Establishing and validating an *in vivo* rodent model of chronic restraint stress

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Thesis presented in partial fulfilment of the requirements for the degree of Master of Science (Physiology) in the Faculty of Natural Science at Stellenbosch University.

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December 2022

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ABSTRACT

Introduction. Psychological stress has emerged as one of the health epidemics of the 21st century and provides an impetus for increased investigation into the effects of a dysregulated stress response on whole body physiology. Although previous studies helped to clarify the association between chronic psychological stress and the onset and progression of cardiovascular diseases, a paucity of mechanistic insights underlying this association remain. Considering the complex nature of the stress system and the similarities that exist between humans and animals, it is therefore ideal to use rodent models to investigate stress-related disorders. Although the incidence and onset of various disorders in humans are gender-specific, clinical, and preclinical research using male subjects still far outnumber those using females. This study therefore aimed to establish and validate an *in vivo* model of chronic restraint stress in male and female Wistar rats.

Materials and Methods. Male and female Wistar rats were subjected to a 4-week restraint stress protocol versus matched controls. Following this, behavioral tests (elevated plus maze [EPM] and tail flick task) were performed together with an assessment of body weight changes and biochemical biomarkers to ascertain whether the model was successfully established.

Results & Findings. Our data revealed that male stressed rats displayed a decreased percentage change in body weight over time versus controls ($p<0.01$). Furthermore, the male stressed group exhibited increased plasma corticosterone levels compared to controls ($p<0.01$), while no significant differences were detected for plasma adrenocorticotropic hormone (ACTH) concentrations. Male brain-derived neurotrophic factor levels (biomarker for neuronal survival and growth) were lower in the stress group versus controls ($p<0.05$). Stressed males also displayed a reduced number of attempts into the open arms of the EPM versus controls ($p<0.05$). There were no significant weight changes for female rats. However, stressed females exhibited lowered plasma corticosterone levels versus controls ($p<0.05$), while also displaying higher plasma ACTH concentrations compared to the control group ($p<0.05$). Stressed females also displayed increased rears (as assessed by EPM test) versus matched controls ($p<0.01$). Our findings reveal intriguing sex-based differences in response to a chronic restraint stress protocol, with males displaying a depressive-type phenotype while females exhibited a post-traumatic stress disorder phenotype. Sex-specific

preclinical research can provide unique insights into the various mechanisms driving stress-related diseases and should eventually lead to the identification of novel diagnostic and therapeutic targets.

Footnote: this thesis will employ US-based spelling and grammar notations.

OPSOMMING

Inleiding. Chroniese spanning kom tans na vore as die gesondheid epidemie van die 21ste eeu en lei tot ‘n toename in navorsing oor die implikasies van 'n chroniese stres reaksie op die hele liggaam se fisiologie. Vorige studies het gehelp om die verwantskap tussen chroniese spanning en die aanvang en bevordering van kardiovaskulêre siektes te verduidelik. Daar is egter steeds onvoldoende kennis oor die onderliggende meganismes wat hierdie verwantskap verduidelik. Inaggenome die kompleksiteit van die stres sisteem en die ooreenkomste wat tussen mense en diere bestaan, is dit ideaal om knaagdier modelle te gebruik om stres verwante kondisies te ondersoek. Alhoewel die voorkoms en aanvang van verskeie kondisies in mense geslag spesifiek is, gebruik navorsing steeds beduidend meer manlike as vroulike deelnemers. Hierdie studie het dus ten doel gehad om 'n *in vivo* model van chroniese beperkings stres in Wistar-rotte te vestig.

Materiale en Metodes. Manlike en vroulike Wistar-rotte is gedurende die studie gebruik. Die eksperimentele groep is blootgestel aan 'n inperkings stres-protokol van vier weke. Gedragstoetse (verhoogde plus doolhof [EPM] en stert wip toets) is hierna uitgevoer tesame met assessering van liggaamsgewig en plasma merkers, om sodoende vas te stel of die model suksesvol gevestig is.

Resultate en bevindinge. Ons data dui aan dat manlike rotte wat aan die stres-protokol blootgestel is, ‘n kleiner persentasie in gewigstoename teenoor die kontrole groep getoon het ($p<0.01$). Verder het die manlike stres groep verhoogde plasma kortikosteroon vlakke getoon in vergelyking met die kontroles ($p<0.01$), terwyl geen beduidende verskille vir plasma adrenokortikotropiese hormoon (ACTH) konsentrasies gevind is nie. Manlike brein-vervaardigde neurotrofiese faktor vlakke (merker vir neurale oorlewing en groei) was laer in die stres groep teenoor die kontroles ($p<0.05$). In vergelyking met die kontrole groep, het die mannetjies wat aan die stres-protokol blootgestel is ‘n vermindering in die aantal pogings getoon met betrekking tot die oop arms van die EPM ($p<0.05$). Daar was geen betekenisvolle veranderinge in gewig vir die vroulike rotte nie. Verlaagde plasma kortikosteroon vlakke is in die vroulike stres groep opgemerk in vergelyking met die kontroles ($p<0.05$), terwyl hulle ook hoër plasma ACTH-konsentrasies teenoor die kontrolegroep ($p<0.05$) getoon het. Wyfies blootgestel aan die stres-protokol het meer male op hul agterpote gestaan (soos geassesseer deur EPM-toets) in teenstelling met die kontroles ($p<0.01$). Ons bevindinge

onthul interessante geslags gebaseerde verskille in reaksie op 'n chroniese inperkings stres-protokol. Hier, het mannetjies 'n depressiewe fenotipe getoon terwyl wyfies 'n post traumatiese stresversteuring-fenotipe getoon het. Geslag spesifieke navorsing met diere kan unieke insigte verskaf met betrekking tot die verskeie meganismes wat spannings verwante kondisies en siektes bevorder. Dit behoort aanvullend te lei tot die identifisering van nuwe diagnostiese en terapeutiese teikens.

Voetnota: hierdie tesis sal gebruik maak van VS-gebaseerde spelling en grammatika notasies.

ACKNOWLEDGEMENTS

To my supervisor Prof Faadiel Essop, thank you for always believing in me and giving me the opportunity to learn from Prof throughout the past few years. Prof was a big part of the reason why I wanted to pursue MSc and I have enjoyed every day of working with Prof. Your passion for science and dedication to your students are contagious and you are someone I aspire to someday be like. Thank you for your unique mentorship, calming nature and words of encouragement. Prof has helped me grow immensely and I would not have been able to get to where I am today without such a good influence in life. Prof taught me to always be willing to learn and keep striving towards excellence.

To my co-supervisor, a very knowledgeable Dr Danzil Joseph, thank you for answering the phone every time I called to ask for advice about a lab crisis and always being available to answer my questions or help with thesis editing. You were always there to lend a helping hand despite your very busy schedule, and I appreciate it so much.

To the CARMA research group aka the lab rats, you have been the most amazing support system and have all become like family. From day one you were all there, ready to jump in and help wherever I needed it. Thank you for all the pep talks, snacks and laughs we shared on long days in the lab. I appreciate you all! To Logan, best lab buddy and partner in crime. The way you approach life is so inspiring. Thank you for your presence throughout the last three years and for sticking with me through all the many emails… and WhatsApp messages. Your jokes and positive energy were always able to turn the bad days into good ones.

To my mom and dad. Thank you for giving me the opportunity to study and to pursue my dreams. You have always gone out of your way to ensure that I have everything I need. Without your unwavering love and support I would not be where I am today. Thank you for always showing an interest in my studies and for encouraging me to become my best self. To my sister, you have been there through the highs and the low's and have always supported me in my pursuits. Thank you for all the late-night motivational conversations and for celebrating my accomplishments with me.

Finally, I thank God for all His blessings and for His presence in everything I do.

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LIST OF ABBREVIATIONS

ACTH	adrenocorticotrophin
AGE	advanced glycation end products
ANOVA	analysis of variance
AP	activator protein
AR	adrenergic receptors
ATP	adenosine triphosphate
BDNF	brain-derived neurotrophic factor
COVID-19	coronavirus disease of 2019
CRH	corticotrophin-releasing hormone
CRS	chronic restraint stress
CMDs	cardiometabolic diseases
CVD	cardiovascular diseases
DNA	deoxynucleic acid
ELISA	enzyme-linked immunosorbent assay
EPM	elevated plus maze
ETC	electron transport chain
FADH	reduced flavin adenine dinucleotide
GAPDH	glyceraldehyde 3-phosphate dehydrogenase
GAS	general adaptation syndrome
GC	glucocorticoid
GFAT	glutamine fructose-6-phosphate amidotransferase
GLUT	glucose transporter
GR	glucocorticoid receptor
GRE	glucocorticoid response element
GSH	reduced glutathione
G3P	glyceraldehyde-3 phosphate
HBP	hexosamine biosynthetic pathway
HK	hexokinase
HPA	hypothalamus-pituitary-adrenal
IL	interleukins
IR	insulin resistance
IRS	insulin receptor substrate
JAK/STAT	janus kinase/signal transducer and activator of transcription

LC	locus coeruleus
LDL	low-density lipoprotein
MAPK	mitogen-activated protein kinases
MR	mineralcorticoid receptor
MS	maternal separation
NADH	reduced nicotinamide adenine dinucleotide
NADPH	nicotinamide adenine dinucleotide phosphate
NCD	non-communicable diseases
NF-κB	nuclear factor kappa B
NOGP	non-oxidative glucose pathway
NOX	nitric oxide
NPY	neuropeptide Y
O-GlcNAc	*O*-linked *N*-acetylglucosamine
PARP	poly adenosine diphosphate ribose polymerases
PFC	pre-frontal cortex
PKA	protein kinase A
PKC	protein kinase C
PLA	phospholipase A
POMC	pro-opiomelanocortin
PNS	parasympathetic nervous system
PVN	paraventricular nucleus
RAGE	receptor of advanced glycation end-products
ROS	reactive oxygen species
SAM	sympathetic-adreno-medullary
SANS	South African National Standard
SD	standard deviation
SOD	superoxide dismutase
TF	transcription factor
TFRE	transcription factor response element
TNF	tumor necrosis factor
UCMS	unpredictable chronic mild stress
UDP-GlcNAc	uridine diphosphate N-acetylglucosamine

1. INTRODUCTION

Although the focus used to be largely on communicable diseases over the last decades, the burden of non-communicable diseases (NCDs) is ever rising, fueled by increased urbanization, a changing diet, and sedentary lifestyles (Sharman & Bachmann, 2019). Non--communicable diseases including hypertension, cardiovascular diseases (CVD) and diabetes currently represent 71% of deaths globally with low- and middle-income countries like sub-Saharan Africa bearing 86% of this burden (Sharman & Bachmann, 2019; World Health Organization, 2021). Stress is an emerging, modern-day "epidemic" with devastating effects on the global economy, for example increased strain on health care systems and/or reduced productivity (Fink, 2017; World Health Organization, 2017). Of note, South Africa is regarded as the second most stressed nation worldwide and hence this creates a particular impetus to better understand the underlying reasons driving this phenomenon (Bloomberg, 2012).

Psychological stress further increased during the coronavirus disease (COVID-19) pandemic, for example a high prevalence of serious psychological distress among US adults was reported during 2020 (McGinty *et al.*, 2020). This suggests that the pandemic's long-term disruptions are crucial drivers of distress. South Africa is not exempt, as a recent survey revealed that more than half of the respondents displayed higher levels of distress than before the pandemic (Pharma Dynamics, 2020). Here, 9% of respondents experienced feelings of anxiousness, 48% reported frustration and ~ 31% indicated depression (Pharma Dynamics, 2020). It is therefore clear that the burden of chronic stress represents a growing concern and supports an increase in stress-related research to better understand its toll on health and disease.

A chronically activated stress response can contribute to psychopathology (anxiety and depression) and pathophysiology (inflammation, diabetes, and atherosclerosis) through dysregulation of the usual, adaptive stress response and can increase susceptibility to mood disorders such anxiety-like and depression-like phenotypes (Godoy et al., 2018; Kumar et al., 2013a; Sher et al., 2020; van Oort et al., 2017; Verma et al., 2011). Such alterations are linked to the onset and progression of cardio-metabolic diseases (CMDs), the focus of this study (Fink, 2017; Kumar et al., 2013a).

Cardiometabolic diseases is a well-characterized term used to describe various complications that can affect the vasculature and intracellular metabolism and function of different tissues and

organs. It is currently the leading cause of global morbidity and mortality and includes complications such as CVD and diabetes mellitus (Rabasa & Dickson, 2016; Noncommunicable disease countries profile, 2018). Of note, the prevalence of CVD doubled over the last 29 years to ~523 million individuals currently burdened with it (Roth *et al.*, 2020). According to the World Health Organization (2017), CVD alone accounts for up to 31% of global mortality and remains the predominant cause of premature morbidity worldwide (Roth *et al.*, 2020). Thus, with the parallel rise in chronic stress, it essential to investigate the underlying mechanisms driving stress-related CVD onset and progression. Although the mechanisms driving stress-related CVD are still being investigated, our laboratory's overall postulate is that oxidative stress and increased activation of non-oxidative glucose pathways (NOGPs) in the heart may be implicated in this process.

This review will therefore discuss the stress system and its mediators, as well as the sex differences that exist in the stress response. Thereafter, the dysfunctional stress response will be examined. The focus is on animal-based studies aiming to establish and validate an *in vivo* rodent model of chronic stress. Finally, the role of cardiac oxidative stress and increased cardiac NOGP activation in stress-related CVD onset and progression will be briefly discussed as the investigation of such pathways is the larger goal of our laboratory.

2. THE STRESS SYSTEM AND ITS MEDIATORS

According to historians and researchers, the biological formulations of stress can be traced back to a speculative article written by Hans Selye in 1936. Here, Selye described a three-phase response termed the general adaptation syndrome - consisting of an alarm phase that was followed by a phase of resistance or adaptation and finally a phase of exhaustion (Jackson, 2014; Tan & Yip, 2018). Many in the field maintain that his article constituted a turning point in the history of stress. Selye has since become known as the creator (or father) of stress owing to his prominence in the field (Fink, 2017; Jackson, 2014). Selye defined stress as a non-specific response to any demand placed on the body (Fink, 2017; Tian *et al.*, 2014; Viner, 1999). Here, he integrated stress concepts established by Claude Bernard (milieu inte´rieur), Walter B. Cannon (homeostasis) and others, to provide a unifying model of stress and adaptation (Jackson, 2014; Tan & Yip, 2018; Viner, 1999). The lack of specificity in Selye's definition of stress has been the subject of considerable criticism and research since the stress response is more specific than originally thought (Fink, 2017). Each stressor has its own neurological

signature and thus activates different brain regions and stress biomarkers (Fink, 2017). While it is reasonable to critically evaluate any paradigm, those dismissive of the general adaptation syndrome have not fully succeeded to dismantle Selye's link between stress and adaptation (Cunanan *et al*., 2018). His discussion of the stages of adaptation and exhaustion also introduced an element of chronicity that was missing from previous studies (Jackson, 2014). Although Selye's work has been thoroughly contested, his notion of biological stress and his emphasis on the role of steroid hormones in this context provides a useful conceptual matrix for understanding the relationship between stress, health and disease and has therefore been adopted and adapted by researchers in a variety of related fields (Jackson, 2014).

Stress is currently defined as an essential multilevel physiological response that occurs when demands placed on an individual exceed the capacity to manage them (Hannibal & Bishop, 2014; Kumar *et al.*, 2013; van Oort *et al.*, 2017). Stress therefore represents the physiologic response to environmental factors that may be encountered (Vale, 2005). A wide variety of stimuli can trigger the stress response, including factors that are both physical (extreme temperatures, pain, infection) and psychological (unemployment, work pressure, family difficulties) in nature.

The brain plays a key role in coordinating such a response to effectively cope with changes and thereby restore homeostasis and well-being (Hannibal & Bishop, 2014; Nirupama *et al.*, 2018; van Oort *et al.*, 2017). Here, various dynamically interacting brain regions located in the limbic system form the stress system and are responsible for triggering a response to changing stimuli (Godoy *et al*., 2018; Sher *et al.*, 2020). Despite some debate regarding the specific regions, the consensus is that the main limbic system components include the thalamus, hippocampus, prefrontal cortex (PFC), amygdala, and hypothalamus (Buijs & Van Eden, 2000; Godoy *et al*., 2018). This response is triggered when visual, auditory, and somatic input are received in the thalamus, which acts as the relay station filtering sensory information between the brain and body (Torrico & Munakomi, 2021).

The hippocampus and the PFC establish stress perception before sensory information passes on to the amygdala for processing. The hippocampus plays a role in memory retrieval. Here, it can use an individual's memory to provide context to incoming stimuli and to determine whether a stressor is indeed a threat (Godoy *et al.*, 2018; Hannibal & Bishop, 2014). The PFC also ensures an appropriate response to stressors by detecting potentially harmful stimuli and enabling behavioral plasticity (Buijs & Van Eden, 2000; Godoy *et al.*, 2018; Sharpley, 2009).

The PFC therefore regulates the limbic 'alarm system' by acting as an inhibitory system if the alarm is not justified (Arnsten *et al.*, 2015; Godoy *et al.*, 2018).

Information regarding stressful stimuli is eventually relayed to the amygdala. Here, it is interpreted in such a way to determine whether it constitutes a real or potential threat (stressor) (Godoy *et al.*, 2018; Hannibal & Bishop, 2014). The amygdala's extensive connections throughout the brain allows it to initiate and orchestrate the emotional stress response, the center of which is in the hypothalamus (Figure 1) (Arnsten *et al.*, 2015; Buijs & Van Eden, 2000; Godoy *et al.*, 2018).

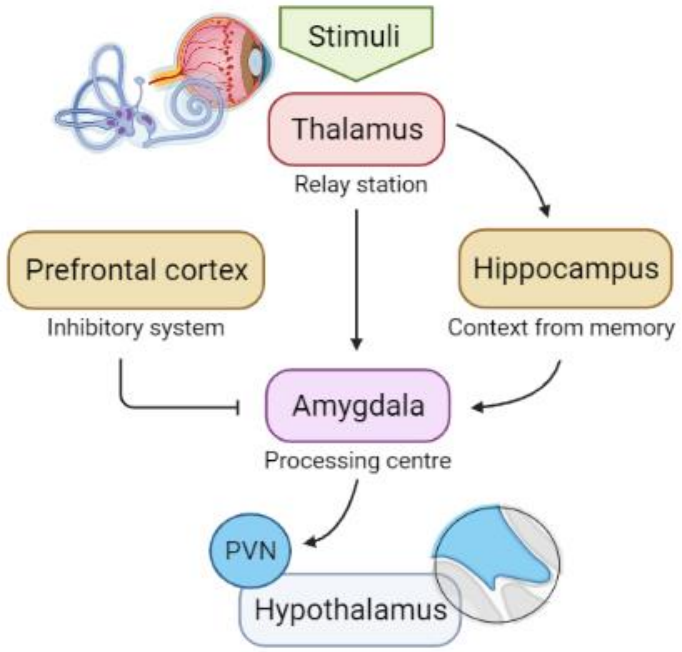

Figure 1. **Activation of the limbic system. Abbreviations:** PVN, paraventricular nucleus. Image adapted from Murison, 2016; Beselaar, 2020 and created using Biorender.com.

The hypothalamus is responsible for integrating emotional stress information from higher brain structures such as the hippocampus, amygdala, and PFC and organizing the final homeostatic stress response (Buijs & Van Eden, 2000; Godoy *et al.*, 2018). The incoming signals trigger the activation of the paraventricular nucleus in the hypothalamus and the subsequent secretion of group-releasing hormones (Vale, 2005; Zänkert *et al.*, 2019). Here, corticotrophin-releasing hormone (CRH) triggers two crucial pathways i.e. the sympathetic-adreno-medullary (SAM) pathway and the hypothalamic-pituitary-adrenal (HPA) axis (Hannibal & Bishop, 2014;

Sharpley, 2009; Zänkert *et al.*, 2019). These two pathways are crucial allostatic responses that are triggered to initiate adaptation (Lundberg, 2005; McEwen, 2009). Allostasis refers to the process of maintaining homeostasis by anticipating needs and preparing to satisfy them before they arise (Guidi *et al.*, 2021; McEwen, 2009). Once the challenge passes, allostasis is "switched off" and these responses return to baseline conditions (Guidi *et al.*, 2021; Lundberg, 2005; McEwen, 2009). The SAM and HPA pathways are interconnected and communicate at the brain level, ultimately functioning as major effector systems through which the brain regulates the body (Figure 2) (Godoy et al., 2018; Kumar et al., 2013a; Vale, 2005).

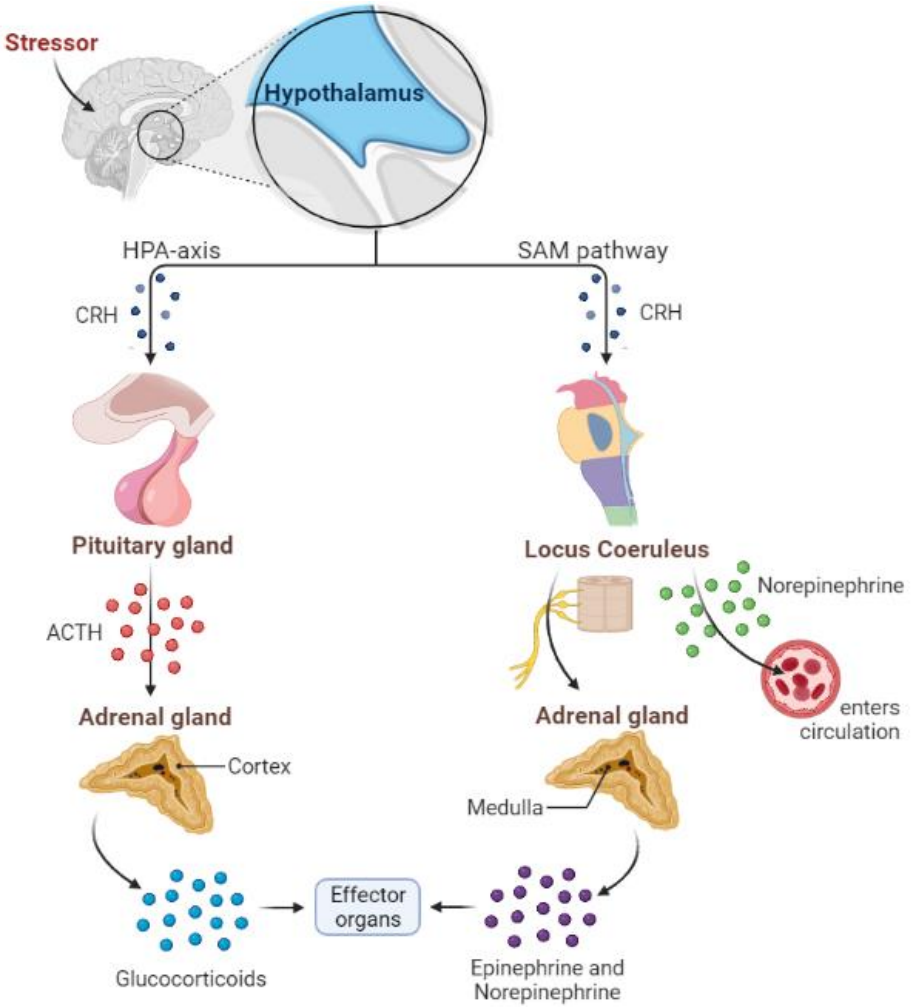

Figure 2. **Activation of the stress system. Abbreviations**: HPA, hypothalamic-pituitary-adrenal; SAM, sympathetic-adreno-medullary; ACTH, adrenocorticotropic hormone. Image adapted from Nirupama *et al.*, 2018; Sher *et al.*, 2020; Rabasa & Dickson, 2016 and created using Biorender.com.

The SAM pathway constitutes the first phase of the stress response and acts via the sympathetic nervous system and the adrenal medulla to bring about a rapid response to stressors (Godoy *et*

al., 2018; Sharpley, 2009; Vale, 2005). The locus coeruleus (LC) is a small structure of the brainstem that is stimulated to produce a rate-limiting enzyme (tyrosine hydroxylase) following the release of CRH (Vale, 2005). Tyrosine hydroxylase is involved in norepinephrine production and secretion directly from sympathetic neurons in the brain (Godoy *et al.*, 2018; Rabasa & Dickson, 2016; Vale, 2005). Furthermore, direct projections from the LC to pre-ganglionic sympathetic neurons in the spinal cord exist. These neurons synapse directly with chromaffin cells in the adrenal medulla, allowing stress response signals to be further passed on to effector tissues (Godoy *et al.*, 2018; Sharpley, 2009). As a result, the glandular tissue of the adrenal medulla is stimulated to synthesize and release both epinephrine and norepinephrine, well-known catecholamines that serve as essential mediators of the stress response (Godoy *et al.*, 2018; Rabasa & Dickson, 2016).

Such catecholamines can bind α-adrenergic receptors (α-AR) and β-adrenergic receptors (β-AR) in target tissues to prepare the body for the fight-or-flight response (Godoy *et al.*, 2018; Rabasa & Dickson, 2016; Thau *et al.*, 2020). Catecholamines act on adipose tissue by binding mainly to β-ARs and this activates protein kinase A (PKA) and triggers lipolysis and thermogenesis (Ryu & Buettner, 2019). Within the smooth muscle of the airways, epinephrine binds β_2-ARs and activates PKA which ultimately induces smooth muscle relaxation and bronchodilation to maximize oxygen consumption for the fight-or-flight response (Alvarado, 2017; Sherwood, 2016). Blood vessels are also affected by catecholamines where those vessels that supply some tissues undergo vasoconstriction, while others undergo vasodilation (Sharma & M. Flood, 2019; Sherwood, 2016). For example, epinephrine binds to vascular smooth muscle cells via the α_2-AR to induce vasoconstriction whereas binding to β_2-AR elicits vasodilation. During the stress response vasoconstriction occurs in vessels that supply the digestive system and skin, while vasodilation occurs in vessels that supply the skeletal and cardiac muscles (Sharma & M. Flood, 2019). Other effects include an increase in blood pressure, and heart rate, as well as ensuring increased glucose availability to muscles (Godoy *et al.*, 2018; Hannibal & Bishop, 2014; Lundberg, 2005; Rabasa & Dickson, 2016; Sharpley, 2009; Thau *et al*., 2020).

The parasympathetic nervous system (PNS) is responsible for the rest-and-digest response (Fink, 2017; Hannibal & Bishop, 2014). The PNS system utilizes acetylcholine and operates via the vagus nerve and parasympathetic neurons to re-establish homeostasis by reducing blood pressure, heart rate, and respiration, increasing digestion and conserving energy through

relaxation (Fink, 2017; Sharpley, 2009).

The second phase of the stress response takes more time and elicits longer-lasting effects (Godoy *et al.*, 2018; Hannibal & Bishop, 2014; Sharpley, 2009; Zänkert *et al.*, 2019). Here, activation of the HPA-axis eventually results in the release of glucocorticoids (GCs) - critical mediators of the stress response (Figure 2) (Sharpley, 2009; Vale, 2005). Following its release from the PVN, CRH travels to the anterior pituitary where it triggers pro-opiomelanocortin (POMC) cleavage into several, smaller biologically active fragments (Zänkert *et al.*, 2019). For example, adrenocorticotropic hormone (ACTH) can be released through the hypophyseal portal system (Godoy *et al.*, 2018; Sharpley, 2009). ACTH acts systemically to trigger the synthesis and release of GCs from the zona fasciculata, the middle layer of the adrenal cortex (Godoy *et al.*, 2018; Sharpley, 2009; Thau *et al.*, 2020; Zänkert *et al.*, 2019).

