and Social Support	Mean Difference (I-J)	Std. Error	Sig.
Total Object Investigation Pre-Stress	Not Deprived Visual	Not Deprived Non-Visual	-8.59	7.37	0.651
		Deprived Visual	-10.75	7.37	0.469
		Deprived Non-Visual	-11.59	7.37	0.403
	Not Deprived Non-Visual	Deprived Visual	-2.16	7.37	0.991
		Deprived Non-Visual	-3.00	7.37	0.977
	Deprived Visual	Deprived Non-Visual	-0.84	7.37	0.999
Total Object Investigation Post-Stress	Not Deprived Visual	Not Deprived Non-Visual	-14.45	8.54	0.337
		Deprived Visual	-24.23*	8.54	0.031
		Deprived Non-Visual	-23.32*	8.54	0.041
	Not Deprived Non-Visual	Deprived Visual	-9.77	8.54	0.664
		Deprived Non-Visual	-8.87	8.54	0.728
	Deprived Visual	Deprived Non-Visual	0.91	8.54	1.0
Object 1 Investigation Pre-Stress	Not Deprived Visual	Not Deprived Non-Visual	-4.97	4.12	0.622
		Deprived Visual	-6.05	4.12	0.459
		Deprived Non-Visual	-8.27	4.12	0.195
	Not Deprived Non-Visual	Deprived Visual	-1.08	4.12	0.994
		Deprived Non-Visual	-3.29	4.12	0.853
	Deprived Visual	Deprived Non-Visual	-2.21	4.12	0.949
Object 1 Investigation Post-Stress	Not Deprived Visual	Not Deprived Non-Visual	-7.68	5.09	0.440
		Deprived Visual	-15.39*	5.09	0.019
		Deprived Non-Visual	-14.25*	5.09	0.034
	Not Deprived Non-Visual	Deprived Visual	-7.71	5.09	0.437
		Deprived Non-Visual	-6.57	5.09	0.573
	Deprived Visual	Deprived Non-Visual	1.13	5.09	0.996
Object 2 Investigation Pre-Stress	Not Deprived Visual	Not Deprived Non-Visual	-1.64	1.10	0.449
		Deprived Visual	-0.49	1.10	0.970
		Deprived Non-Visual	-0.60	1.10	0.947
	Not Deprived Non-Visual	Deprived Visual	1.15	1.10	0.725
		Deprived Non-Visual	1.04	1.10	0.780
	Deprived Visual	Deprived Non-Visual	-0.11	1.10	1.0
Object 2 Investigation Post-Stress	Not Deprived Visual	Not Deprived Non-Visual	-1.52	1.06	0.484
		Deprived Visual	-1.37	1.06	0.569
		Deprived Non-Visual	-1.36	1.06	0.577
	Not Deprived Non-Visual	Deprived Visual	0.15	1.06	0.999
		Deprived Non-Visual	0.16	1.06	0.999
	Deprived Visual	Deprived Non-Visual	0.01	1.06	1.0
Object 3 Investigation Pre-Stress	Not Deprived Visual	Not Deprived Non-Visual	-0.91	1.97	0.967
		Deprived Visual	-3.63	1.97	0.265
		Deprived Non-Visual	-3.03	1.97	0.423

	Not Deprived Non-Visual	Deprived Visual	-2.72	1.97	0.516
		Deprived Non-Visual	-2.12	1.97	0.705
	Deprived Visual	Deprived Non-Visual	0.60	1.97	0.990
Object 3 Investigation Post-Stress	Not Deprived Visual	Not Deprived Non-Visual	-3.99	2.56	0.410
		Deprived Visual	-6.35	2.56	0.074
		Deprived Non-Visual	-7.71*	2.56	0.020
	Not Deprived Non-Visual	Deprived Visual	-2.36	2.56	0.793
		Deprived Non-Visual	-3.72	2.56	0.471
	Deprived Visual	Deprived Non-Visual	-1.36	2.56	0.951
Object 4 Investigation Pre-Stress	Not Deprived Visual	Not Deprived Non-Visual	-1.07	1.30	0.845
		Deprived Visual	-.60	1.30	0.967
		Deprived Non-Visual	0.37	1.30	0.992
	Not Deprived Non-Visual	Deprived Visual	0.47	1.30	0.984
		Deprived Non-Visual	1.44	1.30	0.688
	Deprived Visual	Deprived Non-Visual	0.97	1.30	0.878
Object 4 Investigation Post-Stress	Not Deprived Visual	Not Deprived Non-Visual	-1.16	1.25	0.791
		Deprived Visual	-1.01	1.25	0.850
		Deprived Non-Visual	0.13	1.25	1.0
	Not Deprived Non-Visual	Deprived Visual	0.15	1.25	0.999
		Deprived Non-Visual	1.29	1.25	0.731
	Deprived Visual	Deprived Non-Visual	1.15	1.25	0.797

