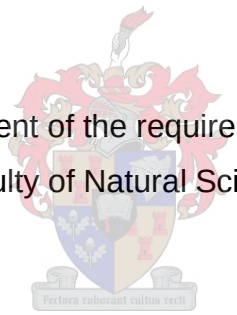
ESTABLISHING AND VALIDATING AN UNPREDICTABLE CHRONIC MILD STRESS RAT MODEL IN A SOUTH AFRICAN LABORATORY

Leandrie Corné Beselaar



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.

Supervisor: Prof M. Faadiel Essop

March 2021

DECLARATION

By submitting this assignment/thesis/dissertation electronically, I declare that the entirety of the work contained therein is my own, original work, that I am the sole author thereof (save to the extent explicitly otherwise stated), that reproduction and publication thereof by Stellenbosch University will not infringe any third-party rights and that I have not previously in its entirety or in part submitted it for obtaining any qualification.

Date: March 2021

ACKNOWLEDGMENTS

This thesis would not have been possible without certain influences in my life. Above all, the credit for my work goes to the Almighty God, for carrying me through two of the most challenging years of my life. Thank you for the endless blessings You sent in midst of it all.

The biggest thank you goes to Prof Faadiel Essop, my mentor and supervisor, who was a big part of the reason I pursued a master's degree in the first place. Prof, you were not only the best MSc supervisor I could have asked for, but the way in which you managed your students through the hardships and uncertainty of 2020 was exceptional. Over the course of being your student you never failed to support me and develop me as a researcher and human being. Thanks to the projects you involved me in, I discovered a hidden passion that ended up changing my career choices for the better. Thank you for the endless philosophical discussions, reminding me why we do what we do, and for the countless times you went out of your way for me. It is so appreciated, and your influence will stay with me forever.

To Nina Truter, my research partner and loyal friend, goes the credit of being the reason that I made it through this degree with my sanity intact. You've become such an integral part of my life that our co-dependence went from a joke to a truth. Thank you for dragging me along with you when it was needed, and for providing me with endless validations which motivated me to keep going. I'm so thankful for your kindness and support during the times when I felt like giving up, and I treasure the memories we made throughout all this. Words cannot describe how thankful I am that I had you and your sense of humor throughout these two years. I could never have done it without my favorite ham.

To the best teammates and friends, Hannah Geddie and Megan Cairns, thank you for being such valuable team players throughout the study. You were always ready to step in where needed and served as my teachers in so many ways. Thank you for pushing me where needed and the value you added to the research. It was a pleasure working with you.

To the CARMA group, and Dr Danzil Joseph in particular, thank you for the help and support over the course of the two years, and the measures that was taken to make me a better researcher. It was an honor to be a part of the team.

To the occupants of the Data Hub and the friends I've made in the department, thank you for being a home to me. Some of my fondest memories and the funniest jokes were made in the Nine-Nine, and I appreciate all the friendships that were formed over endless coffee runs to Lulu.

To the Department of Physiological Sciences and the Postgraduate Office, thank you for the financial support that was provided, without which my two years of study would not have been possible. This thesis is based on the research supported in part also by the National Research Foundation

To my family, namely my father, who will always be my rock, my two brilliant and loving mothers and to the best sister I could ever ask for, thank you for being my home base and safe place. Your support and care have been so valued, and I love you all so much.

Lastly, I have been blessed with extraordinary people and influences in my life. To all who have had an impact in my life, for better or for worse, you have shaped me, and I am a better person for it. Thank you for what you have meant to me over these two years.

ABSTRACT

There is growing concern regarding the societal implications of the increasing burden of chronic stress. In light of this, there are countless animal studies that are currently pursuing the underlying mechanisms of chronic stress-induced disease onset and/or evaluating therapeutic interventions. The unpredictable chronic mild stress (UCMS) model is widely used globally, however to the best of our knowledge it has not yet been employed in South Africa. The present study was therefore aimed at successfully establishing the UCMS model at Stellenbosch University, with the lesser goal of evaluating to which extent the validation tests succeed in confirming a chronically stressed state in the animals.

Male Wistar rats (n=14) were subjected to a nine-week UCMS protocol. The rats were randomly exposed to one or more mild stressors per day and underwent a sucrose preference test (SPT) weekly, aimed at establishing levels of anhedonia. Additionally, detailed weekly monitoring was performed to observe aggressive behaviors and determine general well-being. Following the UCMS protocol, the rats underwent an elevated plus maze (EPM) test to establish the presence of anxiety-like behaviors, after which they were euthanized by decapitation. The results revealed distinct differences in individual responses to stress, therefore the Stress group was subdivided into Stress susceptible and Stress resilient groups, based on specific criteria.

Despite no change in plasma corticosterone levels, molecular analyses showed that plasma adrenocorticotropic hormone levels were significantly increased in the Stress susceptible group. However, the sucrose preference of both Stress susceptible and Stress resilient groups increased over the experimental period. The EPM results revealed anxiety-like behaviors in the Stress susceptible rats, as they spent significantly more time in the closed arms of the EPM and made significantly less entries into open arms, compared to the Stress resilient group. We hypothesize that the Stress resilient rats have some protective mechanism against the effects of chronic stress. Another theory suggests that these rats are more resistant to such effects and take longer to experience the damaging effects thereof.

The results of the behavioral tests used to validate the model showed that the EPM is a more robust validation of the UCMS model than the SPT. Overall, this study contributes to the existing theory that the UCMS model is difficult to establish across different laboratories. Although anxiety-like behaviors were observed in the Stress susceptible groups, the lack of plasma corticosterone changes and anhedonia in the same group suggests that the model might not have been entirely effective at creating a state of chronic stress. The study concludes that rodent models of chronic stress should be validated by multiple tests that focus on evaluating the animal as a whole and not just rely on a single behavioral or molecular parameter.

OPSOMMING

Daar is toenemende kommer oor implikasies wat die toenemende las van chroniese spanning. In die lig hiervan is daar tallose dierestudies wat tans die onderliggende meganismes van die ontstaan van chroniese stres-geïnduseerde siektes en/of terapeutiese intervensies evalueer. Die onvoorspelbare chroniese ligte spanning (UCMS) -model is regoor die wêreld gevestig, maar na ons beste wete is dit nog nie in Suid-Afrika gevestig nie. Hierdie studie was dus daarop gemik om die UCMS-model suksesvol aan die Universiteit Stellenbosch te vestig, met die mindere doel om te evalueer tot watter mate die valideringstoetse daarin slaag om ’n toestand van chroniese spanning in die diere te bevestig.

Manlike Wistar-rotte (n=14) aan 'n onvoorspelbare chroniese stresprotokol van nege weke onderwerp. Die rotte is daagliks blootgestel aan een of meer ligte stressors en het weekliks 'n sukrose-voorkeurtoets (SPT) ondergaan. Daarmee saam is is die rotte weekliks in detail gemonitor om enige aggressiewe gedrag waar te neem en algemene welstand te bepaal. Na die stres-protokol het die rotte 'n verhoogde plus doolhof (EPM) toets ondergaan om die teenwoordigheid van angstige gedrag vas te stel. Die algehele studie-resultate het duidelike verskille getoon in individuele rotte se reaksies op stres, daarom is die Stres-groep onderverdeel in Stres-vatbare en Stres-bestande groepe, gebaseer op spesifieke kriteria.

Molekulêre ontledings het geen verandering is in plasmakortikosteroonvlakke getoon nie, alhoewel dit gewys het dat plasmadrenokortikotropiese hormoonvlakke beduidend verhoog was in die Stres-vatbare groep. Gedurende die eksperimentele periode het die sukrose-voorkeur in beide die Stres-vatbare en Stres-bestande groepe toegeneem. Die EPM-resultate het gewys dat Stres-vatbare rotte anstige gedrag getoon het, aangesien hulle aansienlik meer tyd in die geslote arms van die EPM deurgebring het. Dié groep het ook aansienlik minder kere in die oop arms ingetree, in vergelyking met die Stres-bestande groep. Ons veronderstel dat die Stres-bestande rotte 'n beskermende meganisme ontwikkel het teen die effekte van chroniese spanning. 'n Ander teorie dui daarop dat hierdie rotte meer bestand is en dus langer neem om die skadelike effekte daarvan te ervaar.

Die resultate van die gedragstoetse wat in dié studie gebruik is om die model te onderstuen, het getoon dat die EPM 'n meer robuuste validering van 'n chroniese stresmodel is as die SPT. Oor die algemeen dra hierdie studie by tot ‘n bestaande teorie wat stel dat die UCMS-model moeilik is om tussen verskillende laboratoriums te vestig. Alhoewel angstige gedrag waargeneem is in die Stres-vatbare groep, dui die gebrek aan plasmakortikosteroonveranderings en anhedonie in dieselfde groep daarop dat die model nie heeltemal effektief sou wees om 'n toestand van chroniese spanning te skep nie. Die studie het tot die gevolgtrekking gekom dat knaagdier-modelle van chroniese stres gevalideer moet word deur veelvuldige toetse wat daarop fokus om die dier as geheel te evalueer, en nie net 'n enkele gedrags- of molekulêre eienskap nie.

CONTENTS

FIGURES

TABLES

EQUATIONS

ABBREVIATIONS

α	Alpha
β	Beta
ACTH	Adrenocorticotropic hormone
AIDS	Acquired immunodeficiency syndrome
AVP	Arginine vasopressin
BDNF	Brain-derived neurotrophic factor
BNST	Bed nucleus of the stria terminalis
CMS	Chronic mild stress
CNS	Central nervous system
CRF	Corticotropin-releasing factor
CRH	Corticotropin-releasing hormone
CRS	Chronic restraint stress
CUMS	Chronic unpredictable mild stress
CUR	Curculigoside
CUS	Chronic unpredictable stress
CVD	Cardiovascular disease
CVS	Chronic variable stress
E	Epinephrine
EPM	Elevated plus maze
EZM	Elevated zero maze

FST	Forced swimming test
FOSB	Protein fosB
GABA	Gamma Aminobutyric acid
GC	Glucocorticoid
GCPR	G-coupled protein receptor
GR	Glucocorticoid receptor
HIV	Human immunodeficiency virus
HPA	Hypothalamic-pituitary-adrenal
LC	Locus coeruleus
LC/NE	Locus coeruleus/norepinephrine
L-DOPA	Levodopa
LH	Learned helplessness
MC	Mineralocorticoid
MR	Mineralocorticoid receptor
MS	Maternal separation
NAc	Nucleus accumbens
NCD	Non-communicable disease
NE	Norepinephrine
NORT	Novel object recognition test
NPY	Neuropeptide Y
OFT	Open field test
PFC	Prefrontal cortex
PNS	Parasympathetic nervous system

PTSD	Post-traumatic stress disorder
PVC	Polyvinyl chloride
PVN	Paraventricular nucleus
RAAS	Renin-angiotensin-aldosterone system
SAM	Sympathetic adreno-medullary
SNS	Sympathetic nervous system
SPT	Sucrose preference test
SR	Stress resilient
SS	Stress susceptible
TST	Tail suspension test
UCMS	Unpredictable chronic mild stress
WYK	Wistar-Kyoto

1. INTRODUCTION

1.1. OVERVIEW

Chronic stress has been inextricably linked to disease onset for decades. Psychosocial stress is implicated in conditions varying from cardiovascular and metabolic diseases, to psychiatric and neurological disorders (Chandola *et al.*, 2006; Cohen *et al.*, 2007; Low *et al.*, 2009). Due to the increasing complexity of the contemporary human lifestyle, such diseases are becoming increasingly prevalent. For example, currently more than 70% of global human mortality is attributed to such non-communicable diseases (NCDs) (World Health Organization, 2013). The South African population is not exempt from this, as NCD-related mortality rates are now higher than those for tuberculosis and human immunodeficiency virus (HIV)/acquired immunodeficiency syndrome (AIDS) combined (Nojilana, Bradshaw, Pillay-van Wyk, Msemburi, Somdyala, *et al.*, 2016).

There is growing concern regarding the societal implications of the burden of chronic stress. According to the European Agency for Safety and Health at Work, 80-90% of all industrial workplace accidents occur due to personal problems or an employee's inability to handle stress, while 50% of job absenteeism can be attributed to stress (Salleh, 2008). Additionally, an analysis done in 2006 showed that 13.4 million working days are lost per year due to stress, depression or anxiety (Jones *et al.*, 2003). In light of this, there are countless studies that are currently pursuing the underlying mechanisms of chronic stress-induced disease onset and/or evaluating therapeutic interventions. Here, many researchers employ animal models to best simulate the human chronic stress phenotype and hence enabling further investigations into related psychiatric disorders (Salgado *et al.*, 2013).

However, no animal model of disease is capable of perfectly reproducing the complexity of a human psychiatric disorder (Patchev *et al.*, 2006). As such, there exists a plethora of animal models of chronic stress with various protocols and validation methods (Campos *et al.*, 2013). The aim of this review is therefore to provide a comprehensive overview of the stress response and how a state of chronic stress develops. Following this, the physiological response to chronic stress will be reviewed, followed by a discussion regarding its dysregulation. Subsequently, a discussion of how this chronically stressed state can be best modeled in rodents will follow, as this

thesis focuses on the establishment of a rat model of chronic stress. The most validated rodent models of chronic stress will be thoroughly reviewed to gain insights into those best suited to model the human chronic stress phenotype.

1.2 The stress response and its complexity

General overview of the stress concept

The concept of stress was originally defined by Hans Selye (1936) as the body's "General Adaptation Syndrome", referring to the non-specific response elicited by any type of noxious stimulus that disrupts homeostasis. The body subsequently elicits a suitable response that allows for the necessary restoration of balance (Golbidi *et al.*, 2015). Although this definition (Selye, 1998) has been widely discussed, criticized and debated, it still serves as a useful starting point for any discussion in this context. The idea that links stress to the disruption of homeostasis shows that stress is not inherently a negative response, but more an evolutionary adaptation to ensure survival (Murison, 2016).

The functioning and survival of an organism depends on its ability to maintain homeostasis, which is a term used to describe a stable internal environment in response to fluctuating circumstances (Cannon, 1929). This is brought about through various dynamic processes that establishes stability by secreting mediators aimed at returning the body to a normal state. However, this is not a single linear process but rather a complex network of regulatory systems that allow adequate response(s) to the challenges faced on a daily basis (McEwen, 2007). The concept of "allostasis" was therefore introduced by Sterling and Eyer (1988) to describe the plethora of active processes that the body undergoes to maintain homeostatic stability over a period of time. A more encompassing definition was put forward by Bruce McEwen, who referred to allostasis as the process which maintains physiological stability through constantly changing parameters of its internal milieu by matching them to the changing environmental demands (Juster *et al.*, 2010). The role of allostasis and the overload of the allostatic system during a chronically stressed state will be discussed in length during a later section of this review.

From an evolutionary perspective, a stressor is defined as a stimulus which threatens the survival of the organism and therefore requires energy mobilization to sustain a

subsequent reaction(s) (Murison, 2016). The reaction needs to be initiated, sustained for as long as is necessary, and then successfully shut down after passing of the threat. In this way the stress response forms an integral part of an organism's allostatic process, through the two main arms of the stress response: the hypothalamic-pituitary-adrenal (HPA) axis and the sympathetic adreno-medullary (SAM) pathway (McEwen *et al.*, 2003; Van Oort *et al.*, 2017). Although the stress response is exceptionally varied and wide-spread, the task of responding to a stressor and the subsequent return to stability is mainly facilitated by the two aforementioned pathways (Allen *et al.*, 2014). The neural SAM pathway facilitates the instantaneous "fight-or-flight" response which takes place within seconds of initiation. By contrast, the endocrine HPA pathway triggers a somewhat slower cascade of events which can include neural, hormonal or chemical effects that work in conjunction with the neural SAM pathway to elicit a combined response to the stress stimulus (Smith, 2012).

Different parts of these networks are engaged for various stressor types, with a distinction between physical and psychological stressors (Godoy *et al.*, 2018). For example, an infection or internal hemorrhage is considered a physical stressor, whereas psychological stressors encompass events or circumstances that elicit an emotional response, equally affecting the organism's ability to function optimally (Schneiderman *et al.*, 2005). Despite existing as separate pathways, there exists a large amount of overlap between these systems. This holds true especially for the limbic system as it is the point of origin for all stress responses (Ulrich-Lai *et al.*, 2009).

The stress response has components belonging to either the central nervous system (CNS) or to peripheral systems (Charmandari *et al.*, 2005). Central components are found in the brainstem and hypothalamus. These include (a) neurons releasing both corticotropin-releasing hormone (CRH) and arginine vasopressin (AVP) (mostly found in the paraventricular nucleus (PVN) of the hypothalamus), (b) the medulla and locus coeruleus (LC), and (c) other noradrenergic cell groups in the medulla and pons, that make up the locus coeruleus/norepinephrine (LC/NE) system. The peripheral components include (a) the peripheral parts of the HPA axis, (b) the efferent part of the SAM pathway, and (c) peripheral components of the parasympathetic system (Charmandari *et al.*, 2005).

Involvement of the limbic system

The stress response is initiated in the brain, as the stimulus must first be perceived and evaluated on both subcortical and cortical levels before the appropriate response can be initiated (Herman, 2013). This process is exceptionally complex as the limbic system (main facilitator in this context) consists of various distinct but interconnected brain regions (Ford *et al.*, 2015). These regions play crucial roles in terms of fear, memory, learning, and stress (the focus of this review and thesis) (Ford *et al.*, 2015; Godoy *et al.*, 2018). Although there is some debate regarding the specific regions that make up the limbic system, the general scientific community agrees that the main limbic system components include (among others) the hippocampus, thalamus, amygdala, pre-frontal cortex (PFC) and hypothalamus (Godoy *et al.*, 2018; Murison, 2016; Russo *et al.*, 2013). These can be divided into input (thalamus, PFC, amygdala, hippocampus) and output systems (hypothalamus) (Swenson, 2006).

The sensory input can be sensed and processed on various neural levels upon perceiving a stressor (McAlonan *et al.*, 2000; Murison, 2016). Here the thalamus plays an important role, as the various thalamic nuclei receive the majority of input signals, including visual, auditory and somatic stimuli (Swenson, 2006). The stimuli are then relayed to the amygdala, where the information is analyzed at a basic level. The amygdala serves as an integral structure in terms of mediating the stress response, i.e. by stimulus processing and by facilitating the stress response from other brain regions (Murison, 2016). According to Rajmohan & Mohandas (2007) the main function of the amygdala includes “anxiety, aggression, fear conditioning; emotional memory and social cognition”. It is for this reason that the amygdala is considered the main site of emotional processing in the brain.

The lateral amygdala is the gateway for various inputs. Not only does it receive signals from the thalamus, but cortical structures such as the PFC are also connected to it (Isaacson, 2001). Although the involvement of the PFC is complex, it plays an invaluable role in the regulation of the limbic “alarm system” under stressful conditions (Ford *et al.*, 2015; Godoy *et al.*, 2018). The PFC frequently serves as an inhibitory system, which becomes crucial when the body needs to “apply the brakes” and prevent an overreaction in response to a stimulus of lesser importance (Ford *et al.*, 2015). If the PFC is not functioning optimally, a person may experience impaired

concentration or focus. This can lead to difficulty in controlling their emotions, as well as their ability to think clearly enough to formulate and execute plans to deal with the stressor (Arnsten *et al.*, 2015).