Glucocorticoids are steroid hormones, i.e., cortisol in humans and corticosterone in rodents. They can act as primary messengers and possess the ability to influence several tissues through glucocorticoid receptors (GRs) and mineralcorticoid receptors (MRs) (Fink, 2017; Nirupama *et al.*, 2018; Rabasa & Dickson, 2016; Thau *et al.*, 2020; Zänkert *et al.*, 2019). The lipophilic nature of GCs allows for the easy traversing of cell membranes where they can bind to cytosolic GRs (Ehrchen *et al.*, 2019; Liberman *et al.*, 2018; Nirupama *et al.*, 2018). The GC-GR complexes subsequently translocate to the nucleus to regulate gene expression (Ehrchen *et al.*, 2019; Liberman *et al.*, 2018; Nirupama *et al.*, 2018). Here, this complex can bind to the GC-response element (GRE) of GC-responsive genes (Ehrchen *et al.*, 2019). Binding of dimerized complexes to positive GREs results in enhanced transcription of target genes (transactivation) while monomeric binding to negative GREs suppress target gene transcription (transrepression) (Ehrchen *et al.*, 2019; Liberman *et al.*, 2018; Louw, 2019). During this process of transcriptional regulation, transactivation and transrepression occur in genes responsible for anti-inflammatory and pro-inflammatory effects, respectively. Furthermore, monomeric GC-GR complexes can inhibit transcription without DNA interaction. This process is known as tethering and is accomplished by directly binding transcription factors (TFs) such as nuclear factor-κB (NF-κB) and activator protein-1 (AP-1) (Ehrchen *et al.*, 2019; Liberman *et al.*, 2018; Louw, 2019). Here, the GR can influence the activity of such TFs (considered a prevailing mechanism of transrepression) (Liberman *et al.*, 2018). GC-GR complexes can also simultaneously interact with DNA and neighboring TFs (composite binding) to activate or repress transcription of target genes (Ehrchen *et al.*, 2019; Liberman *et al.*, 2018; Louw, 2019)

(Figure 3).

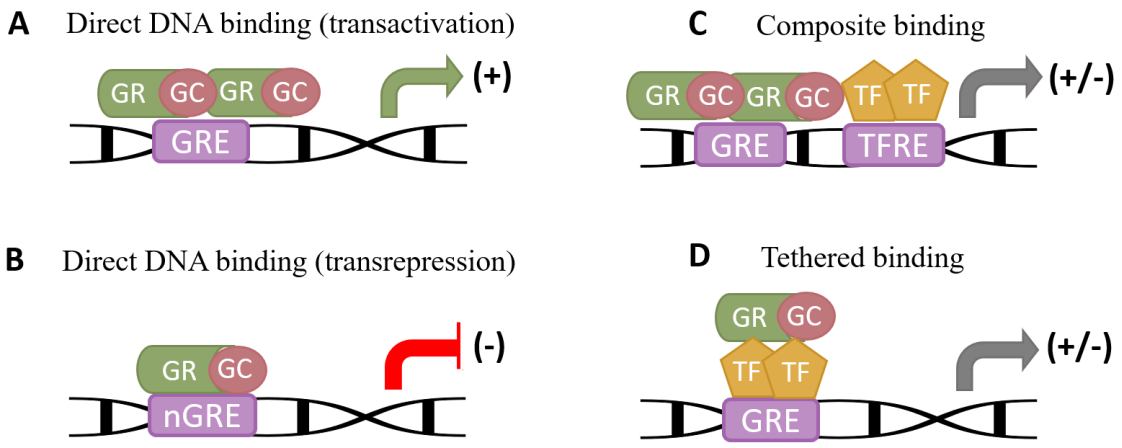

Figure 3. Glucocorticoid mechanisms of transcriptional regulation. (A) GRs can directly activate gene expression by interacting with GREs. (B) GRs can also interact with nGREs to inactivate gene expression. (C) Dimerized GRs can bind to GREs and modulate gene transcription by interacting with neighboring DNA-bound TFs while **(D) Monomeric GRs can regulate gene transcription by attaching itself to DNA-bound TFs. Abbreviations**: DNA, deoxyribonucleic acid; GC, glucocorticoid; GR, glucocorticoid receptor; GRE, glucocorticoid response element; TF, transcription factor; TFRE, transcription factor response element. Image adapted from Liberman *et al*., 2018.

Cortisol is the main GC and is widely known as the primary stress hormone (Thau *et al.*, 2020; Zänkert *et al.*, 2019). Additionally, it also possesses many functional roles. For example, it regulates metabolism and blood pressure, assists in memory formation, and acts as an anti-inflammatory agent (Hannibal & Bishop, 2014; Lundberg, 2005; Thau *et al*., 2020). One of its critical functions is exerting negative feedback on the HPA-axis in order to suppress the stress response (Harris, 2015; Lundberg, 2005; Rabasa & Dickson, 2016).

Although cortisol can reach all neurons in the brain, it only exerts effects on the neurons expressing GRs (Godoy *et al.*, 2018). Multiple regions of the limbic system have been shown to express such receptors including the hippocampus, PFC, and amygdala (Myers *et al.*, 2015). Here, GCs activate a variety of key limbic-neuroendocrine circuits, ultimately coordinating physiological and behavioral output in response to stress (Myers *et al.*, 2015). As previously mentioned, the hippocampus is critical in processes related to memory, particularly contextual learning, and memory retrieval and as GRs are abundantly expressed in the hippocampus stress influences memory processing (Godoy *et al.*, 2018; Kim *et al*., 2015; Myers *et al.*, 2015). Here, GCs cause morphological changes, reduce neurogenesis and long-term potentiation and

decrease dendritic arborization (fine branching at nerve endings). Such alterations can give rise to hippocampus-dependent memory impairments and anxiety- and depression-like behavior (Kim *et al.*, 2015). Activation of the GR also causes delayed suppression of neuronal excitability and synaptic plasticity, possibly to normalize hippocampal activity after stress and thus protect information acquired during the stressful experience (Kim *et al.*, 2015; Myers *et al.*, 2015). The PFC plays a central role in cognitive control functions by acting as an inhibitory system (Godoy *et al.*, 2018) and is another limbic structure susceptible to stress. For example, morphological changes in neurons and impairments in long-term potentiation have been observed in response to stress (Kim *et al.*, 2015). As a result, GCs in the PFC can have a region- and context-specific inhibitory role in HPA-axis regulation. The amygdala integrates sensory information to orchestrate a fear response (Godoy *et al.*, 2018; Myers *et al.*, 2015). It undergoes changes in response to stress, however, in contrast to the hippocampus this includes enhanced long-term potentiation and increased dendritic arborization (Kim *et al.*, 2015). Such changes are proposed as mechanisms underlying stress-associated anxiety disorders. Furthermore, the amygdala releases more neurotransmitters like glutamate, GABA, noradrenaline, and serotonin and can enhance GC secretion in response to stress (Kim *et al.*, 2015; Myers *et al.*, 2015).

Additional biochemical markers including plasma brain-derived neurotrophic factor (BDNF) and neuropeptide Y (NPY) will also be assessed during this study due to their involvement in the etiology and pathophysiology of mood and anxiety disorders (Suliman *et al*., 2013; Wu *et al*., 2011). BDNF is a neurotrophin that is mainly synthesized by neurons and is involved in the survival, proliferation, and differentiation of neurons in the central and peripheral nervous system (Béjot *et al.*, 2011; Suliman *et al.*, 2013). Moreover, it is crucial for synaptic plasticity and dendritic growth (Grande *et al*., 2010). BDNF exerts its effects by binding and activating its receptor tyrosine kinase type-B receptor (Grande *et al*., 2010). While BDNF is concentrated in the brain, it can cross the blood-brain-barrier and can thus also be detected in circulation. Here, some reported that circulating BDNF concentrations may reflect its levels in the brain (Suliman *et al.*, 2013). Notably, stress-responsive GCs can play a role in the regulation of BDNF expression and function, where increased GC levels such (for example cortisol) can lead to attenuated BDNF expression (Grande *et al*., 2010; Suliman *et al.*, 2013). Thus, chronic stress can lead to lower circulating BDNF levels because of this mechanism.

A functional interaction between BDNF and GCs also exist. This suggests a possible role for BDNF in regulating the stress response through interactions with neurotransmitter systems that

in turn regulate HPA-axis activity (Grande *et al*., 2010; Suliman *et al.*, 2013). Recent evidence suggests that BDNF may be a potential biomarker of neuronal dysfunction and subsequent cognitive impairment, and that lower BDNF levels can increase susceptibility to mood disorders such anxiety-like and depression-like phenotypes (Grande *et al*., 2010; Suliman *et al*., 2013; Yu & Chen, 2011). In support, studies in post-mortem human brains revealed BDNF's involvement in the pathological processes of stress-related psychopathologies (for example mood and anxiety disorders) (Suliman *et al*., 2013).

NPY is a neuropeptide that is found in the central and peripheral nervous system where it is involved in regulating cognition, metabolism, and the stress response (Farzi *et al*., 2015; Fletcher *et al*., 2010; Reichmann & Holzer, 2016; Wu *et al*., 2011). Five different Y receptors (Y1, Y2, Y4, Y5, y6) can bind NPY in mammals, of which all are functional expect one (y6) (Farzi *et al*., 2015; Reichmann & Holzer, 2016; Wu *et al*., 2011). The role of NPY in the etiopathology of mood and anxiety disorders has been extensively studied and there is evidence of its involvement in the post-traumatic stress disorder and depression (Wu *et al*., 2011). The literature indicates that NPY is crucial for adaptation to stress as it counteracts the actions of CRH, therefore exerting anxiolytic and anti-stress effects (Farzi *et al*., 2015; Reichmann & Holzer, 2016; Wu *et al*., 2011). Of note, stress can also potentially influence NPY expression through GC action, although downstream effects depend on the type and duration of the stressor(s) (Farzi *et al*., 2015; Reichmann & Holzer, 2016). Here, research indicates that exposure to repeated or chronic stress leads to increased NPY levels (Gellman & Turner, 2013; Ip *et al*., 2019).

3. SEX DIFFERENCES IN THE STRESS RESPONSE

Although the physiological stress response is shared across species, differences within this response exist between males and females (Verma *et al*., 2011). Here, studies show that HPA-axis and SAM pathway patterns differ markedly based on sex (Kajantie & Phillips, 2006; Rincón-Cortés *et al*., 2019; ter Horst *et al*., 2012; Verma *et al*., 2011). Both show significant differences in function and regulation at baseline and in response to stress. The evidence collectively indicates that sex is a crucial determinant of disease susceptibility and there is a clear pattern for the sex-specific prevalence of various disorders (Kudielka & Kirschbaum, 2005; Rincón-Cortés *et al*., 2019; Verma *et al*., 2011). For example, conditions such as autoimmune diseases and chronic pain are more prevalent in women, while susceptibility to hypertension and infectious diseases manifest more in men (Kajantie & Phillips, 2006;

Kudielka & Kirschbaum, 2005). More studies are therefore needed that include female subjects to better understand the variation in the stress response between males and females. This could provide valuable insights into sex-specific prevalence and treatment of stress-related disorders.

Despite the robustness of the physiologic stress response (as detailed above), prolonged stress can cause it to become dysfunctional and maladaptive with several downstream pathophysiological changes.

4. CHRONICITY IN THE STRESS RESPONSE

Although the acute stress response may serve in an adaptive capacity to promote survival, chronic activation may trigger damaging effects (Hannibal & Bishop, 2014). Here, the continued presence of stressful stimuli can stimulate the SAM pathway and the HPA-axis to produce catecholamines and GCs, respectively (Tsyglakova *et al.*, 2019). The allostatic response is no longer limited to the period of challenge due to the constant exposure to elevated levels of stress hormones (McEwen, 2009). This can result in an allostatic overload which is defined as the cumulative effect of chronic exposure to heightened responses from repeated challenges (Guidi *et al*., 2021; McEwen, 2009). For example, higher levels of epinephrine, norepinephrine, and cortisol can lead to dysregulation within the stress system (Tsyglakova *et al.*, 2019). The SAM pathway may be partly responsible for detrimental effects in this instance. Although elevated catecholamine levels are linked to hypertension, stroke, and myocardial infarction (Lundberg, 2005), we postulate that HPA-axis dysregulation plays a more prominent role. Evidence supports the subsequent overload and resistance of cortisol as the primary mediator of downstream stress-related pathology (Hannibal & Bishop, 2014; Lundberg, 2005; Sher *et al*., 2020; Verma *et al*., 2011).

Chronic reactivation of the stress response and repeated cortisol surges cause compensatory GR downregulation or altered tissue levels (Hannibal & Bishop, 2014; Tsyglakova *et al.*, 2019). This blocks cortisol binding and attenuates its effects (Hannibal & Bishop, 2014; Tsyglakova *et al.*, 2019). The resultant decreased GC sensitivity (GC resistance) prompts increased cortisol production and secretion that can lead to adrenal burnout and HPA-fatigue (Hannibal & Bishop, 2014; Tian *et al.*, 2014).

The HPA-axis can thus become dysfunctional with chronic stress and hence an allostatic overload can occur (Guidi *et al*., 2021; Kudielka & Kirschbaum, 2005; Nirupama *et al*., 2018;

Rincón-Cortés *et al*., 2019; Sher *et al*., 2020). Alterations in the limbic neuroarchitecture and function can also occur, which can alter the basic mechanisms of the stress response and contribute to various pathological conditions that negatively affect the heart (Marin *et al.*, 2007; Rahal *et al.*, 2014). Such conditions include changes in glucose metabolism and inflammation, which will be discussed next (Hannibal & Bishop, 2014; Lundberg, 2005).

4.1.Glucose homeostasis

The stress-linked changes collectively induced by cortisol can liberate glucose, amino acids, and fatty acids to ensure their availability for mitochondrial oxidative phosphorylation and energy production (Hannibal & Bishop, 2014; Kudielka & Kirschbaum, 2005; Rabasa & Dickson, 2016). Here, cortisol stimulates proteolysis and lipolysis in the liver, muscle, and adipose tissues to provide substrates for hepatic gluconeogenesis (Figure 4) (Kawahito *et al.*, 2009; Rabasa & Dickson, 2016; Thau *et al.*, 2020). Although such changes will promote glucose synthesis, cortisol can also decrease glucose utilization by binding to glycogen synthase. This decreases its activity and lowers glycogenesis in the liver and skeletal muscle (refer Figure 4) (Nirupama et al., 2018).

Excessive amounts of cortisol under chronic stress conditions significantly alter glucose homeostasis by enhancing such processes and by mobilizing large amounts of substrates (Harris, 2015; Thau *et al.*, 2020). Chronic stress ultimately creates a state of hypermetabolism with increased circulating glucose levels and the possibility of insulin insensitivity (Harris, 2015; Nirupama *et al.*, 2018; Rabasa & Dickson, 2016). This may prove to be problematic as non-insulin-dependent tissues are susceptible to glucotoxicity. Glucotoxicity refers to the adverse effects of elevated glucose levels on cells and tissues (McClain, 2004) that can induce detrimental damage to intracellular components and signaling pathways (Giri *et al.*, 2018; Kawahito *et al.*, 2009).

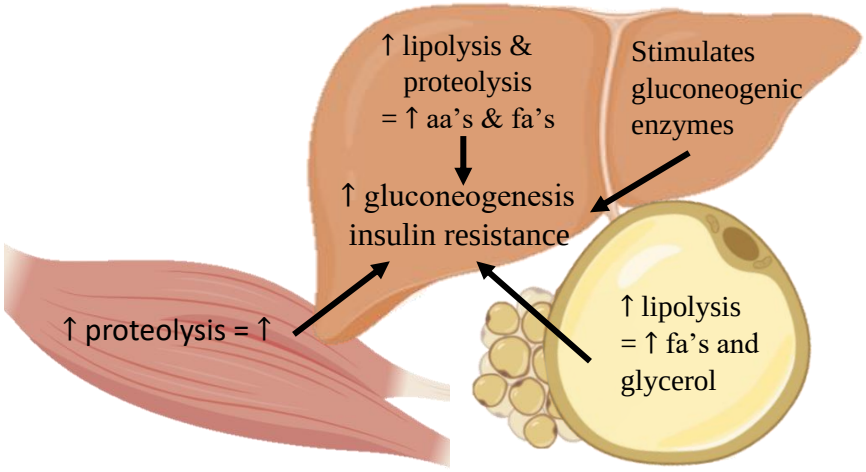

Figure 4. The effects of GC overload on glucose metabolism. Prolonged GC exposure can liberate large amounts of substrates including aa's, fa's, and glycerol. This promotes gluconeogenesis and leads to subsequent insulin resistance. **Abbreviations**: aa's, amino acids; fa's, fatty acids. Image self-constructed using Biorender.com.

These mechanisms of glucose-induced toxicity include changes in gene expression and increased oxidative stress, which in turn enhances the activation of NOGPs (Giri *et al.*, 2018). NOGPs are usually activated at a relatively low rate to metabolize glucose under normal physiological conditions (discussed later).

4.2.Inflammation

Cortisol has another crucial role in the body as a potent anti-inflammatory hormone (Ehrchen *et al.*, 2019; Hannibal & Bishop, 2014; Liberman *et al.*, 2018; Nirupama *et al.*, 2018). During the early stages of stress, cortisol acts as an anti-inflammatory agent to mitigate the production of pro-inflammatory cytokines while enhancing anti-inflammatory cytokine production (Ehrchen *et al.*, 2019; Liberman *et al.*, 2018; Tsyglakova *et al.*, 2019). GC-GR complexes bind and suppress pathways like NF-κB, AP-1, Janus kinase/signal transducers and activators of transcription factors (JAK/STAT), and mitogen-activated protein kinases (MAPK) (refer Figure 5) (Ehrchen *et al.*, 2019; Liberman *et al.*, 2018). These pathways are responsible for the transcription of pro-inflammatory genes, while their inactivation reduces the production of inflammatory mediators such as tumor necrosis factor alpha (TNF-α) and various interleukins (ILs). Additionally, GC-GR complexes can bind and promote the transcription of anti-inflammatory mediators including IL-4, IL-10, and IL-13 (Ehrchen *et al.*, 2019; Liberman *et al.*, 2018).

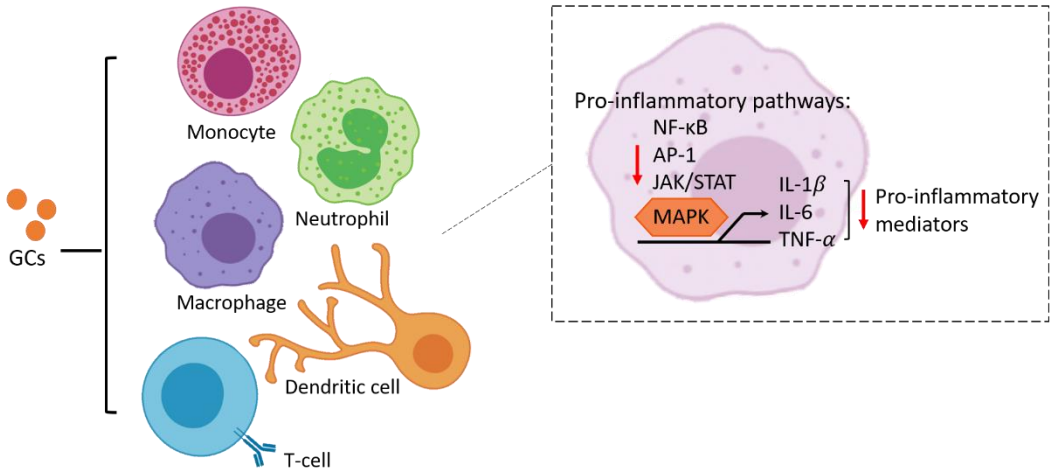

Figure 5. The anti-inflammatory actions of GCs such as cortisol. The dotted square indicates the anti-inflammatory pathways activated by GCs and occur in the nucleus of most immune cells. **Abbreviations**: GCs, glucocorticoids; NF-κB, nuclear factor kappa B; AP-1, activator protein 1; JAK/STAT - Janus kinase/signal transducers and activators of transcription factors; MAPK, mitogen-activated protein kinases; TNFα, tumor necrosis factor alpha; IL, interleukin. Image self-constructed using Biorender.com.

The GR downregulation with chronic stress causes GC resistance and the formation of fewer GC-GR complexes (Hannibal & Bishop, 2014; Tian *et al.*, 2014). Considering that GCs act on almost all immune cells, this can lead to decreased sensitivity of such cells to their anti-inflammatory effects (Ehrchen *et al.*, 2019; Liberman *et al.*, 2018). For example, GCs will no longer prevent the release of pro-inflammatory mediators by monocytes and macrophages. GCs will also be unable to inhibit adhesion and oxidative bursts in macrophages. Furthermore, increased neutrophil adhesion and activation can occur and GCs will no longer act on dendritic cells to suppress their ability to activate T-cells (Liberman *et al.*, 2018). This causes increased recruitment and functionality of such immune cells within the immune system and promotes the release of pro-inflammatory mediators (Ehrchen *et al.*, 2019). Tsyglakova *et al.* (2019) stated that pro-inflammatory cytokines such as IL-6, IL-1β, and TNF-α can activate the HPA-axis and act as a form of positive feedback on this system. Evidence also suggest that extreme surges in cortisol may increase its affinity for the MR, and when bound to it can elicit pro-inflammatory effects (Hannibal & Bishop, 2014). Cortisol's anti-inflammatory effects are thus diminished, and it can no longer fulfill its vital role. The inflammatory response thus becomes increased in duration and intensity, and over time the milieu shifts towards a more permanent pro-inflammatory state (Cohen *et al.*, 2012; Hannibal & Bishop, 2014). Inflammation and glucose dysregulation can contribute to enhanced free radical production and subsequent oxidative stress that can lead to tissue damage (as discussed later) (Hannibal & Bishop, 2014;

Rahal *et al.*, 2014). Further downstream, relatively higher circulating glucose levels and cardiac oxidative stress can increase myocardial NOGP activation (Mapanga & Essop, 2016). Oxidative stress and increased NOGP activation therefore emerge as promising, putative pathways linking chronic stress to downstream pathology and disease progression.

Although the previous discussions helped to clarify the association between chronic stress and CVD, a paucity of mechanistic insights underlying this association still remains. Here, it is useful to employ preclinical animal models of chronic stress in order to gain additional insights in this regard.

5. LABORATORY ANIMAL MODELS OF CHRONIC STRESS

As previously discussed, the mammalian stress system is complex and comprises many different organs, tissues and hormones coordinating an integrated response. Such a response exists at multiple levels with crosstalk between the nervous system, target organs, and hormones. Due to such complexity, replicating stress-induced cardiovascular pathology using cellular and/or computational models is not really feasible. Charles Darwin made an initial observation that animals share the same range of emotion as humans, i.e. similar behaviors when expressing emotions and analogous physiological responses (Campos *et al.*, 2013; Teng *et al.*, 2021). This similarity raises the possibility of studying the mechanisms of stress-related disorders in mammals other than humans (Campos *et al.*, 2013; Teng *et al.*, 2021). More specifically, a rodent model is therefore ideal in this regard as it is a good mammalian system to mimic the human condition.

Several rodent models of stress have thus far been developed keeping Darwin's assumption in mind (Campos *et al.*, 2013). Such models attempt to investigate behavioral, biochemical, and physiological alterations associated with specific emotional states (Campos *et al.*, 2013; Grippo *et al.*, 2003; Maggio & Segal, 2019). Findings generated from animal models have considerably contributed to the current understanding of mechanisms underlying the role of stress in health and disease (Golbidi *et al.*, 2015; Maggio & Segal, 2019; Teng *et al.*, 2021).

Animal models of stress can be categorized into physical or physiological stress paradigms, and psychological or emotional stress paradigms (Golbidi *et al.*, 2015; Radley *et al.*, 2017). Animal models of physical stress include thermal stress, immobilization stress, electric shock stress and behavioral despair stress, while models of psychological stress include social stress,

neonatal stress, noise-induced stress, changing light-dark cycles and a combination of such socioenvironmental stressors (Golbidi *et al*., 2015). For this study, immobilization was employed over a long period of time to induce chronic stress. Immobilization stress can be applied in two ways. The first requires the animals to be placed in an appropriately sized transparent tube to restrict their movement. During the second, animals are completely immobilized by fixing their head and limbs to an underlying board. Although a single repeated stressor (immobilization) can mimic stressful, inescapable situations, adaptation that may occur is less likely to modify the physiological response (Golbidi *et al*., 2015).

Despite the theoretical idea that a model should reproduce all features of the phenomena under investigation, this is rarely the case (Campos *et al.*, 2013). The complexity of the stress response and the multifaceted manifestations that go along with it prevent animal models from replicating all features and symptoms of a specific stress-related disorder (Campos *et al.*, 2013). Instead, it can generate behavioral and physiological aspects associated with specific emotional states and disorders (Golbidi *et al.*, 2015). Each model still suffers from limitations in spite of the progress already made in animal models of stress and thus the perfect model does not exist (Golbidi *et al.*, 2015). However, if one is aware of potential confounders, the protocol may be optimized and results may be interpretated with the necessary caution, making *in vivo* models quite valuable tools. It is also worth noting that specific contributors to human distress cannot be recreated in rodent models, for example feelings of guilt and loss and/or suicidal thoughts and financial stress (Golbidi *et al.*, 2015).

As mentioned above, there are clear sex differences in the stress response - both psychologically and physiologically (Verma *et al*., 2011). Furthermore, the incidence and onset of various disorders in humans are gender-specific for example post-traumatic stress disorder, major depressive disorder, and autoimmune diseases occur more frequently in women (Kajantie & Phillips, 2006; Kudielka & Kirschbaum, 2005; ter Horst *et al*., 2012; Verma *et al*., 2011; Zucker & Beery, 2010). In contrast, men often develop anti-social behavior, substance abuse, and coronary heart diseases (Kajantie & Phillips, 2006; Kudielka & Kirschbaum, 2005; Verma *et al*., 2011). Yet rodent models using male animals far outnumber those using females. For example, a 2009 survey of almost 2,000 animal studies revealed male bias in 8 out of 10 biological disciplines. Here, the ratio of male-only studies to female-only studies in physiology was 3.7 to 1. In 6 of the 10 disciplines, 80 % of the studies were performed only on male animals (Beery & Zucker, 2011; Lee, 2018; Zucker & Beery, 2010). Even in cases where the disease of

interest is more prevalent in women, the male-bias was apparent with only 12% of studies focused on females or both sexes (Karp & Reavey, 2019). Of note, the studies that did include both sexes, only 34% analyzed data separately by sex (Lee, 2018).

The main argument for not using female animals is that their estrous cycle creates additional complexity and logistical problems (Beery & Zucker, 2011; Prendergast *et al*., 2014; ter Horst *et al*., 2012; Zucker & Beery, 2010). One estrus cycle lasts four to five days and consists of four stages (ter Horst *et al*., 2012). The distinct secretion pattern of female sex hormones per stage was suggested to render females intrinsically more variable than males (Prendergast *et al*., 2014; ter Horst *et al*., 2012; Zucker & Beery, 2010). Some evidence suggests that these variations in sex hormone levels can complicate the results (ter Horst *et al*., 2012). Moreover, some maintain that interpretable data only emerge when females are tested in each of the four stages of the estrus cycle. This multiplies the number of subjects per experiment, is time-consuming and significantly increases the cost of the study (Prendergast *et al*., 2014).