Appendix D – Rat Sensory Inputs

Introduction

There are some facts about the Wistar rat that were used as the basis for the development of this model design. The facts were used to interpret the results of the behavioural testing protocol. Wistar rats prioritise the senses differently to those of a human. A human prioritises sight first; objects are processed by appearance before anything other sense. Rats, in contrast, prioritise using auditory and olfactory cues (Burn, 2008). It is important to understand how a rat views the world furthermore to understand how certain manipulations can affect them. Many of these perceptual differences tend to be forgotten by humans.

Auditory

Rats can hear across a range from 200 Hz to 80 or 90 kHz, higher than a human's range of 16Hz to 20 kHz (Kelly & Masterton, 1977). Unlike smell or sight, albinism does not appear to affect rat hearing, with albino rats possessing a normal hearing range. Wistar rats (albinos) can also discriminate between different frequency and intensity of sounds to the same level as pigmented rats, and the sound localisation is similar for pigmented and albino rats (Heffner & Heffner, 1985; Heffner & Heffner, 1992).

Olfactory

Rats live in a richly complex world of smell and their sense of smell registers not only average scents but also the chemicals that can indicate a change in atmosphere or emotion (Doty, 1986). Rats also have a second method to detect scents referred to as the vomeronasal organ (VNO). The VNO is a cigar-shaped passage in a small pouch next to the septum (Zufall, Kelliher & Leinders-Zufall, 2002). This secondary organ primarily detects pheromones, critical for chemical

communication between rats (Brennan, 2001; Holy *et al.*, 2000; Cheal, 1975). Albino rats have been noted to have an impaired sense of smell or reduced responsiveness to olfactory cues, taking twice as long to back away from a pungent-smelling piece of garlic when compared to normally pigmented rats (Keeler, 1945). A separate study by Sachs (1996) found that only four percent of albino rats responded to the scent of a female rat in heat.

Tactility

A rat's sense of touch works through their vibrissae; this is one of their primary methods of getting information about the environment around them. Vibrissae are a type of mammalian hair, often called whiskers, characterised by the large size and special hair follicle that incorporates a capsule of blood, called a blood sinus (Rice, Mance & Munger, 1986; Ebara *et al.*, 2002). These sorts of whiskers are specialised for tactile sensing, much like human fingertips (Carvell & Simons, 1990). Rats sweep their vibrissae back and forth constantly, on average seven times per second (Semba & Egger, 1986; Fanselow & Nicolelis, 1999). As they sweep back and forth their whiskers can hit the same object several times in different places, this allows a rat to determine a three-dimensional picture of their surroundings (Ahissar & Knutsen, 2008). The vibrissae are used to compensate for the lack of visual depth perception (Schiffman *et al.*, 1970).

Vision

Rats generally have poor vision, especially evident in albino rats. Vision can be divided into several components; light receptors, visual acuity, and eye orientation. In humans and rats there are two types of light receptors in the retina; rods, which are sensitive to light only and cones, which are sensitive to colour (Nathans, Thomas & Hogness, 1986; Jacobs, Fenwick & Williams, 2001). There are significant differences in the types and density of these light receptors. Humans have three types of cones; "blue", "green", and "red", giving us trichromatic vision (Bowmaker &