The hippocampus is also an important brain region that has a large effect on the amygdala. The hippocampus is responsible for applying context to incoming stimuli and therefore also plays an important role in memory and its retrieval (Murison, 2016). The hippocampus is thus responsible for recalling a memory regarding a potentially stressful situation, and additionally using that memory to provide context to subsequently determine whether the stressor is indeed a threat (Ford *et al.*, 2015; Murison, 2016). According to research done by Ulrich-Lai & Herman (2009), lesions in the hippocampus lead to increased corticosterone release that indicates a lack of the negative feedback cycle that is usually required to shut down the system. This is particularly observed following exposure to psychological stressors, and not in response to any systemic stressor exposure. These findings are consistent with the role that the hippocampus fulfills in providing context-specific modulation of the body's stress responses (Ulrich-Lai *et al.*, 2009).

It is due to these and many other neural inputs into the amygdala that this region is considered the point where the stress response is initiated, following the completion of the necessary processing (Arnsten *et al.*, 2015). Supporting this is the fact that multiple projections connect the amygdala and hippocampus to the hypothalamus (largely indirectly), which is the main output portion of the limbic system. More specifically, there are two input pathways that can be followed. The first pathway allows inputs from various brain regions to converge on the PVN of the hypothalamus (Jimenez *et al.*, 2019). The second pathway involves the bed nucleus of the stria terminalis (BNST), which is a forebrain structure with important involvements in motivational and stress-related responses and serves as a link between the amygdala and the PVN (McEwen *et al.*, 2010; Murison, 2016). The fact that the PVN is critically important in both pathways is why it is considered the "principal integrator of stress signals" (Ulrich-Lai *et al.*, 2009). Upon stimulation, the PVN initiates a cascade of effects centered around the HPA axis and SAM pathway (Jimenez *et al.*, 2019). The limbic activation, together with the pathways triggered upon activation of the stress response, can be seen in Figure 1.

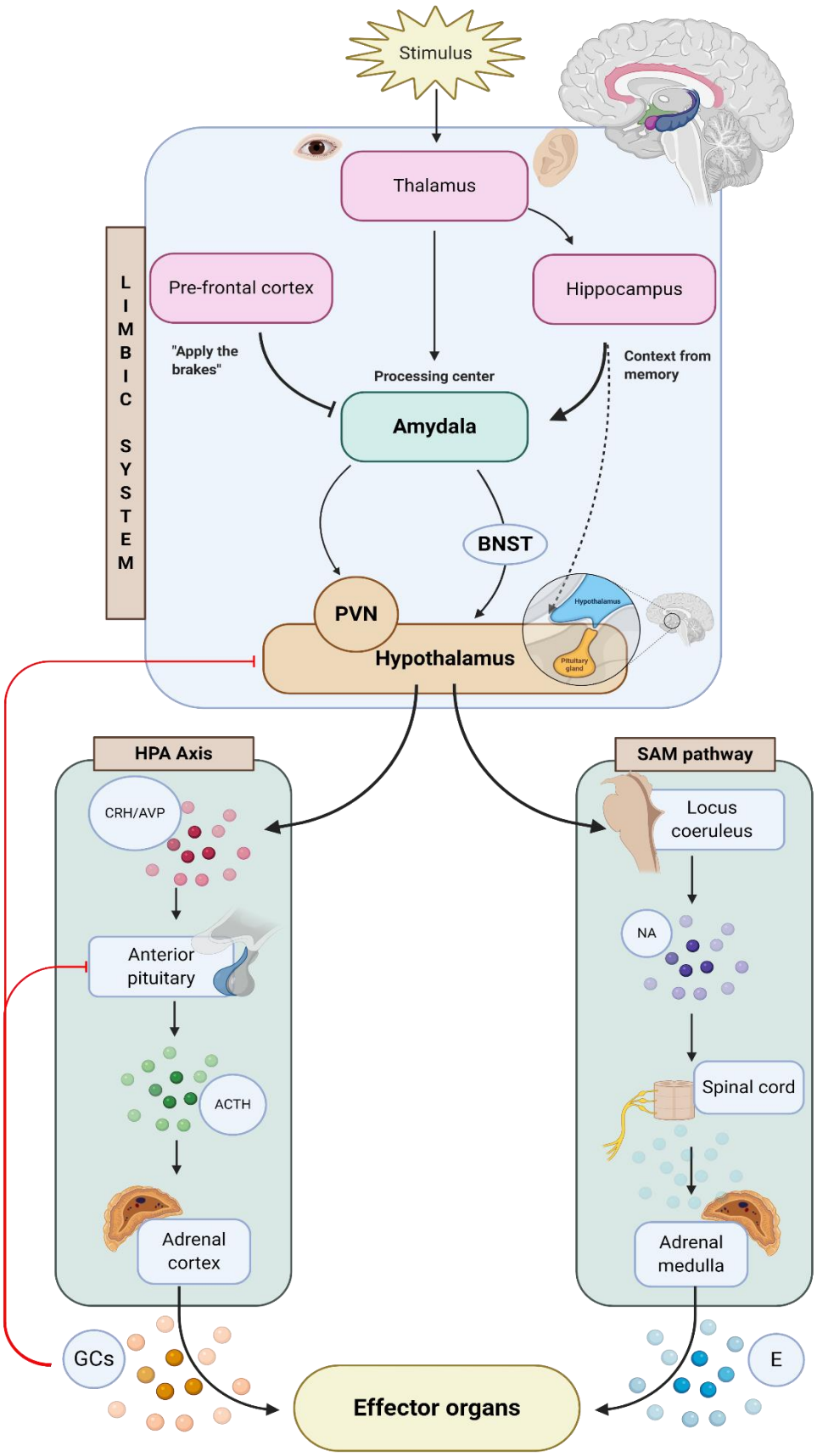


Figure 1: Limbic system activation upon perception of a stressor, after which the HPA axis and SAM pathway is activated. ACTH – adrenocorticotropin hormone; AVP – arginine vasopressin; BNST – bed nucleus of stria terminalis; CRH – corticotropin-releasing hormone; E – epinephrine; GCs – glucocorticoids; HPA - hypothalamic-pituitary-adrenal NE – norepinephrine; PVN – paraventricular nucleus; SAM – sympathetic adreno-medullary. Figure made in BioRender.

Sympathetic stress response

The SAM pathway elicits the most instantaneous response via the CNS and the employment of its sympathetic nervous system (SNS) and parasympathetic nervous system (PNS) arms (Ulrich-Lai *et al.*, 2009). The PNS is responsible for the withdrawing and inhibition of the activity in cases where the SNS activates the response (Murison, 2016). This is also the system that is most closely linked to Cannon's "fight-or-flight" response as previously discussed.

The main mediator of the sympathetic stress response is a system comprised of the LC (a small brainstem structure) and other noradrenergic cell groups, all of which are responsible for the secretion of norepinephrine (NE) (Ford *et al.*, 2015; Murison, 2016). The LC contains the majority of NE-expressing neurons in the brain and therefore serves as a major component of the central arousal network (Myers *et al.*, 2017). The NE released elicits effects on various brain regions such as the amygdala and hypothalamus (Tsigos *et al.*, 2002). Upon activation, the hypothalamus releases CRH from the PVN into the LC. This hormone is responsible for the production of tyrosine hydroxylase, which is the rate-limiting enzyme in NE synthesis (Vale, 2005). At a catecholaminergic synapse, this enzyme catalyzes the reaction that hydroxylates tyrosine to the dopamine-precursor levodopa (L-DOPA), after which L-DOPA is decarboxylated to form dopamine (Molinoff *et al.*, 1971). Dopamine is subsequently transported to the synaptic vesicle where the synthesis of catecholamines occurs. Following the influx of calcium into the synaptic cleft, catecholamines are released and allows the signal to be propagated (Daubner *et al.*, 2012). The result of this process is increased NE in the LC that subsequently leads to sympathetic activation.

The SAM pathway is activated by projections from the PVN and LC to pre-ganglionic sympathetic neurons in the spinal cord. Each pre-ganglionic fiber connects to post-ganglionic fibers which transfers the necessary signal to the effector organ, i.e. the adrenal medulla (Godoy *et al.*, 2018). The chromaffin cells in the inner part of the adrenal gland are responsible for the manufacturing and secretion of both epinephrine and NE, although epinephrine is produced is much larger quantities (Godoy *et al.*, 2018; Murison, 2016). The adrenal medulla is responsible for all the epinephrine production in the body, whereas NE is mainly secreted by the brain (Charmandari *et al.*, 2005). The role of such catecholamines is to prepare the various systems for a

"fight-or-flight" reaction (Ulrich-Lai *et al.*, 2006; Vale, 2005). These catecholamines exert a profound excitatory effect via second messenger pathways on various organs and organ systems, such as the heart, vascular smooth muscle, skeletal muscles, gut, fat, and the kidneys (Murison, 2016).

Once secreted into circulation the catecholamines target adrenergic receptors that are expressed on a variety of cell membranes (Paravati *et al.*, 2019). These receptors are classified as G coupled-protein receptors (GCPRs) and can be further subclassified into alpha- (α) and beta- (β) adrenergic receptors. They are responsible for activating ion channels to mediate an immediate sympathetic response to stress (Paravati *et al.*, 2019). Norepinephrine and epinephrine both possess a high affinity for α_1 and β_1 receptors that are found near terminal sympathetic neurons (Kvetnansky *et al.*, 2009). When bound to these receptors they can exert effects that are aimed at preparing the body for the "fight-or-flight" response (Murison, 2016). These include increasing alertness, raising heart rate and blood pressure, and directing energy towards critical stress responders such as skeletal muscle (Godoy *et al.*, 2018).

HPA axis activation

The HPA axis exerts a slower endocrine response to stress in comparison to the neural SAM pathway, and spans minutes/hours rather than seconds (Murison, 2016). The activation of the HPA axis starts in the amygdala which stimulates PVN neurons to secrete CRH and AVP (Figure 1) (Vale, 2005). The principle hypothalamic hormone is CRH, and its function is to stimulate the anterior pituitary gland to release adrenocorticotropic hormone (ACTH) (Charmandari *et al.*, 2005). Adrenocorticotropic hormone does not work in isolation as the literature reports links between CRH and AVP, showing that AVP is a synergistic factor in the secretion of ACTH. However, AVP possesses limited ability to cause ACTH secretion on its own. Therefore AVP and CRH each stimulate the other to bring about ACTH secretion (Tsigos *et al.*, 2002).

The main target of ACTH is the adrenal gland and it stimulates the zona fasciculata (one of three layers in adrenal cortex) to synthesize glucocorticoid (GC) hormones, considered to be the final effectors of the HPA axis (Charmandari *et al.*, 2005). Arguably the most important secretion is cortisol, a well-known stress biomarker (known as corticosterone in rodents) (Ulrich-Lai *et al.*, 2006). Glucocorticoids specifically facilitate the stress response by increasing the amount of energy available

to the body, and therefore adds to the “fight-or-flight” effects caused by catecholamines (Kvetnansky *et al.*, 2009). They largely accomplish this by ensuring an increased supply of glucose via glycogenolysis and gluconeogenesis, as well as by lipolysis and thermogenesis (Godoy *et al.*, 2018). Additionally, GCs can elicit a primarily inhibitory effect on the immune system as high levels are known to decrease the production of cytokines and mediators of inflammation, as well as lowering antibody production (Murison, 2016; Vale, 2005). Apart from ensuring GC secretion, ACTH separately stimulates the production and release of a mineralocorticoid (MC) called aldosterone from the adrenal cortex (zona glomerulosa) that contributes to the stress response by activating the renin-angiotensin-aldosterone system (RAAS) to raise blood pressure (Lagraauw *et al.*, 2015).

There are two types of receptors capable of facilitating the binding and cellular uptake of GCs, namely glucocorticoid receptors (GRs) and mineralocorticoid receptors (MRs) (Gomez-Sanchez *et al.*, 2014). Mineralocorticoids display a particularly high affinity for GCs, and this means that such receptors are consistently occupied even in the presence of relatively low GC circulating levels. As GRs possess a much lower affinity for the hormone than MRs, they largely become occupied only as the circulating GC levels increase, as is the case when the body responds to stress (Joëls *et al.*, 2010). A delicate balance between these two receptors is therefore crucial for maintaining homeostatic stability in the body (Gomez-Sanchez *et al.*, 2014).

Arguably one of the most critical processes that takes place as part of the stress response is the shutdown of the response once the threat has passed. Prolonged exposure to stress mediators can elicit detrimental effects such as chronic immunosuppression, prolonged inhibition of vegetative systems, and consistent elevated heart rate and blood pressure (Murison, 2016). Thus specific shutdown systems are required, with GCs the main facilitators of this process (Miller *et al.*, 2002; Sapolsky *et al.*, 2000). This response begins at a limbic level, namely the hippocampus and hypothalamus, leading to increased GRs expression (Figure 2) (McEwen *et al.*, 2010). However, secreted cortisol is largely bound to corticosteroid-binding globulin in circulation, meaning that only about 5% of the free circulating cortisol is available to initiate negative feedback inhibition at target tissues (Johnson *et al.*, 1992). With chronic stress the relatively limited number of GRs become desensitized to GCs and may result in failure to stop the stress response (Figure 2) (Merkulov *et al.*, 2017). This

is one of the reasons that it is imperative that the GRs located in the brain remain sensitive enough to potentiate the necessary reaction.

In a stressed state, increased GC levels will cause activation of GRs located in the hypothalamic PVN and the anterior pituitary gland, directly inhibiting the continued secretion of CRH and ACTH, respectively (Charmandari *et al.*, 2005). However, GCs can indirectly inhibit the stress response as well, as they enhance the secretion of

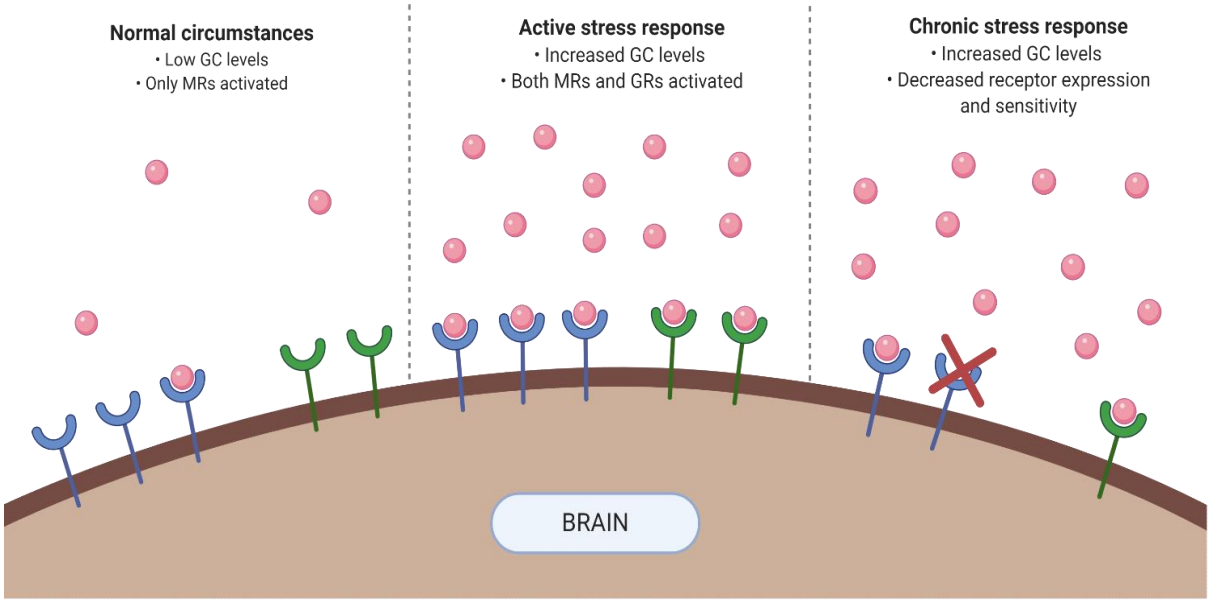


Figure 2: Mechanism of GRs/MRs under different conditions. Blue receptors represent MRs while green receptor represent GRs. GC – glucocorticoids; GR – glucocorticoid receptor (green); MR – mineralocorticoid receptor (blue). Figure made in BioRender.

neuropeptide-Y (NPY). When present in the hypothalamus NPY is a potent inhibitor of NE release in the brain (Hirsch *et al.*, 2011). The inhibition of GC release is aimed at bringing the body back to a homeostatic environment and prevent prolonged exposure to the effects of GCs, as these effects are designed to be short-lived (Merkulov *et al.*, 2017). It is important that the stress response is also shut down at the sympathetic level. As mentioned previously, there is a significant amount of crosstalk between the HPA axis and the SAM pathway. Inhibiting feedback loops therefore also exist in the noradrenergic neurons that contribute to both CRH and LC/NE inhibition (Tsigos *et al.*, 2002). This inhibition is mainly bought about by gamma-aminobutyric acid (GABA) and other opioid peptides, which are aimed at inhibiting activation of the LC/NE system (Charmandari *et al.*, 2005). Aside from the negative feedback inhibition, it is also important to consider the role that the PNS plays in the stress response. After

a stressor has passed and the body begin to return to a normal state, the PNS employs acetylcholine to aid in the return to homeostasis by acts such as decreasing the heart rate and decreasing metabolism. It also promotes the re-activation of previously inhibited systems such as digestion (Everly Jr. *et al.*, 2019).

Due to the conjoint actions of the HPA and SAM pathway, as well as the significant level of overlap and crosstalk between the systems, the overall stress response elicits a profound effect on the body as it adapts to survive (Kyrou *et al.*, 2009). Although described as a top-down process, the mediators of these systems work in a non-linear fashion to up- and downregulate each other (McEwen *et al.*, 2010). The responses discussed above are all considered as part of the normal stress response that is necessary to restore a state of homeostasis. However, such responses may become detrimental within the context of chronic stress that results in its continuous activation.

1.3 Chronic stress and allostatic load

Bruce McEwan, a pioneer in the field of stress research, redefined the concept of stress using modern jargon to divide the concept of stress into “good stress” and “bad stress (McEwen, 2006). By this distinction, “good stress” refers to the normal functioning of the body when adapting to adverse circumstances by employing the HPA axis and SAM pathways (McEwen, 2007). However, the concept of “bad stress” is centered around an individual being continually exposed to an active stress response. This is known as chronic stress and this comes at a physiological price which is referred to as “allostatic load” (McEwen *et al.*, 1993). Allostatic load refers to the wear and tear of physiological systems that results from a chronically overactive or underactive allostatic response, or the ineffective management of allostatic systems in response to a continuously stressed state (Beckie, 2012; McEwen, 2008). Although linked, allostasis and allostatic load function display the paradox of stress mediators such as cortisol and catecholamines. These mediators are necessary to successfully adapt to stress (the process of which is called allostasis). However, the exposure to these stress mediators can cause damage (allostatic load) to the systems responsible for managing allostasis (McEwen, 2017).