A strong argument for including female animals in research is that male sex hormones may also display temporal variation. Such variability in males can arise when rodents are group-housed and dominant behaviors such as chasing, biting, and fighting develop (Prendergast *et al*., 2014). This enables rodents to attain and maintain social status within the social hierarchy (Prendergast *et al*., 2014; Williamson *et al*., 2017a). Findings indicate that the average dominant male rodent exhibits significantly higher plasma testosterone than subordinate males (Williamson *et al*., 2017b). This degree of hormonal fluctuation is like that found in female rodents during the estrus cycle. Furthermore, the consensus is that females (without regard to the estrus cycle) are no more variable than males (Becker *et al*., 2016; Prendergast *et al*., 2014). This is true across various physiological, morphological, and behavioral traits (Becker *et al*., 2016; Prendergast *et al*., 2014). Female utilization therefore does not require monitoring of the estrus cycle and means that less subjects are needed per experiment which saves time and decreases the cost of the study. Sex-specific preclinical research can provide a wealth of data on the regulation of the stress response in both males and females. It can further add valuable insights regarding the sex differences in models which reflect stress-related disorders (ter Horst *et al*., 2012). For example, understanding how different sexes react to medication can aid researchers in predicting possible adverse effects in men and women.

Choosing an animal model of disease is a crucial aspect for study and strict criteria should therefore be applied to determine the validity and utility of models (Golbidi *et al*., 2015). There

are now some useful criteria to assess the validity of animal models of stress. These include face validity (behavioral manifestations in the model should resemble the signs and symptoms observed in patients), construct validity (pathophysiological alterations in humans should also be present in the model), and predictive validity (therapeutic interventions that are effective in humans should also be able to reverse the changes induced by the model) (Abelaira *et al.*, 2013; Campos *et al.*, 2013; Golbidi *et al.*, 2015; Wang *et al.*, 2018). Furthermore, the model should be easy to utilize and exhibit consistent molecular and biochemical phenotypic manifestations (reliability) (Golbidi *et al.*, 2015).

5.1.Types of animal stress models

5.1.1. Social defeat model

Exposing rodents to an aggressive or dominant peer for fixed periods is known to induce a state equivalent to psychosocial stress experienced by humans (Golbidi *et al.*, 2015; Milic *et al.*, 2021). This approach is mainly used in rodents (for example mice and rats) and involves a resident-intruder encounter (Golbidi *et al*., 2015; Golden *et al*., 2015; Schöner *et al*., 2017). Here, the resident can be housed with a female prior to the resident-intruder encounter to establish a sense of territorial authority and possession. This ultimately promotes aggression (Golbidi *et al.*, 2015). The aggressor (resident) is initially put into a divided cage known as the home cage, whereafter the intruder is placed into the aggressor's home cage compartment. The intruder is then attacked and forced into subordination by the aggressor, leading to its social defeat (Campos *et al.*, 2013; Golden *et al.*, 2015; Milic *et al.*, 2021).

The social defeat model is based on its ability to reduce social interaction and enhance anxiety-like behavior in the submissive animal, demonstrating the model's face validity (Golbidi *et al.*, 2015; Golden *et al.*, 2015; Milic *et al.*, 2021). With respect to construct validity, the social defeat model exhibits increased body weight and plasma corticosterone levels as well as metabolic disturbances such as altered lipid regulation (Golden *et al*., 2015; Schöner *et al*., 2017). Golden *et al.* (2015) also demonstrated that social avoidance induced by social defeat is reversible by chronic (but not acute) administration of antidepressants, providing this model with predictive validity. The social defeat model is considered a strong stress model as it utilizes social stress, which is an inherent aspect of rodents and humans.

5.1.2. Maternal separation (MS) model

The disruption of social contacts in rodents during early life development, also known as maternal separation, is a significant stressor across species and is used to mimic early life stress (Campos *et al*., 2013; Patchev & Patchev, 2006; Wang *et al.*, 2020, 2018). This model is crucial for investigating the pathophysiology and treatment of depression (Abelaira *et al*., 2013; Wang *et al*., 2018). It involves separating rat pups from their mothers for fixed periods each day, usually from the second day of life up to 14 days of age (Campos *et al.*, 2013; Golbidi *et al.*, 2015).

This stress model is based on its capacity to evoke long-lasting behavioral and structural alterations (Abelaira *et al.*, 2013; Campos *et al.*, 2013; Patchev & Patchev, 2006). Rodents exhibit high anxiety and defensive-exploratory behavior as well as HPA-axis alterations, increased GC and decreased neurotrophin levels, thus providing both face and construct validity (Abelaira *et al*., 2013; Campos *et al*., 2013; Patchev & Patchev, 2006; Schöner *et al*., 2017). Furthermore, the MS model shows predictive validity as treatment with clinical drugs such as antidepressants reversed most changes observed in behavior and molecular parameters (Abelaira *et al.*, 2013).

Although most stress models make use of adolescent rodents, the MS model provides a unique opportunity to study the adverse effects of early life stress. While this is regarded as a well-recognized stress model, it is crucial to note that its effects may vary between immediate behavioral changes (vocalization) and delayed endocrine changes (HPA-axis). In addition, the magnitude of the alterations is dependent on the length of separation and the exact age of the animals at the time of stress induction (Patchev & Patchev, 2006).

5.1.3. Unpredictable chronic mild stress (UCMS) model

The UCMS model was designed to mimic unpredictable, small everyday life stressors and hence ensure its translatability from the animal to the human context (Campos *et al.*, 2013). This protocol involves daily exposure to a host of mild stressors that are randomly applied to minimize habituation (Campos *et al.*, 2013; Golbidi *et al.*, 2015; Maggio & Segal, 2019; Teng *et al.*, 2021). Table 1 highlights the various mild stressors and their different targets. The UCMS model provides a good basis to investigate stress-related complications and is mainly used by researchers to study the neurobiology of depression (Abelaira *et al.*, 2013; Zhu *et al.*, 2014).

Table 1. Mild stressors included in the UCMS model.

Mild stressor	Target of stressor
Damp bedding	Touch sense
No bedding	Touch sense
Tilted cage	Balance
Confined area	Restraint
Switching between cages	Social stress
Altered dark/light cycles	Sleep-wake cycle
Strobe light	Sight sense
White noise	Hearing sense
Bobcat urine granules placed in cages & predatory sounds	Hearing and smell sense

Rodents chronically exposed to different stressors exhibit behavioral modifications such as altered sleep, increased anxiety and anhedonic behavior which is related to the face validity of this model (Abelaira *et al.*, 2013; Campos *et al.*, 2013; Golbidi *et al.*, 2015; Teng *et al.*, 2021; Zhu *et al.*, 2014). Alterations in the HPA-axis such as increased plasma corticosterone levels and adrenal weight support the model's construct validity (Abelaira *et al.*, 2013; Golbidi *et al.*, 2015). Rodents further exhibit decreased antioxidant enzymes and higher pro-inflammatory cytokines (Abelaira *et al.*, 2013; Golbidi *et al.*, 2015). Moreover, many of the changes elicited in the UCMS model can be reversed with antidepressants, demonstrating the predictive validity of this model (Abelaira *et al.*, 2013; Campos *et al.*, 2013; Schöner *et al.*, 2017). The UCMS is thus a reasonably established model of stress and one of the most translationally relevant models for studying the psychological and pathophysiological effects of chronic stress. However, the complex nature of this paradigm complicates reproducibility in different laboratories and makes its universal adoption quite difficult at times (Becker *et al.*, 2021a).

5.1.4. Chronic restraint stress (CRS) model

Restricting locomotor activity and exploration of rodents (better known as restraint) is probably the most extensively used method of stress induction (Patchev & Patchev, 2006; Zhu *et al.*, 2014). Despite its duration (acute, intermittent, or chronic), restraint is perceived as a severe stressor and successfully induces the entire spectrum of known allostatic responses (Patchev & Patchev, 2006). The stress associated with the CRS model is mild, continuous and predictable in order to mimic everyday human behavior, such as daily repetition of stressful work and/or

relationship-related issues (Seewoo *et al.*, 2020; Wang *et al.*, 2018). Here, rodents are restrained in transparent tubes for one hour on a daily basis for either an acute or chronic period, depending on the protocol (Brivio *et al.*, 2020; Marin *et al.*, 2007; Zhu *et al.*, 2014). Restraint as a method of stress induction is not limited to the CRS model as it is also employed in others such as the UCMS model.

Following a period of chronic restraint, animals exhibit anxiety-like behavior which is indicative of the model's face validity (Campos *et al.*, 2013; Rahal *et al.*, 2014; Schöner *et al.*, 2017; Seewoo *et al.*, 2020). With regards to construct validity, the CRS model typically displays decreased body weight, elevated corticosterone levels, and altered hepatic glucose homeostasis (Chen *et al.*, 2020; Hannibal & Bishop, 2014). Furthermore, the model demonstrates predictive validity as the alterations induced is effectively reversed with pharmacological treatment (Ampuero *et al.*, 2015; Chen *et al.*, 2020).

In contrast, others showed that predictable chronic stress such as daily restraint for an extended period could decrease anxiety-like behaviors (Parihar *et al.*, 2011). Such inconsistent behavioral responses may be attributed to adaptations of the HPA-axis that may desensitize or stabilize the HPA to such physical stressors (Marin *et al.*, 2007). However, the restraint in the Parihar *et al.* (2011) study was only applied for five minutes each day, which is far less severe than the one hour of restraint usually employed daily (Parihar *et al.*, 2011). It is thus clear that some studies can elicit contrarian outcomes, although such contradictions stem from a much shorter duration of restraint per day. Such relatively short durations are, however, not sufficient to accurately mimic daily repetitive stress experienced by humans and should not be considered as outcomes of the CRS model. The consensus is therefore that the CRS model succeeds in producing behavioral changes such as anxiety in rodents (Campos *et al.*, 2013; Rahal *et al.*, 2014; Seewoo *et al.*, 2020).

Thus, various reliable rodent models of stress exist, their strengths and weaknesses summarized in Table 2. In particular, the CRS model mimics predictable, inescapable daily stressors experienced by humans and thereby induces the entire spectrum of allostatic responses. It is therefore considered a strong rodent model of stress, although it is difficult to reproduce in different laboratories. This provides a unique opportunity to study chronic stress and the subsequent behavioral and physiological alterations.

Table 2. The strengths and weaknesses of popular rodent models of stress.

Types of stress models	**Strengths**		**Weaknesses**	
Social defeat	• Utilizes social stress, an inherent aspect of rodents and humans • Demonstrates face, construct, and predictive validity	Millic *et al.*, 2021; Golden *et al.*, 2015	• Injuries resulting from social defeat could threaten the rodent's well-being • Requires the use of many rodents and is labor-intensive • Suitable only to male rodents	Becker *et al.*, 2021; Golden *et al.*, 2015
Maternal separation	• Provides the opportunity to study early life stress • Demonstrates face, construct. and predictive validity	Abelaira *et al.*, 2013; Becker *et al.*, 2021; Wang *et al.*, 2018	• Results vary depending on the exact age of stress induction and the rat strain • The use of different methodologies results in an altered state of stress and long-term outcomes	Becker *et al.*, 2021; Patchev & Patchev, 2006.
UCMS	• Useful to investigate the effect of mild unpredictable stress • Translationally relevant • Minimizes habituation • Good face, construct, and predictive validity	Abelaira *et al.*, 2013; Becker *et al.*, 2021; Campos *et al.*, 2013; Golbidi *et al.*, 2015; Maggio & Segal, 2019	• Space demanding and labor-intensive • Varying reproducibility in different research centers • Studies use different applied stressors resulting in variations in the degree of stress	Abelaira *et al.*, 2013; Becker *et al.*, 2021
CRS	• Useful to study the effect of chronic psycho-emotional stress • Translationally relevant • Induces the entire spectrum of known allostatic responses • Good face, construct, and predictive validity	Becker *et al.*, 2021; Chen *et al.*, 2020; Patchev & Patchev, 2006; Seewoo *et al.*, 2020	• Possibility of habituation • Results vary depending on the duration of restraint	Marin *et al.*, 2007; Parihar *et al.*, 2011

5.2.Validation tests for rodent models of chronic stress

As previously noted, stress models need to be properly validated to ensure that the protocol is indeed inducing chronic stress. Here, the construct validity (representing physiological alterations) can be assessed by using enzyme-linked immunosorbent assay (ELISA) assays to determine relative levels of key circulating biomarkers. Animal welfare and related behavioral changes (face validity) are also important to determine the chronic stress phenotype in animal models employed.

Standard welfare monitoring is performed (SANS 10386:2008) to provide an overview of animal welfare by assessing changes in both physiologic components and behavioral responses (Beaver & Bayne, 2014; Hawkins *et al*., 2011; van der Meer *et al*., 2001). All experimental animals must be monitored at least once per day (including weekends) throughout the course of the study (Faculty of Health Sciences, University of Cape Town, 2020; Mähler *et al*., 2014). Here, a standard scoring system is used to monitor animal welfare based on a panel of indicators such as body and coat condition, behavior, body functioning and locomotor activity (Beaver & Bayne, 2014). Accurate documentation of the physical condition of animals according to standard scoring systems may help identify animals in poor clinical condition (rough hair coat, discharge around the eyes or nose, weight loss, dehydration, abnormal postures, weakness, and abnormal breathing) (Beaver & Bayne, 2014; Faculty of Health Sciences, University of Cape Town, 2020). Where relevant, the grimace scale can additionally be used to assess changes in facial expressions related to pain (Appendix B) (Burkholder *et al*., 2012; Deuis *et al*., 2017). The body condition score is another specific scoring system that may be used in conjunction with weight loss. It evaluates the subcutaneous fat layer covering the spine and pelvis and is a useful, rapid tool for assessing the overall condition and health of rodents (Appendix C) (Burkholder *et al.*, 2012; van der Meer *et al*., 2001). Documented behavior can help identify signs of morbidity such as alterations in expected rodent behavior, avoidance, decreased activity or hyperactivity, withdrawal, freezing and aggression (Beaver & Bayne, 2014; Faculty of Health Sciences, University of Cape Town, 2020). In this way researchers can gain insights into the physical and emotional state of the experimental animals and take measures to alleviate suffering (Burkholder *et al*., 2012; Hawkins *et al*., 2011; van der Meer *et al*., 2001).

As stress can precipitate or exacerbate anxiety-like disorders and pain disorders in humans (da Silva Torres *et al*., 2003; Jennings *et al.*, 2014; Lezak *et al*., 2017), stress research endeavors to evaluate such parameters as measures of stress-like behavior. This study will therefore

validate the efficacy of the CRS model by utilizing ethological based tasks centered on anxiety-like behavior and pain perception. The specific behavioral tasks included in this study will be discussed next. The interpretation of behavioral test results requires an understanding of the cause of the behavior observed. An increased understanding of behavior may be achieved by studying natural rodent behavior, evaluating the ethological validity of the test, determining the source of motivation in the test, and using the knowledge of the rodents' sensory capacity to view the test from a rodent's perspective (Belovicova *et al*., 2017).

5.2.1. Elevated plus maze task

The elevated plus maze (EPM) is one of the most common behavioral assays used to evaluate anxiety-related behavior in rodents (Campos *et al.*, 2013; Komada *et al.*, 2008; Schneider *et al*., 2011; Seewoo *et al*., 2020; Walf & Frye, 2007). This was originally developed by Montgomery (1955), who designed a Y-shaped apparatus and included two open sections and an enclosed section. Montgomery observed that a unique stimulus (for example a novel environment) can evoke both an exploratory and a fear drive creating approach-avoidance conflict behavior. Here, the rats consistently showed high levels of exploration and preference for the enclosed arms (Campos *et al*., 2013; Montgomery, 1955). He concluded that since the open and enclosed arms should evoke the same exploratory drive, the greater avoidance of the open arms was due to increased fear of open and elevated spaces (Campos *et al*., 2013; Komada *et al.*, 2008; Montgomery, 1955).

Handley & Mithani (1984) later modified this task into an elevated maze (at least 50 cm above the ground) with two open and two enclosed arms. These arms were arranged to form a plus shape, with the open arms facing each other (Handley & Mithani, 1984). Here, they aimed to detect either the anxiogenic-like (increases anxiety) or the anxiolytic-like (reduces anxiety) effect of drugs. One year later Pellow *et al.* (1985) conducted an extensive pharmacological, physiological, and behavioral validation of the EPM as a test of anxiety in rats, which was extended to mice by Lister (1990).

The test starts when the rat is placed in the center of the plus shape, facing the same open arm and takes a total of five minutes (Handley & Mithani, 1984; Schneider *et al*., 2011; Seewoo *et al*., 2020; Walf & Frye, 2007). The EPM task previously spanned over ten minutes, but the duration was shortened to five minutes by Pellow (1985) when researchers discovered that avoidance behavior was relatively high until the five-minute mark, after which it decreased

toward the end of the ten-minute session. The EPM task is used to record attempts into (latency to enter), entries into, and time spent in the open and closed arms (Handley & Mithani, 1984; Pellow *et al*., 1985). Rodents tend to avoid open, illuminated spaces while at the same time they tend to explore new spaces (Belovicova *et al.*, 2017). Such measures are thus ultimately used to determine to what extent the fear of the open arms overrules the natural exploration desire of the rodent (Pellow *et al*., 1985a). The latency to enter, number of entries and time spent within each arm type can be used as indices of anxiety, with less time spent in the open arms inferring higher anxiety levels (Campos *et al*., 2013; Komada *et al.*, 2008; Schneider *et al.*, 2011).

The EPM is widely used owing to its numerous advantages. According to Pellow *et al.* (1985) the task is rapid and simple, it does not require the use of expensive equipment, and is based on spontaneous behavior thereby avoiding noxious stimuli, lengthy training and food/water deprivation (Pellow *et al.*, 1985; Rodgers & Dalvi, 1997). The EPM is considered an ethologically based task as it assesses spontaneous rodent behavior without the presence of motivation (Belovicova *et al*., 2017; Carobrez & Bertoglio, 2005). It is therefore essential to perform a full behavioral analysis in addition to the preference for open or closed arms. Here, many other ethological parameters are also observed in order to assess anxiety-like behavior and therefore provide more insight into the emotional state of the rodent and more validity to the task (Table 3) (Rodgers & Dalvi, 1997; Rodgers & Johnson, 1995).

Table 3. Ethological parameters used to assess anxiety-like behavior during the EPM task.

Additional ethological parameters	**References**
Grooming – Licking of paws to clean the face or body	Cruz *et al*., 1994; Pellow *et al.*, 1985; Rodgers & Dalvi, 1997; Rodgers & Johnson, 1995
Stretched-attend posture (risk assessment) – Limbs and abdomen elongated horizontally, back arched	Carobrez & Bertoglio, 2005; Cruz *et al*., 1994; Rodgers & Dalvi, 1997; Rodgers & Johnson, 1995; Weiss *et al*., 1998
Freezing – Stiffening of whole body, immobility of whiskers	Pellow *et al*., 1985; Rodgers & Dalvi, 1997
Immobility – In a fixed position, with whisker movement	Pellow *et al*., 1985; Rodgers & Dalvi, 1997
Rearing – Back legs on ground, upper body raised (unsupported in air or resting against wall)	Cruz *et al*., 1994; Pellow *et al*., 1985; Rodgers & Dalvi, 1997; Rodgers & Johnson, 1995; Weiss *et al*., 1998

Head dips – Dropping the head over the maze edges	Pellow *et al.*, 1985

5.2.2. Tail flick task

The tail flick task is a test of nociception in rodents and was first described by D'Amour and Smith (1941). According to the International Association for the Study of Pain, nociception refers to the neural processes of encoding and processing noxious stimuli whereas pain is an unpleasant sensory and emotional experience associated with actual or potential tissue damage (Barrot, 2012). Although pain and nociception can be dissociated from one another they are closely linked (Barrot, 2012). In patients, pain is assessed by verbal expression, which is not possible in animals - instead pain is extrapolated (inferred) from pain-like behaviors (Barrot, 2012; Deuis *et al*., 2017). What is therefore usually referred to as pain-tests in animals are tests of nociception (Barrot, 2012). Of note, stress can potentially modulate pain perception depending on the nature, duration, and intensity. Such changes can either reduce or exacerbate the perception and response to pain (da Silva Torres *et al*., 2003; Ibironke & Mordi, 2011; Jennings *et al.*, 2014; Umar *et al.*, 2015). For example, acute stress can induce a reduction in the pain response and this phenomenon is known as stress-induced analgesia (Costa *et al*., 2005; da Silva Torres *et al*., 2003; Ibironke & Mordi, 2011; Jennings *et al*., 2014; Thomson *et al.*, 2020; Umar *et al.* 2015). Alternatively, chronic stress can cause stress-induced hyperalgesia which is an increased sensitivity to pain (Costa *et al*., 2005; da Silva Torres *et al*., 2003; Ibironke & Mordi, 2011; Jennings *et al*., 2014; Thomson *et al.*, 2020; Umar *et al.* 2015).

This test requires a heat stimulus to be applied to the tail of the rat. The heat stimulus can include radiant heat, where a beam of light is focused on the tail, or hot water where the distal end of the tail is immersed in a water bath (Deuis *et al*., 2017). The latter does not require specialized equipment and is therefore relatively quick and easy to perform (Deuis *et al*., 2017). Here, a hot water bath maintained at 50°C ± 2°C is used to immerse the distal end of the rat's tail until tail withdrawal (flicking response) or signs of struggle are observed (Deuis *et al*., 2017; Hanlon & Vanderah, 2010; Ibironke & Mordi, 2011; Umar *et al*. 2015). A cut-off time of 10 seconds is used to prevent tissue damage (Costa *et al*., 2005; Da Silva Torres *et al*., 2003 Hanlon & Vanderah, 2010). Tail withdrawal latency is then measured. The latency period in the chronic stress group is expected to be decreased when compared with the control, indicating

a state of stress-induced hyperalgesia (Costa *et al.*, 2005; Umar *et al.*, 2015).

5.2.3. Measures of validity in the EPM and tail flick test

Face validity describes the ability of a task to measure what it is supposed to measure (Hanell & Marklund, 2014; Walf & Frye, 2007). Here, the EPM measures the anxiety and fear related to open, elevated spaces. In this task the open arms are avoided and rodents spend most of their time in the closed arms (Walf & Frye, 2007). The EPM therefore demonstrates face validity. During the tail flick test a rodent is exposed to a noxious stimulus and the rodent's ability to detect it is determined by measuring their tail flick latency. In this task the rodent flicks its tail seconds after it was placed into a warm bath. The tail flick test therefore possesses face validity.

Construct validity refers to whether the observable dependent variable used measures the intended unobservable construct (Hanell & Marklund, 2014; Walf & Frye, 2007). With regards to the EPM, rodent anxiety levels are the unobservable variable, and it is measured with an observable variable which is the time spent on the open arms. This is can be observed in anxiogenic drugs reducing time spent on the open arms and anxiolytic drugs increasing the time spent on the open arms of the maze (Walf & Frye, 2007). The EPM therefore has construct validity. With regards to the tail flick test, the unobservable variable is detection of a noxious stimulus by the rodent, and it is measured with an observable variable which is the tail flick latency. This is demonstrated by an increase in tail flick latency after morphine-nimodipine co-administration which in turn is reversed by naloxone administration (Gupta *et al.*, 2007), attributing construct validity to the tail flick test.

Predictive validity is defined as the extent to which the dependent measure predicts behavior on a related measure (Hanell & Marklund, 2014; Walf & Frye, 2007). During the EPM, increased open arm activity due to lower anxiety levels should predict similar behavior in other regards. Frye *et al.* (2000) showed that higher open arm activity occurs in rodents that also demonstrate increased central square entries in a brightly lit open field. Furthermore, plasma corticosterone is increased with open arm exposure and is positively correlated with risk assessment behavior (Walf & Frye, 2007). The EPM therefore demonstrates predictive validity. After careful investigation, literature describing or demonstrating the predictive validity of the tail flick test could not be found and therefore this cannot be verified in this instance.

The results obtained from the above-mentioned tests can be used to assess the presence and

extent of stress-like behaviors associated with the CRS model. Although behavioral tests are an integral part of stress research, it is crucial to consider biological factors that can affect the behavior of the animal when interpreting such results. It is important to support behavioral test results with other methodologies such as biochemical and molecular measurements. This study will therefore assess plasma ACTH, corticosterone, BDNF and NPY measurements. The route through which stress exerts downstream pathophysiological alterations is still largely debated. However, there is robust evidence that implicates oxidative stress as a crucial role player in this instance. Our postulate is therefore that oxidative stress, elevated NOGP flux and downstream sequelae also play a key role in this process.

6. STRESS AND ITS LINKS TO CARDIOVASCULAR DISEASES

6.1.Oxidative stress

Oxidative stress is a common phenomenon in the body and was first defined by the redox pioneer Helmut Sies as an imbalance between systemic prooxidants and antioxidants favoring the prooxidants (Giustarini *et al.*, 2009). Prooxidants include all reactive, free radical containing molecules collectively known as reactive oxygen species (ROS) (Rahal *et al.*, 2014). These reactive species are natural by-products of intracellular metabolism, but are produced in relatively higher amounts under conditions of stress (D'Oria *et al.*, 2020; Rahal *et al.*, 2014; Stier *et al.*, 2019).

A variety of sources generate ROS, with the mitochondrion being the most prominent site (~90 %) (D'Oria *et al*., 2020; Rahal *et al.*, 2014; Srivastava & Kumar, 2015; Stier *et al*., 2019). Here, the electron transport chain is responsible for the formation of the majority of superoxide (Mapanga & Essop, 2016). Other examples of ROS include hydrogen peroxide, hydroxyl radicals and peroxyl radicals. Of note, a certain amount of ROS is required under physiological conditions to sustain optimal cellular functioning, for example to modulate multiple cell signaling pathways and regulate the expression of various genes (D'Oria *et al.*, 2020; Rahal *et al.*, 2014). Despite the physiological importance of ROS, excessive amounts of radicals readily react with other molecules like nucleic acids, lipids, carbohydrates, and proteins and can induce detrimental damage (D'Oria *et al.*, 2020; Dubois-Deruy *et al.*, 2020; Rahal *et al.*, 2014; Stier *et al.*, 2019). The detoxification of ROS is therefore essential for normal intracellular functioning and thus an antioxidant defense system is in place to ensure a balance between its production and removal (D'Oria *et al.*, 2020; Dubois-Deruy *et al.*, 2020).

The previously discussed alterations in glucose homeostasis and the body's inflammatory profile resulting from chronic stress also contribute to ROS generation, overwhelming the detoxifying capacity of tissues (Rahal *et al.*, 2014). This surge in ROS over a prolonged period may deplete the natural antioxidant capacity and can thus give rise to a relatively high oxidative state in the heart. Subsequently, persistent cardiac oxidative stress can elicit damaging effects, including cardiomyocyte dysfunction and apoptosis, impaired cardiac remodeling, fibrosis, and contractile dysfunction (D'Oria *et al.*, 2020).