Dartnall, 1980; Nathans, Thomas & Hogness, 1986; Curcio *et al.*, 1991). Rats on the other hand only have two types; "blue-UV" and "green", giving them dichromatic vision (Jacobs, Fenwick & Williams, 2001). The distribution of the rods and cones in human retina is 95% rods and 5% cones, whereas in the rat retina it is 99% rods and 1% cones (Curcio *et al.*, 1990; LaVail, 1976). The light intensity is hypothesised to be more important than the colour due to this distribution of cones and rods (LaVail, 1976). Albino rats tend to have impaired vision even in low light due to fewer rods within the retina (Jacobs, Fenwick & Williams, 2001). The lack of melanin precursor results in 30% of rods failing to develop in albino rats (Ilia & Jeffery, 2000).

Visual acuity refers to the clarity of vision, measured in cycles per degree (cpd) (Prusky *et al.*, 2002). In humans, acuity is about 30 cpd, for normally pigmented rats its 1 cpd and albino rats have visual acuity of 0.5 cpd. Translated into vision chart measurements; normally pigmented rats have about 20/600 vision and albino rats have about 20/1200 vision. In addition to their poor visual acuity rats also have an enormous depth of focus. The depth of focus is the range of distances an object remains in focus for an unaccommodated eye (Green, Powers & Banks, 1980). Additionally, albino rats lack melanin, which allows light to pass through the iris and dazzle the retina, in bright light, they would not be able to see anything at all (LaVail, 1976).

In comparison to human vision, pigmented rat vision is blurry and dichromatic. Albino rats have very blurry, light dazzled vision (see Figure 1). Most albino rats are severely visually impaired or blind within a few weeks of opening their eyes. Both pigmented and albino rats rely less on visual and more on smell and hearing when compared to humans.