McEwen divided allostatic load into four types, according to the situation within which it occurs (Figure 3) (McEwen, 1998). The first type is frequent stress exposure and describes a situation where an individual is repeatedly exposed to various stressors,

leading to a normal stress response taking place too often. The second type involves repeated exposure to the same stressor, without any adaptation to the stressor, while the third type of allostatic load is centered around an inability to shut off the allostatic responses to a stressor after the stressor is no longer present. It is hypothesized that this failure to turn off the HPA and SAM pathways are the result of exhaustion of the allostatic systems (Wilkinson *et al.*, 1997). The fourth type of allostatic load describes a situation where inadequate responses by some allostatic systems cause compensatory measures in others. When the one system fails to respond appropriately, there is an increase in activity of other systems, as they are no longer being counter-regulated by the underactive system (McEwen, 1998).

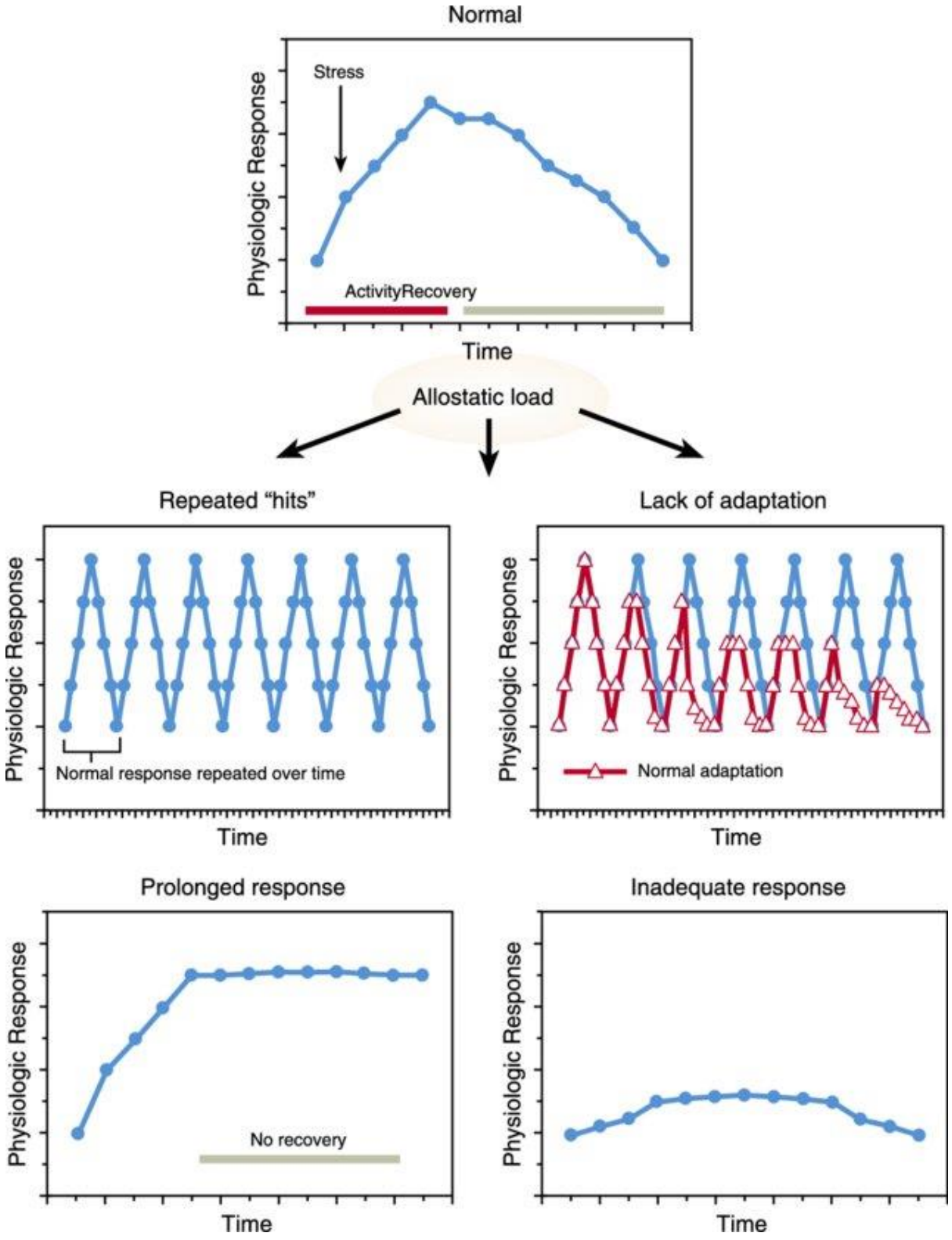


Figure 3: Four types of allostatic load (McEwen, 1998).

Aside from these specific scenarios, allostatic load can also result due to other factors. Here feelings of anticipation and worry can also contribute to the wear and tear of the allostatic systems (Schulkin *et al.*, 1994). Such feelings prepare an individual for a

threat and can drive the secretion of stress mediators such as epinephrine and cortisol and are therefore likely to contribute to allostatic load (Schulkin *et al.*, 1994).

Allostatic load and the implications thereof can be clearly described within the context of cardiovascular and metabolic diseases. Increased job strain that encompass a lack of control in working circumstances and high psychosocial demands can result in elevated blood pressure (at home) and increased atherosclerosis progression (Everson *et al.*, 1997; Schnall *et al.*, 1992). Such factors are thus ultimately brought about by chronic psychosocial stress and can subsequently cause the onset of coronary artery disease (Yao *et al.*, 2019). Chronic stress situations that encompass feelings of fatigue, irritability and demoralization are also associated with increased activation of platelets and the fibrinogen system that can both contribute to myocardial infarctions (Markowe *et al.*, 1985; Räikkönen *et al.*, 1996).

The high concentrations of cortisol receptors located in the brain means that it is extensively affected (specifically the hippocampus) by chronic stress (McEwen *et al.*, 1986). As discussed in an earlier section, the hippocampus uses memory retrieval to provide context for stressful situations that have an emotional bias (Ford *et al.*, 2015). Glucocorticoids are heavily involved in this process and therefore the stress-mediated impairment of the hippocampal region can decrease the accuracy and reliability of contextual memories (McEwen, 1998). In simpler terms, the chronic stress-induced allostatic load on the hippocampus can impair the brain's ability to access information that is required to classify a situation as non-threatening (Sapolsky, 1990). Stress-induced hippocampal dysfunction is initiated during acute stress, as this response increases the presence of GCs and results in the suppression of short-term hippocampal functions (Kirschbaum *et al.*, 1996; McEwen *et al.*, 1995). Repeated stress can also cause atrophy of dendrites in the hippocampus, which is reversible if the exposure is short-lived. However, stress that persists for months to years can cause permanent destruction of these neurons, resulting in damage that has been associated with recurrent depressive illness, post-traumatic stress disorder and Cushing's disease (McEwen *et al.*, 1997; Sapolsky *et al.*, 1996).

The implications of chronic stress and allostatic load are linked to metabolic disorders as well. For example, the Whitehall studies examined the relationship between stress in the British civil service workplace and the prevalence of the metabolic syndrome

(Chandola *et al.*, 2006). These findings showed a positive correlation, as participants with a greater exposure to job stress over 14 years were linked to increased onset of the metabolic syndrome. The same study also reported increased abdominal obesity (an important contributor to metabolic syndrome onset) at the lowest civil service grades (Chandola *et al.*, 2006). Hypertension was also shown to be a useful index of job stress by McEwen (1998), who reported a higher prevalence in factory workers with time pressured and repetitive job actions. However, these health problems go beyond industry-related findings as increased morbidity and mortality were reported in societies where instability and conflict were common factors (McEwen, 1998). For example, cardiovascular disease (CVD) was reported as a major contributor to the 40% increase in death rate in Russian men following the fall of Communism (Bobak *et al.*, 1996).

To summarize, the effects of the stress response can be divided into changes brought about on a scale of seconds to minutes. Here the SAM pathway acts as the first immediate response, with the HPA following shortly with hormonal effectors (Murison, 2016). These physiological effects prepare the body for a rapid response to the threat and include the following: a) increasing cardiovascular tone (e.g. increased heart rate and blood pressure) for rapid fuel substrate delivery to target organs, b) increased cognitive awareness and state of readiness, c) mobilization of stored energy and inhibition of energy storage, d) stimulation of specific immune functions, and e) inhibition of unnecessary functions such as digestion and reproduction (Sapolsky *et al.*, 2000).

Responding to such stressful experiences can lead to growth and adaptation as the body learns resilience for similar future circumstances. However, continuous exposure to such chronically stressful experiences can lead to the exhaustion of body systems responsible for maintaining homeostasis, both physiologically and psychologically (McEwen *et al.*, 2010). The exposure to chronic stress has far-reaching consequences, impacting body systems such as the cardiovascular, metabolic and neural systems (McEwen, 1998).

1.4 Rodent models used for chronic stress

Stress research has evolved beyond the understanding of basic physiological mechanisms. As eloquently stated by McEwen & Stellar, “To begin to understand

mechanisms involved in the interactive effects of acute and chronic stress on health, a multilevel, interdisciplinary approach must be used" (McEwen *et al.*, 1993). This statement was supported by Oken, Chamine & Wakeland (2015), who advocated for approaching the topic of stress research from a systems science perspective, as this would help develop a deeper understanding of the physiology and psychology of stress. In light of this, several animal models of chronic stress have been established to more accurately study complex disease states caused by chronic stress such depression, anxiety disorder, and post-traumatic stress disorder (PTSD). These disorders are frequently the target of stress research, as stress-related dysfunction of the limbic system is a key trigger for the development of such psychiatric conditions (Jaggi *et al.*, 2011; Jankord *et al.*, 2008).

The theoretical motivation behind animal models is that the model needs to reproduce all features of the illness that is being investigated (Campos *et al.*, 2013). Unfortunately, this is rarely achieved in stress research as researchers are not only tasked with recreating the complexities of human psychiatric disorders, but also the complexity of the stress response (Patchev *et al.*, 2006). This is further complicated by physiological differences that exist between humans and laboratory animals (Salgado *et al.*, 2013). Therefore, when establishing an animal model of chronic stress the focus is not necessarily to perfectly simulate pathologies, but instead attempt to establish a state of anxiety or depression that is related to such disorders (Lister, 1990). This can be achieved in various ways and hence leading to the establishment of a number of different models, with significant variations (Campos *et al.*, 2013). The latter include factors such as specific animal species used, stressor types, stress protocol length, and the psychiatric condition that models aim to establish. Here a review of the most widely used animal stress models and variations was recently published by our research team (Sher *et al.*, 2020).

Two major goals of animal models are a) to study symptoms and underlying causes of stress-induced diseases, and b) to assess therapeutic interventions that target such complications (Chadman *et al.*, 2009). The models developed also depend on whether physiological or psychological stress is examined (Campos *et al.*, 2013). Although both types of stressors are suitable to acute and chronic stress models, psychological stressors offer a distinct ethical advantage as there is less physical harm involved (Jaggi *et al.*, 2011). The overlapping use of both physical and psychological stressors

offers promise as it prevents habituation of the animal to the various stressors and allows for better translation to the human context (Sher *et al.*, 2020).

Physical stressors often target the temperature control of the rodent, as evidenced in stressors that involve immersion in cold water and cold environment isolation (Jaggi *et al.*, 2011). Other commonly used stressors include physical restraint, electric foot shock-induced stress, and forced swimming stress (Campos *et al.*, 2013). Psychological stressors are included in models such as social defeat, maternal separation, circadian rhythm disruption and predator threats (Chiba *et al.*, 2012; Frisbee *et al.*, 2015; Lezak *et al.*, 2017). Thus, different models use a variety of the aforementioned stressors to induce a state of stress in animals. However, this review will focus on chronic stress models and consider their validation in a critical fashion.

The selection of a stress model is a crucial aspect of any study and hence certain factors need to be considered before an appropriate and informed decision can be made. In order to be classified as an accurate and trustworthy model the model needs to possess face validity, construct validity and predictive validity (Willner, 1984).

- Face validity is defined as the analogy between the symptoms of psychiatric disorders in humans and the behaviors exhibited by the experimental rodents (Bhat *et al.*, 2014). It can also be described as “phenomenological similarity” (Steimer, 2011). The face validity of a stress model is therefore a measure of how effectively the model replicates the core symptoms and characteristics of depression and anxiety (Willner *et al.*, 2002).
- Construct validity refers to the cause of the disease and requires analogy between human and animal regarding the etiology and biochemistry thereof, as well as symptomology and treatment (Bhat *et al.*, 2014; Chadman *et al.*, 2009). This is also described as “theoretical rationale” (Steimer, 2011). The measurement of this validity requires an examination of not only the superficial pathology of the disease, but also the underlying physiological mechanisms (Akiskal, 1986).
- Predictive validity revolves around ensuring that the performance in the test/model will predict the condition it models (Steimer, 2011). It includes the ability of an animal model to elicit the same effects each time it is employed, and the capacity of a successful treatment to also be effective in humans (Bhat

et al., 2014; Chadman *et al.*, 2009; Hogg, 1996). A model with excellent predictive validity therefore needs to be conscious of recognizing true positives and negatives, but also be aware of false results in this regard. While no model has 100% predictive validity, the aim is to develop one that gets as close as possible (Bhat *et al.*, 2014).

As mentioned before, there are no “perfect models” due the complexity of stress and related effects, and thus each of the discussed models present with their own unique challenges. Some models are more effective at replicating a state of anxiety and depression in rodents and frequently used ones include chronic mild stress (CMS)/unpredictable chronic mild stress (UCMS) (Willner *et al.*, 1987), chronic restraint stress (CRS) (Chiba *et al.*, 2012), maternal separation stress (MS) (Nylander *et al.*, 2013), learned helplessness (LH) (Seligman *et al.*, 1975) and social defeat stress (Kabbaj *et al.*, 2001). As the focus of the study is centered on the UCMS model, this will be discussed in more detail while the other models will only be briefly discussed.

Frequently used rodent models of chronic stress

The CRS model is based on the well-understood concept that submitting rodents to constant restraint elicits a depressive effect (Wang *et al.*, 2017). Using restraint as a stressor is not limited to the CRS model as short periods of restraint can form part of CMS protocols (30 minutes to four hours), while the restraint periods are longer in a CRS model (more than six hours) (Chiba *et al.*, 2012; He *et al.*, 2020; Jaggi *et al.*, 2011). Such periods of restraint can also vary depending on which conditions are being simulated. To better recreate predictable chronic stress the restraint sessions are usually longer than two hours for a period of 14 to 21 days (Wang *et al.*, 2017). The majority of results reveal not only increased corticosterone levels in the stressed rats, but also shows depressed behavior and aggression (Wood *et al.*, 2003). The model is therefore considered as a strong rodent model of stress, as the changes observed are not only behavioral but also include the genetic and protein changes observed in patients burdened with depression (Wang *et al.*, 2017).

The social defeat stress model is another one that is effective at simulating depression in rodents (Meerlo *et al.*, 1996). As humans and rodents are both inherently social beings, social stress is a significant factor involved in the psychopathology of various

depressive disorders (Agid *et al.*, 2000; Huhman, 2006). In this model, a test rodent is placed into the cage of an older, aggressive and dominant rodent in an attempt to induce an attack on the “intruder” and leading to its social defeat (Wang *et al.*, 2017). Following the threat/attack, the defeated rodent is separated from the dominant rodent with a barrier, after which the test rodent is subjected to the same procedure several times with different dominant rodents. The effect of this protocol on test rodents includes signs of anhedonia, anxiety, defensive behaviors, and changes in food intake (Meerlo *et al.*, 1996).

Linking closely to the social defeat model is the LH model of stress, characterized by the rodent’s state of “helplessness” following periods of inescapable and uncontrollable electric shock stress (Krishnan *et al.*, 2011). When faced with a similar stressor, but with an added escape route, the rodent would fail to escape or show a marked delay in escaping the stressor (Seligman *et al.*, 1975). Although the physiological effects of this model include altered HPA activity, disrupted circadian patterns and weight loss, these effects can be reversed by antidepressants (Cryan *et al.*, 2004; Henn *et al.*, 2005). Learned helplessness can be induced within a day or several days of repeated stress exposure, revealing the acute and chronic stress utility of this model. Unfortunately, this model has a large degree of variability in whether or not the state of helplessness is developed, as studies reported that 10-80% of rodents simply fail to exhibit such escape deficits (Krishnan *et al.*, 2011). This model is validated by analyzing their escape behavior, for example their hesitance to press a lever or to cross through a door (Yan *et al.*, 2010).

Although the majority of existing stress models involve the use of adult rodents, the MS model examines the effects of early life stress on the development of psychiatric disorders (Wang *et al.*, 2020). Here the principle is that the stressors experienced during the development phase of a child’s life can cause the development of depression or psychosis later-on in life (Kendler *et al.*, 2002; Morgan *et al.*, 2007). The procedure involves separating mother from their pups during the postnatal period. However, the length of separation can vary between laboratories and range from hours to days (Wang *et al.*, 2017). The model causes a complete break in crucial mother-pup interaction and the effects of the stress are then measured and observed in the pup’s later life stages (Jaggi *et al.*, 2011). Studies show that its use leads to an impaired HPA response as well as altered habituation and inhibited exploratory

behaviors in the pups (Jaggi *et al.*, 2011). It also causes memory and learning deficits that are (in part) caused by decreased neurotrophins and increased functioning of stress-processing pathways in the amygdala (Planchez *et al.*, 2019). The maternal separation models are often validated by tests such as elevated plus maze (EPM) tests and open field tests (OFTs) (Wang *et al.*, 2020).

Unfortunately, the majority of chronic stress models possess inherent disadvantages that diminish their use as these problems diminish their efficacy and translational capabilities (Frisbee *et al.*, 2015). However, the CMS/UCMS model has been identified as one of the most translationally-relevant models for studying the varying effects of depression and anxiety in rodents (He *et al.*, 2020; Wiborg, 2013).

UCMS model

A stress model focused on chronic exposure to mild stressors was first developed by Katz (1982), but firmly established as a rodent model of chronic stress-induced depression by Paul Willner and his team (Willner *et al.*, 1987). The model is considered by many to be the most validated models of depression and reportedly has excellent face, construct and predictive validities (Campos *et al.*, 2013; O'Leary *et al.*, 2013; Papp, 2012; Pucilowski *et al.*, 1993; Tian *et al.*, 2013). The model has since been used to emulate the effects of long-term exposure to mild human stressors such as job insecurity and dissatisfaction, political unrest, deteriorating relationships and other socio-economic influences known to cause depression (Frisbee *et al.*, 2015; Golbidi *et al.*, 2015). The name of the model can be quite confusing: CMS (Katz, 1982), UCMS (Pothion *et al.*, 2004), chronic unpredictable mild stress (CUMS) (Willner *et al.*, 1987), chronic unpredictable stress (CUS) (Cox *et al.*, 2011) and chronic variable stress (CVS) (Ostrander *et al.*, 2006) are all terms used the describe a similar chronic stress model that incorporates mild or unpredictable stressors, or both. Changes between protocols are often miniscule, however these changes have recently been thoroughly reviewed (Willner, 2017a).