6.2.Non-oxidative glucose pathways

Glucose metabolic perturbations also occur downstream of oxidative stress. Here, oxidative stress-mediated DNA damage activates poly adenosine diphosphate ribose polymerases (PARPs) (Mapanga & Essop, 2016; Schalkwijk & Stehouwer, 2020). However, higher PARP activity can suppress glyceraldehyde-3 phosphate dehydrogenase (GAPDH), a key enzyme of the glycolytic pathway. This results in an upstream accumulation of glycolytic intermediates that are then shunted into various NOGPs and thereby increasing their flux (Mapanga & Essop, 2016; Schalkwijk & Stehouwer, 2020).

The NOGPs metabolize glucose and display a constant, relatively low flux under normal glycemic conditions (Mapanga & Essop, 2016). The main NOGPs include the polyol pathway, the protein kinase C (PKC) pathway, the hexosamine biosynthetic pathway (HBP) pathway, and the AGE pathway. All the NOGPs are briefly described in Figure 6. Under physiological conditions, such pathways induce nutrient-driven post-translational modifications of various molecules to ensure the proper functioning of signaling pathways (Fishman *et al*., 2018; Mapanga & Essop, 2016; Schalkwijk & Stehouwer, 2020). However, the high glucose availability resulting from chronic stress triggers an increase in such processes (Fishman *et al*., 2018; Giri *et al*., 2018; Hegab, 2012; Schalkwijk & Stehouwer, 2020). This greatly promotes the formation of the molecules produced in NOGPs and thereby contributes to myocardial oxidative stress, inflammation, and possible atherosclerosis (Fishman *et al.*, 2018; Hegab, 2012).

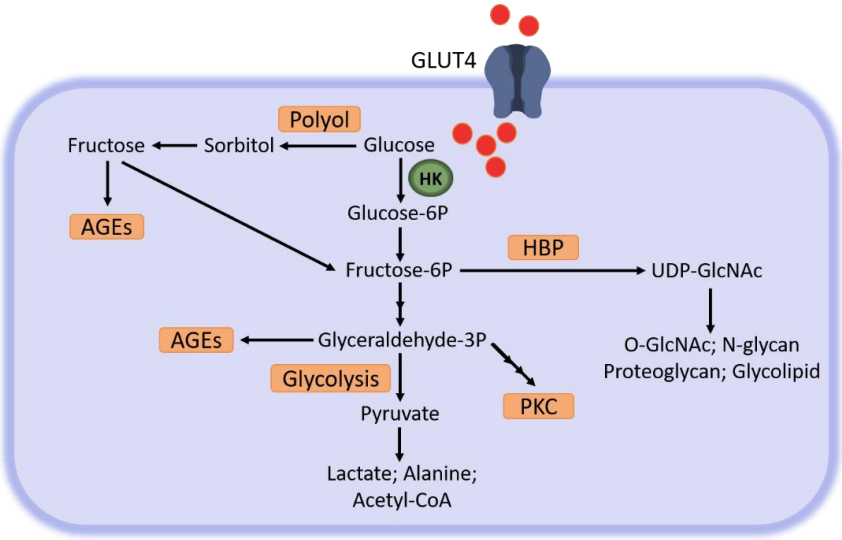

Figure 6. Glucose metabolic pathways in the adult heart. Abbreviations: GLUT, glucose transporter; HK, hexokinase; AGEs, advanced glycated end products; HBP, hexosamine biosynthetic pathway; PKC, protein kinase C; 6P, 6-phosphate; UDP-GlcNAc, uridine diphosphate N-acetylglucosamine; *O*-GlcNAc, *O*-linked *β*-N-acetylglucosamine; CoA, coenzyme A. Image adapted from Mapanga & Essop, 2016 and Schalkwijk & Stehouwer, 2020.

The previous discussions clearly highlight the possible relationship between chronic stress and the development of cardiac pathophysiology. It further emphasizes the role of oxidative stress and increased NOGP flux in this process. Such molecular alterations will be further investigated during future studies in our laboratory. The current study will primarily focus on establishing and validating a chronic rodent model of restraint stress through anthropometric, biochemical, and behavioral assessments. This will enable future studies in our laboratory to investigate the pathophysiological alterations associated with chronic psychological stress.

7. RESEARCH FOCUS

7.1. Aims and objectives

We aim to establish and validate an *in vivo* model of chronic restraint stress in male and female Wistar rats. Our main objectives were the following:

- Assess anthropometric alterations through body and organ weight measurements
- Determine behavioral alterations with the use of two behavioral tests namely, the tail flick task and the EPM
- Evaluate biochemical alterations in plasma corticosterone, ACTH, BDNF and NPY with the use of ELISAs

8. METHODS AND MATERIALS

8.1. Study Ethics

This study was approved by the Animal Ethics Committee of Stellenbosch University (ACU-2021-19400) (Appendix A). Moreover, the animals used in this study were treated in accordance with the Guidelines for the Care and Use of Laboratory Animals of the National Academy of Science (National Institute of Health publication No. 85-23, revised 1996).

8.2. Animals and Habituation Period

A total of 32 Wistar rats (250-350 g) were included in this study and group-housed in rooms at the Animal Laboratory at the Stellenbosch University's medical school (Tygerberg, South Africa). These rooms included controlled temperature and lights. Wistar rats were selected for the study as they are the most widely used rat strain due to their multipurpose characteristics, making them a powerful species for medical research. The Wistar rat is an outbred rat strain and provides the study with a divergent experimental population in order to draw more valid extrapolations to other strains and species. Moreover, there were also logistical reasons for selecting the Wistar strain, i.e., readily available to us from the breeding facility with a relatively rapid turnover time. As our research team only obtained limited access to specialized rooms in the animal facility, the Wistar strain offered good utility in this context. Both male (n=16) and female (n=16) rats were included in the study and initially underwent a 2-week habituation period to allow them to acclimatize to their cage mates, the novel environment, handlers, and a behavioral test. All animals were weighed once a week and their weight were recorded.

A power analysis was conducted by Prof. Martin Kidd (Director: Centre for Statistical Consultation, Stellenbosch University), using data collected from previously conducted stress studies our own labs as well as insight from our collaborator, Prof. Carine Smith (stress biology expert based at Stellenbosch University). Means and standard deviations of important parameters for model validation (plasma corticosterone and a behavioral measure of anxiety) from our previous study were submitted to Prof Kidd and the analyses were performed off of the assumption that the new model would result in about half of the variation that was seen previously. This assumption was validated by Prof. Carine Smith who has worked extensively with restraint models. Cohen (1988, 1992) suggested that d = 0.2 be considered a 'small' effect

size, 0.5 represents a 'medium' effect size and 0.8 a 'large' effect size. The analyses revealed that we would require 25 rats per group to detect an effect size of 0.8. Any less than that, for example, 15 animals per group will only give us 80% power to detect an effect size of 1.1 as a significant difference - an effect size that is considered large by statistical standards. It would therefore be more challenging to make solid assumptions from the data that are collected unless the differences between groups are very large (not usually the case in chronic stress studies). However, through ethical clearance the group received only 120 animals and therefore the sample size was slightly decreased for males (n=16) and females (n=16) to be able to assign animals to each of the different projects in the group.

8.3. Housing conditions

Rats are social animals and were thus housed four per cage, to allow for social interaction and indulgence in normal behaviors such as grooming (Boggiano *et al*., 2008). Three days after habituation started, the male and female rats were randomly divided into two equal groups based on sex (n=8; control and stress). Once divided into the different cohorts, the rats continued to be group-housed in the same room with controlled temperature and lights. Appropriate bedding was provided, and all animals had *ad libitum* access to a standard chow diet and water (except during the period of restraint). Environmental enrichment is crucial to allow animals to express species-typical behavior, promote mental and physical health and therefore enhance overall animal welfare (Baumans, 2005). Environmental enrichment was provided for the duration of this study in the form of wood shavings (allows for burrowing behavior), group-housing (allows for social interaction), food pellets placed on the wire bar lid (enables rodents to stretch upright and facilitates interaction between the rodent and its environment through visual and olfactory inputs) and red plastic cylinder-shaped tubes (provides shelter and can serve as a gnawing item) (Baumans, 2005).

8.4. Experimental procedure

The stress protocol was started when the rats reached 9-10 weeks to reflect the age of adulthood, known as a period during which humans are likely to experience increased life stressors (Sengupta, 2013). The male and female stress groups were subjected to one hour of restraint each day, seven days a week for four weeks (Figure 7) (Brivio et al., 2020; Marin et al., 2007; Zhu et al., 2014). Here, the rats were placed in Perspex cages which were obtained from Prof Carine Smith’s group and built to tightly restrain one rat in each of the six compartments. Of

note, the Perspex cages prevent the rats from having a stress response due to isolation as they can still see their peers during restraint. The body heat from their peers also warms the sides of the cage to create a similar effect to when rats sleep clumped together. Restraint took place during the light cycle between 09H00 and 11H00, while corticosterone levels would be expected to be in the falling phase of the diurnal rhythm. This ensures that the changes observed are due to the restraint stress and not the natural rhythmic increase in corticosterone levels. Unrestrained animals (control groups) were left in their home cages without any intervention and all rats received regular check-ups from a registered veterinarian to ensure overall well-being. The rats were subjected to a baseline tail flick test prior to the start of experimental procedure and a final tail flick test one day following the completion of the experimental procedure. An EPM task was also performed two days post restraint stress. All behavioral tests were done during the light cycle from 09H00 onwards, while corticosterone levels would be expected to be in the falling phase of the diurnal rhythm.

Previous exposure to the EPM apparatus in rats and mice elicits a learning component in the first trial which modifies performance in subsequent trials (Bertoglio & Carobrez, 2000). This phenomenon is called 'one-trial tolerance' and leads to a marked decrease in open arm exploration despite treatment with anxiolytic-acting benzodiazepines upon retesting (Schneider et al., 2011). Here, the prior exposure is thought to induce the release of endogenous inverse agonists that bind to, and alter, benzodiazepine receptors in brain areas – further desensitizing such receptors. This ultimately enhances memory processes and induces a shift in the anxiety/fear response from an unconditioned to an acquired phobic response (Bertoglio & Carobrez, 2000). A baseline measurement was thus only taken for the tail flick test and not for the EPM.

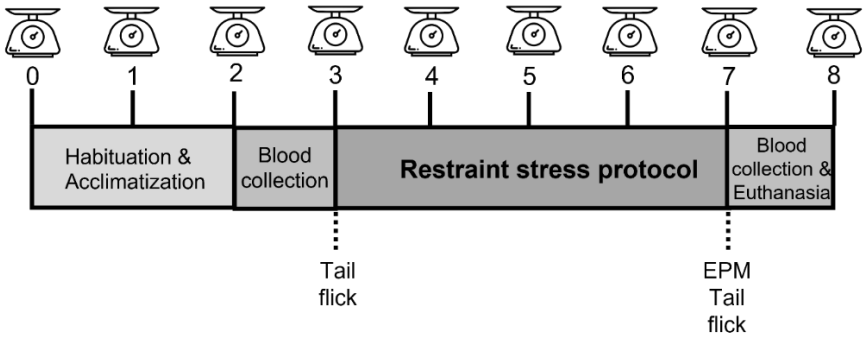

Figure 7. Restraint stress experimental timeline. Indicates the full study duration, habituation, and acclimatization periods. It also contains weekly weighing, blood collection at the start and end of the CRS protocol, behavioral tests, and euthanasia. **Abbreviations:** EPM, elevated plus maze. Image self-constructed.

8.5. Blood collection and euthanasia

Blood sample collection was done at the start and the end of the CRS protocol. During the last four days of habituation, the rodents were placed under anesthesia (2% isoflurane for 3 minutes) to enable blood collection for baseline biochemical measurements. At the end of the experimental protocol, the rodents were again placed under anesthesia (2% isoflurane for 3 minutes) in order to collect blood for final biochemical measurements. Here, the tubes were precoated with ethylenediaminetetraacetic acid to prevent clotting (456034, Greiner Bio-One, Frickenhausen, Germany). Following collection, the whole blood samples were centrifuged (1,500 x g for 10 minutes, at 4°C) using the Optima MAX-XP Ultracentrifuge (CTZZ18006, Beckman Coulter, California, USA) for the preparation of plasma samples and were then aliquoted, snap-frozen and stored at -80°C.

Following the experimental procedure, all rats were euthanized through decapitation. Immediately after decapitation trunk blood was collected through a plastic funnel into serum blood tubes (456018, Greiner Bio-One, Frickenhausen, Germany) that was used for another master's project. Various organs were collected and weighed to detect major macroscopic and/or ultrastructural changes that can then be investigated further. Here, the brain, heart, liver, kidneys, adrenals, and spleen were harvested and subsequently snap-frozen in liquid nitrogen. During the collection of the brain, different regions were dissected out and stored separately. Such regions include the pre-frontal cortex, hypothalamus, hippocampus, amygdala, and rest of brain. All harvested samples were stored at -80°C until further use.

8.6. Behavioral tests

8.6.1. Elevated plus maze (EPM) task

The elevated plus maze task was performed as previously described by Pellow *et al.* (1985). The maze used during behavioral testing consisted of two open arms (50 cm x 10 cm) and two closed arms of the same size with 40-cm-high walls (Figure 8). The whole maze is elevated to a height of 50 cm above the ground and had a central square (10 x 10 cm) where the arms intersects to form a plus shape, with the open arms facing each other (Handley & Mithani, 1984). The entire maze is constructed out of wood.

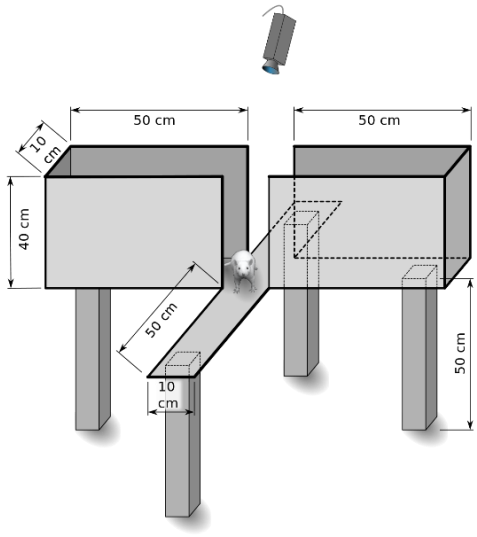

Figure 8. **An illustration depicting the set-up of the elevated plus maze test.** Image obtained from Free Software Foundation, Incorporated.

The EPM started when a rat was placed in the center of the plus shape, facing the same open arm each time and took a total of five minutes. The test was performed during the light cycle while corticosterone levels would be expected to be in the falling phase of the diurnal rhythm. Animals were habituated to the behavioral testing room prior to the start of the test and the maze remained in the same position during each test performed. The following parameters were recorded: attempts into, entries into, and time spent in the open and closed arms. Other ethological behaviors such as stretch-attend posture, rearing, and head dips were also assessed. The procedure was recorded on a GoPro HERO7 (Woodman Labs Inc., California) that was positioned directly above the EPM and mounted to a beam that spanned across the ceiling of the room The data was collected from the recorded videos and then analyzed by blinded investigators.

8.6.2. Tail flick task

The tail flick task was performed as previously described by Umar *et al*. (2015) and required the tail of the rat to be immersed in a hot water bath until tail withdrawal (flicking response) or signs of struggle were observed (Umar *et al*., 2015). The rat was manually and loosely restrained by the researchers and placed perpendicular to the water bath. A 500 mL beaker was filled up to the 450 mL mark with warm water (50°C ± 2°C). Thereafter 3 cm (or 2/3) of the tail (measured from the distal tip) was submerged in the water. A cut-off time of 10 seconds was used to prevent tissue damage and tail withdrawal latency was then measured. While one

researcher loosely restrained the rat and placed the distal end of its tail in the water bath, the other person monitored the flicking response through observation and recorded the results. During this procedure, tail withdrawal latency was used as an indication of nociception.

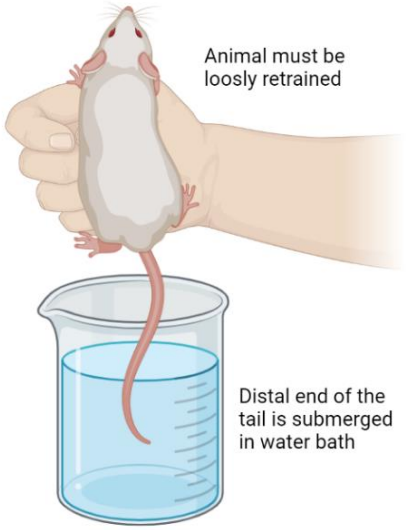

Figure 9. An illustration depicting the set-up of the tail flick test. Image self-constructed using Biorender.com.

8.7. Biochemical analysis

ELISA kits were used to assess baseline and final plasma corticosterone (Elabscience®, Houston, Texas, USA; #E-EL-0160) as well as final plasma ACTH levels (Elabscience®, Houston, Texas, USA; #E-EL-R0048), as they are considered as reliable markers of HPA-axis activity. Final plasma BDNF (Elabscience®, Houston, Texas, USA; #E-EL-R1235), and NPY levels (Elabscience®, Houston, Texas, USA; #E-EL-R0655) were also assessed as they are reliable markers involved in mood regulation.

Baseline measurements were only performed and analyzed for plasma corticosterone, while final measurements were obtained and analyzed for plasma corticosterone, ACTH, BDNF and NPY. Ideally, the study should have included baseline and final measurements for all four the biochemical markers. However, due to limited resources that had to be divided between several projects, this study could only make use of five ELISA kits. With this we assessed final plasma corticosterone, ACTH, BDNF and NPY. Thereafter, only one kit remained which is why baseline measurements were only taken for plasma corticosterone.

A concentration run was initially done by running the highest and the lowest concentrations in the standard series provided by each kit concurrently with male and female, control and stressed samples made up to different concentrations. This was done to determine which sample concentrations fell within the kit's range. For each kit the concentration that fell comfortably within the standard range for all the samples were chosen for the full kit run. Once the appropriate sample concentration was determined, the assay procedure could commence. Firstly, all the reagents were prepared according to the kit instructions and 50 µL of diluted standard and sample were added to the wells. Thereafter, 50 µL Biotinylated Detection Antibody working solution was immediately added to each well, the plate was covered with a sealer and incubated for 45 minutes at 37°C. Following incubation, the plate was decanted and washed for 1 minute with 350 µL of wash buffer. This step was repeated 3 times. 100 µL of HRP Conjugate working solution was then added to each well. Once again, the plate was covered and incubated for 30 minutes at 37°C. Following incubation, the plate was decanted and washed for 1 minute with 350 µL of wash buffer. This step was repeated 5 times. Next, 90 µL of Substrate Reagent was added to each well, the plate was covered and incubated for 15 minutes at 37°C. Finally, 50 µL of Stop Solution was added to each well and the optical density was determined using the FLUOstar Omega Multimode Microplate Reader (415-1364, BMG Labtech, Offenburg, Germany) set to 450 nm. All standards and samples were run in duplicate.

8.8. Statistical analysis

All statistical analyses were performed using GraphPad Prism version 7.0 (GraphPad Software Inc., San Diego, California) and Statistica (Humburg, Germany) and was done in conjunction with Prof. Martin Kidd at the Centre for Statistical Consultation at Stellenbosch University. Firstly, the distribution of data was determined with the Shapiro-Wilks test. Parametric data were then analyzed with either a Student's t-test or by analysis of variance (ANOVA) tests. The non-parametric data were analyzed by the Mann-Whitney test. In all cases a p-value <0.05 was considered significant and any outliers were excluded. Levene's test for homogeneity of variance was used to determine equal variances and Fisher's LSD test was used as post hoc analysis to correct for multiple comparisons. All data is presented as mean ± standard deviation (SD).

9. RESULTS

9.1. Anthropometric measurements

All rats were weighed on a weekly basis for the duration of the study. Although the male control rats displayed slightly higher body weights than their stressed counterparts from week 2 onwards, this change was not statistically significant (**Figure 10a**). The male stressed group (34.25 ± 2.37 % body weight, n=8) exhibited a decrease versus the controls (41.00 ± 6.32 % body weight, n=8) in the percentage change in body weight over time ($p<0.01$; **Figure 10b**). In addition, the female control rats did not show a statistically significant difference when compared to the stressed rats (**Figure 11a**) despite exhibiting a slightly lower body weight from week 2 onwards. There was no significance between the female controls (24.50 ± 3.54 % body weight, n=8) and stressed rats (22.75 ± 2.65 % body weight, n=8) in terms of the percentage weight change over time. (**Figure 11b**).

9.1.1. Body weight

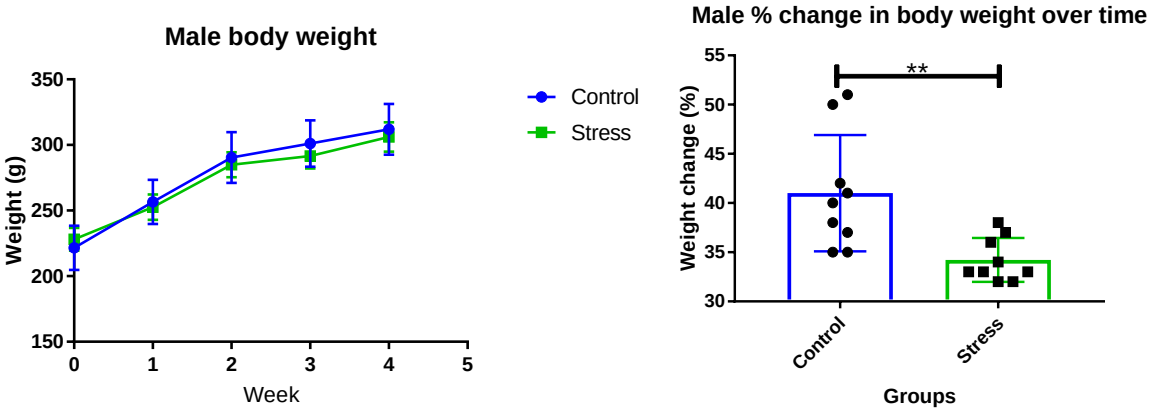

Figure 10. (a) Body weight over time. The male rats were weighed on a weekly basis for the duration of the protocol. Although the control group showed slightly higher body weights from week one, there were no statistically significant differences between the two groups by the end of the study. Analysis done by two-way ANOVA; values represented as mean ± SD, n=8. **(b) Percentage change in body weight over time.** The experimental (stressed) group (34.25 ± 2.37 % body weight, n=8) displayed a decrease versus controls (41.00 ± 6.32 % body weight, n=8) in the percentage change in body weight over time ($p<0.01$). Analysis done by Students t-test; values represented as mean ± SD.

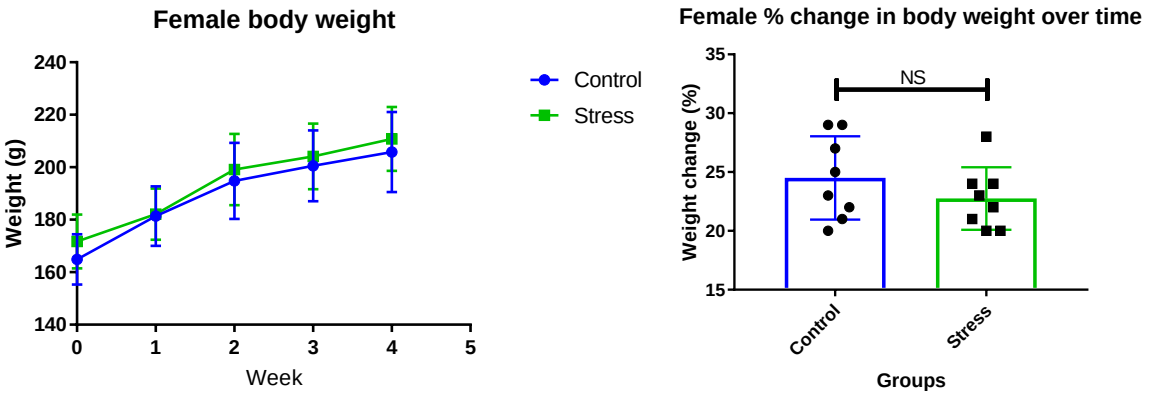

Figure 11. (a) Body weight over time. The female rats were weighed on a weekly basis for the duration of the protocol. Although the experimental (stress) group showed a slightly higher body weight from week two, there were no statistically significant differences between the two groups by the end of the study. Analysis done by two-way ANOVA; values represented as mean ± SD, n=8. **(b) Percentage change in body weight over time.** There was no significant percentage change in body weight over time for the stressed rats (22.75 ± 2.65 % body weight, n=8) versus controls (24.50 ± 3.54 % body weight, n=8). Analysis done by Students t-test; values represented as mean ± SD, n=8.

9.1.2. Organ weights

Following the completion of the experimental period, rats were euthanized, and several organs dissected out and weighed before freezing and storage. No significant changes were observed for heart, liver, spleen, left and right adrenal glands, pre-frontal cortex, hypothalamus, hippocampus, and amygdala weights in males (**Appendix D**). However, female control rats (0.03 ± 0.01 % body weight, n=8) displayed an increased hypothalamus weight ($p<0.05$) versus the stressed rats (0.05 ± 0.02 % body weight, n=8) (**Figure 12**). No significant changes were observed for heart, liver, spleen, left and right adrenal glands, pre-frontal cortex, hippocampus, and amygdala weights in the females (**Appendix D**). The male and female organ weights are summarized in **Table 4**.

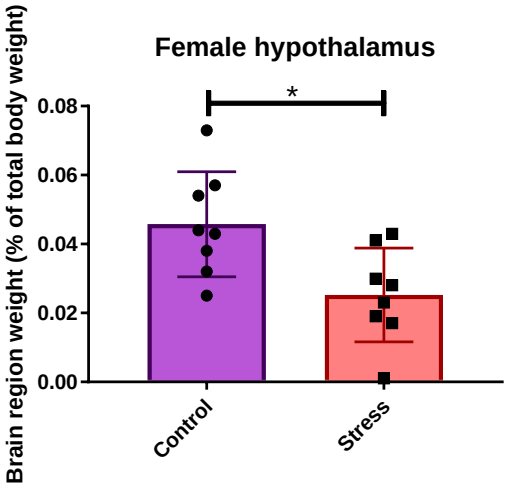

Figure 12. **Female hypothalamus weight**. The experimental (stress) group (0.03 ± 0.01 % body weight, n=8) showed a difference ($p<0.05$) compared to the control group (0.05 ± 0.02 % body weight, n=8). Organ weight was calculated as a percentage of body weight. Analysis done by Student's t-test; values represented as mean ± SD.

Table 4. Male and female organ weights following the CRS protocol. Organ weight was calculated as a percentage of total body weight. *Values rounded to two decimals spaces.

Organ	Weight (mean ± SD; % body weight)					
	Male control	Male stress	Significant difference (Yes or No)	Female control	Female stress	Significant difference (Yes or No)
Heart	0.36 ± 0.03	0.37 ± 0.01	No	0.41 ± 0.02	0.40 ± 0.02	No
Liver	3.52 ± 0.14	3.61 ± 0.23	No	3.68 ± 0.10	3.72 ± 0.10	No
Spleen	0.27 ± 0.03	0.27 ± 0.04	No	0.37 ± 0.08	0.34 ± 0.06	No
Left adrenal	0.03 ± 0.02	0.03 ± 0.01	No	0.06 ± 0.02	0.05 ± 0.01	No
Right adrenal	0.03 ± 0.01	0.03 ± 0.01	No	0.06 ± 0.01	0.05 ± 0.01	No
Pre-frontal cortex	0.03 ± 0.01	0.03 ± 0.01	No	0.06 ± 0.01	0.05 ± 0.01	No
Hypothalamus	0.03 ± 0.01	0.03 ± 0.01	No	0.05 ± 0.02	0.03 ± 0.01	Yes ($p<0.05$)
Hippocampus	0.04 ± 0.01	0.03 ± 0.01	No	0.04 ± 0.01	0.04 ± 0.01	No
Amygdala	0.03 ± 0.02	0.02 ± 0.01	No	0.03 ± 0.01	0.02 ± 0.01	No

9.2. Biochemical measurements

To ascertain whether the model successfully induced a state of chronic stress, well-known biochemical parameters can be assessed as markers of the underlying physiological processes. For example, corticosterone and ACTH concentrations are regarded as sound biological markers of HPA-axis activity, while BDNF and NPY are useful ones to indicate the regulation of stress and mood.