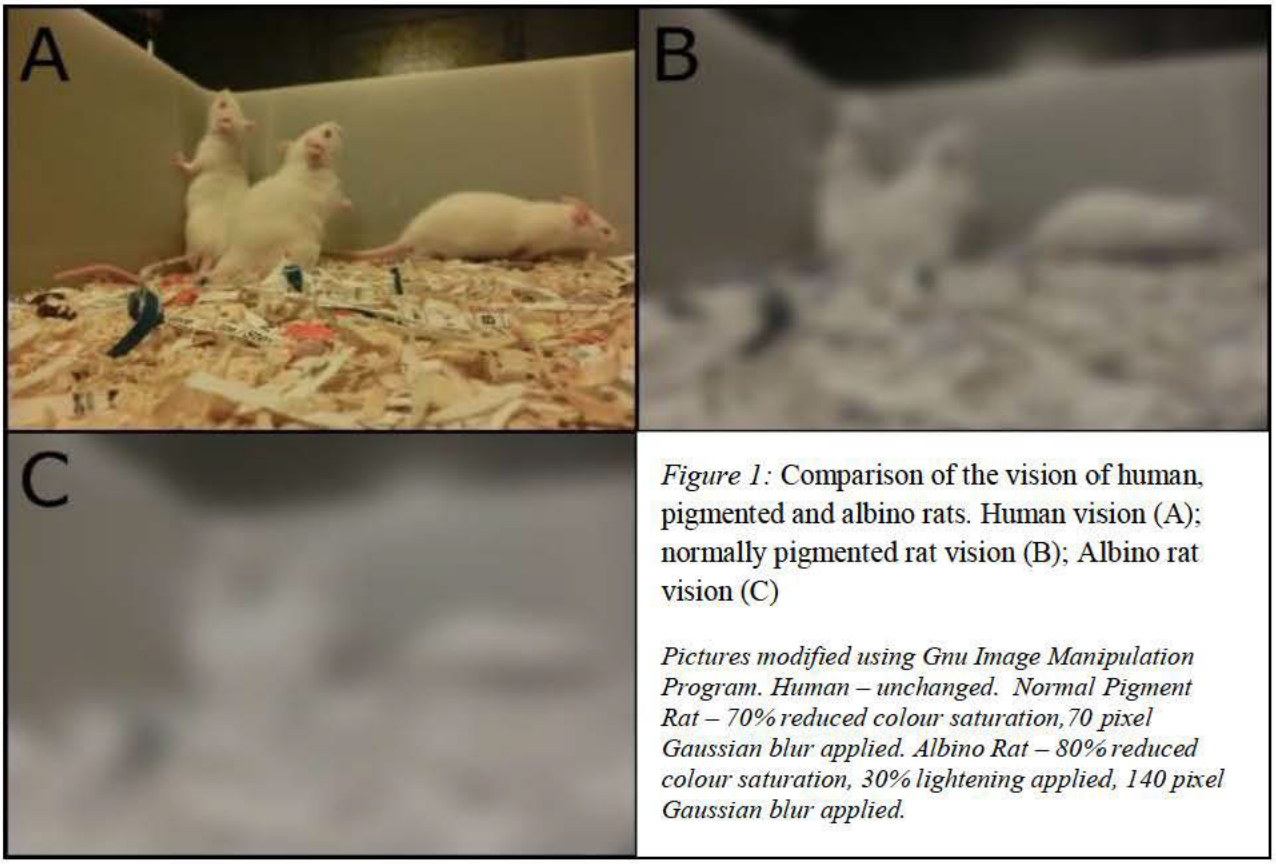

Figure 1: Comparison of the vision of human, pigmented and albino rats. Human vision (A); normally pigmented rat vision (B); Albino rat vision (C)

Pictures modified using Gnu Image Manipulation Program. Human – unchanged. Normal Pigment Rat – 70% reduced colour saturation, 70 pixel Gaussian blur applied. Albino Rat – 80% reduced colour saturation, 30% lightening applied, 140 pixel Gaussian blur applied.

Appendix E – Comparison Tables of Methodologies for Maternal Deprivation and Chronic Mild Stress Studies

Table 1: Comparison of Maternal Deprivation study parameters and outcomes.

Reference	Deprivation time	Post-Natal Days	Rat Strain	Sex	Outcome
Colorado *et al.*, 2006	15 min/day Early Handling or 6 hr/day Maternal Separation.	PND2-6; PND9-13	Sprague Dawley	Male	↓ orienting behaviour, ↑ impulsive behaviour. Hyperactivity in a novel environment (MS rats)
Eklund & Arborelius, 2006	15 min or 3 hr/day Maternal Separation. Twice daily	PND1-13	Wistar	Both	LMS-no effect on males, ↓ anxiety behaviour in females. BMS-↓ anxiety behaviour in males, no effect on females.
El Khoury *et al.*, 2006	3 hr/day Maternal Separation	PND2-14	FSL + FRL	Male	FSL-↓ swim duration in FST, escitalopram ↑ swim duration for MS and non-MS. FRL-no effect of MS on swim duration, no effect of anti-depressant
Ellenbroek & Cools, 2000	24 hr separation. Litter together	PND9	Wistar, Lewis, Fischer 344	Male	Wistar-↑ apomorphine susceptibility. Lewis-↓ basal startle amplitude. Fischer344-↓ apomorphine susceptibility
Kalinichev *et al.*, 2002	3 hr/day Maternal Separation	PND2-14	Long-Evans	Both	Males-MS over secrete CORT, less likely to explore open arms in EPM, ↑ startle amplitude, ↑ vocalisations in response to startling. Females-MS less likely to explore open arms in EPM
Kohda *et al.*, 2006	8 hr Maternal Deprivation every	PND2-10	Fisher 344	Male	15 up-regulated and 9 down-regulated genes for hippocampal samples. Reduced expression of