The UCMS model exposes rodents (most commonly rats) to a variety of mild physical and psychological stressors in an unpredictable fashion for an extended time period (Jaggi *et al.*, 2011; Willner, 1997). The theoretical motivation behind the model is that the procedure will induce a state of chronic stress and trigger the onset on anhedonia (described in full in the next section) (Scheggi *et al.*, 2018). In short, anhedonia can

be defined as the unresponsiveness to pleasurable events or activities and is a defining characteristic of depression (Willner, 1997). The link between chronic stress and depression stemmed from the observation that rodents were less inclined to increase their fluid consumption of a sucrose or saccharine solution following exposure to a chronic stress regime (Katz, 1982). In support, disrupted reward pathways in mice following exposure to uncontrollable foot-shocks could be reversed with anti-depressants (Zacharko *et al.*, 1991). The UCMS model was designed to model these depressive effects and hence exposes rodents to daily stressors such as cage tilting, predator scents and sounds, damp bedding, removal of bedding, disruption of the light/dark cycle, paired housing, exposure to reduced temperatures, water-filled cages, stroboscopic light, white noise and food and water deprivation (Mineur *et al.*, 2006; Pucilowski *et al.*, 1993; Willner *et al.*, 1987). The stress regime is continued for several weeks and the effects thereof evaluated throughout and also validated at the end of the experiment by using several tests (discussed in the next section) (Willner, 1997).

Despite the benefits of the CMS concept, the model retains a disadvantage which is the habituation to, or development of resistance, against the stressors employed (Jaggi *et al.*, 2011). Upon exposure to chronic stress, the HPA axis can undergo stabilization or desensitization and seemingly inhibit the negative feedback regulating the stress response (Franco *et al.*, 2016). Others confirmed this finding by showing that rodents exhibited a habituated corticosterone response during acute restraint stress following exposure to consistent handling, restraint and crowding stress (Gadek-Michalska *et al.*, 2003; Magarinos *et al.*, 1995). In light of these adaptability concerns, the UCMS model was developed and relies on the unpredictable nature of its stress protocol to overcome the habituation phenomenon. The protocol involves the same stressors used in the CMS model but presents them in a pre-determined, randomized fashion (Campos *et al.*, 2013). While some protocols call for a single stressor to take place at different times each day, others are characterized by rodents being exposed to two or more stressors in a single day (Bekris *et al.*, 2005; Jaggi *et al.*, 2011).

Aside from inducing anhedonia, the UCMS model triggers other behavioral abnormalities that are often difficult to quantify, such as reduced grooming habits and changes in sexual and aggressive behaviors (Krishnan *et al.*, 2011). Moreover, such rodents also display obvious signs of anxiety, impaired movement, slow responses to

stimuli, and decreased memory and learning abilities (He *et al.*, 2020). Such findings therefore extended the use of the UCMS model beyond merely a chronic stress model for depression, but also validates it as a model for anxiety (Lezak *et al.*, 2017).

1.5 Validation of rodent models used for chronic stress

Animal models of stress and anxiety are centered around replicating psychological symptoms in laboratory animals. When measuring the extent to which an experimental stress regime successfully replicated the effect of chronic stress, the behavioral changes in the animals need to be regarded as highly as the physiological mechanisms. There is a plethora of behavioral tests that can be employed to measure the extent to which the chronic stress affected the rodent and whether the desired state is being recreated. Depending on the animal and the type of model used, different tests can be applied under different circumstances, however there are specific behaviors in rodents that are inextricably linked to an increased state of stress.

Daily behavioral monitoring is required by almost all ethical and regulatory bodies in animal research, however many of the standard behaviors observed can provide an insight into the mental state of the rodent. Many of the same behaviors that are seen in human anxiety disorder can be observed in rodents, such as avoidance, hypervigilance, non-verbal vocalization and escape behaviors (Rodgers *et al.*, 1997b). Rodents are also capable of displaying a wide variety of defensive actions when faced with threats, which include freezing, defensive attack, decreased investigative behaviors and even death-feigning in some cases (Rodgers *et al.*, 1997b).

Behavioral monitoring also takes into account the absence of normal behaviors. In studies that use a grooming score as a measure of stress, specific areas of the rodent's coat (head, neck, belly, back, tail paws) are assessed to determine grooming behaviors (Yan *et al.*, 2010). It is expected that exposure to chronic stress regimes cause a decrease in fur state caused by deteriorated grooming behaviors (Mineur *et al.*, 2006). A variation of this is known as a sucrose splash test during which a rodent is splashed with a sucrose solution, after which researchers observe the grooming behaviors induced by this (Planchez *et al.*, 2019). In humans, eating disorders are often linked to depression and therefore the monitoring of food intake is included in the validation process to account for disturbances in eating behaviors (Planchez *et al.*, 2019). Aside from forming part of standard requirements for the care of laboratory

animals, body weight measurements are also done to determine the rate of weight loss during stress regimes (Hasler *et al.*, 2004).

Measures of depression and anxiety in rodents

Specific evaluations and tests have been developed to assess mental health in rodents, each designed to examine a particular area of behavioral interest. The validation methods that were used in this experimental study will be discussed and evaluated in full, with brief descriptions of additional validation methods commonly used in similar contexts. These tests are usually done in addition to assessing general behavioral changes and can aid in determining the validity of the specific stress model employed (Table 1) (Chadman *et al.*, 2009). As the UCMS model is centered around replicating aspects of mental disorders such as depression or anxiety disorder, it is frequently validated by measuring the levels of anxiety-like behavior and anhedonia in rodents (Willner, 1997).

According to Lister (1990), anxiety can be divided into trait and state anxiety. State anxiety is seen as the anxious feeling experienced by a subject at a specific moment, acutely caused by an anxiogenic stimulus. By contrast, trait anxiety describes a chronic feature of a subject which does not vary between different time points (Lister, 1990). It is difficult to accurately reproduce anxiety in an animal model and therefore the methods of validation are centered around evaluating how well the model recreated features of trait anxiety (Campos *et al.*, 2013). These tests are designed to create an approach-avoid conflict in rodents and focus on mazes, novel environments and novel objects (Campos *et al.*, 2013). The test used most often to assess anxiety is the EPM test and will be discussed at length in later paragraphs.

Another major sign of mental health frequently tested is anhedonia, which is considered to be one of the main psychiatric symptoms of various mental disorders, particularly common in individuals suffering from major depressive disorder (Hoffman, 2016). From a psychological viewpoint, anhedonia can be described as a decreased ability to experience pleasure (Scheggi *et al.*, 2018). It is a common practice in stress studies to evaluate an organism's response to rewarding activities in order to determine the extent of pleasure derived. For example, some found that rats exposed to a period of chronic stress exhibit lowered pleasure-seeking behavior (Matthews *et al.*, 1995). The evaluation of anhedonia is thus a central feature of most animal-based

stress studies and in this instance the sucrose preference test (SPT) is the most commonly used method for its determination (Table 1) (Willner, 2017a).

Table 1: Summary of widely used chronic stress rodent models, focusing on protocols, validation methods and the success of the validation methods

Study	STRESS MODEL & SPECIES	STUDY RATIONALE	BRIEF PROTOCOL	BEHAVIORAL VALIDATION	BIOLOGICAL VALIDATION	VALIDATION RESULTS
(José Jaime *et al.*, 2016)	UCMS Young male Wistar rats	Young rats exposed to chronic stress that do not develop anhedonia, show other behavioral alterations that suggest depression or anxiety.	1. 4 weeks of CMS. 2. Body weight and sucrose preference measured. 3. Exposed to behavioral testing.	• EPM • SPT • Defensive burying • Forced swimming	• Serum corticosterone	• No signs of anhedonia • Increased open arm entries by stress group • Cumulative burying behavior increased in stressed group • No difference in forced swimming immobility • No difference in serum corticosterone measurements
(Ducottet *et al.*, 2005)	UCMS BALB/c ByJ & C57BL/6 J mice	Studied coping mechanisms of mice subjected to a stress regimen and how they relate to emotional reactivity	1. Separated animals into high and low emotionality states based on initial EPM and free exploration tests. 2. Half of each mouse strain subjected to 2-week UCMS protocol.	• Holeboard tests • EPM • Free exploratory paradigm *Stress tests:* • Vanilla pasta test • Physical test (piloerection/ dirty coat)	• None	• High emotionality group showed fewer entries into EPM open arms • Exploration decreased in high emotionality group • Stressed mice ate less vanilla pasta • Stressed mice showed increased

			3. Subjected to various behavioral tests.	• Grooming test • Conflict situation • Light/dark box test		physical state, and decreased time spent grooming • Stressed group less inclined to enter dark box
(Henningsen *et al.*, 2012)	CMS Male Wistar rats	Used CMS protocol to identify stress-susceptible/resilient rats and used proteomics to determine neural proteins associated with each state.	1. Rats grouped according to baseline sucrose preference. 2. Experimental group exposed to 8 weeks of CMS regime.	• SPT	• Proteomic analysis • Immunohistochemistry	• Significantly decreased sucrose consumption following CMS • 30% of rats decreased in sucrose preference → anhedonic-like • 23% of rats did not decrease sucrose preference → resilient
(Willner *et al.*, 2019)	CMS Male Wistar & WKY rats 4-5 weeks	Tested the effectiveness of three antidepressant drugs, using a chronic stress model, validated by behavioral testing.	1. Tested Wistar and WYK rats separately. 2. Exposed rats to CMS regime with weekly SPT and tested the ability of the drugs to reverse effects of CMS.	• SPT • EPM • NORT	• None	• WYK made less entries into open arms and spent less time in open arms compared to Wistar rats. • WKY spent less time exploring novel objects • WKY classified as more 'emotional' strain.

(Seewoo *et al.*, 2020)	CRS Male Sprague Dawley rats	Tested the validity of CRS in the study of depression.	1. Rats were individually restrained in transparent plastic tubes for 2.5 hours daily over a period of 13 days. 2. Exposed to different behavioral tests.	• EPM • FST	• Resting-state functional magnetic resonance imaging (rs-fMRI).	• EPM test confirmed presence of anxiety-like behaviors • FST confirmed depression-like symptoms • rs-fMRI showed hypoconnectivity between several large brain regions • Reported decreased hippocampal volume
(Tian *et al.*, 2013)	UCMS Male Sprague Dawley rats	Investigate anti-depressant mechanisms and effects of genipin on a chronic stress model.	1. Rats divided into a control group, a UCMS + vehicle group, and five UCMS + test compound groups. 2. UCMS procedures conducted for four weeks, drug administered two hours before stressors during the last two weeks. 3. Rats exposed to behavioral testing.	• SPT • OFT	• Nuclear magnetic resonance-based metabolomics	• Body weight decreased in stress group, however increased after drug administration. • Stressed rats showed decreased sucrose consumption at the end of third and fourth weeks • Stressed rats showed decreased locomotor activity in OFT after four weeks of stress exposure,

						however the results were reversed by drug administration. • Study showed UCMS was a reliant model and metabolomics a valid approach for studying depression.
(Meerlo *et al.*, 1996)	Social defeat Tyron Maze Dull S3 rats 4 months old	Examine the effects of single and double social defeat stress on rats and their subsequent social behavior.	1. Experimental rats were exposed to either a single, or double session of social defeat. 2. Social defeat was created by placing animals in the home cage of an aggressive rat. 3. Behavioral tests were performed two days after stress.	• OFT • SPT • Social behavior	• None	• OFT showed that decreased locomotion significantly; no change between single/double defeat. • No significant SPT results. • Defeated rats showed less subsequent contact with unfamiliar rats.
(Marais *et al.*, 2008)	MS & CRS Male Sprague Dawley rats	Used a MS model to examine the effects of early life stress on stress hormones, neurotrophins, behavior and antioxidant potential.	1. Pups were separated form mothers for three hours daily from days 2-14. 2. The same rats were chronically restrained for four hours daily in adulthood for a period of 5 days.	• FST	*HPA activity:* • Plasma corticosterone /ACTH • Hypothalamic CRH	• MS and restrained rats had significantly increased immobility in FST compared to control group. • Increased CRF in MS rats. • Increased corticosterone

						levels in MS rats.
(Yang *et al.*, 2019b)	LH Male C57BL/6 mice 3 months old	Established a LH model to study the protective anti-depressive effects of curculigoside (CUR) and associated mechanisms.	1. Mice were injected with CUR and exposed to a fear memory and extinction protocol, after which they were exposed to behavioral tests. 2. The LH paradigm was created using the fear conditioning box, and by using inescapable foot-shocks.	• TST • FST • Morris water maze	• Multiple	• LH induced depressive states, such as prolonged immobility in FST and TST. • Higher-dose CUR aided in fear extinction, and improved fear-associated depressive behaviors.

ACTH, adrenocorticotropic hormone; CMS, chronic mild stress; CRF, corticotropin-releasing factor; CRS, chronic restraint stress; EPM, elevated plus maze; FST, forced swimming test; LH, learned helplessness; MS, maternal separation; NORT, novel object recognition test; OFT, open field test; SPT, sucrose preference test; TST, tail suspension test; UCMS, unpredictable chronic mild stress; WYK, Wistar-Kyoto

Sucrose Preference Test

a. Basic concept

The SPT developed for rodents is centered around their instinctive preference for sweet-tasting things (Scheggi *et al.*, 2018). The test measures the amount of sweet-tasting solution that is consumed over a fixed time period compared to water consumption during the same time. Considering that no active measures or motor activities are required, the test reflects the rodent's capacity to experience pleasure and also how rewarding stimuli are affected in response to specific experimental procedures (Hoffman, 2016). There are two options when deciding upon a protocol for the SPT, namely a one- or two-bottle system. The two-bottle testing system is typically used by researchers, with one containing a sucrose/saccharin solution and the other plain tap water (Alkhlaif *et al.*, 2017; Brenes Sáenz *et al.*, 2006; D'Aquila *et al.*, 1997; Katz, 1982; Strekalova *et al.*, 2004; Willner *et al.*, 1987). The rodent has a free choice and following the designated time period the intake of each bottle is recorded, either by weight or volume. By contrast, some methods employ a one-bottle testing system, limiting the rodent choice, however this method is less effective as it does not take into account any potential effects that the experimental procedure has on total fluid intake (Hoffman, 2016; Willner *et al.*, 1987). The preference of the animal is then calculated using the following formula:

$$Preference = \frac{sucrose\ consumption}{sucrose\ consumption\ \times water\ consumption}$$

Equation 1: Formula used to determine individual sucrose preference (Willner *et al.*, 1987).

b. Habituation

Animals are habituated to the sucrose solution prior to testing and trained to drink the solution. Although the methods used can vary from an overnight introduction to two-week long periods of habituation, the majority follow a three-day protocol (Juczewski *et al.*, 2020; Kumar *et al.*, 2011; Scotton *et al.*, 2019). Here, rats are exposed to two bottles of pure water for 24 hours, then 24 hours exposure to two bottles of the sucrose

solution to be used in the SPT, and finally 24 hours simultaneous exposure to one bottle of water and one bottle of sucrose solution (He *et al.*, 2020).

c. Food/water starvation

Rats are deprived of food and/or water for a period of time prior to the start of the SPT in order to motivate them to drink. However, there is much debate surrounding the duration of such periods. The first study to expose rats to a chronic stress model and that subsequently tested their sucrose preference only deprived the rats of food and water for 4 hours (Willner *et al.*, 1987). However, more recent studies starved the rats for significantly longer periods of time for example a review of the 91 published articles over the last three years found that 76.9% of published SPT protocols included a starvation period of 24 hours, 12.1% used a 12-hour starvation protocol, and 4.4% starved rats for 48 hours (He *et al.*, 2020). The remainder of studies starved their rats for a period of between 15 and 40 hours, as reported in the review performed by He *et al.* (2020). By contrast, others did not apply any period of food or water deprivation in this case by arguing that the acute stress and metabolic influences of starvation may elicit adverse effects on data generated (Sequeira-Cordero *et al.*, 2019; Strekalova *et al.*, 2004).

d. Test duration

SPT protocols often differ in the duration of sucrose exposure and this may be a cause for variation in experimental results. For example, Willner *et al.* (1987) set the test duration at one hour - that is in agreement with a comprehensive review done by He *et al.* (2020) where they found that the majority of SPT protocols employed the one hour test duration (after starving rats for 24 hours). However, following such a lengthy period of starvation many rats are in a desperate state of thirst and would drink whichever solution they found first, not distinguishing between pure water and sucrose. Under normal physiological conditions, rats consume between 9-12 mL of water per 100 grams of body weight, meaning that one hour may be too short to correct for the initial increase in fluid consumption and therefore accurately reflect actual sucrose preference (Claassen, 1994; Strekalova *et al.*, 2004). Longer SPT testing periods are therefore increasingly employed by researchers, ranging between 12 and 48 hours (Brenes Sáenz *et al.*, 2006; Matthews *et al.*, 1995; Pothion *et al.*, 2004;

Sequeira-Cordero *et al.*, 2019; Strekalova *et al.*, 2004). Tests with a longer duration are considered to be more accurate as they better reflect the mental state of the rodent (He *et al.*, 2020)

It is important to note the time of day during which starvation and testing takes place as this may influence data generated. He *et al.* (2020) hence aimed to determine the best time of day to perform a SPT by starving and testing during both the light and dark phases of the rat's circadian cycle. Here, they showed that 12 hours of food deprivation during the light phase did not elicit any effect on body weights, but a similar protocol during the dark phase resulted in significant weight-loss. Similar results were obtained when examining testing times and they concluded that sucrose consumption measurements during the dark phase would be the most indicative mood indicator. The explanation for both observations is that rats are distinctly nocturnal animals; as they are more active during the dark phase, they are more likely to be consuming food and water during this time (He *et al.*, 2020). This finding is supported by Hoffman (2016) who stated that the SPT starting time should typically be close to the beginning of the dark phase.

e. Concentration of sucrose solution

There have been variations regarding sucrose concentrations used in SPTs with no conclusive "correct" concentration. Some examined the effects of chronic stress on rodents and verified it using various sucrose concentrations that were introduced in a random pattern (Matthews *et al.*, 1995). These data revealed that the rats did not display any particular preference for a specific sucrose concentration (Matthews *et al.*, 1995). However, others found that rodents exhibit a sucrose preference that falls within an inverted U-shaped curve, meaning that the optimal sucrose uptake occurs in an intermediate concentration range (Willner, 1997). However, despite such important findings the majority of SPTs are conducted using a 1-2% sucrose solution (Alkhlaif *et al.*, 2017; Bekris *et al.*, 2005; Juczewski *et al.*, 2020; Kumar *et al.*, 2011; Strekalova *et al.*, 2011), with only select studies using a more concentrated solution (37% sucrose) (Brenes *et al.*, 2008; Brenes Sáenz *et al.*, 2006). Those adopting the lower sucrose concentrations argue that a 1% solution is more sensitive to the influence of the stress protocol and not by the caloric content of the solution, which is a concern when using more concentrated solutions (Scheggi *et al.*, 2018). Here, a higher caloric content

(more concentrated solutions) may lead to some metabolic motivation behind its increased consumption and therefore cannot be used as a reliable measure of anhedonia (Scheggi *et al.*, 2018). The survey done by Willner (2017b) supports this idea as his findings showed that the use of a more concentrated sucrose solution tends to be associated with less reliable results. In line with this, it is recommended that a sucrose solution should not exceed 2% (Papp, 2012).