Our data revealed no significant differences in baseline corticosterone levels between male control (6.96 ± 1.12 ng/ml, n=8) and stressed rats (6.73 ± 1.24 ng/ml, n=8) (**Figure 13a).** The final male plasma corticosterone levels were significantly higher ($p<0.01$; **Figure 14a**) in the stressed group (15.08 ± 1.37 ng/ml, n=8) compared to the control group (12.58 ± 1.43 ng/ml, n=8), while no significant changes were detected in plasma ACTH levels for controls (463.80 ± 50.41 pg/ml, n=7) versus stress (498.20 ± 37.32 pg/ml, n=8) (**Figure 15a**). BDNF concentrations in the male stressed rats (27.08 ± 24.28 pg/ml, n=7) were lower ($p<0.05$; **Figure 16a**) compared to the controls (169.80 ± 168.60 pg/ml, n=8). No significant differences in

plasma NPY levels were detected between the male control (1522.00 ± 307.30 pg/ml, n=8) and stressed rats (1645.00 ± 189.10 pg/ml, n=8) (**Figure 17a**).

No significant differences were detected between female control (5.76 ± 0.87 ng/ml, n=8) and stressed rats (6.25 ± 0.65 ng/ml, n=7) for baseline plasma corticosterone concentrations (**Figure 13b**). However, female final plasma corticosterone levels were lower ($p<0.05$; **Figure 14b**) in the stressed group (13.13 ± 1.48 ng/ml, n=8) compared to the controls (15.09 ± 1.84 ng/ml, n=8), while plasma ACTH levels were higher ($p<0.05$; **Figure 15b**) in the stressed group (566.90 ± 91.80 pg/ml, n=8) versus controls (485.70 ± 43.36 pg/ml, n=8). No significant changes were detected between the control (44.62 ± 52.52 pg/ml, n=8) and stress groups (59.74 ± 59.05 pg/ml, n=7) for plasma BDNF levels (**Figure 16b**) as well as between the control (1536.00 ± 356.50 pg/ml, n=8) and stress groups (1527.00 ± 224.10 pg/ml, n=8) for plasma NPY concentrations (**Figure 17b**).

8.2.1. Baseline plasma corticosterone (CORT)

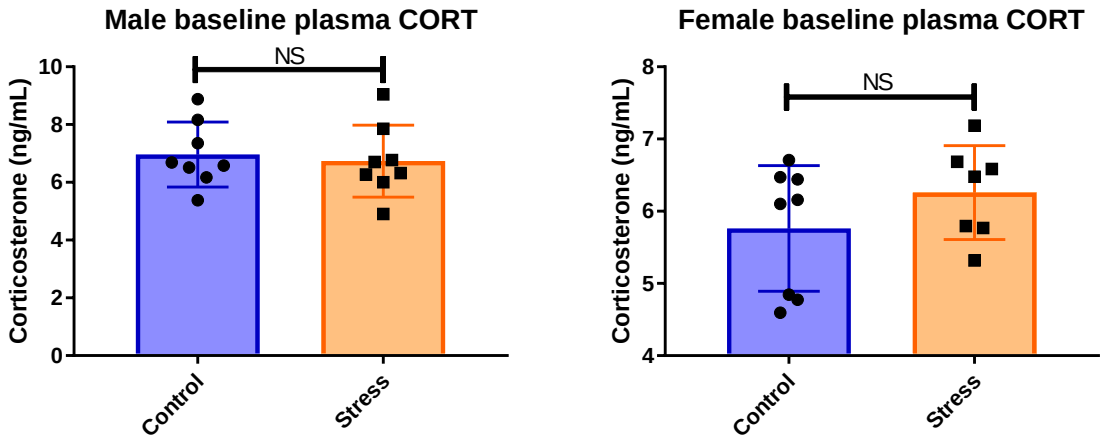

Figure 13. (a) Male plasma CORT levels at baseline. There was no statistical significance between the experimental (stress) group (6.73 ± 1.24 ng/ml, n=8) and the control group (6.96 ±, 1.12 ng/ml, n=8). **(b) Female plasma CORT levels at baseline.** The experimental (stress) group (6.25 ± 0.65 ng/ml, n=7) displayed no difference compared to the control group (5.76 ± 0.87 ng/ml, n=8). Analysis done by mixed model ANOVA in R; values represented as mean ± SD.

8.2.2. *Final plasma corticosterone (CORT) levels*

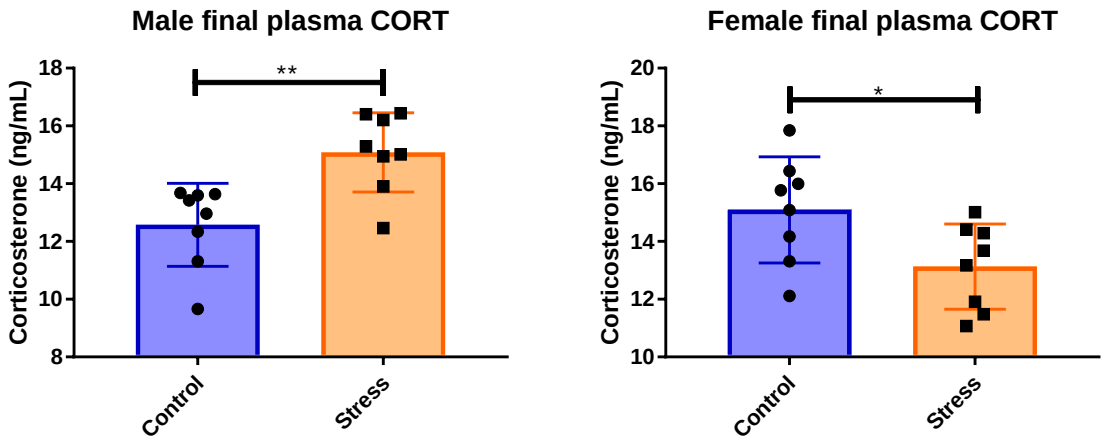

Figure 14. (a) Male plasma CORT levels at the end of the experimental protocol. A statistical significance ($p<0.01$) was observed between the experimental (stress) group (15.08 ± 1.37 ng/ml, n=8) and the control group (12.58 ± 1.43 ng/ml, n=8). **(b) Female plasma CORT levels at the end of the experimental protocol.** The experimental (stress) group (13.13 ± 1.48 ng/ml, n=8) showed a significant difference ($p<0.05$) compared to the control group (15.09 ± 1.84 ng/ml, n=8). Analysis done by mixed model ANOVA in R; values represented as mean ± SD.

8.2.3. *Plasma adrenocorticotropic hormone (ACTH) levels*

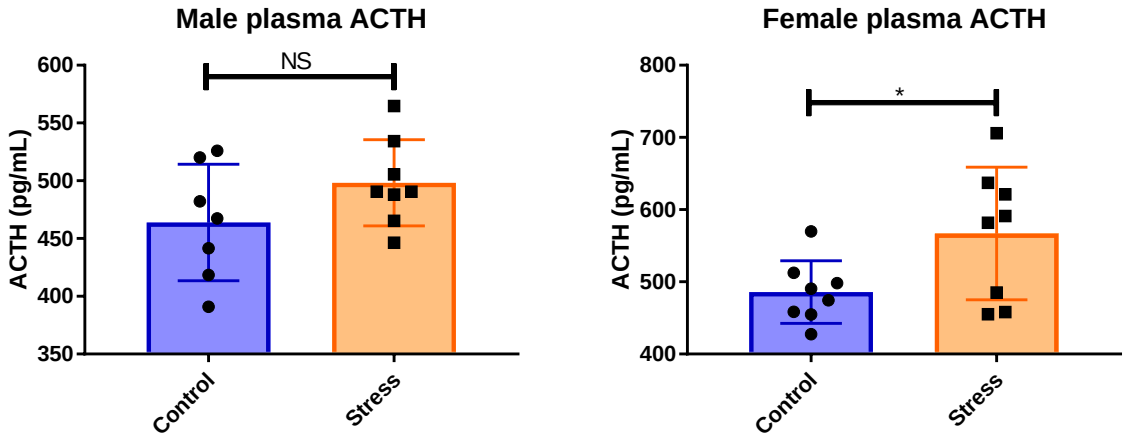

Figure 15. (a) Male plasma ACTH levels. No statistical significance was observed between the experimental (stress) group (498.20 ± 37.32 pg/ml, n=8) and the control group (463.80 ± 50.41 pg/ml, n=7). **(b) Female plasma ACTH levels**. The experimental (stress) group (566.90 ± 91.80 pg/ml, n=8) showed a significant difference ($p<0.05$) compared to the control group (485.70 ± 43.36 pg/ml, n=8). Analysis done by two-way ANOVA; values represented as mean ± SD.

8.2.4. Plasma brain-derived neurotropic factor (BDNF) levels

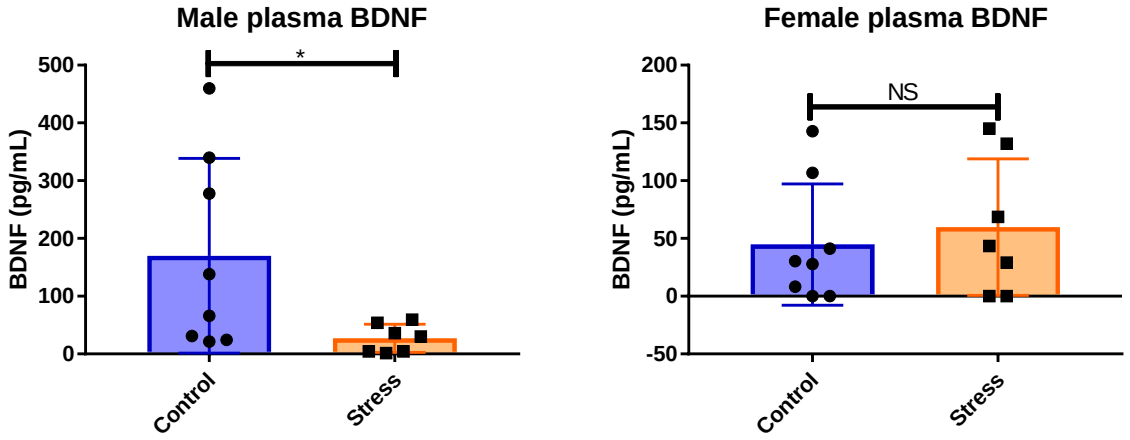

Figure 16. (a) Male plasma BDNF levels. A statistical significance ($p<0.05$) was observed between the experimental (stress) group (27.08 ± 24.28 pg/ml, n=7) and the control group (169.80 ± 168.60 pg/ml, n=8). **(b) Female plasma BDNF levels.** The experimental (stress) group (59.74 ± 59.05 pg/ml, n=7) showed no significant difference compared to the control group (44.62 ± 52.52 pg/ml, n=8). Analysis done by two-way ANOVA; values represented as mean ± SD.

8.2.5. Plasma neuropeptide Y (NPY) levels

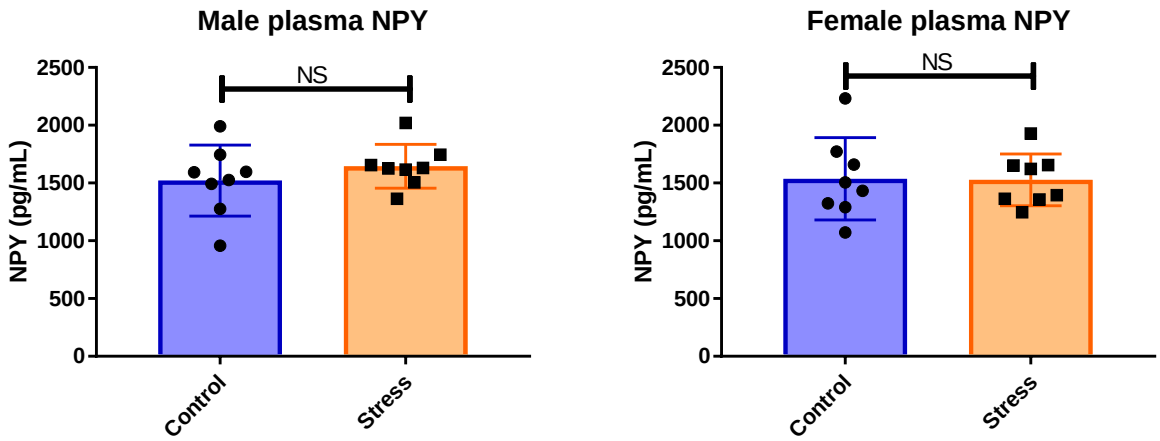

Figure 17. (a) Male plasma NPY levels. A statistical significance ($p<0.01$) was observed between the experimental (stress) group (1645.00 ± 189.10 pg/ml, n=8) and the control group (1522.00 ± 307.30 pg/ml, n=8). **(b) Female plasma NPY levels.** The experimental (stress) group (1527.00 ± 224.10 pg/ml, n=8) had no significant difference compared to the control group (1536.00 ± 356.50 pg/ml, n=8). Analysis done by two-way ANOVA; values represented as mean ± SD.

9.3. Behavioral measurements

To further ascertain whether the model successfully induced a state of chronic stress, behavioral parameters can also be assessed together with the biochemical parameters. The EPM is a behavioral test commonly used to assess anxiety-like behavior in rodents. Open and closed arm activity in addition to various other parameters such as head dips, rears and stretch-attend postures were assessed.

Here, the male control group spent more time ($p<0.001$; **Figure 18**) in the closed arms of the maze (197.60 ± 77.76 s, n=8), compared to the open arms (50.71 ± 48.79 s, n=8), while the stressed group also spent more time ($p<0.001$; **Figure 18**) in the closed arms of the maze (195.00 ± 70.50 s, n=8), compared to the open arms (51.90 ± 43.78 s, n=8). Additionally, the male stressed rats (4.87 ± 1.88 s, n=8) made less attempts ($p<0.05$; **Figure 19**) into the open arms of the maze versus the control rats (7.87 ± 4.51 s, n=8). No significant differences were observed between the male control and stressed rats in the other parameters (**Appendix E**).

The female control group spent more time ($p<0.001$; **Figure 20**) in the closed arms of the maze (195.40 ± 54.79 s, n=8), compared to the open arms (55.65 ± 35.15 s, n=8), while the stressed group also spent more time ($p<0.001$; **Figure 20**) in the closed arms of the maze (179.00 ± 50.05 s, n=8), compared to the open arms (57.7 ± 37.80 s, n=8). Additionally, the female stressed rats (28.13 ± 4.58 s, n=8) exhibited more rears ($p<0.001$; **Figure 21**) versus the female control rats (18.63 ± 2.82 s, n=8). No significant changes were observed between the female control and stressed rats in the other parameters (**Appendix E**).

The tail flick task was also performed as an exploratory test to investigate the possible effects of chronic stress on nociception. However, no significant changes in either male or female nociception were detected between the control and stressed rats (**Appendix E**).

9.3.1. Elevated plus maze

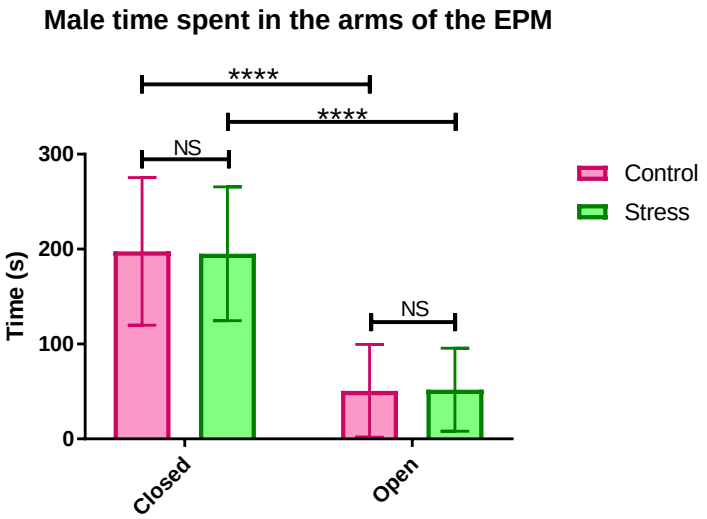

Figure 18. Average time spent in the closed arms versus the open arms of the maze for the male control and stressed rats. A statistical significance ($p<0.001$) was observed in the control group between time spend in closed arms (197.60 ± 77.76 s, n=8) versus open arms (50.71 ± 48.79 s, n=8). The experimental (stress) group also showed a significant difference ($p<0.001$) in time spent in closed arms (195.00 ± 70.50 s, n=8) compared to the open arms (51.90 ± 43.78 s, n=8). No significant difference was detected in the time spent in the closed or the open arms of the maze for either control or stressed rats. Analysis done by Student's t-test; values represented as mean ± SD.

Figure 19. Male number of attempts into the open arms of the maze. A statistical significance ($p<0.05$) as observed between the experimental (stress) group (4.87 ± 1.88, n=8) and the control group (7.87 ± 4.51, n=8). Analysis done by Student's t-test; values represented as mean ± SD.

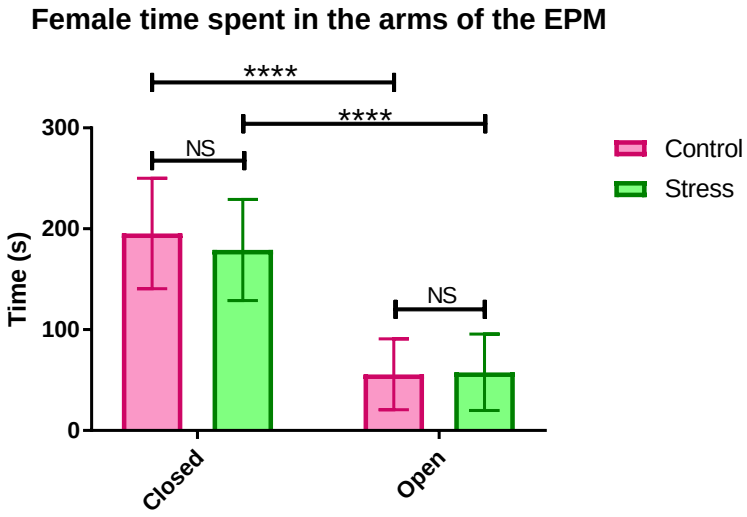

Figure 20. Average time spent in the closed arms versus the open arms of the maze for the female control and stressed rats. A statistical significance ($p<0.0001$) was observed in the control group between time spend in closed arms (195.40 ± 54.79 s, n=8) versus open arms (55.65 ± 35.15 s, n=8). The experimental (stress) group also showed a significant difference ($p<0.0001$) in time spent in the closed arms (179.00 ± 50.05 s, n=8) versus the open arms (57.70 ± 37.80 s, n=8). No significant difference was detected in the time spent in the closed or the open arms of the maze for either control or stressed rats. Analysis done by Student's t-test; values represented as mean ± SD.

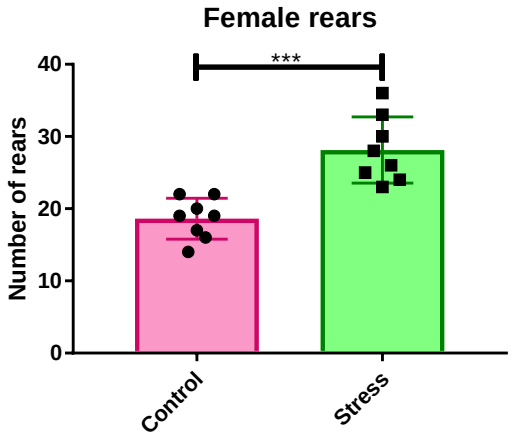

Figure 21. Number of rears for the female rats during the EPM. A statistically significant difference ($p<0.001$) was observed between the experimental (stress) group (28.13 ± 4.58, n=8) and the control group (18.63 ± 2.82, n=8). Analysis done by Student's t-test; values represented as mean ± SD.

9.4.Measurements in males versus females

Anthropometric and biochemical results obtained in the study were not only compared between control and stressed rats, but also between male and female rats to assess possible sex-based

differences. Significant sex-based differences were observed in the weights of the pre-frontal cortex, hypothalamus, left and right adrenal gland and the heart. Here, control females (0.06 ± 0.01 % body weight, n=8) exhibited a significantly larger ($p<0.001$) pre-frontal cortex than control males (0.03 ± 0.01 % body weight, n=7) (**Figure 22**). This effect remained in the stressed female rats (0.05 ± 0.01 % body weight, n=8) when compared to stressed male rats (0.03 ± 0.01 % body weight, n=8), although it was sightly diminished ($p<0.01$). The hypothalamus weight of the female rats in the control group (0.05 ± 0.02 % body weight, n=8) was also significantly higher ($p<0.05$) than control male rats (0.03 ± 0.01 % body weight, n=8) (**Figure 23**). This difference disappeared when comparing stressed females (0.03 ± 0.01 % body weight, n=8) to their male counterparts (0.03 ± 0.01 % body weight, n=8). The female control group (0.41 ± 0.02 % body weight, n=8) displayed significantly larger ($p<0.01$) heart weights than the male control group (0.36 ± 0.03 % body weight, n=8) (**Figure 24**). This difference was slightly lowered ($p<0.05$) in the female stressed rats (0.40 ± 0.02 % body weight, n=8) versus the male stressed rats (0.37 ± 0.01 % body weight, n=8). Additionally, the female control rats (0.06 ± 0.01 % body weight, n=8) displayed larger ($p<0.001$) right adrenal glands (**Figure 25**) compared to the control males (0.03 ± 0.01 % body weight, n=8). The left adrenal glands (**Figure 26**) were also larger ($p<0.01$) in the female controls (0.06 ± 0.02 % body weight, n=8) compared to the male controls (0.03 ± 0.02 % body weight, n=8). This difference remained for only for the right adrenal glands ($p<0.01$) in the stressed groups, female (0.05 ± 0.01 % body weight, n=8) and male (0.03 ± 0.01 % body weight, n=8). Sex-based differences were also observed in the biochemical measurements. Our data revealed that control females (15.09 ± 1.84 % body weight, n=8) exhibited significantly higher final plasma corticosterone level ($p<0.05$) than control males (12.58 ± 1.43 % body weight, n=8) (**Figure 27**). This difference disappeared when comparing stressed females (13.13 ± 1.48 % body weight, n=8) to the stressed male group (15.08 ± 1.37 % body weight, n=8). Finally, BDNF levels from the female controls (44.62 ± 52.52 % body weight, n=8) were lower ($p<0.05$) compared the male controls (169.80 ± 168.60 % body weight, n=8) (**Figure 28**). This difference was not observed in the stressed female (59.74 ± 59.05 % body weight, n=7) and male (27.08 ± 24.28 % body weight, n=7) groups.

9.4.1. Organ weights

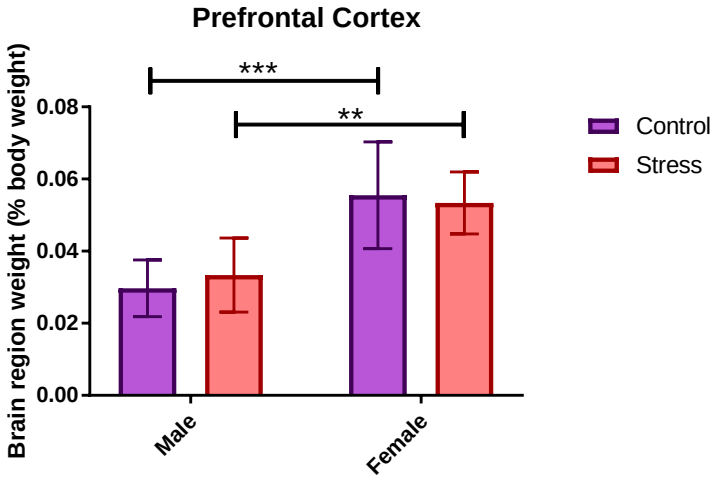

Figure 22. Male and female prefrontal cortex weights. The male control group (0.03 ± 0.01 % body weight, n=7) displayed a significant difference ($p<0.001$) compared to the female control group (0.06 ± 0.01 % body weight, n=8). A significant difference ($p<0.01$) was also observed between the male experimental (stress) group (0.03 ± 0.01 % body weight, n=8) and the female experimental (stress) group (0.05 ± 0.01 % body weight, n=8). Organ weight was calculated as a percentage of body weight. Analysis done by two-way ANOVA; values represented as mean ± SD.

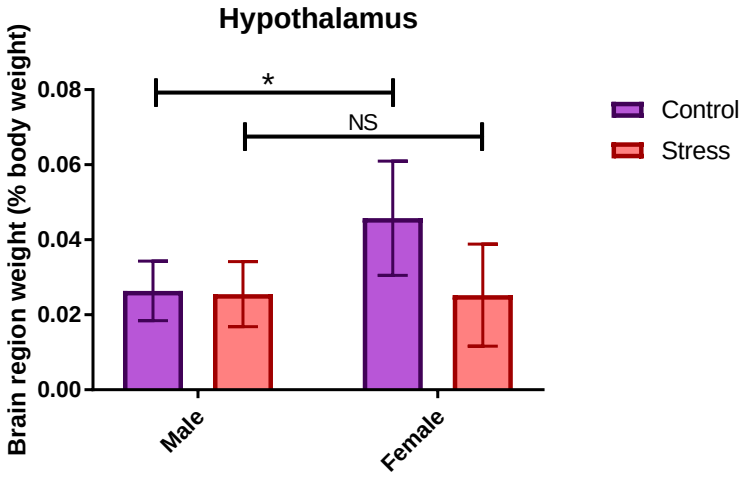

Figure 23. Male and female hypothalamus weights. The male control group (0.03 ± 0.01 % body weight, n=8) showed a significant difference ($p<0.01$) compared to the female control group (0.05 ± 0.02 % body weight, n=8). No statistical difference was observed between the male experimental (stress) group (0.03 ± 0.01 % body weight, n=8) and the female experimental (stress) group (0.03 ± 0.01 % body weight, n=8). Organ weight was calculated as a percentage of body weight. Analysis done by two-way ANOVA; values represented as mean ± SD.