	second day.				choroid plexus enriched genes
Ladd *et al.*, 2004	15 mins/3 hr/day Maternal Separation.	PND2-14	Long-Evans	Male	HMS180 rats had ↑ hippocampal MR mRNA density, ↓ cortical and hippocampal GR mRNA density
Lambas-Senas *et al.*, 2009	3 hr/day Maternal Separation	PND2-15	Sprague Dawley	Male	↑ Anxiety and depressive behaviours in open field and FST. ↑ Hypothermic response to 8-OH-DPAT no effects otherwise.
MacQueen *et al.*, 2003	3 hr/day Maternal Separation	PND4-22	C57Bl/6J mice	Both	↓ swim times in FST, ↓ BDNF in dentate gyrus and CA3.
Marco *et al.*, 2012	24 hr Single Maternal Separation	PND9	Wistar	Both	Females-Impaired recognition memory. Males-↑ hippocampal GFAP expression. Both-general ↓ NeuN expression, ↓ BDNF, PSD95, synaptophysin levels in frontal cortex, ↓ BDNF, PSD95, NCAM in the hippocampus
Marais *et al.*, 2008	3 hr/day Maternal Separation	PND2-14	Sprague Dawley	Male	Alteration to NGF and NT-3 in the dorsal and ventral hippocampus. ↑ Basal CORT, ↓ ACTH, lower serum antioxidant potential. Depressive-like behaviour in FST
Matthews *et al.*,1999	6 hr/day Maternal Separation	PND5-20 (10 separations)	Lister-hooded	Both	Males-self-administered less cocaine than controls. Females-self-administered more cocaine than controls
Mintz *et al.*, 2005	4 hr/day. Early	PND1-14	Wistar	Male	Cold-ED induced a preference for a separate

	Deprivation at room and warm temps				home base, limiting social interactions. Warm-Ed had no effect on social behaviour
Pryce, Bettschen & Feldon, 2001	4 hr/day. Early Deprivation	PND1-21	Wistar	Both	EH increases arched-back nursing, ED stimulated licking and arched-back nursing at the reunion. No effect in offspring weaning weight
Reus *et al.*, 2011	3 hr/day. Litter together	PND1-10	Wistar	Male	↑ Immobility in FST, ↓ climbing time in the open field, Reduced BDNF in the amygdala, reduced NT-3 and NGF in hippocampus and amygdala.
Ruedi-Bettschen *et al.*, 2005	4 hr/day. Early Deprivation	PND1-14	Wistar	Both	Dark-Cold ED reduced sucrose preference, less mobile in FST. Light ED had no effect.
Ruedi-Bettschen *et al.*, 2006	4 hr/day. Early Deprivation	PND1-14	Fischer	Both.	The reduced motivation for and consumptions of sucrose, ↑ activity in FST, ↓ coping behaviour in the aversive environment, attenuated plasma CORT, ↑ hypertensive response to novel environment and ↑ PFC serotonin
Volodina *et al.*, 2012	5 hr/day Early Deprivation	PND1-14	White outbred rats	Both	Lower body weight, ↓ in CORT response, Semax administration weakened these effects.

Table 2: Comparison of chronic stress study parameters and outcomes.

Reference	Rat strain	Stressors Used	Time	Housing	Behavioural assessment	Outcome
Andresen *et al.*, 2004	Wistar	Swim, restraint, cold, PSD and foot shock	1-4 days	Unknown	N/A	↓TEST in PSD, foot shock, cold. ↑ PROG in foot shock, PSD. ↓ Estrone + Estradiol in PSD, foot shock, restraint. ↑ CORT in PSD, foot shock
Andrus *et al.*, 2010	Wistar-Kyoto, Fischer 344, Brown Norway	Restraint	2 hr/day. 2 weeks	Unknown	Elevated Plus Maze	No difference in monoaminergic transmission related genes
Barbazanges *et al.*, 1996	Wistar	Restraint	45 min 3x/day	Single housed	N/A	Decrease in type 1 hippocampal CR after blocking stress induced CORT response. Injected CORT reinstates effects of prenatal stress
Bravo *et al.*, 2014	Sprague Dawley	CCI (pain) or Chronic Mild Stress	2 weeks	Single housed	von Frey test, modified Forced Swim Test	CMS led to ↓ electrophysiological activity of LC. Effects of CMS exacerbated combined with CCI.
Carobrez *et al.*, 2002	Wistar	Social defeat	2 defeats	Single housed	N/A	Deficient CORT response in defeated rats. High mortality in defeated rats.
Chen *et al.*, 2012	Sprague Dawley	Chronic swim stress	5 min/day. 2 weeks	Single housed	Forced Swim Test, Open Field	↓ levels of p-GSK3β and β-catenin in mPFC.
Chiba *et al.*, 2012	Wistar	Restraint	6 hr/day. 4 weeks	Pair housed	Sucrose Preference, Open Field, Elevated Plus Maze, Forced Swim Test	↓ entries in open arms EPM, ↑ immobility FST, ↓ GR expression