Although SPTs formed part of depression studies since the early 1980s (Katz, 1982), it was not until 1994 that researchers considered the effects of body weight changes on sucrose consumption (Matthews *et al.*, 1995). Here, chronic stress experimental regiments elicit detrimental effects on the normal growth rate of the rodent and the subsequent weight loss would in turn reduce its caloric needs (Scheggi *et al.*, 2018). Therefore, it is crucial to measure the amount of sucrose consumed per gram of body weight.

Elevated Plus Maze (EPM)

a. Basic concept

The EPM is aimed at measuring unconditioned anxiety in rodents. The idea to use a maze as a measure of anxiolytic behavior takes an ethological approach, and was originally developed by Montgomery (1955). It was generally recognized at the time that rodents naturally explored novel stimuli, but also that the same novelty could inspire fear and anxiety (Weiss *et al.*, 1998). Montgomery's research aimed to investigate whether rodent exposure to a novel environment would elicit a fear response as well as a desire to explore. His maze design consisted of a Y-shape, which included an open (exposed) portion, and an enclosed portion. In the original test, it was speculated that the rats' preference for the closed arm stemmed from an increased fear response evoked by the open arm (Montgomery, 1955). Based on this research, another research team designed an X-shaped maze in 1984, with two open and two closed arms, elevated at least 50 cm off the floor (Handley *et al.*, 1984). The study not only successfully proved that anxiolytic drugs would increase the exploration of the open arms but was also the origin of what is known today as the 'elevated plus maze'. The EPM test has since been used to screen various anxiolytic drugs, however its use has extended to the understanding of anxiety disorders such as depression

and post-traumatic stress (Bannerman *et al.*, 2004; Carobrez *et al.*, 2005; Rasmussen *et al.*, 2001).

b. Testing procedure

The aim of the EPM test is to observe and quantify rodent behavioral responses (Rodgers *et al.*, 1997b). In short, the rodent is placed on an elevated maze with no roof and four arms of equal length: two open arms and two closed arms (enclosed by solid walls on the outside edges). These arms are cross each other in the middle to create a plus (+) shape (Handley *et al.*, 1984). The test is initiated by placing the rodent in the center of the two arms, facing either an open or enclosed arm (Handley *et al.*, 1984). However, the majority of studies have the rodents facing an open arm as first stated by Pellow, Chopin, File & Briley (1985). While there are a plethora of observations that can be made during the test, the most important factor to record is the time spent in, and entries into the open and closed arms (Handley *et al.*, 1984; Pellow *et al.*, 1985). An approach-avoid conflict is created in the rodent and the test aims to determine to what extent the fear of the open arm overrules the natural exploration desire of the rodent (Ohl, 2003). Increased avoidance of the open arms is indicative of anxiety (Rodgers *et al.*, 1997b). The amount of time spent in open arms is expressed as a percentage of total time spent in arms, as well as the number of entries into open arms expressed as a percentage of the total number of entries into both arms, are therefore the most important calculations needed to determine the test results (Handley *et al.*, 1984; Montgomery, 1955; Pellow *et al.*, 1985).

The earliest testing protocols spanned for 10 minutes and not the 5 minutes that has since become standard for contemporary studies (Handley *et al.*, 1984; Montgomery, 1955). The test duration was shortened to five minutes in 1985, as it was observed that avoidance behavior was particularly increased until the 5 minute mark, but decreased toward the end of a 10 minute session (Pellow *et al.*, 1985).

The EPM test was first suggested to have ethological potential in 1990 by Richard Lister, due to the test's inherent involvement of spontaneous exploration by the rodent, with no external reward motivation (Lister, 1990). However, to make the EPM a truly ethological model would require not only observations of the arm preference, but also a full behavioral analysis of the subject (Carobrez *et al.*, 2005). Therefore, many other

ethological parameters are commonly observed which could be more reflective of anxiety and motor activity and provide better insight into the affective state of the animal (Cruz *et al.*, 1994; Schneider *et al.*, 2011) (Table 2).

Table 2: Ethological parameters frequently observed during EPM test

Ethological parameter	**References**
Displacement activity *Grooming, gnawing, chewing, etc.*	(Cruz *et al.*, 1994; Pellow *et al.*, 1985; Sandbak *et al.*, 2001)
Freezing *Absolute stillness, no whisker movement*	(Pellow *et al.*, 1985)
Immobility *Stillness with whisker movement*	(Pellow *et al.*, 1985)
Defecation *Production of faecal boli*	(Pellow *et al.*, 1985)
Head dipping/Scanning *Dropping the head over maze edges*	(Cruz *et al.*, 1994; File *et al.*, 1975; Schneider *et al.*, 2011)
Rearing *Lifting forepaws off the ground to stand only on hind paws*	(Cruz *et al.*, 1994; Schneider *et al.*, 2011)
Risk assessment *Scanning danger areas from enclosed space, stretched attend/flatback postures,*	(Cruz *et al.*, 1994; Rodgers *et al.*, 1997b; Schneider *et al.*, 2011)
End-exploring *Moving to the edges of the maze arms*	(Cruz *et al.*, 1994)

The data can be collected either by observers present in the room during the test, or by automated software which analyzes video footage recorded during the test (Walf *et al.*, 2007). There are definite benefits and drawbacks to both. Having observers in the room provides a real-time analysis, done from a closer perspective than a camera would allow. However, extreme care should be taken to ensure that the observers make no movements or noise, as this could affect rodent behavior (Walf *et al.*, 2007).

However, one may sacrifice some of the accuracy of data collected as video cameras often cannot distinguish smaller movements and behaviors, such as the difference of whisker twitching between immobility and freezing (Cruz *et al.*, 1994). Before the analyses are started, firm criteria need to be established regarding each parameter in order to minimize inter-observer bias, e.g. whether it counts as a rear if only one front paw is lifted off the floor instead of both.

c. Important considerations

Due to the number of studies that use the EPM test to evaluate anxiety, there are almost as many protocols for the test as there are laboratories studying anxiety (Rodgers *et al.*, 1997b). However, in order to achieve consistent and comparable results certain factors need to be taken into consideration.

One of the most important factors to consider is what time of the day to conduct the test. Studies have been published where the effects of circadian rhythm on EPM performance was analyzed, many with significant results (Yannielli *et al.*, 1995, 1996). These studies mainly reported that the percentage of time spent on the open arms and the number of entries made into the open arms were increased during the dark phase, reflecting a lower level of anxiety during this time. The total number of entries into both arms were also increased, indicating a higher locomotor activity level than in the light phase (Yannielli *et al.*, 1995, 1996). These findings were supported by Golombek, Rosenstein, Yannielli, Keller Sarmiento & Cardinali (1997). However, the same results were disputed by Jones & King (2001) who compared the results of an EPM test performed in the dark phase to one performed in the light phase and found no significant differences. This does not seem to be a reliable finding as Andrade *et al.* (2003) performed a comprehensive review of the timing of EPM test sessions and reported differences between light- and dark phase testing. Most importantly, they found that studies which tested in the light phase reported findings more consistent with data obtained from rats that were previously exposed to some form of stress. Light phase testing could therefore cause skewed results and indicate a higher level of stress and anxiety than is actually present (Andrade *et al.*, 2003).

An examination of the literature reveals that Montgomery did not state the original testing time in his experiment (Montgomery, 1955). Handley & Mithani (1984) reported

that all their tests were completed between 10:00 and 14:00 and other studies similarly reported their testing time to fall within the early light phase of the animal (Cruz *et al.*, 1994; Schneider *et al.*, 2011). However, there is a strong argument for testing the animals in their dark phase as rodents are most active during this time with more hormonal fluctuations than during the light phase (Walf *et al.*, 2007). Thus it can be concluded that the best time to conduct the EPM test would be during the rodent's dark phase (Andrade *et al.*, 2003). This is supported by Albani, Andrawis, Abella, Fulghum, Vafamand & Dumas (2015) who stated that by two months of age the peak activity of a rodent falls within the dark phase.

A separate consideration focuses on the type and intensity of artificial lighting during the test. Elevated light levels can affect behavior in the EPM and particularly cause increased avoidance of open arms, while low lux lighting caused increased entries into the open arms (Bertoglio *et al.*, 2002; Griebel *et al.*, 1993; Jones *et al.*, 2001; Milena *et al.*, 2005). However, there are studies that contest this as Handley, McBlane, Critchley & Njung'e (1993) reported that lighting had no effect on EPM performance. This argument was supported by a study done on male Wistar rats where EPM tests conducted at three different light intensities found that illumination levels had no effect on rat behavior (Becker *et al.*, 1996). The evidence is clearly contradictory and therefore the illumination implemented during the EPM test vary greatly. While testing in the dark phase seems to be the most reflective of the rodent's normal activity levels, this creates the problem of inaccurate observations due to the darkness in the room. Based on a more recent study's findings, bright illumination increases the baseline anxiety levels of rodents, therefore the resultant EPM data would be skewed (Violle *et al.*, 2009). However, without sufficient lighting, observers or cameras would not be able to accurately record the rodent's movements and behavior. This problem was first circumvented by Cruz *et al.* (1994) who made use of a red light for illumination. As red lights emit wavelengths over 600 nm, which are poorly visible for rats, the animals are kept in a "dark" environment and allows them to execute their normal behaviors and also allows for accurate observations. This method was also used by Violle *et al.* (2009) who reported that the red light illumination increased the number of open arm entries made by the rats.

Another seemingly simple factor to consider is the material from which the plus maze is constructed. Since the establishment of the maze test, wood has been a popular choice (Albani *et al.*, 2015; Andrade *et al.*, 2003; Bertoglio *et al.*, 2002; Cruz *et al.*, 1994; Golombek *et al.*, 1997; Montgomery, 1955; Pellow *et al.*, 1985; Sandbak *et al.*, 2001) but other options include metal (Montgomery, 1955), clear or tinted PVC plastic (Becker *et al.*, 1996; Belzung *et al.*, 2001; Schrader *et al.*, 2018), fiberboard (Walf *et al.*, 2007), and plexiglass (Lister, 1987). Handley & Mithani (1984) lined the floor of their maze with wire mesh, while Montgomery (1955) covered theirs with hardware cloth and sheet metal. According to a review done by Hogg (1996) various studies reported covering the maze floor with rubber, most likely to avoid rodents slipping off the edge. Another measure employed to prevent rodents falling off the maze involved adding small ridges to the open arms of the maze (Cruz *et al.*, 1994; Martínez *et al.*, 2002; Schneider *et al.*, 2011). According to the same review done by Hogg, the use of raised edges on the open arms are mainly found in studies that uses a maze made of Perspex, therefore correcting for the slippery nature of the Perspex floors. Although the material used does not seem to have a major impact on the results of the EPM test (Hogg, 1996), certain changes made to the maze can elicit a distinct influence. In the study done by Lister (1987), clear plexiglass walls were used for the closed arms with the motivation that it will keep the illumination levels consistent throughout the maze. Another study tested the difference in behavioral responses between rats subjected to an EPM with either clear or opaque walls on the closed arms (Violle *et al.*, 2009). These results showed that the use of clear walls on the closed arm decreased the rodent's preference for the closed arm when compared to that of the open arm.

d. Validity of test

The EPM complies with the same three distinct criteria used to classify stress models, therefore classifying it as a reliable behavioral test. Firstly, the EPM has face validity, which is defined in this context as the ability of a task to seemingly measure what it is intended to measure (Walf *et al.*, 2007). In this case, the maze is aimed at measuring the willingness of a rodent to explore open areas, or differently stated, the anxiety or fear created by open spaces. As rodents tend to avoid the open spaces and show

more stressed behavior on the open arms (e.g. defecation, immobility, freezing) the test therefore shows face validity (Lister, 1990).

The second criterion is construct validity that refers to whether an unobservable construct can be measured by using an observable dependent variable (Walf *et al.*, 2007). For the EPM test, rodent anxiety levels serve as the unobservable construct and it is measured by the observable factor of the time spent by the rodent in the open arms. This is clearly observed in the effects exerted by a) anxiogenic drugs, which most notable decreases the time spent on the open arms, and b) anxiolytic drugs increasing the time spent on the open arms (Pellow *et al.*, 1985), therefore awarding the test construct validity.

The third criterion is predictive validity which can be described as how likely it is for the dependent measure to predict the performances seen in future (Walf *et al.*, 2007). Different studies have shown efficacy of the EPM test and the results now repeated many times over. The test also displays a biochemical link as increased plasma corticosterone levels were associated with increased time spent in open arms, and also positively correlates with various risk assessment behaviors in the maze (File *et al.*, 1994; Rodgers *et al.*, 1999).

Additional methods to validate depression and anxiety

The SPT and EPM tests are the two of the most frequently used validation methods in chronic stress models and the use of these tests often overlap in studies (refer Table 2). There are other tests with the same aims, albeit with variations in the tests or different protocols. These include the open field test (OFT), tail suspension test (TST), forced swimming test (FST), elevated zero maze (EZM), and the holeboard test.

The OFT is often used in conjunction with the EPM test to assess locomotor activity and state of anxiety in rodents (Lezak *et al.*, 2017; Tian *et al.*, 2013). This test has been very popular due its minimal cost and time requirements and as it involves a simple apparatus and a short duration (Lister, 1987). The open field itself consists of a large square or circular arena with marking to indicate specific areas of the arena, in which the rodent is allowed to move freely (Lister, 1990; Wang *et al.*, 2017). The arena is illuminated by lighting focused on the middle section and the floor is marked to allow the quantification of the rodent's movement. The assessment of anxiety/locomotor

activity is made by determining the ratio of time spent in the center areas to the time spent staying close to the walls of the enclosure (“wall hugging”) (Wang *et al.*, 2017). Theoretically, an anxious rodent will spend more time “wall hugging” and avoiding highly lit areas, as well as displaying inhibited movement (Lister, 1990). This reflects the decreased spontaneity and curiosity associated with anxiety-like disorders (He *et al.*, 2020). However, there are some criticisms regarding the test as it can be argued that it has the potential to confound exploration and general locomotor activity (Brown *et al.*, 2008). Differences seen in the results of an OFT can simply be the result of differences in locomotor activity, which is not necessarily connected to the rodent’s exploratory behavior (Brown *et al.*, 2008).

A variation of the EPM procedure is the EZM test which was designed on the same principle as its counterpart except for replacing the plus shape with an elevated ring shape (Chadman *et al.*, 2009). As with EPM, the maze has alternating open and close (light and dark respectively) portions based on the theory that anxious rodents will avoid the brightly lit areas (Campos *et al.*, 2013). The motivation for developing the EZM was the ambiguity caused by the center square of the plus-shaped maze, which the EZM eliminates through its continuous design (Campos *et al.*, 2013; La-Vu *et al.*, 2020).

Another protocol designed to assess the exploratory nature of rodents is the holeboard test, which is based on the rodent’s instinctive curiosity and exploration desire (Lister, 1990). The test apparatus consists of a closed area with holes cut into the floor, large enough for a rodent to poke its head through (Campos *et al.*, 2013). The frequency and length of the head-dipping actions are recorded and accepted as an indication of directed exploration, with anxious rats being more likely to show reduced head-dipping (Brown *et al.*, 2008). When compared to the OFT, the holeboard test is considered a better measure of how attracted the rodent is to novelty and exploration, as active head-dipping is considered as a more valid measure in this case (Brown *et al.*, 2008). Additionally, a modified holeboard test was developed which takes place in an enriched environment and that allows the rodents to exhibit various behavioral patterns (Labots *et al.*, 2015). This modified protocol is designed to serve as a single evaluation to replace a battery of different behavioral tests, as there is concern that exposure to an array of tests may influence the behavior of experimental animals (Ohl

et al., 2001). Many of the ethological validation tests performed are most effective in naïve rodents which would dramatically increase the number of animals required per experiment. In this case, the used of a modified holeboard test would limit such concerns and allow for multiple observations during a single test (Labots *et al.*, 2015; Ohl *et al.*, 2001).

Two other tests that often overlap are the forced swimming and tail suspension tests. However, such tests are really aimed at determining behaviors associated with depression (Chadman *et al.*, 2009; Liu *et al.*, 2018; Yan *et al.*, 2010). Both tests are evaluated by the time the rodent spends on escape behaviors in relation to the immobile time, as immobility behaviors are related to behavioral despair (Scheggi *et al.*, 2018). Such tests are frequently employed in testing the efficacy of anti-depressants. The tests can be used across a broad spectrum of drugs, as the underlying mechanism on which the drug works is not implicated in the test (Yan *et al.*, 2010). The tests are also very easily automated, making them very low cost as well as low effort stress validation methods (Cryan *et al.*, 2004).

For the FST the rodents are placed into an inescapable cylinder filled with water after which the rodents initially display swimming, splashing or struggling actions before becoming more immobile by floating (Krishnan *et al.*, 2011; Mineur *et al.*, 2006). An advantage is its simplicity and low budgetary requirements. However, concerns were raised regarding its validity as these tests only focus on predictive validity (Chadman *et al.*, 2009; Yan *et al.*, 2010). The test has successfully been classified as inducing depressive behaviors, however these can be reversed through the acute administration of almost any anti-depression treatment (Krishnan *et al.*, 2011). Thus it is poorly translatable to humans as the effects of antidepressants take weeks to manifest in this case (Krishnan *et al.*, 2011).

The TST faces the same criticisms due to its theoretical similarities to the FST. For the TST the tail of the rodent is taped to a bar from which they hang suspended for a specific period of time (Chadman *et al.*, 2009). Although the TST may reportedly offer greater sensitivity, the criteria for measuring despair is similar to the FST as the time spent struggling to escape is measured against time spent hanging immobile (Bhat *et al.*, 2014). One benefit of the TST that sets it slightly apart from the FST is the lack of

hypothermic exposure which limits any confounding results due to this influence (Cryan *et al.*, 2004).

In summary, there are various animal models of chronic stress being used across the world, such as the CMS/UCMS, CRS, MS, LH and social defeat stress models. These models are employed to recreate a specific state of chronic stress in animals, allowing the research of more complex diseases that originate from stress dysregulation. The most popular and validated animal model of chronic stress is the CMS/UCMS model as it has predictive, face and construct validity. Regardless of which model is used, the model needs to be properly validated to ensure that the protocol is successfully inducing a state of chronic stress as it is designed to. As shown by this review, there are many methods of validating a chronic stress model. The most widely used of these tests are the SPT and EPM tests, which are designed to validate states of depression and anxiety respectively. However, there is no single test that can be confidently used to determine the success of a particular model. Tests should rather be used in conjunction with one another as not only do they overlap in purpose, but stress models also overlap in recreating depression and anxiety. In light of this, the current study focused on validating the efficacy of the UCMS model by employing an ethological approach to tests that are centered on both anxiety- and depression-like symptoms.