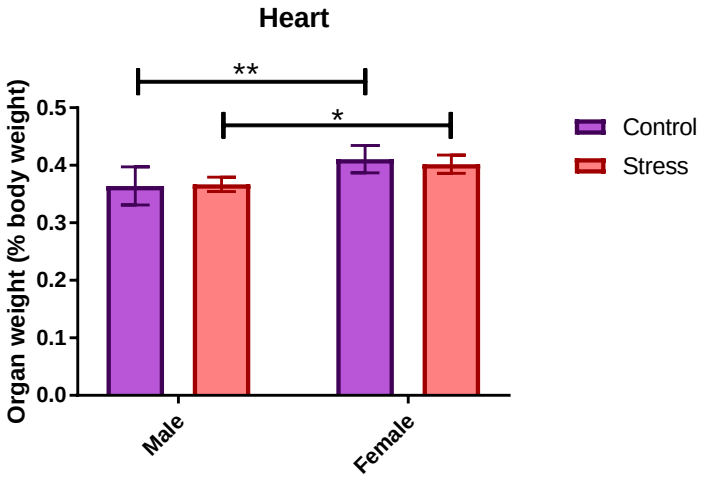

Figure 24. Male and female heart weights. The male control group (0.36 ± 0.03 % body weight, n=8) showed a significant difference ($p<0.01$) compared to the female control group (0.41 ± 0.02 % body weight, n=8). A statistical difference ($p<0.05$) was also observed between the male experimental (stress) group (0.37 ± 0.01 % body weight, n=8) and the female experimental (stress) group (0.40 ± 0.02 % body weight, n=8). Organ weight was calculated as a percentage of body weight. Analysis done by two-way ANOVA; values represented as mean ± SD.

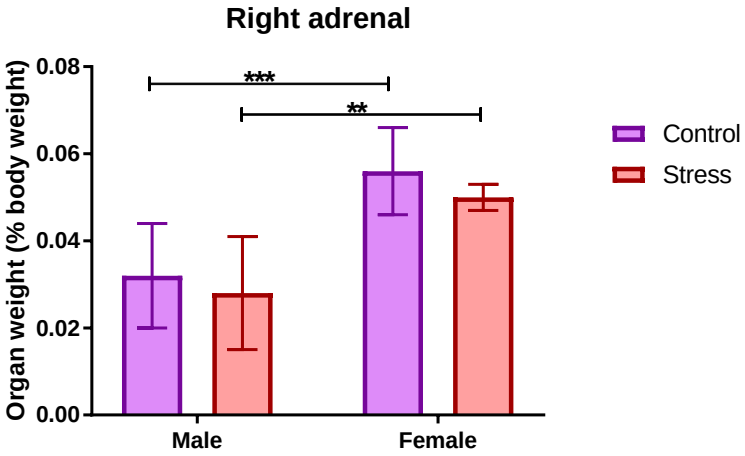

Figure 25. Male and female right adrenal weights. The male control group (0.03 ± 0.01 % body weight, n=8) showed a significant difference ($p<0.001$) compared to the female control group (0.06 ± 0.01 % body weight, n=8). A statistical difference ($p<0.01$) was also observed between the male experimental (stress) group (0.03 ± 0.01 % body weight, n=8) and the female experimental (stress) group (0.05 ± 0.01 % body weight, n=8). Organ weight was calculated as a percentage of body weight. Analysis done by two-way ANOVA; values represented as mean ± SD.

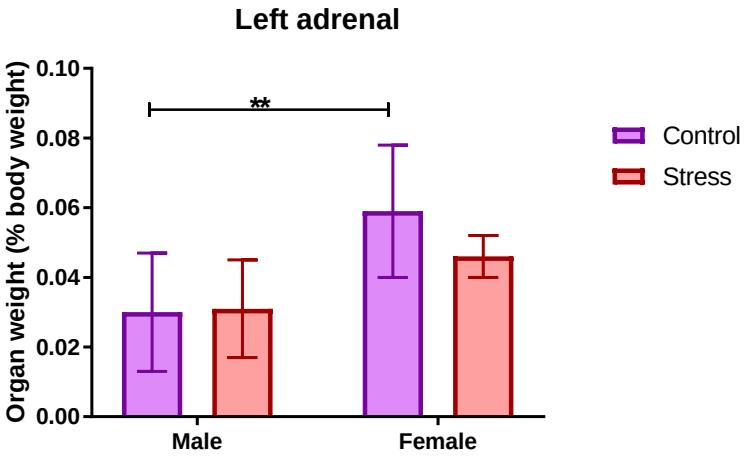

Figure 26. Male and female left adrenal weights. The male control group (0.03 ± 0.02 % body weight, n=8) showed a significant difference ($p<0.01$) compared to the female control group (0.06 ± 0.02 % body weight, n=8). No statistical difference was observed between the male experimental (stress) group (0.03 ± 0.01 % body weight, n=8) and the female experimental (stress) group (0.05 ± 0.01 % body weight, n=8). Organ weight was calculated as a percentage of body weight. Analysis done by two-way ANOVA; values represented as mean ± SD.

9.4.2. Biochemical measurements

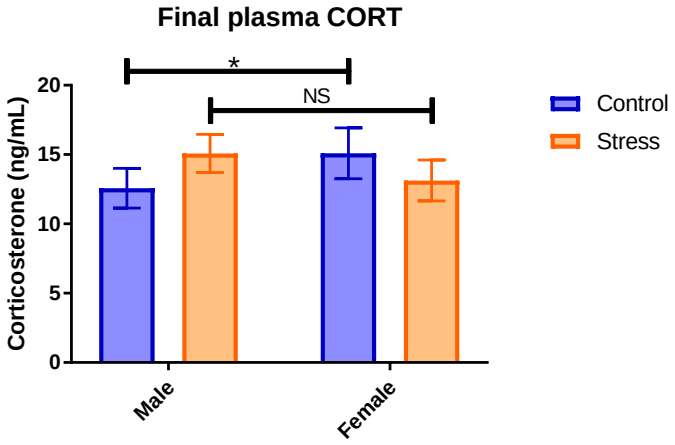

Figure 27. Male and female final plasma corticosterone concentrations. The male control group (12.58 ± 1.43 ng/ml, n=8) showed a significant difference ($p<0.01$) compared to the female control group (15.09 ± 1.84 ng/ml, n=8). No statistical difference was observed between the male experimental (stress) group (15.08 ± 1.37 ng/ml, n=8) and the female experimental (stress) group (13.13 ± 1.48 ng/ml, n=8). Analysis done by mixed model ANOVA in R; values represented as mean ± SD.

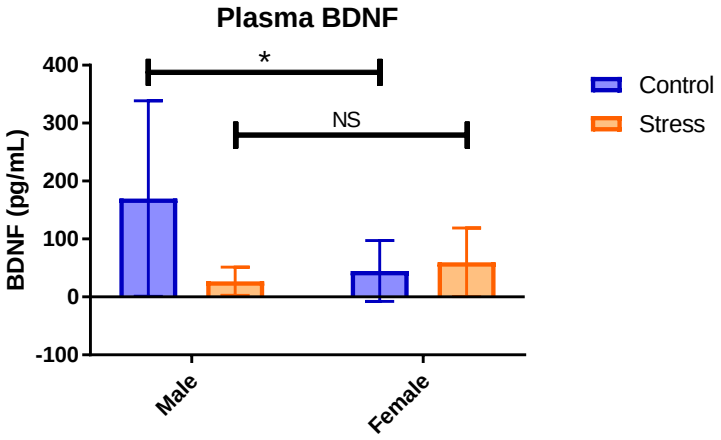

Figure 28. Male and female plasma BDNF concentrations. The male control group (169.80 ± 168.60 pg/ml, n=8) showed a significant difference ($p<0.05$) compared to the female control group (44.62 ± 52.52 pg/ml, n=8). No statistical difference was observed between the male experimental (stress) group (27.08 ± 24.28 pg/ml, n=7) and the female experimental (stress) group (59.74 ± 59.05 pg/ml, n=7). Analysis done by two-way ANOVA; values represented as mean ± SD.

10. DISCUSSION

This study forms part of a larger research project aimed at investigating the mechanisms promoting the onset and progression of stress-related cardio-metabolic diseases. The goal of this thesis was to establish an *in vivo* rodent model of chronic restraint stress in a South African laboratory and to validate this by employing various biochemical and behavioral tests. Of note, the chronic restraint stress model does not induce a specific clinical endpoint and therefore our aim was not to replicate a specific disorder such as anxiety and/ or depression. Rather, we aimed to evaluate the extent to which the model successfully induced a state of chronic stress in the rodents.

Activation of the stress response increases the activity of the HPA-axis and SAM pathway and leads to the subsequent release of mediators from each stress pathway (Lundberg, 2005; McEwen, 2009). These pathways are interconnected and communicate at the brain level, ultimately functioning as major effector systems through which the brain regulates the whole organism (Godoy *et al*., 2018; Kumar *et al*., 2013a; Vale, 2005). Activation of the HPA-axis and SAM-pathway eventually results in the release of GCs and catecholamines that serve as essential mediators of the stress response (Godoy *et al.*, 2018; Rabasa & Dickson, 2016). Although elevated catecholamines are linked to cardio-metabolic pathology such as

hypertension, stroke, and myocardial infarction (Lundberg, 2005), our postulate is that HPA-axis dysregulation plays a more prominent role. In support, evidence implicates the overload and resistance of cortisol as the primary mediator of downstream stress-related pathology (Hannibal & Bishop, 2014; Lundberg, 2005; Sher *et al*., 2020; Verma *et al*., 2011). We therefore primarily assessed HPA-axis activity to determine whether the chosen model successfully induced a state of chronic stress. Here, plasma corticosterone and ACTH concentrations were measured together with plasma BDNF and NPY levels. BNDF is a neurotropic factor involved in a variety of signaling cascades to ultimately regulate mood, metabolism, and neuroplasticity (Béjot *et al*., 2011; Grande *et al*., 2010; Suliman *et al*., 2013), while NPY is neurotransmitter with a role in mood, stress coping, metabolism, and brain activity (Farzi *et al*., 2015; Fletcher *et al*., 2010; Reichmann & Holzer, 2016; Wu *et al*., 2011).

As previously discussed, clear differences within the stress response exist between males and females (Verma *et al*., 2011). For example, studies showed that HPA-axis and SAM pathway patterns differ markedly based on sex, both showing significant differences in function and regulation at baseline and in response to stress (Kajantie & Phillips, 2006; Rincón-Cortés *et al*., 2019; ter Horst *et al*., 2012; Verma *et al*., 2011). Due to the lack of women in clinical research and the heavy bias towards males in pre-clinical research, we included male and female Wister rats in this study to gain unique insights into the mechanisms promoting the onset and progression of cardio-metabolic diseases.

10.1. Results obtained from male rats

Exposure to chronic stress is known to alter basic mechanisms of the stress response. A large body of literature has focused on the anthropometric, biochemical, and behavioral changes associated with chronic stress. For example, exposure to chronic stress can decrease body weight gain (Abelaira *et al*., 2013; Chen *et al*., 2020; Chiba *et al*., 2012; Marin *et al*., 2007), elevate plasma ACTH and corticosterone concentrations (Abelaira *et al*., 2013; Becker *et al*., 2021; Buynitsky & Mostofsky, 2009; Chiba *et al*., 2012; Marin *et al*., 2007) and increase anxiety-like behavior (Ampuero *et al*., 2015; Becker *et al*., 2021; Campos *et al*., 2013; Chiba *et al*., 2012; Reber & Slattery, 2016; Seewoo *et al*., 2020) in male animals.

10.1.1. Effects of chronic stress on anthropometric measurements

Our findings revealed a significant decrease in the percentage change in weight over time in

the male stressed group compared to controls (**Figure 29**). This finding is corroborated by various studies that reported attenuated body weight (or less weight gain) in male but not female animals following chronic stress (Lin *et al*., 2008; Olave *et al*., 2022; Patterson & Abizaid, 2013; Solomon *et al.*, 2011). Here, chronic stress and the subsequent rise in GC concentrations can lead to less body weight gained, through a reduced caloric efficiency (Dallman *et al.*, 2003; Rabasa *et al*., 2019). Another possibility is that the prolonged elevation in corticosterone levels can stimulate the catabolism of skeletal muscle proteins, and lead to muscle loss and thus lowered weight gain (Jeong *et al*., 2013).

No significant changes were observed in the organ weights of the male animals. Together, these results indicate that our stress paradigm was not sufficiently severe to change the weight of organs and hence suggest a more moderate phenotypic outcome. The results regarding the weights of the organs between control and stressed groups should, however, be interpreted with caution as there are no baseline measurements to use as comparison.

10.1.2. Effects of chronic stress on behavioral measurements

Stress can precipitate or exacerbate anxiety-like disorders in humans and therefore stress-research aims to evaluate such parameters as a measure of stress-like behavior (da Silva Torres *et al*., 2003; Jennings *et al.*, 2014; Lezak *et al*., 2017). This study employed the EPM, which is one of the most common behavioral assays used to evaluate anxiety-related behavior in rodents (Campos *et al.*, 2013; Komada *et al.*, 2008; Schneider *et al*., 2011; Seewoo *et al*., 2020). The behavioral data obtained from EPM testing showed significantly more time spent in the closed arms of the maze for all the male rats (**Figure 29**). Rodents are considered animals of prey and thus may not easily display significant behavioral changes. The CRS protocol may therefore not have been severe enough to induce significant and observable changes in anxiety-like behavior. The stressed male rats made significantly less attempts into the open arms of the maze compared to the controls (**Figure 29**). Here, reduced open arm activity could represent higher anxiety levels, where the fear of open, elevated spaces overrules the rodent's natural exploration desire (Belovicova *et al.*, 2017; Pellow *et al*., 1985a). The significant decrease in attempts observed in the stressed male rats could not, however, be corroborated by any of the other measures of anxiety here employed.

10.1.3. Effects of chronic stress on biochemical measurements

As previously mentioned, the activation of the stress response leads to the release of HPA-axis mediators and hence a corresponding increase in such molecules would be expected. Elevations in such markers are considered the primary biological determinant whether a model successfully induced stress (Kudielka & Kirschbaum, 2005). Biochemical data gathered from the male rats revealed elevated plasma corticosterone levels with no significant changes in plasma ACTH levels (**Figure 29**). Although the elevation in plasma corticosterone levels suggests HPA-axis hyperactivity, this may not necessarily be the case as no changes occurred in ACTH levels. The literature indicates that stress and androgens can interact to cause sex-specific alterations in the HPA-axis and affect the subsequent release of ACTH and corticosterone for example, androgens such as testosterone can inhibit HPA-axis activity (Heck & Handa, 2019). However, since stress can attenuate testosterone levels it would be expected to no longer inhibit HPA-axis activation. This can possibly explain the increase in plasma corticosterone observed in the male stressed rats. As Heck & Handa (2019) further states that androgens can attenuate pituitary ACTH, this may help explain the ACTH data for our stressed male rats.

While no significant differences in plasma NPY levels were observed between the control and stressed male rats, plasma BDNF levels were lower in the stressed male rats (**Figure 29**). Of note, GCs such as cortisol can regulate BDNF production and the literature supports a subsequent decrease in brain and plasma BDNF as corticosterone levels rise in response to chronic stress (Suliman *et al*., 2013; Yu & Chen, 2011).

10.1.4. Overall effects of chronic stress on male rats

The data gathered from the stressed male rats following the implementation of the CRS model included a slight but not clear increase in anxiety-like behavior, increased plasma corticosterone and decreased plasma BDNF levels, along with reduced weight gain (**Figure 29**).

Such results clearly show a dysregulated stress response in the stressed male animals following the implementation of the CRS model and collectively suggests a mild depressive-like phenotype in the male rats – although additional studies are required to prove this notion. Studies investigating the effects of chronic stress on anxiety-like behavior mostly report on time spent on the open and closed arms and entries made into the open and closed arms of the

maze (Belovicova *et al.*, 2017; Carobrez & Bertoglio, 2005; Carola et al., 2002; Lezak *et al*., 2017; Komada *et al*., 2008; Schneider *et al*., 2011). As we could not find other studies describing similar behavioral results (reduced attempts into the open arms) the behavioral data could not be linked to the depressive-like phenotype that was suggested.

Regarding the biochemical findings, the HPA-axis is a key endocrine adaptor to various stressors and plays a crucial role in the pathophysiology of stress-related psychiatric diseases such as depression (Chiba *et al*., 2012). Numerous studies demonstrated HPA-axis dysregulation in depressive patients, implicating chronically elevated GCs in this process (Chiba *et al.*, 2012; Grande *et al.*, 2010; Sharpley, 2009; Wang *et al*., 2020). A similar increase in corticosterone was observed in the stressed male rats but together with attenuated plasma BDNF levels. Brain-derived neurotropic factor is a neurotophin that influences various cellular processes, including neuronal survival, synaptic plasticity, and mood (Chiba *et al*., 2012). Notably, stress-responsive GCs can play a role in the regulation of BDNF expression and function, where increased corticosterone levels can lead to attenuated BDNF expression (Grande *et al*., 2010; Suliman *et al*., 2013). BDNF has also been implicated in the etiopathology of depression (Chiba *et al.*, 2012). Its downregulated expression and/or function may therefore be associated with the onset of depression. In support, diminished BNDF levels were found in the brains and plasma of depressed patients (Chiba *et al.*, 2012). Furthermore, treatment with antidepressants (for example selective serotonin reuptake inhibitors) can increase BDNF expression in the brain and alleviate depressive symptoms (Yu & Chen, 2011).

Anthropometric results revealed less overall weight gained by the stressed rats. This finding is corroborated by various studies that reported lower weight gain in male animals following chronic stress (Lin *et al*., 2008; Olave *et al.*, 2022; Patterson & Abizaid, 2013; Solomon *et al.*, 2011). As for the suggested depressive-like phenotype, animals exhibiting anhedonia (decreased sugar consumption, lack of appetite, decreased locomotor activity or hyperactivity and sleep disturbances) also show less overall weight gain (Becker *et al.*, 2021; Du *et al.*, 2020; Wang *et al.*, 2017). Similar biochemical and anthropometric results have been reported by Zardooz *et al*. (2006). They demonstrated that 4-weeks of restraint stress induced low body weight gain along with increased plasma corticosterone levels. Of note, a study by Jeong *et al.* (2013) also found that the CRS model resulted in higher corticosterone levels. Here, the prolonged elevation in corticosterone levels can stimulate the catabolism of skeletal muscle proteins, and lead to lowered weight gain (Jeong *et al*., 2013). It is also proposed that such high

corticosterone concentrations can lead to less body weight gained through a reduced caloric efficiency (Dallman *et al.*, 2003; Rabasa *et al.*, 2019). A more meaningful scientific connection is therefore established by validating the biochemical findings with the underlying anthropometric results, ultimately creating a collective coherence between all the different aspects of the study.

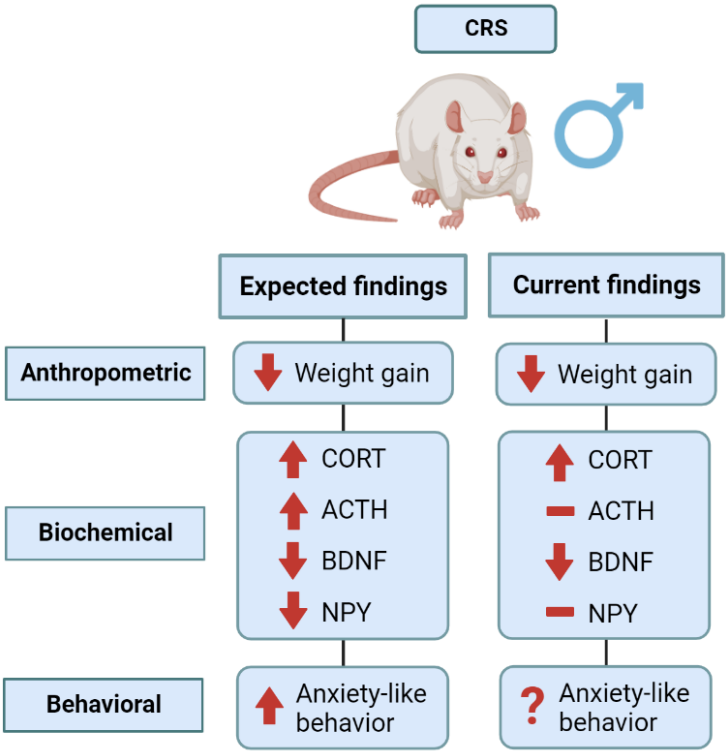

Figure 29. Summary diagram containing the expected anthropometric, biochemical, and behavioral findings as well as the current results obtained from the male animals following an CRS model. Diagram self-constructed using Biorender.com.

10.2. Results obtained from female rats

Anthropometric, biochemical, and behavioral changes associated with chronic stress were also determined in the female rats. Here, studies revealed that the exposure of female animals to chronic stress induced no changes in body weight (Lin *et al.*, 2008; Olave *et al.*, 2022; Patterson & Abizaid, 2013; Solomon *et al.*, 2011), increased plasma ACTH and corticosterone levels (Becker *et al.*, 2021; Buynitsky & Mostofsky, 2009) as well as anxiety-like behavior (Becker et al., 2021a; Campos *et al.*, 2013; Zhu *et al.*, 2014).

10.2.1. Effects of chronic stress on anthropometric measurements

In accordance with such published findings, the anthropometric measurements gathered from the female rats showed no significant changes in body weight over the course of the study (**Figure 30**) (Lin *et al*., 2008; Olave *et al*., 2022; Patterson & Abizaid, 2013; Solomon *et al.*, 2011). Estrogens have been shown to be involved in the regulation of ghrelin secretion, where higher circulating estradiol levels can induce enhanced ghrelin secretion (Kellokoski *et al.*, 2005; Smith *et al.*, 2022). Ghrelin is considered a potent orexigenic peptide hormone and is thus a strong stimulator of food intake. The higher levels of estradiol produced by the female rats opposed to the male rats can lead to increased food intake. The effects of chronic stress on lower overall weight gain in the male animals can thus be counteracted in the females by increased food intake and therefore no change in overall weight gain. Food intake was, however, not monitored which means that this theory cannot be confirmed. Of note, a significant decrease in hypothalamus weight was observed in the stressed female rats. The hypothalamus plays a key role in the stress response as it is responsible for integrating emotional stress information from higher brain structures and organizing the final homeostatic stress response (Buijs & Van Eden, 2000; Godoy *et al.*, 2018). Following its activation, the hypothalamus secretes CRH which can trigger the HPA-axis response (Hannibal & Bishop, 2014; Sharpley, 2009; Zänkert *et al.*, 2019). Lower hypothalamus weight in the stressed female rats could thus possibly result from stress-induced HPA-dysregulation. As a potential mechanism leading to reduced volumes of the hypothalamus, it is speculated that the HPA-axis hyperactivity can induce neuronal atrophy, neurotoxicity and neuroendangerment, leading to the loss of hypothalamic neurons and thus mass (Terlevic *et al*., 2013).

The results regarding the weights of the organs between control and stressed groups should, however, be interpreted with caution as there are no baseline measurements to use as comparison.

10.2.2. Effects of chronic stress on behavioral measurements

Behavioral data recorded during the EPM revealed that all the female rats spent significantly more time in the closed arms of the maze (**Figure 30**). Once again, this lack in significantly altered anxiety-like behavior could be due to the mild nature of the CRS model that was employed. The stressed female rats also displayed a significant increase in the average number of rears versus matched controls (**Figure 30**). Rears are considered a form of risk-assessment

behavior and may thus infer a slight elevation in anxiety-like behavior. The absence of other measures of anxiety together with the greater number of rears indicate that an increase in anxiety-like behavior cannot be reliably concluded in this instance.

10.2.3. Effects of chronic stress on biochemical measurements

A wealth of studies supports an increase in HPA-axis mediators under conditions of stress. However, although such increases in HPA-axis mediators are widely accepted as markers of stress, this is not consistently found in chronic studies (Goldstein & Kopin, 2007; Selye, 1946; Zoladz *et al.*, 2021). Here, some evidence supports the idea that chronic stress, particularly in the case of repeated exposure to the same stressor, can initially increase the release of corticosterone but that this could eventually result in a blunted HPA-axis response (Stephens & Wand, 2012; Thompson *et al.*, 2012). Although some research work focused on female HPA-axis activity following acute stress, it is less well understood under chronic conditions (Heck & Handa, 2019).

The biochemical data obtained from the female rats in the current study revealed decreased plasma corticosterone concentrations together with increased plasma ACTH levels in the stressed female rats (Figure 30). This may indicate a blunted HPA-axis as previously suggested (Stephens & Wand, 2012; Thompson *et al.*, 2012). Different theories exist to explain such dysfunction, including describing it as a maladaptive response to chronic stress (Kumar *et al.*, 2013), or a central abnormality in GC regulation (Vythilingam *et al*., 2010). Here, the influence of estrogens on the female stress system could also potentially explain the results obtained. Evidence suggests that estradiol in rats induces a functional change in MRs and GRs in various brain regions including the pituitary gland. Such alterations can lead to enhanced pituitary responsiveness and thus increased ACTH production (Kirschbaum *et al.*, 1996). Moreover, Weiser & Handa (2018) states that estrogen receptor α, which is the receptor responsible for mediating the effects of estrogens, is only expressed in the peri-PVN. As this region is implicated in HPA-axis inhibition, estradiol can thus impair the sensitivity of the HPA-axis to GC negative feedback, thereby causing CRH and subsequent ACTH levels to remain high (Weiser & Handa, 2018). Alternatively, estradiol has been shown to increase vasopressin levels. Increased synthesis of vasopressin and the subsequent co-release with CRH from hypothalamic neurons could also explain the increase in pituitary ACTH (Kirschbaum *et al.*, 1996; Graugaard-Jenson *et al.*, 2008). Estradiol (primary form of estrogen) can thus increase the ACTH content of the pituitary gland, while also upregulating circulating GCB levels to

lower free corticosterone, thereby lowering negative feedback loops (Heck & Handa, 2019). This may help account for the higher plasma ACTH levels observed after the chronic stress protocol. Although a similar increase in plasma corticosterone would be expected, estradiol can affect the adrenal gland's sensitivity to ACTH (Heck & Handa, 2019) meaning that elevated ACTH availability may not necessarily lead to increased corticosterone levels.

10.2.4. Overall effects of chronic stress on female rats

The data gathered from the stressed female rats following the implementation of the CRS model include a slight but not clear increase in anxiety-like behavior. Moreover, they displayed a reduction in hypothalamus weight, increased plasma ACTH and decreased plasma corticosterone levels (**Figure 30**).

Such results reflect a clear dysregulation in the female stress response and collectively points towards the development of a mild anxiety-like phenotype in the stressed rats – although further studies are required to prove this notion. The results more specifically resemble the PTSD phenotype as such findings align with data generated by others. For example, Shumake *et al*., (2005) demonstrated that congenitally helpless rats (a rat strain selectively bred for elevated susceptibility to learned helplessness) display a significant increase in the number of rears versus control rats in the open field test and light-dark box task. The study states that the increase in rears is consistent with a temperament of high novelty seeking which closely resembles the temperament observed in combat veterans with PTSD (Shumake *et al*., 2005). Rearing behavior is therefore expected to increase in a PTSD phenotype compared to controls.

Regarding the biochemical results obtained, Zoladz *et al*. (2021) demonstrated significantly lower corticosterone concentrations in female rats following predator-based psychosocial stress. These changes manifested despite the absence of anxiety-like behavior and, closely resemble the results obtained from our study. In support, female animals displayed reduced corticosterone concentrations following exposure to a predator-based stress protocol (Schöner *et al*., 2017), while PTSD patients exhibited lower cortisol levels and a higher ACTH/cortisol ratio (Fischer, 2021; Vythilingam *et al*., 2010).