Cook & Wellman, 2004	Sprague Dawley	Restraint	3 hr/day. 3 weeks	Group housed	N/A	Reduction in apical dendritic branch number and length by 18 and 32%. Reduction in terminal branch number and length by 19 and 35%
D'Aquila *et al.*, 1994	Lister hooded	Chronic unpredictable stress	5 weeks	Single and group housed	Elevated Plus Maze, Social Interaction Test, Sucrose Consumption	No effect on social interaction, anxiolytic-like profile in EPM, Isolated rearing furthered reduction in sucrose consumption
Evans *et al.*, 2012	Wistar	Social Isolation	6 weeks, 10 weeks	Group housed, unless under isolation	Novelty Suppressed Feeding, Open Field, Forced Swim Test	Reduction in endogenous ALLO. Depressive/anxiety-like behavioural profile. Impairment of hippocampal neurogenesis. Symptoms can be prevented or normalised with ALLO treatment
Ferraz *et al.*, 2011	Wistar	Restraint	20 min/day 6 weeks	Unknown	Modified Forced Swim Test, Morris Water Maze, Elevated Plus Maze,	PUFA supplementation ↑ exploration in EPM, ↓ immobility in mFST, restored cognitive function. Supplementation ↓ plasma CORT levels
First *et al.*, 2011	Wistar	Chronic Unpredictable Mild Stress	5 weeks	Single housed	Open Field, Morris Water Maze	↓ hippocampal IGF-1R levels, decrease in ERK phosphorylation in hippocampus and FC. Anti-depressants normalised changes
Fischer *et al.*, 2012	Finders Sensitive Line /Flinders Resistant Line	Social Isolation	5 weeks	Single or pair housed	Object Recognition Test, Elevated Plus Maze, Open Field, Force Swim Test	Isolation erased immobility in FST difference b/t strains. Equal impact on ORT, ↑ activity in EPM. ↑ of metabolic intake in isolated FRL rats

Forbes *et al.*, 1996	Lister hooded	Chronic unpredictable stress	6 weeks	Single housed, unless stressor required otherwise	Sucrose Consumption, Sucrose Preference	Body weight and sucrose consumption reduced in stressed and food deprived animals.
Henningsen *et al.*, 2009	Wistar	Chronic Mild Stress	10-14 hr/day. 6-8 weeks	Single housed	Sucrose Consumption, Spontaneous Alternation Behaviour, Fear Conditioning, Avoidance test	CMS caused ↓ in SC, negative effect on cognitive performance on SAB. ↑ freezing behaviour in contextual fear conditioning.
Herzog *et al.*, 2009	Wistar	Social instability stress	4 weeks	Group housed	Sucrose Preference, Forced Swim Test	Increased adrenal weight, increased plasma CORT. Elevated plasma LH. Reduced sucrose preference, no alteration to FST
Karson *et al.*, 2013	Wistar	Chronic Unpredictable Mild Stress	8 weeks	Single housed	Locomotor activity, Elevated Plus Maze, Forced Swim Test, Sucrose Preference	TNF-α inhibitor ↓ depressive- and anxiety-like behaviour.
Katz *et al.*, 1981	Sprague Dawley	Chronic unpredictable stress	3 weeks	Pair housed	Open Field	Reduced basal activity from control levels. And eliminated activity in response to acute stress. Reversed with anti-depressant treatment