1.6 STUDY AIMS

Although the UCMS model is well-established across the world, to the best of our knowledge it has not yet been established in South Africa. The present study was therefore aimed at successfully establishing the UCMS model at Stellenbosch University, with the lesser goal of evaluating to which extent the validation tests succeed in confirming a chronically stressed state in the animals. This effort is part of a larger study in our research group that focuses on investigating the impact of chronic stress in terms of the onset and progression of cardio-metabolic diseases.

2. STUDY DESIGN

2.1 METHODS AND MATERIALS

The UCMS model was established as a protocol that induces a wide array of symptoms typically associated with chronic stress (Frisbee *et al.*, 2015). This study is a continuation of work previously done in our facility and is adapted from a similar UCMS protocol (Sher, 2019). The animals 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). This study was performed at the animal unit located on the main campus of Stellenbosch University, with the approval of the institution's Animal Ethics Committee (Ethics #: ACU-2018-6311).

Acclimatization period and baseline testing

Male Wistar rats (10-11 weeks old) were used in this study, as this reflects an age of ~24-30 years, known as a time-period during which humans are very likely to experience major life stressors (Sengupta, 2013). Following their arrival from the breeding facility on Tygerberg campus of Stellenbosch University (~40 km from main campus), the rats were acclimatized for a period of seven days to become familiar with the handlers and also to recover from any travel stress (Capdevila *et al.*, 2007). During the next two weeks, the rats were acclimatized to the sucrose solution used during the SPT, as well as the Perspex box used during behavioral monitoring (Christiansen, Bouzinova, Palme & Wiborg, 2012; D'Aquila, Newton & Willner, 1997; Pothion, Bizot, Trovero & Belzung, 2004; Scotton, Colombo, Reis, Possebon & Hizo, 2019). See Figure 4 for full study timeline.

Housing specifications

Upon arrival, the rats were immediately separated into individual cages and continued to be singularly housed for the duration of the study. Although rats are social animals and this served as a stressor in itself, the separation of animals was necessary as the present study was part of a larger research project that required individual fecal sampling (Boggiano *et al.*, 2008). Once the rats were divided into cohorts, they continued to be housed in the same room with an ambient room temperature of 22 ±

3°C and humidity levels of ~60 ± 10%, to ensure that environmental conditions remained consistent between groups. Each rat was monitored daily to ensure their wellbeing and to note behavioral aspects. The rats also received a weekly check-up from a registered veterinarian.

Body weight and food consumption

The rats were weighed once per week to monitor growth and general wellbeing. Their food consumption was also monitored by weighing out the amount of food provided following completion of the SPT and weighing what was left by the start of the starvation period before the SPT seven days after. The weekly food consumption was then extrapolated from this data.

Experimental procedure

The rats (now aged 13-14 weeks) were divided into a control group (n=14) and experimental (stressed) group (n=14), matched for weight and baseline sucrose preference (D'Aquila *et al.*, 1997). The experimental group was exposed to a series of unpredictable chronic stressors over a period of nine weeks. These stressors took place in a separate room where the environmental conditions were similar to the

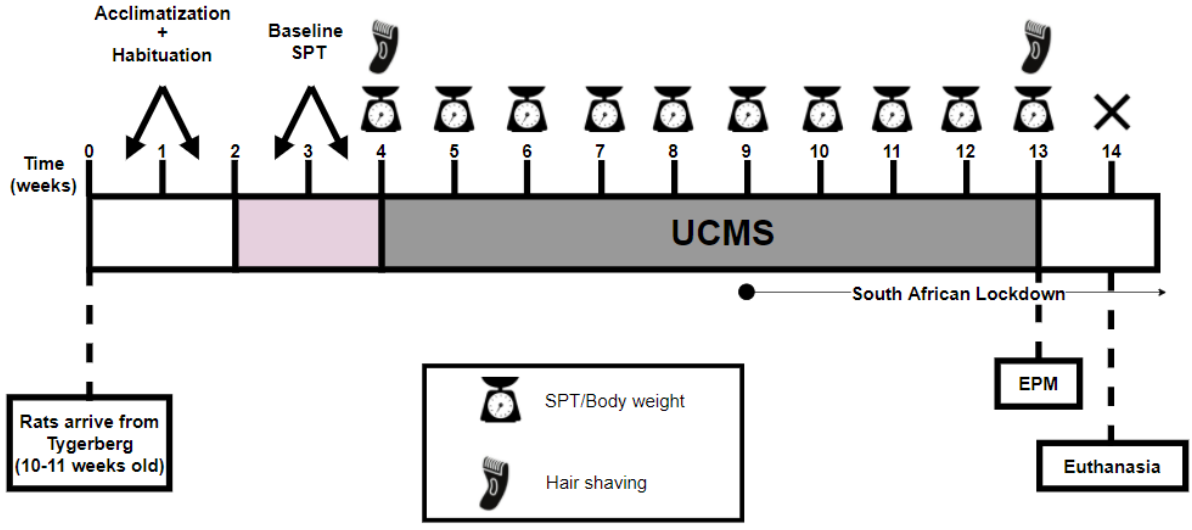


Figure 4: Experimental timeline, indicating study duration, acclimatization and habituation periods, weekly weighing and SPT's, hair shaving, behavioral testing and euthanasia. The South African Lockdown period, due to the global pandemic, is also indicated below the timeline.

housing room. Following the completion of the daily stressor/s, the experimental group was moved to a separate room for “recovery” period where they remained for three to four hours before being returned to the same room as the control group. This was done to ensure that the control group was not affected by the stressed rats in any way, and to limit contact between groups until any acute effects of the stressor had passed (Carnevali *et al.*, 2017; Castelhano-Carlos *et al.*, 2009). For the same reason, the stressed rats were also put into new cages after every stressor, before being returned to the housing room (Bondi *et al.*, 2008).

The stressors took place during the light cycle and their duration lasted between 30 minutes and eight hours per day for six days per week. Food and water were available to the rats for the duration of all the stressors, with the exception of restraint (30 min) and cage tilting (four hours). Stressors were chosen at random from a predetermined list (Table 3), in order to ensure the unpredictable nature of the model. Each rat was evaluated during a behavioral monitoring session (on a weekly basis) and underwent a SPT every seventh day. The rats were also subjected to an elevated plus maze four days prior to termination of the experiment.

Table 3: Exposure of rats to random stressors over an 8-week period.

Stress	Duration	Description
Damp Bedding	8 hours	Bedding (sawdust) was dampened with approximately 500 mL of water. This was done in such a way as to avoid pooling of water within the cage.
Light/Dark	8 hours	The light-dark cycle of the rats was disrupted for the duration of this stressor. Lights were kept on for 30 minutes and switched off for the following 30 minutes, repeatedly, for the duration of 8 hours.
Predator exposure	8 hours	The rats were exposed to the scent of a predator through the presence of concentrated bobcat urine (The Pee Mart, Vassalboro ME) in the cage. Rats were also exposed to pre-recorded sounds of cats (https://www.youtube.com/watch?v=ubfNppg9tao).
No Bedding*	4 hours	Bedding was removed for the duration of this stressor.

Cage Tilt*	4 hours	The rat's cage was tilted at 45° and secured once in the tilted position.
Social stress**	4 hours	Rats were taken out of their home cages and placed into the home cage of another rat.
White noise**	2 hours	Rats were exposed to static white noise at 80 dB (https://www.youtube.com/watch?v=CCnCMHNyny8&t=6s).
Strobe light	2 hours	Rats were kept in a dark room and exposed to a flashing white light to create a strobe light effect.
Restraint	30 min	Rats were placed in a clear Perspex container that can comfortably restrain 6 rats at a time. Rats were able to see each other and feel body warmth through the separations.

Certain shorter stressors were combined on one day, such as cage tilting with no bedding (*), and social stress with white noise/strobe light (**).

Euthanasia and blood/tissue collection

Following the experimental procedure, rats were euthanized through decapitation after which various organs (brain, heart, gastrointestinal tract (GIT), spleen, pancreas, liver, adrenal glands, kidneys, tibia) were harvested. Immediately following decapitation, the various organs were dissected, weighed, placed into appropriately sized microfuge tubes, and subsequently snap-frozen in liquid nitrogen. Immediately after decapitation, trunk blood was collected into a plastic funnel (rinsed with ethylenediaminetetraacetic acid to prevent clotting). The whole blood samples were immediately centrifuged according to the protocol in Appendix A for the preparation of plasma samples. These samples were subsequently aliquoted (~500 µl per 2 mL microfuge tube) and snap frozen as well. All harvested specimens were stored at -80°C until further use.

Hair sampling

As seen on Figure 4, hair samples were collected at the start and end of the UCMS protocol. This process was aided by a registered veterinarian, who placed the rats under anesthesia and shaved a designated area of fur on their left flanks, ultimately amounting to a 5 cm x 8 cm area. The same area of fur was again shaved after

completion of the UCMS protocol to sample the new hair growth. The hair samples were immediately folded into aluminum foil sheets after being shaved off, placed into resealable bags and stored at 4°C until further use.

Sucrose Preference Testing (SPT)

The rats were initially introduced to the 1.5% sucrose solution that was used during the SPT throughout the study. In order to accurately measure changes in sucrose preference, a preference had to first be established during a habituation period. The protocol used for this test was adapted from Willner *et al.* (1987). Here, rats were initially exposed to two 100 mL bottles of a 1.5% sucrose solution for 24 hours, after which the bottles were replaced with a) a 100 mL bottle of the sucrose solution and b) a 100 mL bottle of regular tap water. These bottles were available to the rats for a period for 24 hours. The following day, the rats were exposed to the full SPT protocol. The rats were deprived of food and water for 12 hours prior to the start of the test, which took place at the beginning of their dark phase (18:00). For the test, 100 mL of sucrose solution and 100 mL of regular drinking water were placed into the cages and left in for one hour. The consumption of both bottles was subsequently measured, and rats were again provided with their standard chow and water. The positions of the bottles were switched weekly to control for a side preference (Juczewski *et al.*, 2020).

In total, six baselines tests (which consisted of the full SPT protocol) were done over a period of two weeks, and a standard sucrose preference was established by taking the average sucrose preference of all six tests. After the start of the stress protocol, a SPT was done every seven days (each Sunday) and the study recorded the changes in sucrose preference over the course of the experiment. Individual sucrose preference was calculated using the following formula:

$$Preference = \frac{sucrose\ consumption}{sucrose\ consumption\ \times water\ consumption}$$

Behavioral monitoring (Perspex monitoring)

Over the course of the nine-week period, rats were closely monitored to keep track of certain behavioral parameters that would serve as an indication of their anxiety/stress levels. The parameters observed in this study consisted of the following: rearing, latency, defecation, urination, bruxing, grooming, aggressive/escapist behavior, and

level of piloerection. Such parameters were previously described as good indicators of anxious/stressed behavior in rodents (Cruz *et al.*, 1994). In order to accurately observe such parameters, rats were placed in a 20 cm x 20 cm x 30 cm clear Perspex box with a removable lid for a period of five minutes. The observations took place after 13:00 during the light cycle of the rats. To ensure that the rats were properly accustomed, they were slowly introduced to the Perspex box during a two-week acclimatization period, during which three baseline readings were taken for each parameter. For the remainder of the study, all rats were subjected to this form of behavioral monitoring once per week.

Elevated Plus Maze (EPM)

The elevated plus maze was conducted as previously described by Pellow *et al.* (1985). The maze consisted of two open arms and two closed arms, raised 50 cm off the ground, intersecting each other to create a plus (+) sign. The open arms were each 50 cm x 10 cm, while the closed arms were 50 cm x 10 cm x 30 cm with an open (uncovered) roof. The entire maze was constructed out of wood and was painted black. Additionally, the open arms had no ridges and merely consisted of an open plank. The test lasted for five minutes, recorded from the moment the rat was placed in the center of the maze, facing the same open arm.

The test took place during the dark phase of the rats, and therefore the testing room (the same room in which behavioral monitoring was done) was illuminated by a red light to allow investigators to monitor the rats accurately. Both the light source and a camera was positioned directly above the maze. The following measures were observed during the test: entries into open arms, entries into closed arms, time spent in open arms, time spent in closed arms, head dips, rears, and stretch-attend postures. The data was collected from videos recorded during the procedures and analyzed by two blinded investigators. A third investigator was used to verify inter-observer variability.

Molecular analyses

Enzyme immunoassay kits were used to analyze plasma and hair corticosterone (DetectX Corticosterone EIA Kit; Arbor Assays, Ann Arbor, MI; #K014-H1), as well as plasma ACTH (SimpleStep ELISA® Kit; Abcam, USA; #ab263880) levels. Data were

analyzed using a Four Parameter Logistic (4PL) curve fit program. All samples were analyzed in duplicate, per the manufacturer's instructions (Appendix A, B & C).

Statistical analyses

All statistical analyses were performed using Statistica 13.0 (StatSoft Inc., Dell Software, Tulsa OK) and done in conjunction with Prof. Martin Kidd at the Centre for Statistical Consultation at Stellenbosch University. Parametric data were analyzed mainly by analysis of variance (ANOVA) tests. The repeated measures data were analyzed using mixed model ANOVAs in R, while the remaining data was analyzed by two-way ANOVAs. The non-parametric data were analyzed by Mann-Whitney or Kruskal-Wallis tests. In all cases a p-value <0.05 was considered significant and any outliers were excluded through the use of normal probability plots. However, the data for both plasma corticosterone and ACTH levels were winsorized instead of removing outliers. 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).

2. RESULTS

The results in this study form part of a larger one that focuses on the links between chronic stress and the onset and progression of cardio-metabolic diseases. Although there was robust teamwork, the current thesis focused on assessing whether the UCMS model was successfully established in our laboratory.

A. Control vs Stress

3.1 General measurements

a. Body weight

The rats were weighed on a weekly basis (Figure 5) for the duration of the experiment. There were no statistically significant changes between the two groups by the end of the study, despite the Control group showing a slightly higher body weight from Week 3 onwards.

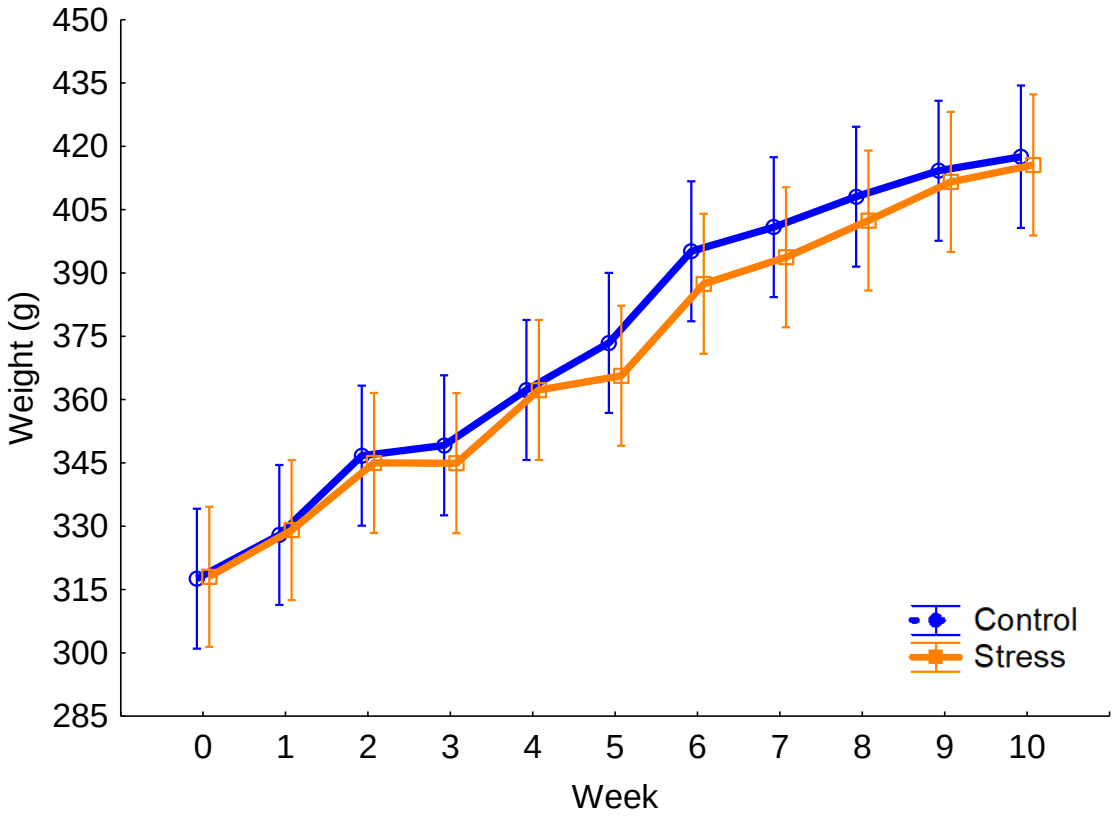


Figure 5: Body weight increase for Control and Stress groups over time. Data displayed as mean ± SD; repeated measures, mixed model ANOVA in R; n=14;

b. Food consumption

Although the rats showed a significant decrease in food consumption over the course of the 10-week period, the graph reveals that the patterns were of an erratic nature (Figure 6). There was a sharp decline in food consumption during Week 2, before a return to baseline consumption during Weeks 3 and 4. Furthermore, there was another rapid decrease during Week 5, after which the Stress rats consumed a considerably high amount of food for the next 2-3 weeks. Both groups ended on a similar food consumption at the end of the 10-week period.