Anthropometric results revealed decreased hypothalamus weight in the stressed rats. The hypothalamus is a crucial hub consisting of a network of neural structures modulating fear conditioning and extinction and is highly relevant to the pathophysiology of anxiety-type

conditions like PTSD (Fischer, 2021). As a potential mechanism leading to reduced volumes of the hypothalamus, it is speculated that the HPA-axis hyperactivity can induce neuronal atrophy, neurotoxicity and neuroendangerment, leading to the loss of hypothalamic neurons (Terlevic *et al.*, 2013). Of note, Terlevic *et al.* (2013) demonstrated that significantly lower hypothalamus volumes were observed in patients with generalized anxiety disorder compared to healthy controls, thus supporting the suggested anxiety-like phenotype with the anthropometric results. Here, a more meaningful scientific connection is established by validating the behavioral results with the underlying biochemical findings and anthropometric data, ultimately creating a collective coherence between all the different aspects of the study.

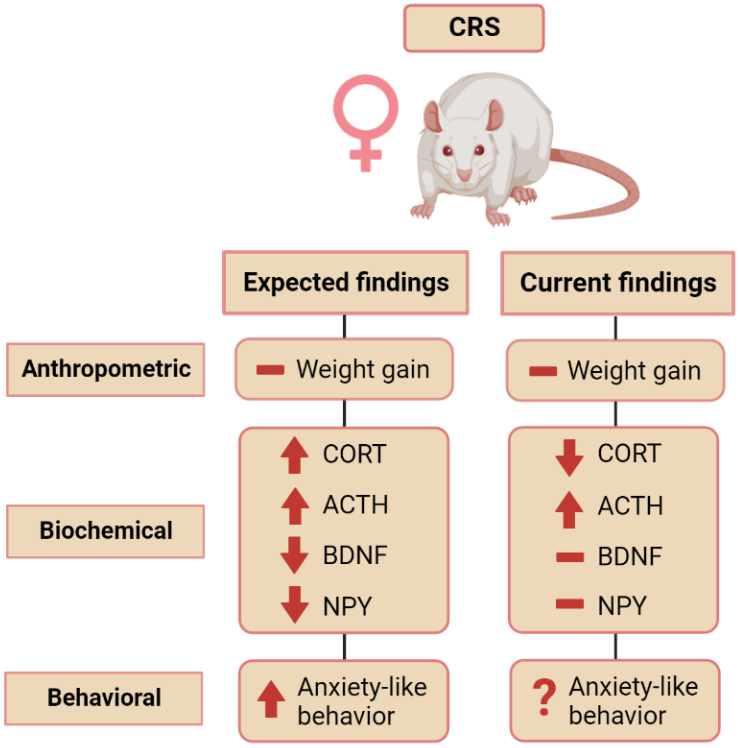

Figure 30. Summary diagram containing the expected anthropometric, biochemical, and behavioral findings as well as the current results obtained from the female animals following an CRS model. Diagram self-constructed using Biorender.com.

10.3. Sex-based differences in response to chronic stress

A comparison was drawn between the anthropometric, biochemical, and behavioral results that were obtained from male and female rats. Here, organs such as the pre-frontal cortex, hypothalamus, heart, and adrenal glands showed significant differences between the male and

female control rats as well as for the stressed ones. The results regarding the weights of the organs between male and female groups should, however, be interpreted with caution as there are no baseline measurements to use as comparison. A significant difference was also found for final plasma corticosterone and BDNF concentrations in the control and stress groups. When comparing the corticosterone levels obtained from the control animals (after the CRS protocol) to a healthy rat, the levels of the female controls were higher than what would be expected at that time of the diurnal rhythm. Male control animals demonstrated corticosterone levels within the normal range, indicating that the females may have been more sensitive to the changes in the environment (Tygerberg animal facility) compared to the males. After careful consideration, it seems that external factors (not originating from the restraint protocol itself) may explain the slightly elevated stress levels of the female rats. As result, it is difficult to draw reliable conclusions in terms of the differences observed between the male and female rats used in this study.

10.4. Study limitations and future recommendations

Despite various improvements from previous runs in our laboratory, our research work is not without limitations. The findings from the present study demonstrated that a state of chronic mild stress was induced following the four-week CRS model. The literature supports the development of the entire spectrum of known allostatic responses following restraint stress (Patchev & Patchev, 2006). Our biochemical results showed a dysregulated stress response in both sexes after the stress model. However, the lack of an increase in anxiety-like behavior from the behavioral results suggest a degree of adaptation to the stressor. A degree of habituation can be expected as this experimental model only employs a single repeated stressor, but the inescapable nature of the stressor ensures that the stress response is constantly triggered – just to a lesser extent (Marin *et al.*, 2007). Future studies implementing the CRS model should thus consider increasing the severity of the protocol by lengthening the duration of stressor exposure to induce a more pronounced phenotypic outcome. Despite such limitations, it is our opinion that the CRS model is designed to induce mild stress as it should mimic small, everyday stressors experienced by humans (Seewoo *et al.*, 2020; Wang *et al.*, 2018) and this should therefore be taken into consideration when increasing the severity of the model. Hence the relatively mild phenotypic outcomes in our model should be considered a strength as it more closely resembles real-life scenarios versus an artificially induced stress-like phenotype.

The use of only one behavioral test (EPM) to determine altered behavior in the rodents may

also be regarded as a study limitation. Results obtained from two or more behavioral tests can provide additional insights into the behavior associated with chronic stress and provide an opportunity to compare and interpret results in unison to gain a more well-rounded picture of animal behavior. As previously stated, stress can increase susceptibility to mood disorders such as anxiety-like and depression-like phenotypes (Godoy *et al.*, 2018; Kumar *et al.*, 2013; Lezak *et al.*, 2017; van Oort *et al.*, 2017). Research studies should therefore utilize ethological-based tasks centered on anxiety and anhedonia to evaluate animal behavior. Although the EPM is the most widely researched and used test to assess anxiety-like behavior (Belovicova *et al.*, 2017; Carobrez & Bertoglio, 2005), other tests also exist including the open-field test (OFT) and the light-dark box test. The OFT makes use of an apparatus consisting of a square arena divided into 36 squares, surrounded by continuous high walls and measures locomotor activity, willingness to explore and anxiety levels (Belovicova *et al.*, 2017; Carola *et al.*, 2002; Lezak *et al.*, 2017). Here, the level of anxiety is inferred by the latency to enter, and time spent in the OFT center and OFT periphery (Belovicova *et al.*, 2017; Carola *et al.*, 2002; Lezak *et al.*, 2017). The light-dark box test uses an apparatus consisting of a box divided into two chambers: a darker, minimally lit side with black walls and a brightly lit side with white walls (Belovicova *et al.*, 2017; Lezak *et al.*, 2017). Anxiety levels are determined with time spent in and latency to enter the light side of the box (Belovicova *et al.*, 2017; Lezak *et al.*, 2017). Tests for depression include the forced swim test and the tail suspension test. The forced swim test is based on the observation that in the case of forced swimming, without the possibility to escape, animals will exhibit immobility after an initial period of intense swimming and climbing (Belovicova *et al.*, 2017). Such behavioral immobility is measured in a temporal manner and is described as a state of despair (anhedonia) (Belovicova *et al.*, 2017; Smith, 2012). Although the tail suspension test induces similar behavior as the forced swim test and involves hanging the animal by its tail, it should be noted that it is not an ethological-based task (Belovicova *et al.*, 2017). Here, the animal will try to escape the stressful situation. However, after some time the animal ceases to struggle and immobility occurs. Immobile phase time is measured as signs of anhedonia (Belovicova *et al.*, 2017; Hanell & Marklund, 2014). However, subjecting animals to various behavioral tests should be limited as this may be problematic since participation in one test can influence the results obtained from subsequent tests. The order in which the tests are carried out is thus important, and performing them on separate days can reduce potential interactions (Hanell & Marklund, 2014).

In the current study, two researchers were acclimatized to the experimental animals and were

allowed to interact with and perform tests on the rats. However, different individuals working in the animal facility did have access to the animal rooms to perform tasks such as providing clean cages, food, and water. This may have induced additional stress in the rats as observed by increased plasma corticosterone levels in the female control group. Future studies should therefore limit the number of individuals that the rats are exposed to by ensuring that the researchers acclimatized to the animals, perform all the necessary tasks themselves.

The control and experimental animals were housed together for the duration of the study. Another limitation may also be that following restraint the experimental group was immediately placed back into their cages and returned to the housing room in the animal facility. Thus, for future studies the restraint protocol should include a period of recovery for the stressed animals following stressor exposures, as rodents undergoing stress release alarm pheromones in their fecal pellets, urine, and glandular secretions (Bind *et al.*, 2013). Pheromones are chemical signals that elicit responses within species and are crucial for intraspecies communication (Bind *et al.*, 2013). Pheromones can therefore be sensed by the control animals in the vicinity, and hence separation of the different groups may help attenuate such effects.

There is consensus in the literature that females are no more variable than males, i.e. across various physiological, morphological, and behavioral traits (Becker *et al*., 2016; Prendergast *et al*., 2014), as a similar degree of hormonal fluctuation (and thus variability) can arise in males when rodents are group-housed and dominant behaviors develop (Prendergast *et al.*, 2014). Despite proof that females are not more variable than males, the literature shows that the estrus cycle does impact on the female stress response and subsequent downstream pathological alterations (Heck & Handa, 2019). Performing vaginal smears on experimental animals to monitor the estrus cycle may therefore help provide greater insights into sex-based differences in this context. For example, female animals in diestrus (low estradiol) are similar to males, where they exhibit low resting GC secretion and a quick on-off stress response. In contrast, females in proestrus (high estradiol and progesterone) and estrus (recent peak in estradiol) can show elevated basal GCs and increased stress-induced corticosterone and ACTH levels (Heck & Handa, 2019). However, such monitoring of the estrus cycle can increase experimental samples numbers, is time-consuming and will significantly increase the overall costs of the study (Prendergast *et al.*, 2014).

Of note, our research group's focus is on the effects of chronic psychological stress on the development of CVD as a paucity of mechanistic insights underlying this association remain.

As discussed earlier in this thesis, our laboratory's postulate is that increased myocardial oxidative stress and NOGP activation may be implicated in this process. In light of this, we proposed a unified hypothesis, i.e., that chronic stress contributes to the overactivation of NOGPs together with a highly intracellular pro-oxidative state. We hypothesize that this will result in an allostatic overload that ultimately promotes cardiac pathophysiology. Although our original goal was to investigate such pathways, the initial characterization of the CRS model required more tests and validations than we originally expected and thus such analyses could not be completed. However, following the establishment of the CRS model in our laboratory, we can now proceed to evaluate the role of the proposed oxidative stress-NOGP axis in terms of stress-related CVD onset and progression in future studies.

11. CONCLUSION

Psychological stress is emerging as one of the health epidemics of the 21st century and it is therefore crucial to investigate the underlying mechanisms driving stress-related diseases onset and progression. The goal of this study was to establish and validate an *in vivo* rodent model of chronic stress in males and females. Our findings revealed intriguing sex-based differences in response to the CRS protocol, with males and females exhibiting distinct phenotypes. Future studies are crucial to further explore such sex-based differences. Here, the CRS model provides a unique opportunity to study the multidimensional effects of chronic psychological stress, as it is known to induce the entire spectrum of allostatic responses. It is also a translationally relevant model and possesses good face, construct, and predictive validity (Becker *et al*., 2021b; Chen *et al*., 2020; Patchev & Patchev, 2006; Seewoo *et al.*, 2020).

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APPENDICES

Appendix A: Ethical approval

UNIVERSITEIT
STELLENBOSCH
UNIVERSITY

REC: Animal Care and Use

Project Approval Letter

Date: 19 November 2021

PI Name: Prof MF Essop

Protocol #: ACU-2021-19400

Title: The effects of chronic stress on cardiovascular, brain and metabolic function

Dear MF Essop ,

Your project application, was reviewed by the Research Ethics Committee: Animal Care and Use (REC: ACU) via committee review procedures and was approved. Please note that this clearance is only valid for a period of twelve months. Ethics approval of projects spanning more than one year must be renewed annually through submission of a progress report, up to a maximum of three years.

Approval Date: 19 November 2021 - 18 November 2022

Animal Species: Wistar Rats

Animal Numbers: 120

Applicants are reminded that they are expected to comply with accepted standards for the use of animals in research and teaching as reflected in the South African National Standards 10386: 2008. The SANS 10386: 2008 document is available on the Division for Research Developments website www.sun.ac.za/research.

As provided for in the Veterinary and Para-Veterinary Professions Act, 1982. It is the principal investigator's responsibility to ensure that all study participants are registered with or have been authorised by the South African Veterinary Council (SAVC) to perform the procedures on animals, or will be performing the procedures under the direct and continuous supervision of a SAVC-registered veterinary professional or SAVC-registered para-veterinary professional, who are acting within the scope of practice for their profession.

Please remember to use your protocol number 19400 on any documents or correspondence with the REC: ACU concerning your research protocol.

Please note that the REC: ACU has the prerogative and authority to ask further questions, seek additional information, require further modifications or monitor the conduct of your research.

Any event not consistent with routine expected outcomes that results in any unexpected animal welfare issue (death, disease, or prolonged distress) or human health risks (zoonotic disease or exposure, injuries) must be reported to the committee, by creating an Adverse Event submission within the system.

We wish you the best as you conduct your research.

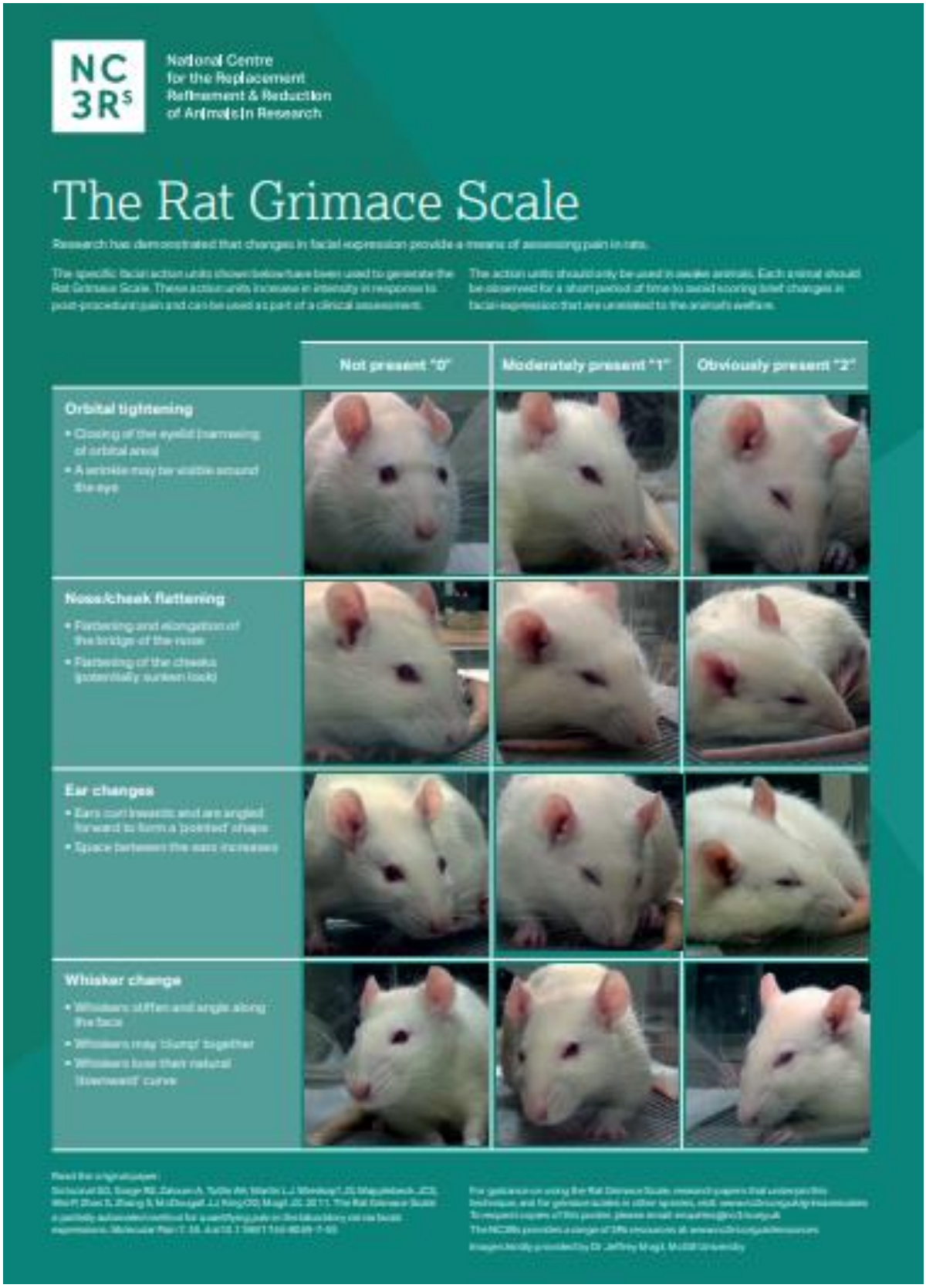
If you have any questions or need further help, please contact the ethics help-desk at applyethics@sun.ac.za or 021 808 9003.
This committee is registered with the National Health Research Ethics Council of South Africa - registration number: AREC-150211-007.

Sincerely,

Mr. WA Beukes
Coordinator: Research Ethics (Animal Use and Biosafety)
E: wabeukes@sun.ac.za

Appendix B: Rat grimace scale

The Grimace Scale uses facial expressions as an indication of pain



Appendix C: Body condition score

The body condition score, used to evaluate the subcutaneous fat layer covering the spine and pelvis. Retrieved from Hickman & Swan, 2010.

BC 1
Rat is emaciated

- Segmentation of vertebral column prominent if not visible.
- Little or no flesh cover over dorsal pelvis. Pins prominent if not visible.
- Segmentation of caudal vertebrae prominent.

BC 2
Rat is under conditioned

- Segmentation of vertebral column prominent.
- Thin flesh cover over dorsal pelvis, little subcutaneous fat. Pins easily palpable.
- Thin flesh cover over caudal vertebrae, segmentation palpable with slight pressure.

BC 3
Rat is well-conditioned

- Segmentation of vertebral column easily palpable.
- Moderate subcutaneous fat store over pelvis. Pins easily palpable with slight pressure.
- Moderate fat store around tail base, caudal vertebrae may be palpable but not segmented.

BC 4
Rat is overconditioned

- Segmentation of vertebral column palpable with slight pressure.
- Thick subcutaneous fat store over dorsal pelvis. Pins of pelvis palpable with firm pressure.
- Thick fat store over tail base, caudal vertebrae not palpable.

BC 5
Rat is obese

- Segmentation of vertebral column palpable with firm pressure; may be a continuous column.
- Thick subcutaneous fat store over dorsal pelvis. Pins of pelvis not palpable with firm pressure.
- Thick fat store over tail base, caudal vertebrae not palpable.

Appendix D: Non-significant anthropometric results

Prefrontal cortex

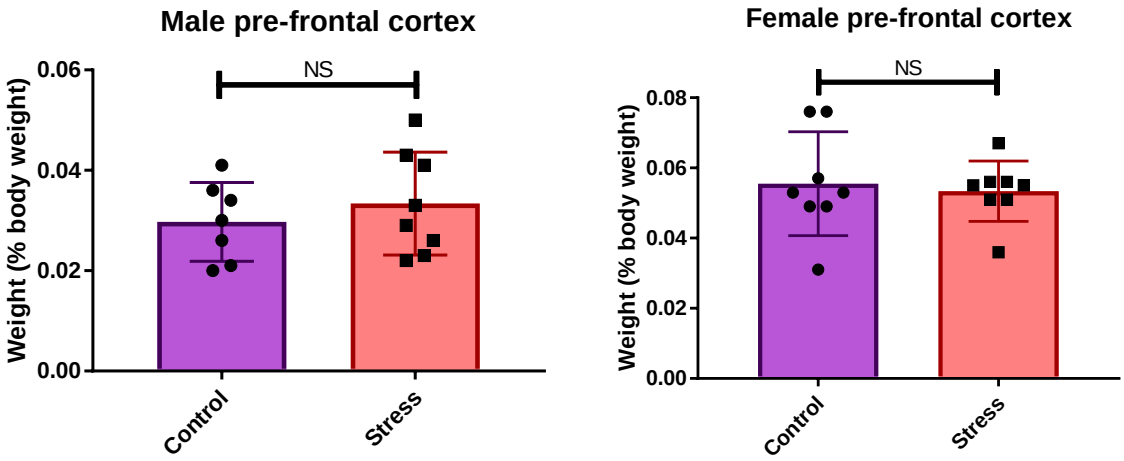


Figure 31. (a) Male pre-frontal cortex weights. No statistical significance observed between the experimental (stress) group (0.03 ± 0.01 % body weight, n=8) and the control group (0.03 ± 0.01 % body weight, n=7). **(b) Female pre-frontal cortex weights.** The experimental (stress) group (0.05 ± 0.01 % body weight, n=8) showed no significant difference compared to the control group (0.06 ± 0.01 % body weight, n=8). Organ weight was calculated as a percentage of body weight. Analysis done by Student's t-test; values represented as mean ± SD.

Hypothalamus

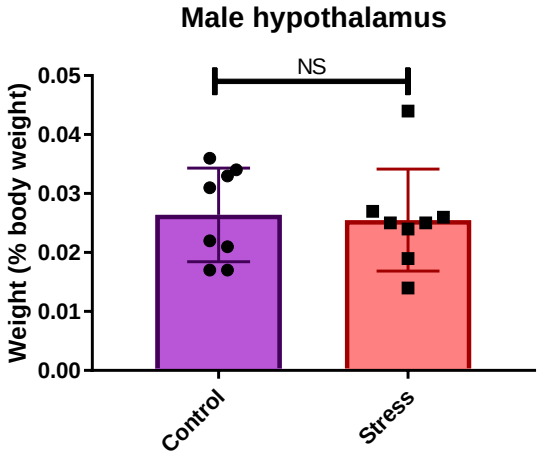


Figure 32. Male hypothalamus weights. No significant difference observed between the experimental (stress) group (0.03 ± 0.01 % body weight, n=8) and the control group (0.03 ± 0.01 % body weight, n=8). Organ weight was calculated as a percentage of body weight. Analysis done by Student's t-test; values represented as mean ± SD.

Hippocampus

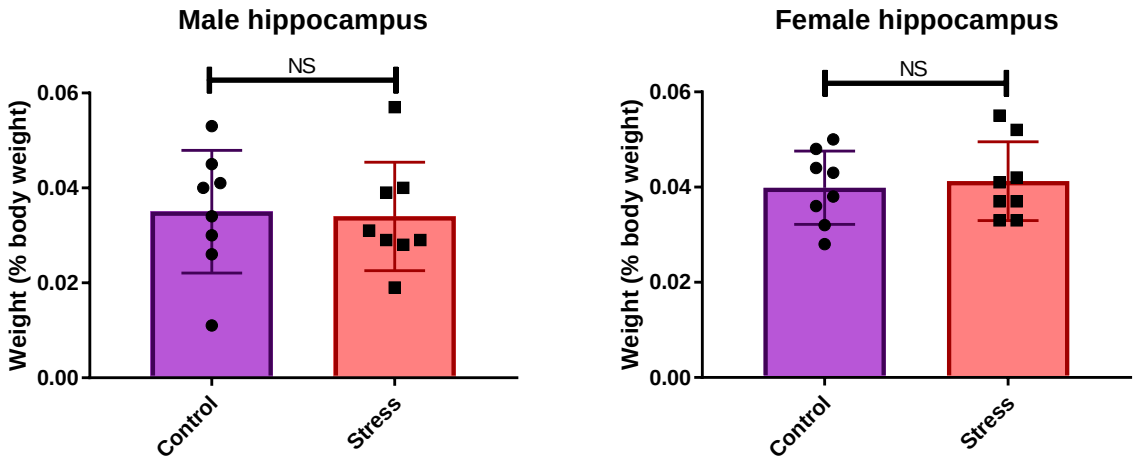


Figure 33. (a) Male hippocampus weights. No statistical significance observed between the experimental (stress) group (0.03 ± 0.01 % body weight, n=8) and the control group (0.04 ± 0.01 % body weight, n=8). **(b) Female hippocampus weights. The** experimental (stress) group (0.04 ± 0.01 % body weight, n=8) showed no significant difference compared to the control group (0.04 ± 0.01 % body weight, n=8). Organ weight was calculated as a percentage of body weight. Analysis done by Student's t-test; values represented as mean ± SD.

Amygdala

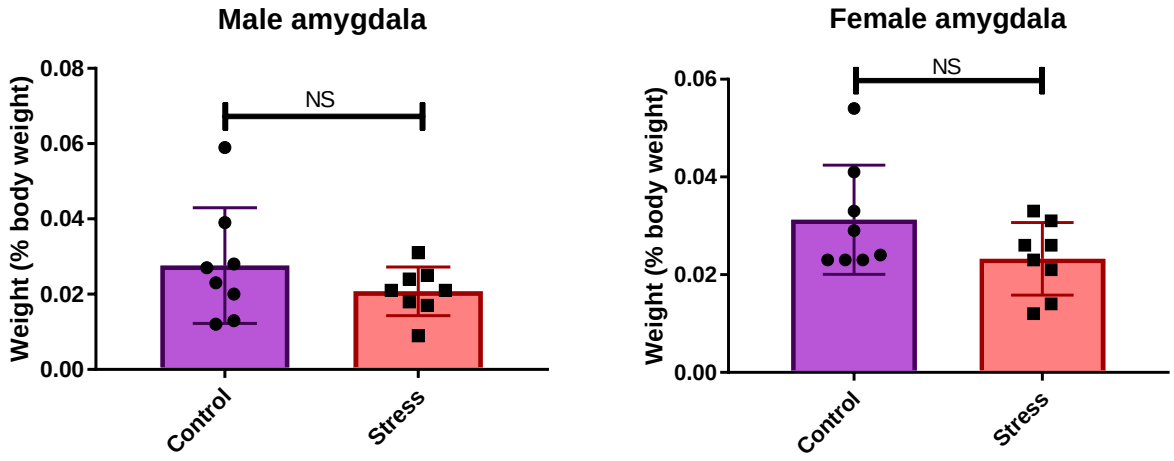


Figure 34. (a) Male amygdala weights. No statistical significance observed between the experimental (stress) group (0.02 ± 0.01 % body weight, n=8) and the control group (0.03 ± 0.02 % body weight, n=8). **(b) Female amygdala weights**. The experimental (stress) group (0.02 ± 0.01 % body weight, n=8) showed no significant difference compared to the control group (0.03 ± 0.01 % body weight, n=8). Organ weight was calculated as a percentage of body weight. Analysis done by Student's t-test; values represented as mean ± SD.