Kompange *et al.*, 2008	Wistar	Chronic Unpredictable Mild Stress	3 weeks. 3 stressors/day	Group housed	Sucrose Preference, Forced Swim Test, Elevated Plus Maze, Social Avoidance test.	↓ SP, ↑ immobility in FST. Induced social avoidance. ↑ grooming. ↓ anxiety in EPM
Koo *et al.*, 2009	Sprague Dawley	Chronic unpredictable stress	3 weeks	Group housed	Sucrose Preference	Stress activates NF-KB signaling and decreases proliferation of neural stem-like cells
Larsen *et al.*, 2010	Sprague Dawley	Chronic Unpredictable Mild Stress	4 weeks	Pair housed	Elevated Plus Maze, Open Field, Novel Object, Forced Swim Test, Sucrose Consumption	Antidepressant treatment ↑SC, ↓ immobility in FST, CUS ↑ BDNF mRNA expression in the hippocampus.
Li *et al.*, 2011	Sprague Dawley	Chronic Unpredictable Mild Stress	3 weeks	Pair housed	Sucrose Preference, Novelty Suppressed Feeding	CUS causes anhedonia and anxiogenic behaviour, ↓ expression levels of synaptic proteins and spine number. Changes reversed by ketamine
Liu *et al.*, 2014	Wistar	Chronic Unpredictable Mild Stress	5 weeks	Unknown	Sucrose Preference, Forced Swim Test, Open Field	Anti-depressant treatment reversed behavioural symptoms and the ↑ serum CORT. Reversible ↓BDNF in hippocampus and amygdala
Liu *et al.*, 2011	Sprague Dawley	Chronic unpredictable stress	5 weeks	Unknown	Open Field	Electroacupuncture reversed depressive-like behaviour induced by stress. EA treatment blocked the stress induced GFAP level decrease in the hippocampus

Liu *et al.*, 2013	Sprague Dawley	Chronic unpredictable mild stress	3 weeks	Unknown	Sucrose consumption, Open Field, Forced Swim Test	Stress exposure induced depression-like behaviour, increased serum CORT, decreased 5-HT, increased IFN-y, TNF-a and elevated IDO in PFC. Swimming exercise helped to reverse these changes.
Lukkes *et al.*, 2009	Sprague Dawley	Isolation	3 weeks	Single and group housed	Open Field, Social Interaction, Fear Behaviour, Tail Withdraw, Tone Response.	↑ anxiety-like behaviour in OF, ↑ fear behaviour, ↓ social contact
Luo *et al.*, 2008	Sprague Dawley	Chronic Unpredictable Mild Stress	3 weeks	Single housed	Sucrose Preference, Open Field, Forced Swim Test	Stress-induced behavioural changes were suppressed or blocked by intrahippocampal injection of 5-HT or NPY.
Mao *et al.*, 2010	Sprague Dawley	Chronic Unpredictable Mild Stress	5 weeks	Unknown	Sucrose Preference, Open Field	↓ SP, ↓ locomotor activity in OF. Treatment with TGP suppressed behavioural and biochemical changes
Mizoguchi *et al.*, 2003	Wistar	Water immersion and restraint	2 h/day. 4 weeks	Group housed	N/A	PFC-Cytosolic GR levels increased.
Muscat *et al.*, 1990	Lister hooded	Chronic unpredictable stress	10-12 weeks	Single housed	Sucrose Preference	Drugs selectively reversed performance improvement in imipramine-treated stressed animals. 5HT antagonist metergoline increased sucrose consumption in all groups.
Muscat *et al.*, 1992	Lister hooded	Chronic unpredictable stress	7-12 weeks	Single housed	Sucrose Preference	Stress induced decrease in sucrose consumption which was reversed by the administration of DA agonists, which had the same effect as anti-depressant administration.