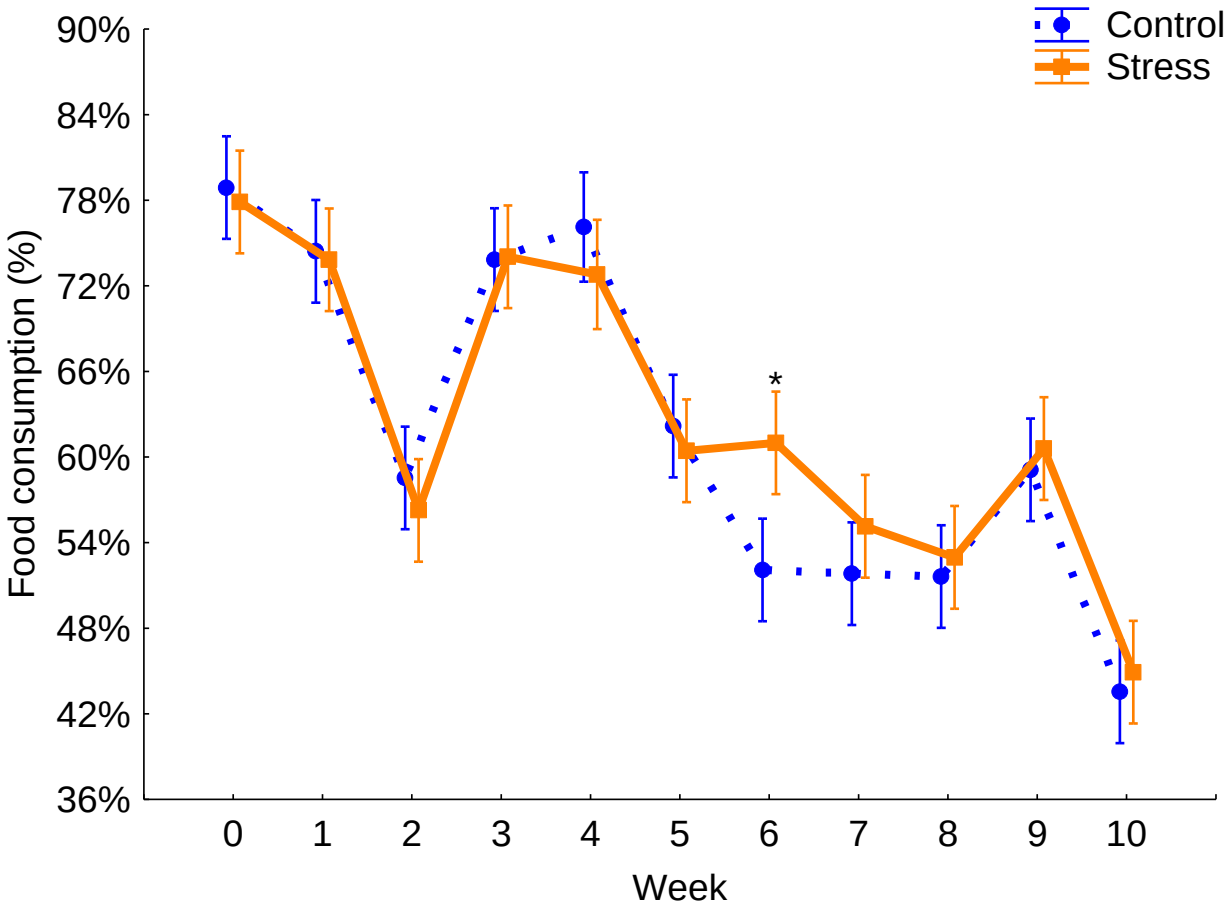


Figure 6: Percentage food consumption for Control and Stress groups over time. Data displayed as mean ± SD; repeated measures, mixed model ANOVA in R; n=14; *p<0.01.

c. Organ weights

After termination of the experiment the organs were dissected out and weighed before storage at -80°C. However, no significant changes were observed when comparing the weights of the brain, heart, left and right kidneys, left and right adrenal glands, spleen, liver, pancreas, GIT and tibia of the Control vs Stress groups (corrected for body weight; data not shown).

3.2 Molecular analyses

Plasma corticosterone and ACTH levels are widely considered to be reliable biological markers of HPA axis activity. However, no significant differences were observed for corticosterone (Figure 7) and ACTH (Figure 8) levels between the Control and Stress groups.

a. Plasma corticosterone

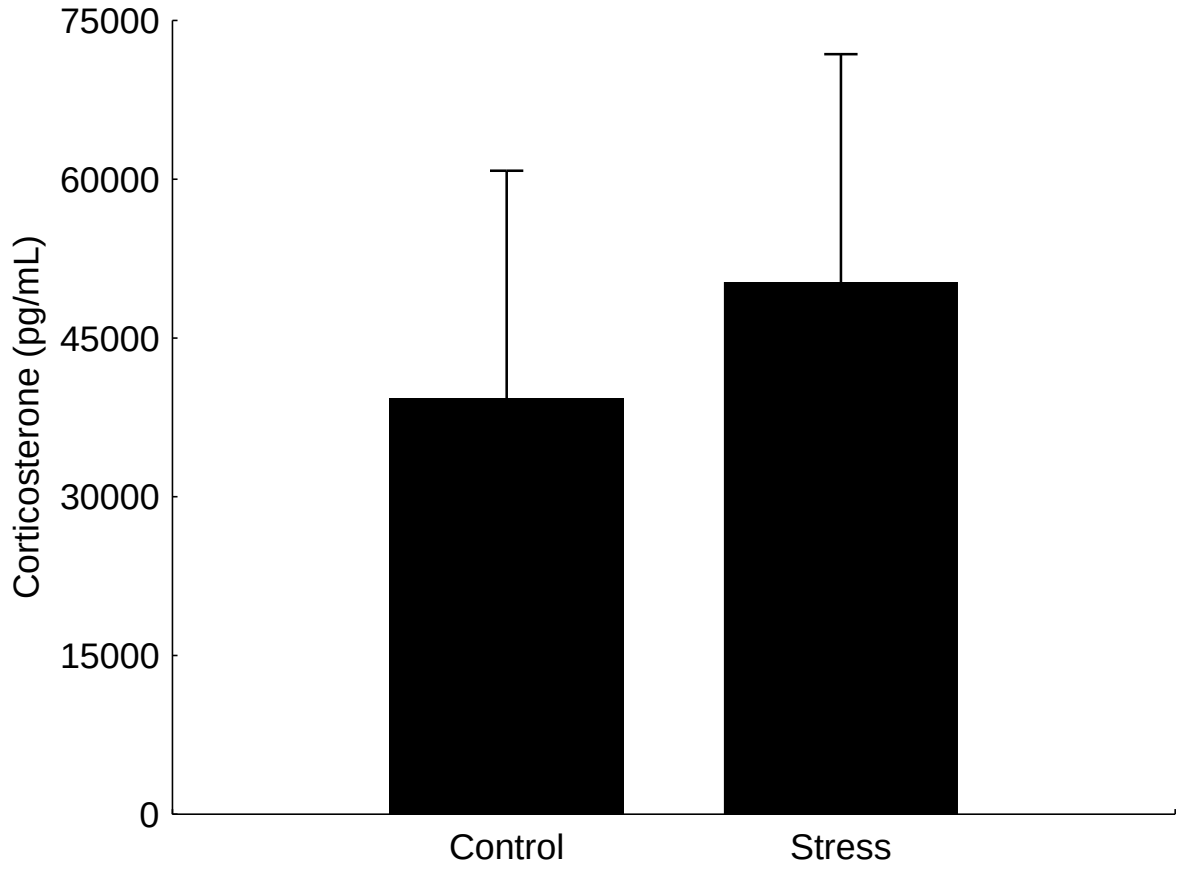


Figure 7: Plasma corticosterone levels compared between Control and Stress groups. Data displayed as mean ± SD; two-way ANOVA; Control n=13, Stress n=14; p=0.38.

b. ACTH

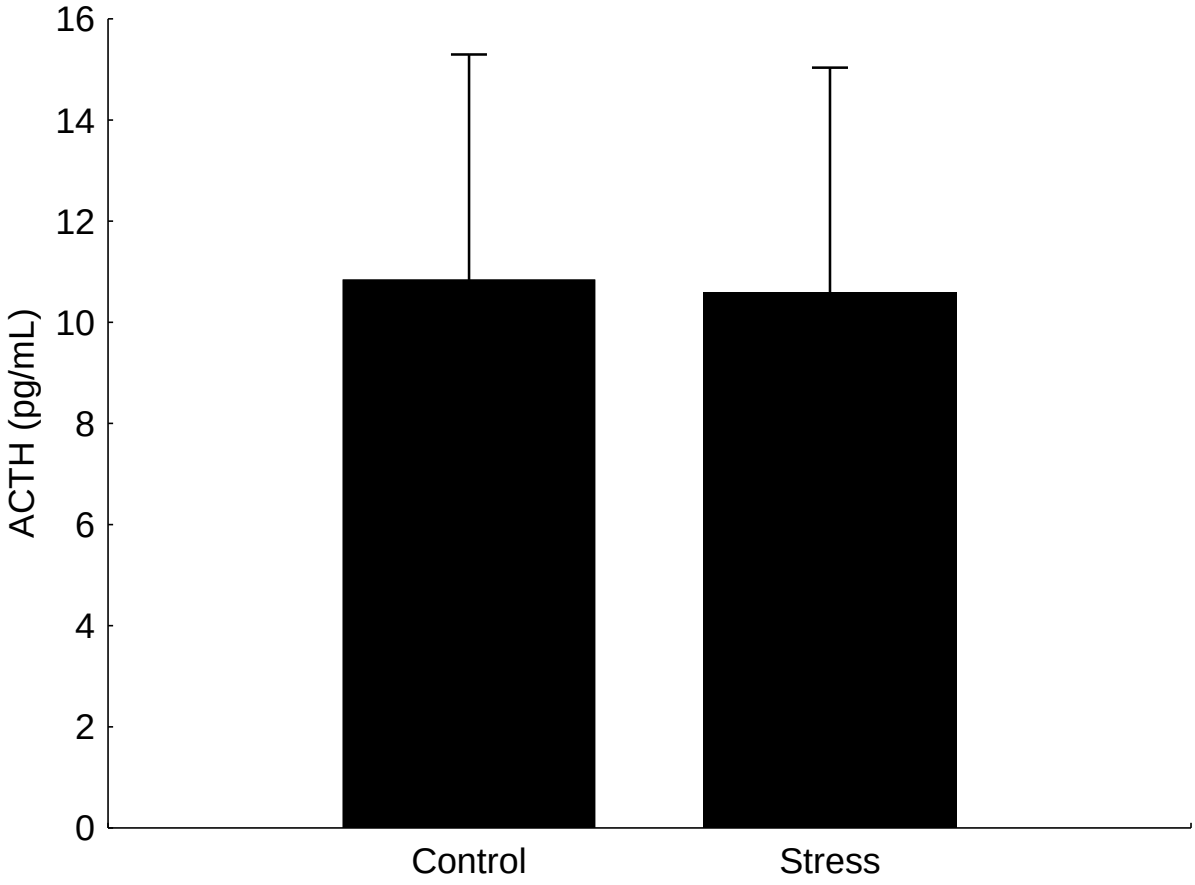


Figure 8: Plasma ACTH levels compared between Control and Stress groups. Data displayed as mean ± SD; two-way ANOVA; Control n=13, Stress n=14; p=0.63.

c. Hair corticosterone

No viable results were obtained from the molecular analysis for hair corticosterone levels, as the absorbance values from the hair fell below the detectable range of the ELISA kit. There were also inconsistencies between the prescribed protocol and advice given by manufacturer technicians during troubleshooting, leading to untrustworthy data from the kit.

3.3 Behavioral validation methods

a. Behavioral monitoring

During weekly detailed monitoring of rat behavior, various parameters were observed and scored according to intensity/frequency. Results showed no significant differences

for any of these parameters except for frequency and intensity of coat piloerection, as indicated in the following graphs. Unfortunately, these results were highly variable and therefore cannot be fully interpreted.

Rearing

Rearing behaviors increased over time in both groups, however, these results did not show any statistical significance (Figure 9). There was an increase from weeks two to four, however not statistically significant. The p-value also did not show any significant interaction between groups and time.

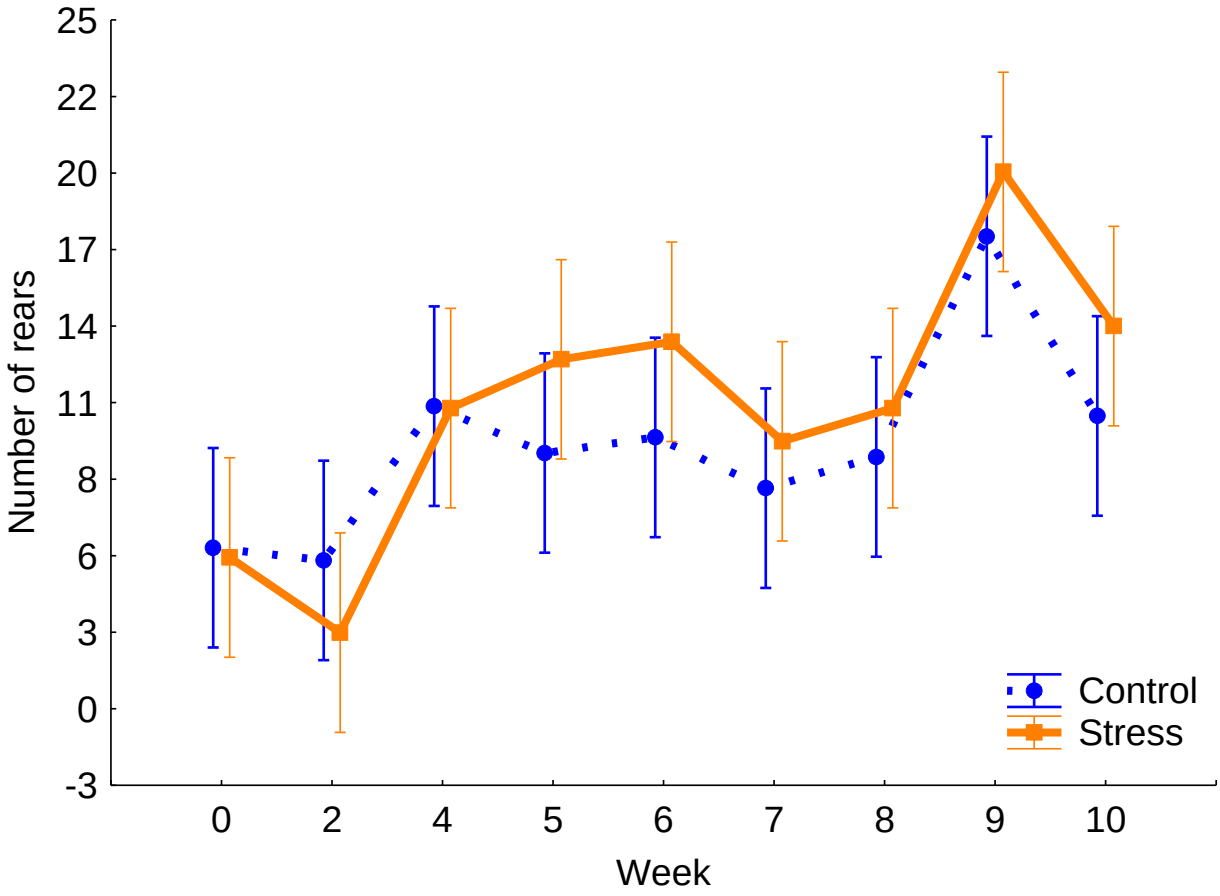


Figure 9: Comparison of average number of rears recorded per session for Control and Stress groups over time. Data displayed as mean ± SD; repeated measures, mixed model ANOVA in R; n=14; p=0.26.

Latency

The control group maintained a steady level of latency before rearing during monitoring sessions in the 10-week testing period (Figure 10). In sharp contrast, the stress group showed spikes during Weeks 4 and 8. There was no significance interaction between time and groups.

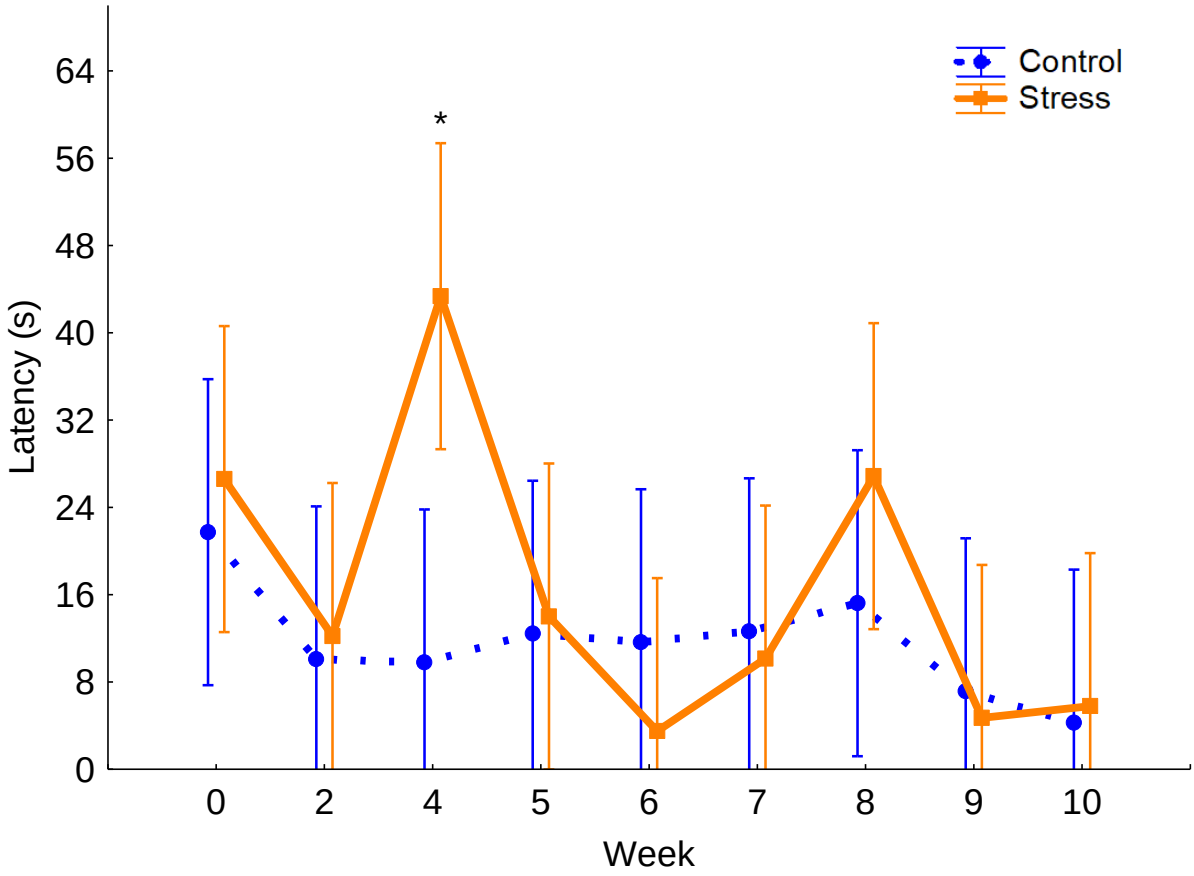


Figure 10: Comparison of average latency period recorded per session for Control and Stress groups over time. Data displayed as mean ± SD; repeated measures, mixed model ANOVA in R; n=14; *p<0.01.

Grooming

Grooming behaviors of the Control group remained relatively constant during the testing period. However, the Stress group again displayed more erratic patterns (Figure 11), including an increase from weeks two to four, however not statistically significant.