Heart

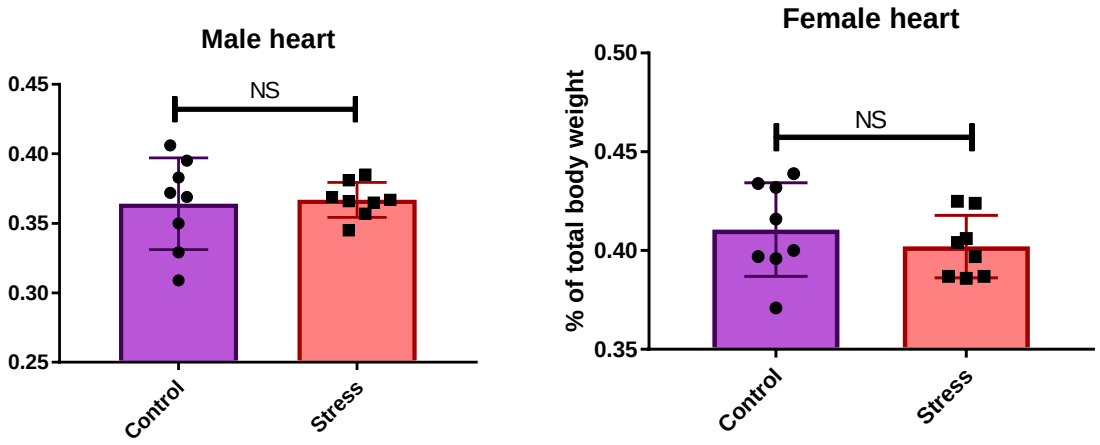


Figure 35. (a) Male heart weights. No statistical significance observed between the experimental (stress) group (0.37 ± 0.01 % body weight, n=8) and the control group (0.36 ± 0.03 % body weight, n=8). **(b) Female heart weights.** The experimental (stress) group (0.40 ± 0.02 % body weight, n=8) showed no significant difference compared to the control group (0.41 ± 0.02 % body weight, n=8). Organ weight was calculated as a percentage of body weight. Analysis done by Student's t-test; values represented as mean ± SD.

Liver

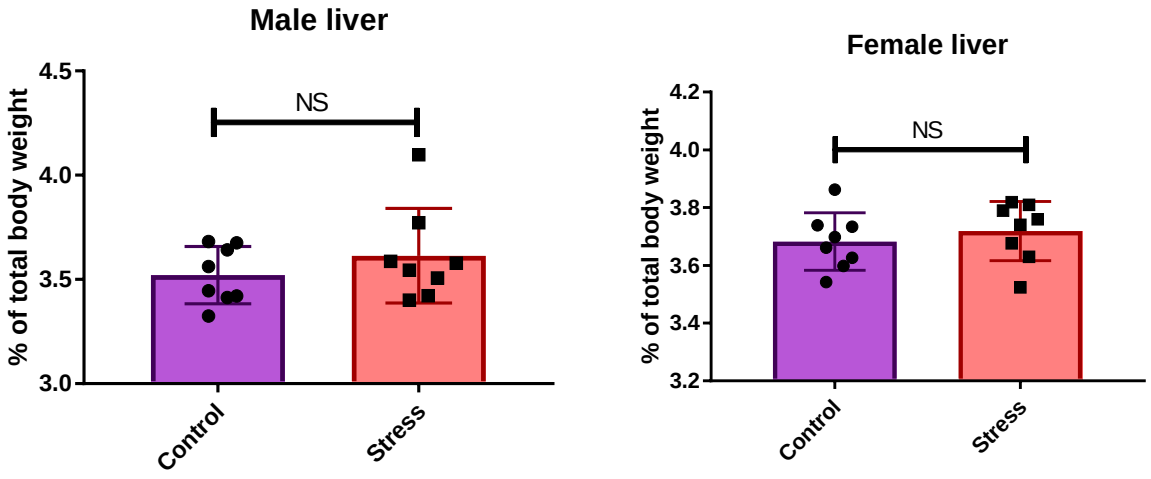


Figure 36. (a) Male liver weights. No statistical significance observed between the experimental (stress) group (3.61 ± 0.23 % body weight, n=8) and the control group (3.52 ± 0.14 % body weight, n=8). **(b) Female liver weights. The** experimental (stress) group (3.72 ± 0.10 % body weight, n=8) showed no significant difference compared to the control group (3.68 ± 0.10 % body weight, n=8). Organ weight was calculated as a percentage of body weight. Analysis done by Student's t-test; values represented as mean ± SD.

Spleen

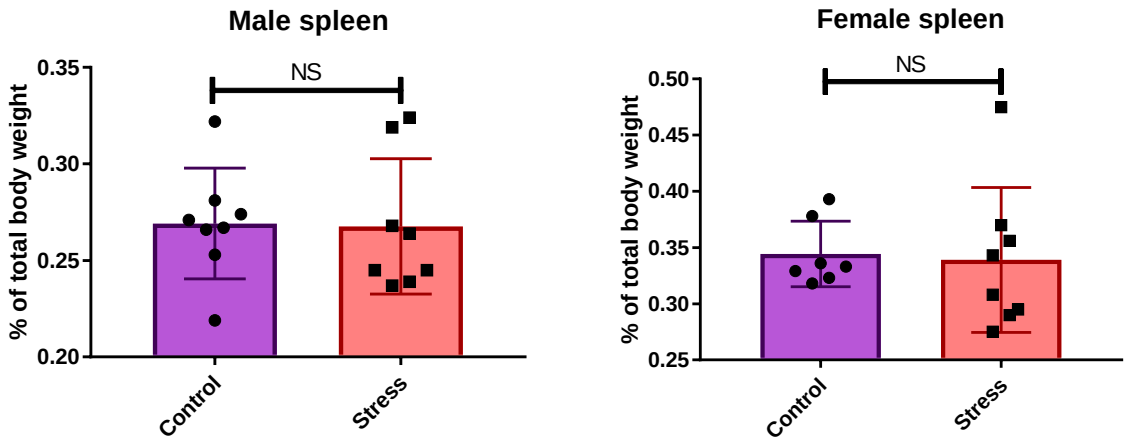


Figure 37. (a) Male spleen weights. No statistical significance observed between the experimental (stress) group (0.27 ± 0.04 % body weight, n=8) and the control group (0.27 ± 0.03 % body weight, n=8). **(b) Female spleen weights.** The experimental (stress) group (0.34 ± 0.06 % body weight, n=8) showed no significant difference compared to the control group (0.37 ± 0.08 % body weight, n=8). Organ weight was calculated as a percentage of body weight. Analysis done by Student's t-test; values represented as mean ± SD.

Right adrenal gland

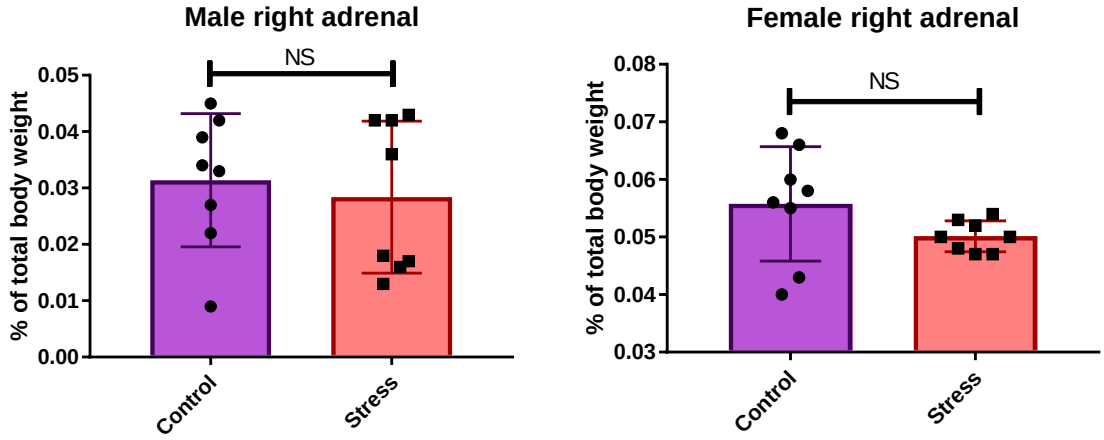


Figure 38. (a) Male right adrenal weights. No statistical significance observed between the experimental (stress) group (0.03 ± 0.01 % body weight, n=8) and the control group (0.03 ± 0.01 % body weight, n=8). **(b) Female right adrenal weights**. The experimental (stress) group (0.05 ± 0.01 % body weight, n=8) also showed no significant difference compared to the control group (0.06 ± 0.01 % body weight, n=8). Organ weight was calculated as a percentage of body weight. Analysis done by Student's t-test; values represented as mean ± SD.

Left adrenal gland

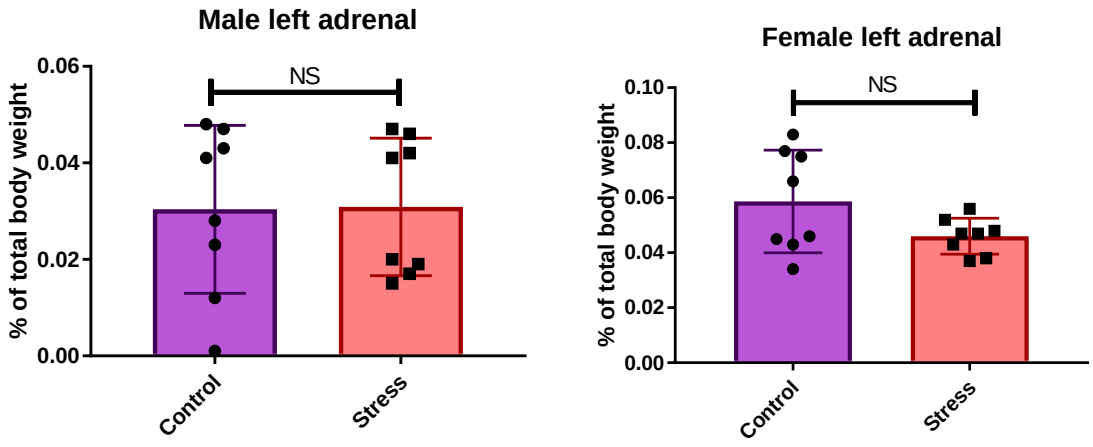


Figure 39. (a) Male left adrenal weights. No statistical significance observed between the experimental (stress) group (0.03 ± 0.01 % body weight, n=8) and the control group (0.03 ± 0.02 % body weight, n=8). **(b) Female left adrenal weights.** The experimental (stress) group (0.05 ± 0.01 % body weight, n=8) also showed no significant difference compared to the control group (0.06 ± 0.02 % body weight, n=8). Organ weight was calculated as a percentage of body weight. Analysis done by Student's t-test; values represented as mean ± SD.

Appendix E: Non-significant behavioral results

Elevated plus maze

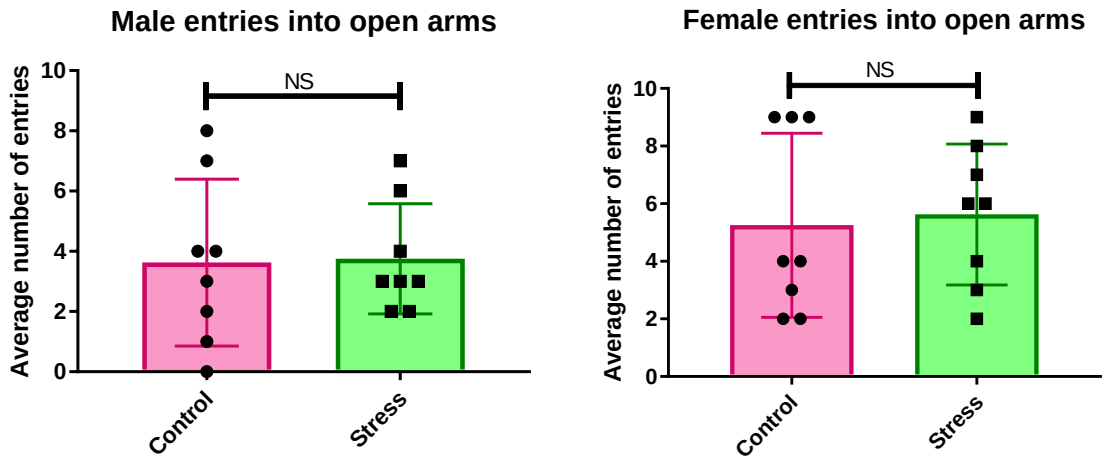


Figure 40. (a) Male number of entries into the open arms of the EPM. No significant difference observed between the experimental (stress) group (3.75 ± 1.83, n=8) and the control group (3.62 ± 2.77, n=8). **(b) Female number of entries into the open arms of the EPM. The** experimental (stress) group (5.62 ± 2.44, n=8) also showed no significant change between the control group (2.25 ± 3.19, n=8). Analysis done by Student's t-test; values represented as mean ± SD.

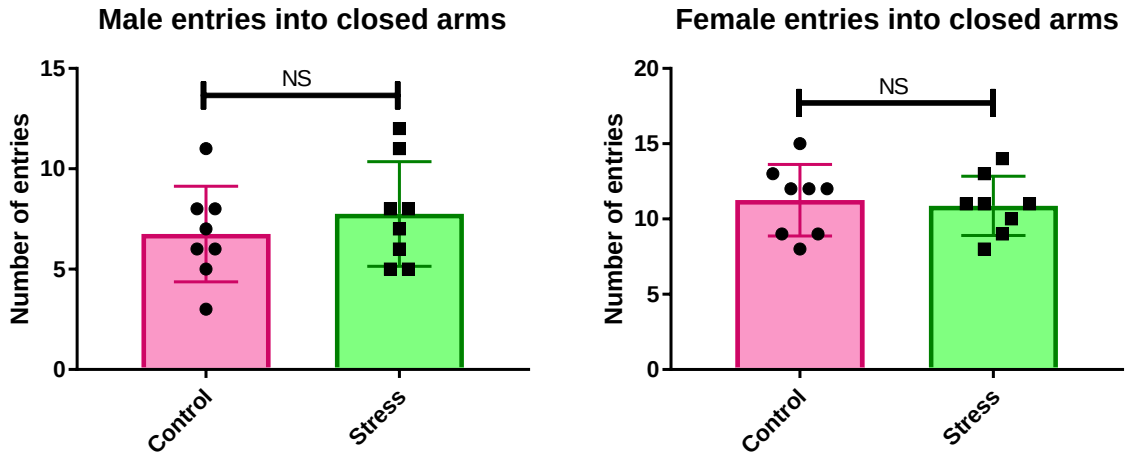


Figure 41. (a) Male number of entries into the closed arms of the EPM. No significant difference observed between the experimental (stress) group (7.75 ± 2.60, n=8) and the control group (6.75 ± 2.37, n=8). **(b) Female number of entries into the closed arms of the EPM.** The experimental (stress) group (10.88 ± 1.95, n=8) also showed no significant change between the control group (11.25 ± 2.37, n=8). Analysis done by Student's t-test; values represented as mean ± SD.

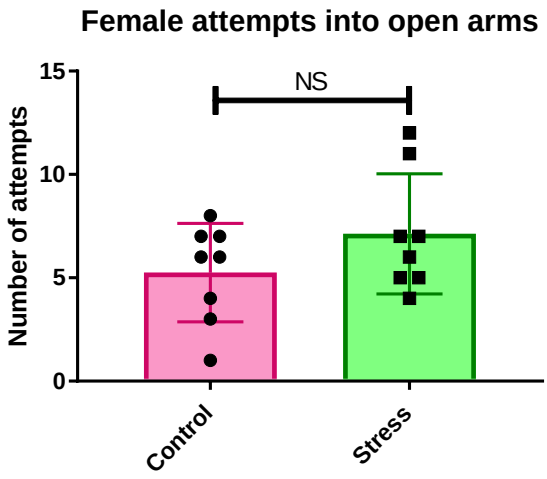


Figure 42. Female number of attempts into the open arms of the maze. No statistical significance ($p<0.05$) was observed between the experimental (stress) group (7.125 ± 2.9, n=8) and the control group (5.25 ± 2.375, n=8). Analysis done by mixed model ANOVA in R; values represented as mean ± SD.

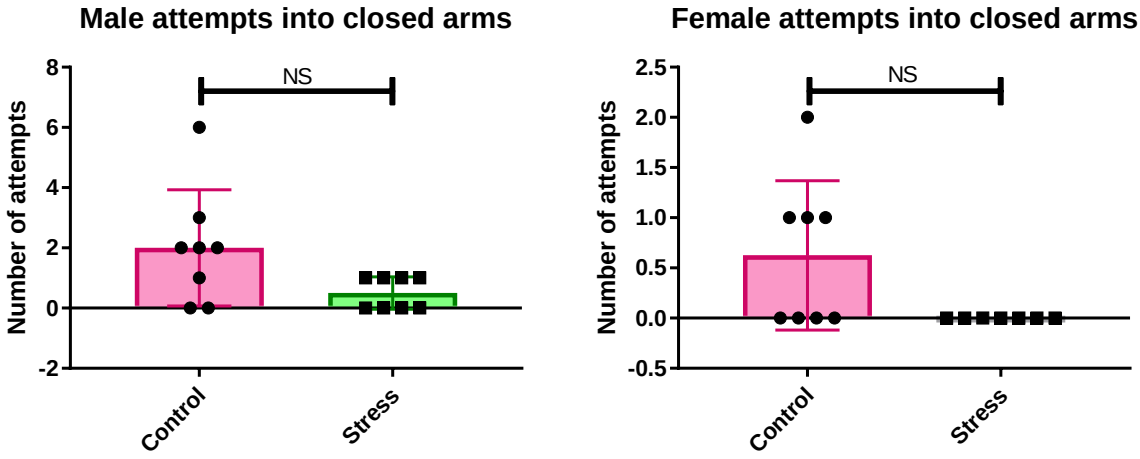


Figure 43. (a) Male number of attempts into the closed arms of the maze. No significant difference observed between the experimental (stress) group (0.50 ± 0.53, n=8) and the control group (2.00 ± 1.92, n=8). **(b) Female number of attempts into the closed arms of the maze.** The experimental (stress) group (1.00 ± 2.82, n=8) also showed no significant change between the control group (0.62 ± 0.74, n=8). Analysis done by Student's t-test; values represented as mean ± SD.

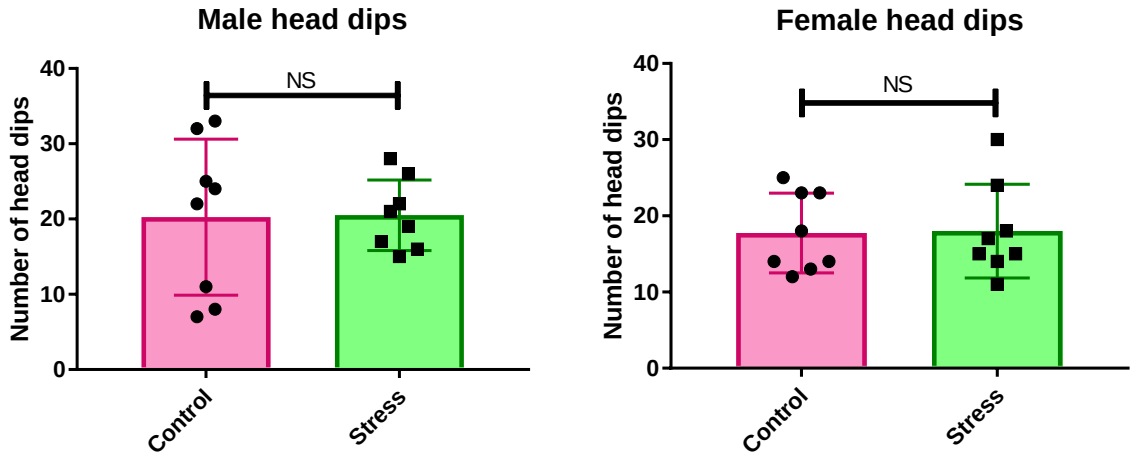


Figure 44. **(a) Male number of head dips in the EPM.** No significant difference observed between the experimental (stress) group (20.50 ± 4.69, n=8) and the control group (20.25 ± 10.36, n=8). **(b) Female number of head dips in the EPM.** The experimental (stress) group (18.00 ± 6.14, n=8) also showed no significant change between the control group (17.75 ± 5.23, n=8). Analysis done by Student's t-test; values represented as mean ± SD.

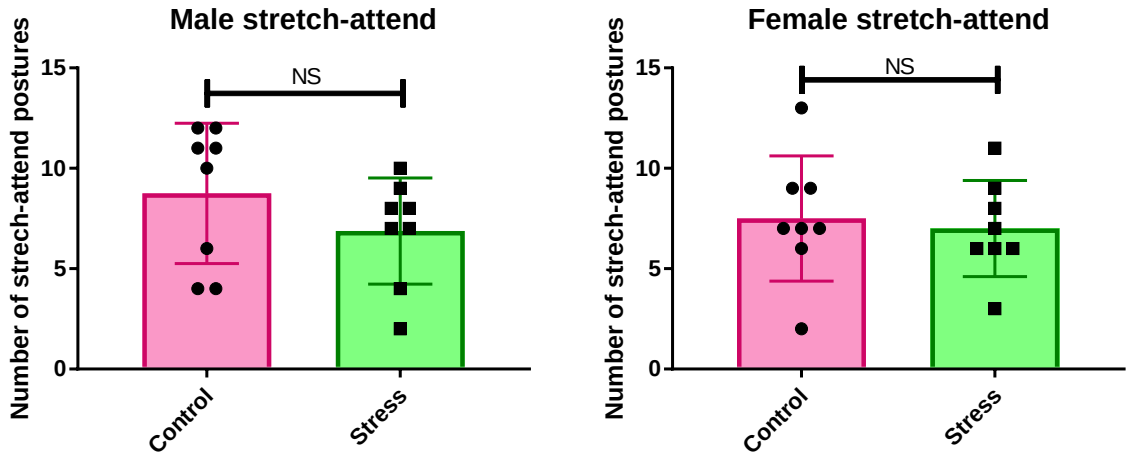


Figure 45. (a) Male number of stretch-attend postures during the EPM. No significant difference observed between the experimental (stress) group (6.88 ± 2.64, n=8) and the control group (8.75 ± 3.49, n=8). **(b) Female number of stretch-attend postures during the EPM.** The experimental (stress) group (7.00 ± 2.39, n=8) also showed no significant change between the control group (7.70 ± 3.11, n=8). Analysis done by Student's t-test; values represented as mean ± SD.

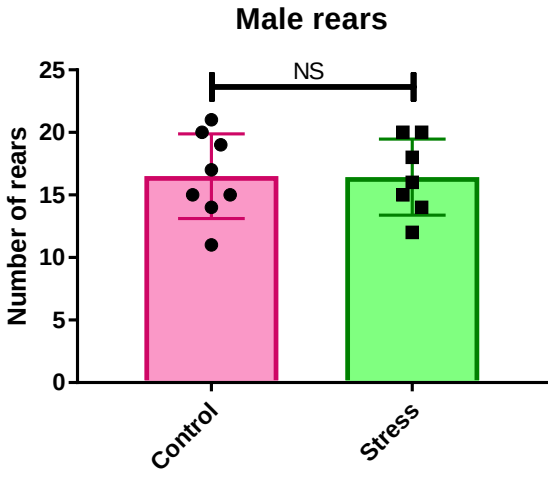


Figure 46. Number of rears for the male rats during the EPM. No statistical significance was observed between the experimental (stress) group (16.43 ± 3.047, n=8) and the control group (16.5 ± 3.381, n=8). Analysis done by Student's t-test; values represented as mean ± SD.

Tail flick task

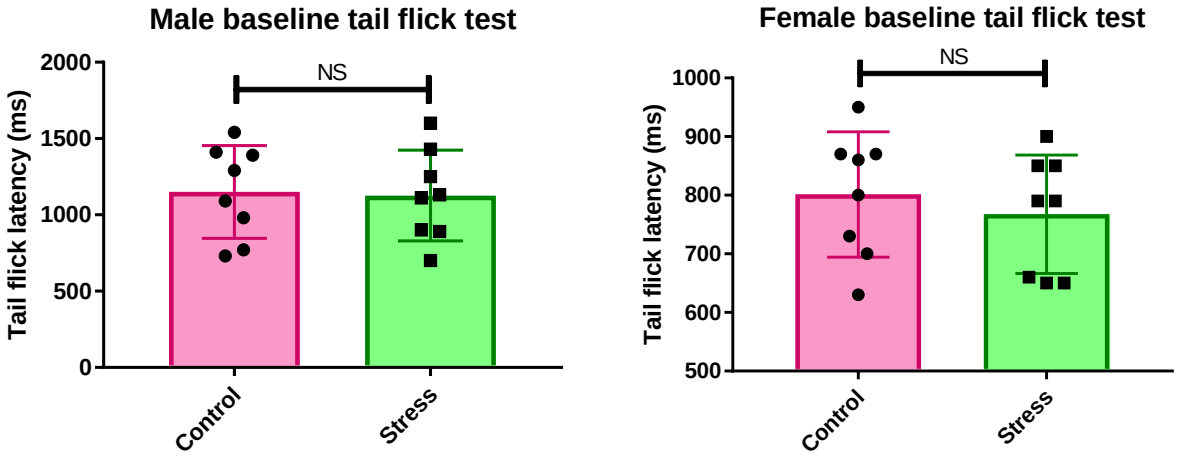


Figure 47. Male tail flick latency at baseline. No significant difference observed between the experimental (stress) group (1126.00 ± 297.80 ms, n=8) and the control group (1150.00 ± 304.00 ms, n=8). **(b) Female tail flick latency at baseline.** The experimental (stress) group (767.50 ± 101.00 ms, n=8) also showed no significant change between the control group (801.30 ± 106.70 ms, n=8). Analysis done by Student's t-test; values represented as mean ± SD.

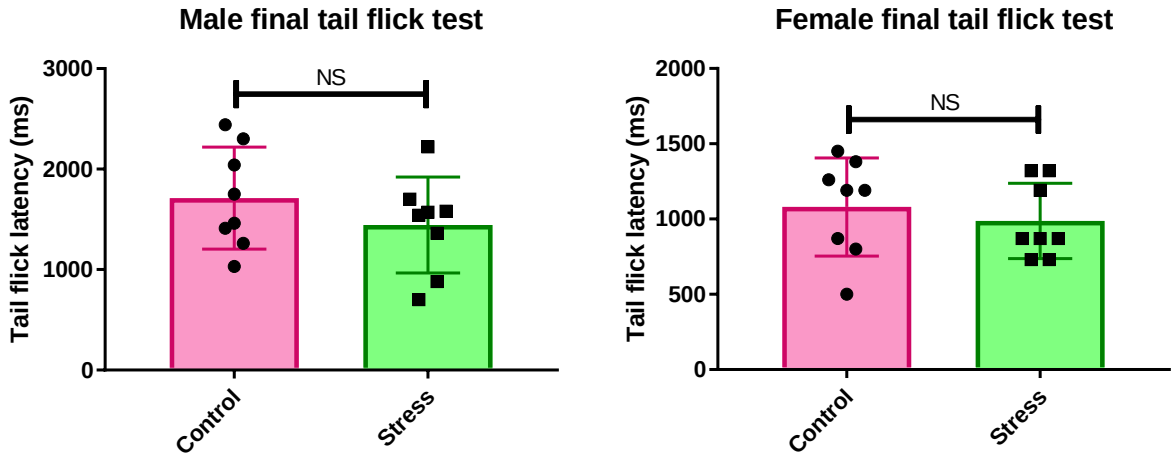


Figure 48. Male tail flick latency at the end of the stress protocol. No significant difference observed between the experimental (stress) group (1444.00 ± 476.60 ms, n=8) and the control group (171.00 ± 508.30 ms, n=8). **(b) Female tail flick latency at the end of the stress protocol.** The experimental (stress) group (987.50 ± 249.60 ms, n=8) also showed no significant change between the control group (1080.00 ± 325.70 ms, n=8). Analysis done by Student's t-test; values represented as mean ± SD.

Appendix F: Turnitin originality report