Qi *et al.*, 2008	Sprague Dawley	Chronic forced swim stress	5 min/day. 3 weeks	Single housed	Open Field, Elevated Plus Maze, Sucrose Preference	Chronic forced swim test induced depressive-like behaviour, decreased levels of P-ERK2, P-CREB, ERK1/2 and CREB in hippocampus and PFC. These changes were reversed with antidepressant treatment
Radley *et al.*, 2008	Sprague Dawley	Restraint	6 hr/day. 3 weeks	Group housed	No tests used	↓ Dendritic spine volume and surface area. The overall shift in spine population, reduction in large spines and increase in small spines.
Rana *et al.*, 2016	Sprague Dawley	Chronic Unpredictable Mild Stress	4 weeks	Group housed	Sucrose Preference, Novelty Suppressed Feeding, Social Interaction, Forced Swim Test	Low Responders maintained high SP levels. ↑ Social explore. ↓ immobility in FST following CMS
Raone *et al.*, 2007	Sprague Dawley	Restraint and electric shock	4 weeks	Group housed	Escape Deficit	↑ basal plasma CORT, escape deficiency from stress. Adrenal hypertrophy and ↓ GR express. in hippocampus, hypothalamus, mPFC and pituitary. Changes reversed w/ antidepressant
Reus *et al.*, 2012	Wistar	Chronic Unpredictable Mild Stress	6 weeks	Group housed	Open Field, Sweet Food Consumption	Stress-induced anhedonia, adrenal hypertrophy, ↑ CORT, no change in BDNF. Changes normalised by antidepressants
Schrijver *et al.*, 2002	Lister hooded	Isolation	From weaning onwards	Single and group housed	Open Field, Novel Object, Light/Dark box, Morris Water Maze,	Isolation enhanced activity under several conditions of novelty. Isolation showed persistent activity in L/D box

Suo *et al.*, 2013	Sprague Dawley	Chronic Predictable Mild Stress and Chronic Unpredictable Mild Stress	4 weeks Predictable. 3 weeks Unpredictable	Group housed	Sucrose Preference, locomotor activity, Novelty Suppressed Feeding, Elevated Plus Maze, Forced Swim Test	Predictable stress produced anti-depressant and anxiolytic-like effects. ↑mTOR signalling. Predictable stress prevented depressive- and anxiety-like behaviour caused by chronic stress.
Trujillo, Durando & Suarez, 2016	Wistar	Chronic variable stress	24 days	Group housed	Elevated Plus Maze	Increase anxiety behaviours, region specific increase/decrease Fos-ir, increased GR-ir
Ulrich-Lai *et al.*, 2006	Sprague Dawley	Chronic variable stress	Variable length. 2 weeks	Pair housed	N/A	Increased adrenal weight, DNA and RNA content and RNA/DNA ratio. Hyperplasia in outer ZF, hypertrophy in inner ZF and Medulla. Reduced cell size in ZG
Van den Hove *et al.*, 2014	Sprague Dawley	Chronic Unpredictable Mild Stress	3 weeks	Group housed	Elevated Zero Maze, Forced Swim Test, Sucrose Consumption	Prenatal stress caused anxiety-like behaviour in EZM, normalised with chronic stress. ↑ Immobility in FST and SC for males. ↓SC in females.
Will *et al.*, 2003	WMI and WLI	Restraint	10 min restraint at 12 week age	Group housed	Forced Swim Test, Open Field, Defensive Burying	Lower and less variable CORT response to restraint in WMI. Desipramine and Phenelzine decreased immobility in FST in WMI
Wood *et al.*, 2015	Sprague Dawley	Social stress (resident-intruder)	30 min/day. 5 days	Single housed	Sucrose Preference	IL-1β ↑ in short-latency and ↓ in long-latency rats. Stress produced anhedonia selectively in SL rats, prevented by the IL-1R antagonist
Yazir *et al.*, 2015	Wistar	Chronic Mild Stress	5 weeks	Group housed	Locomotor Activity, Passive Avoidance Test, Morris Water Maze	Stress-induced ↓ BDNF + c-Fos in hippocampal CA1, CA3 + amygdala. ↑ plasma levels of TNF-α and IL-1β. Antidepressants reversed changes

You *et al.*, 2011	Wistar	Chronic mild stress	4 weeks	Single housed	Sucrose Preference, Locomotor Activity	CMS caused reduction in sucrose preference and locomotion. Real time RT-PCR showed high expression of pro-inflammatory cytokines and low expression of anti-inflammatory cytokines. Decrease in BDNF mRNA
Zalosnik *et al.*, 2014	Wistar	Chronic variable stress	24 days	Group housed	Fear-conditioning test	No main effect
Zhao *et al.*, 2008	Sprague Dawley	Chronic Mild Stress	5 weeks	Group housed	Open Field, Sucrose Consumption, Forced Swim Test,	Stress ↓ OF activity and SC, and ↑ immobility in FST. No effect of antidepressant treatment in OF activity but reversed the other symptoms