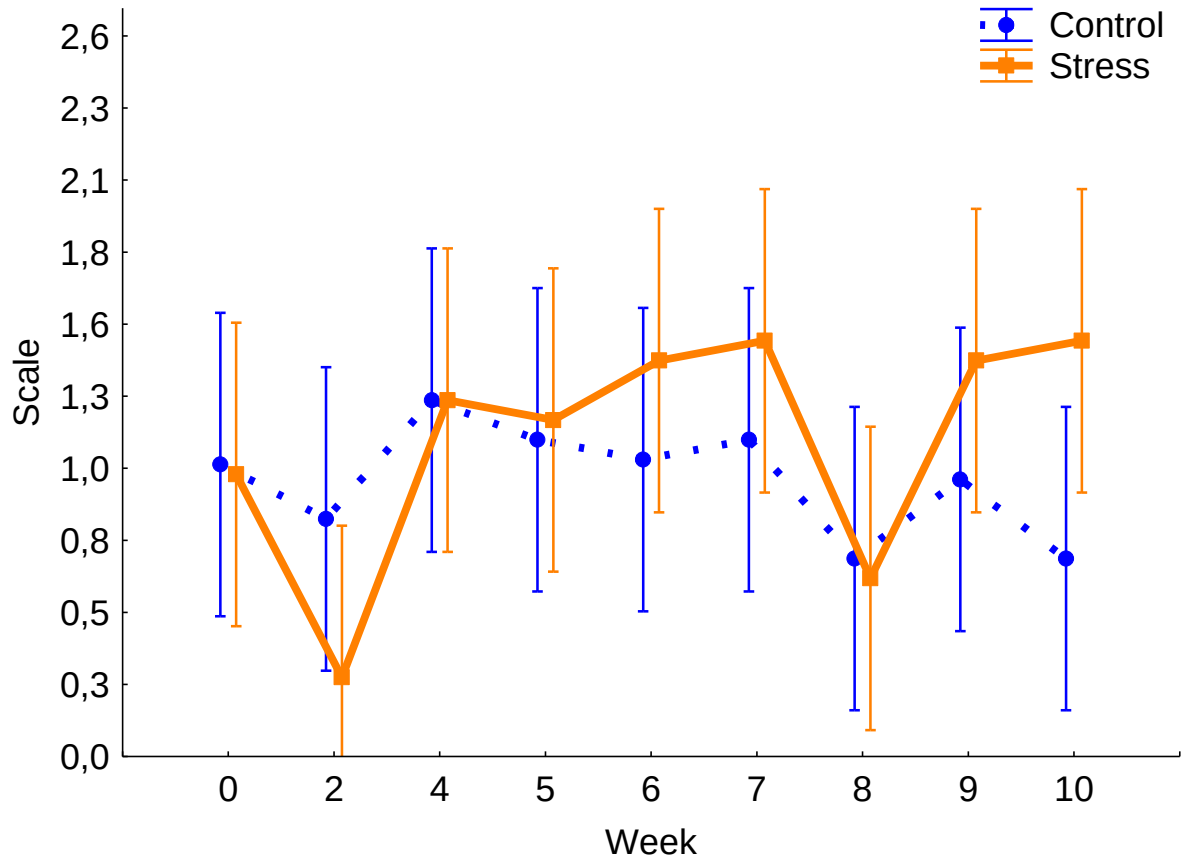


Figure 11: Comparison of average grooming intensity recorded per session for Control and Stress groups over time. Data displayed as mean ± SD; repeated measures, mixed model ANOVA in R; n=14; p=0.28.

Piloerection

Piloerection of the fur increased for both groups over the 10-week period and there was a significant interaction between group and time ($p<0.01$) (Figure 12). There was also an increase from weeks two to four, however not statistically significant.

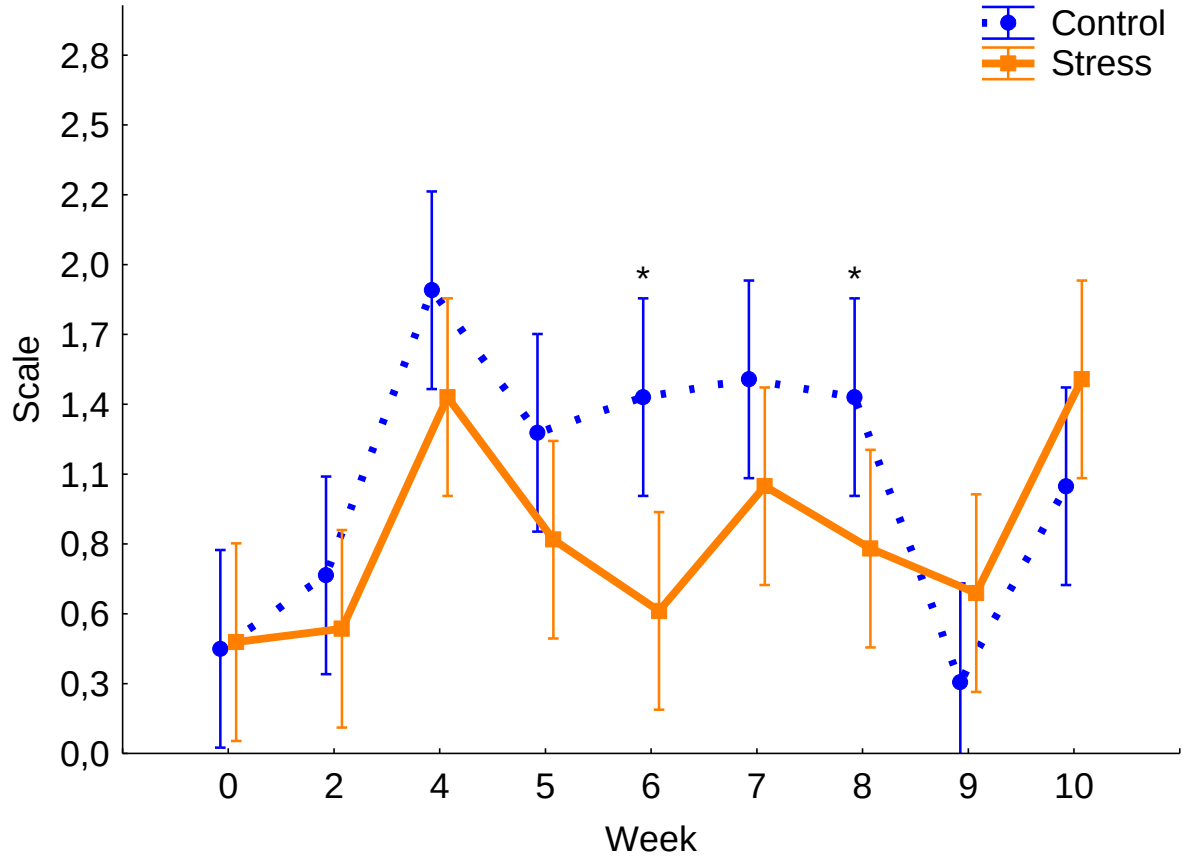


Figure 12: Comparison of average score for piloerection recorded per session for Control and Stress groups over time. Data displayed as mean ± SD; mixed model ANOVA in R; n=14; *p<0.05.

b. Sucrose preference test

Sucrose preference in both groups increased significantly over the 10-week testing period (Figure 13). The biggest disruptions occurred during Week 5 and 6, where the Control group showed a spike in sucrose preference. The Stress group also showed a slight increase but remained relatively stable. However, both groups displayed a very sharp decline in preference during Week 6 after which it returned to relatively normal levels. The Control group ended the testing period with a significant increase in sucrose preference compared to the Stress group.

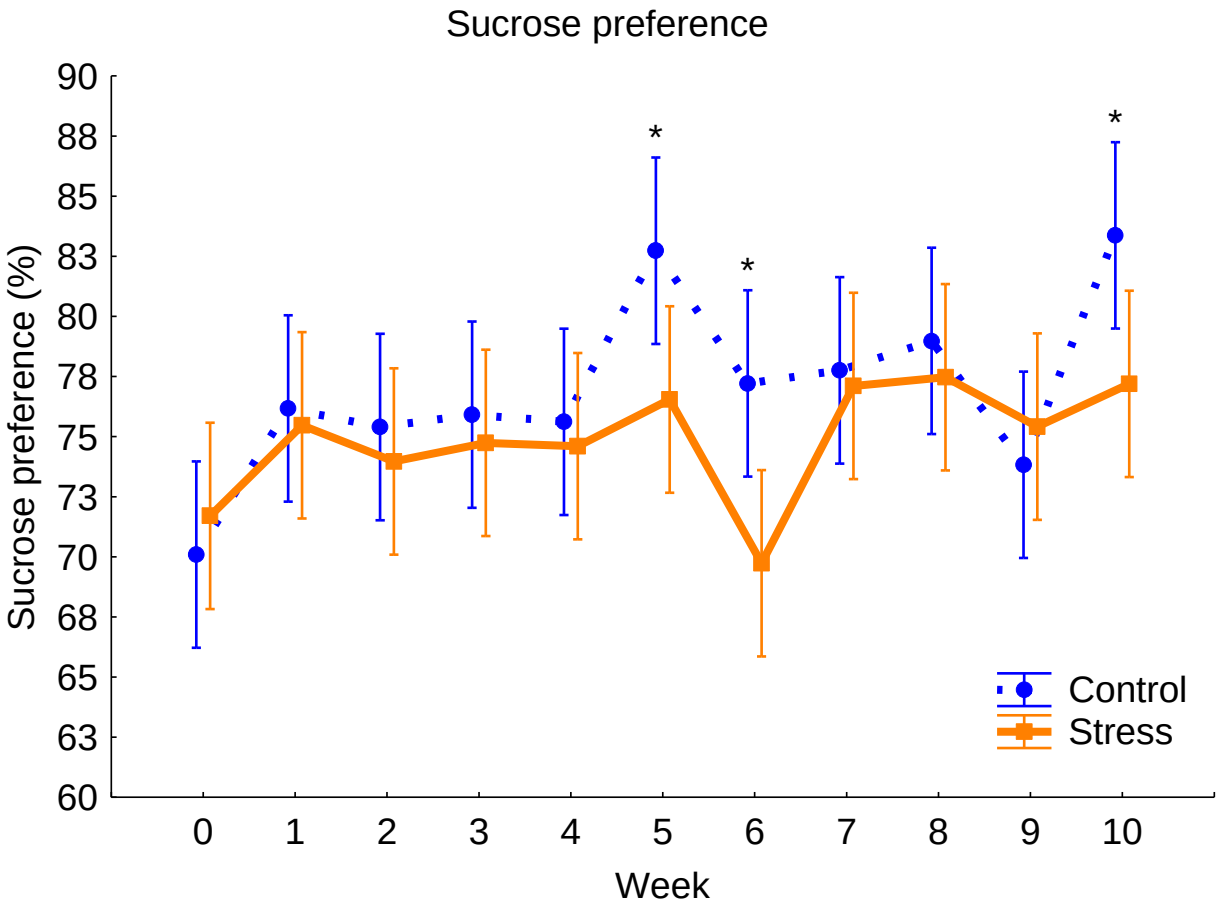


Figure 13: Weekly sucrose preference for Control and Stress groups over experimental period. Data displayed as mean ± SD; mixed model ANOVA in R; n=14; * $p<0.05$.

The sucrose preference was also re-calculated by considering the effect that body weight may have on individual sucrose consumption (Figure 14). Here the analysis showed that there was no significant change throughout the 10-week protocol.

However, the Stress group showed a slight decrease in the grams of sucrose ingested per gram of body weight from Week 0 to Week 10. The P-value indicates no significant interaction between group and time.

Sucrose intake per gram of body weight

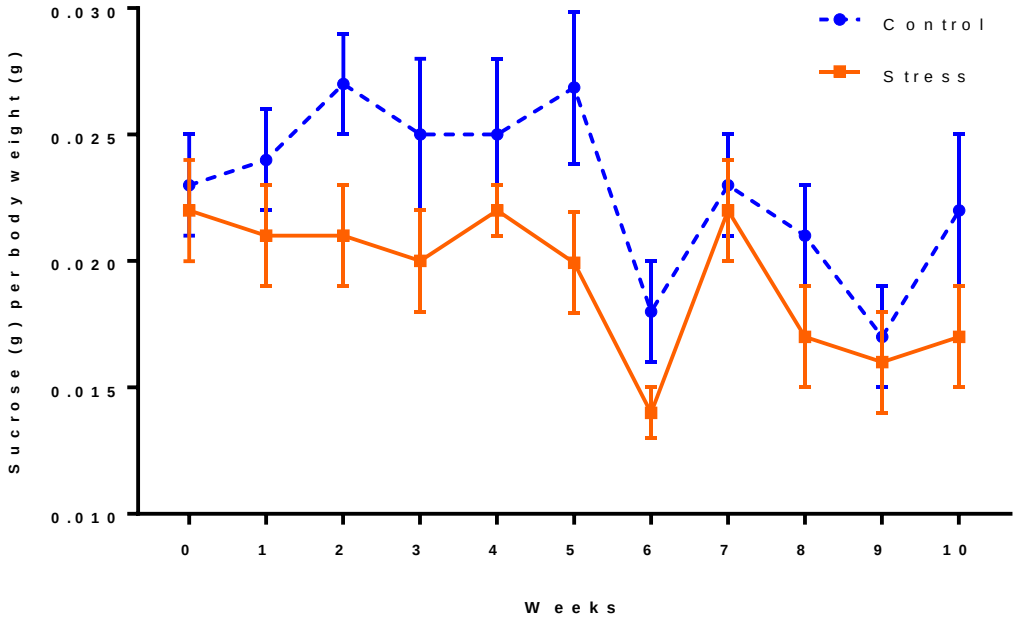


Figure 14: Average individual sucrose intake per gram of body weight for Control and Stress groups. Data displayed as mean ± SD; mixed model ANOVA in R; n=14; p=0.19.

d. Elevated plus maze

The time spent in the different arms of the plus maze is a good indicator of anxiety-like behavior. There was no significant difference between the Control and Stress groups for either time sent in the open arms (Figure 15) or time spent in closed arms (Figure 16). The entries into each arm are recorded as an additional indication of the approach-avoid conflict created by the maze. As with time spent in arms, there was no recorded significant difference between the number of entries into either arm made by the Control group versus the Stress group.

Time spent in open arms

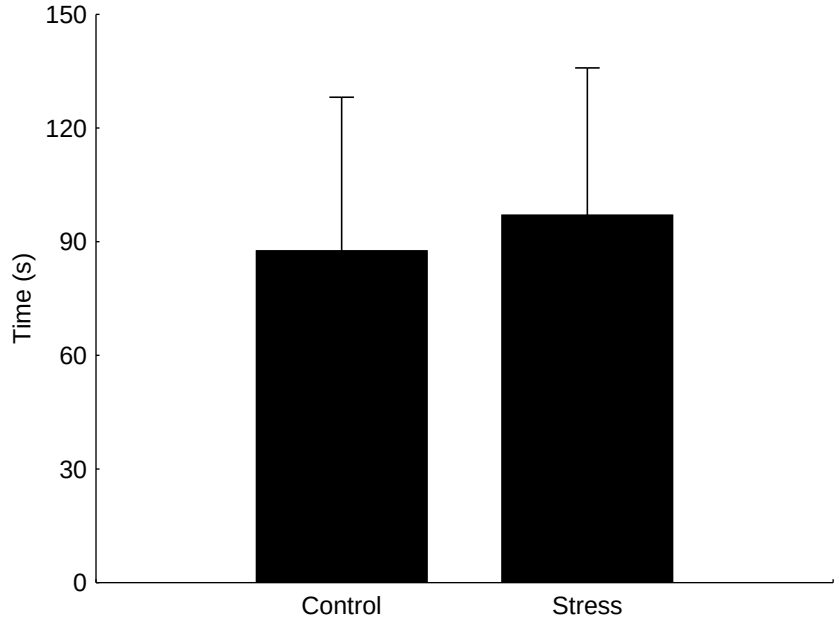


Figure 15: Average time spent in maze open arms for Control and Stress groups. Data displayed as mean ± SD; two-way ANOVA; Control n=11, Stress n=12; p=0.83.

Time spent in closed arms

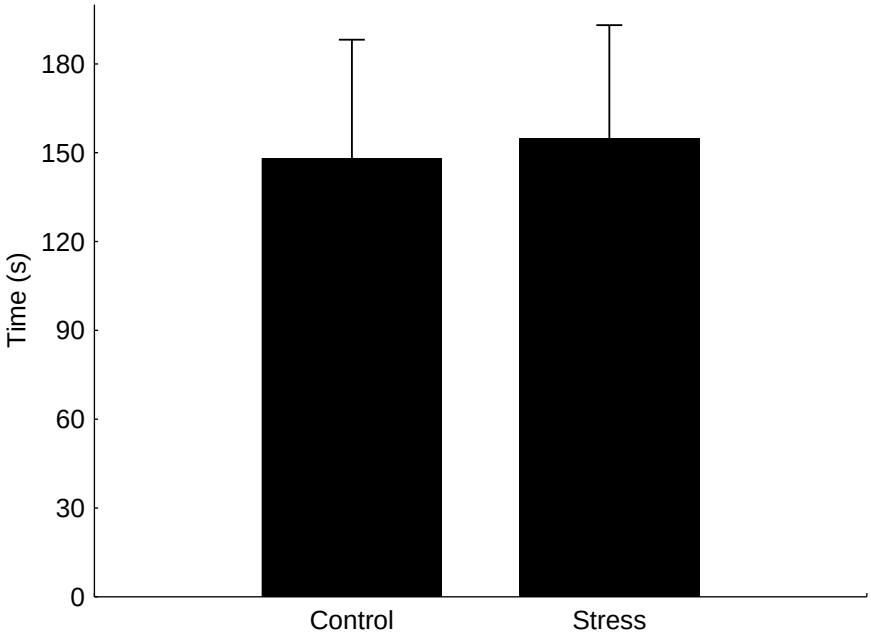


Figure 16: Average time spent in maze closed arms for Control and Stress groups. Data displayed as mean ± SD; two-way ANOVA; Control n=11, Stress n=12; p=0.60.

Entries into open arms

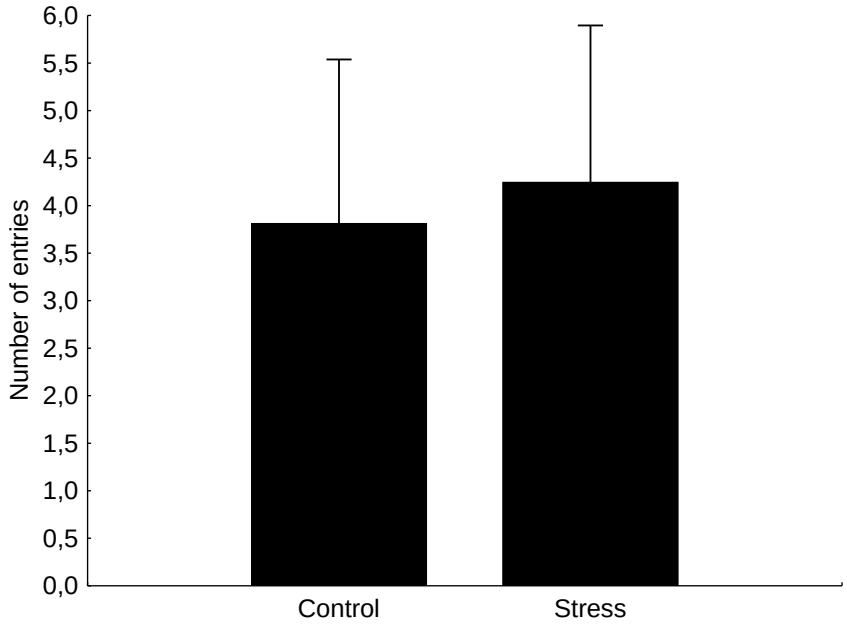


Figure 17: Average entries into maze closed arms for Control and Stress groups. Data displayed as mean ± SD; two-way ANOVA; Control n=11, Stress n=12; p=0.98.

Entries into closed arms

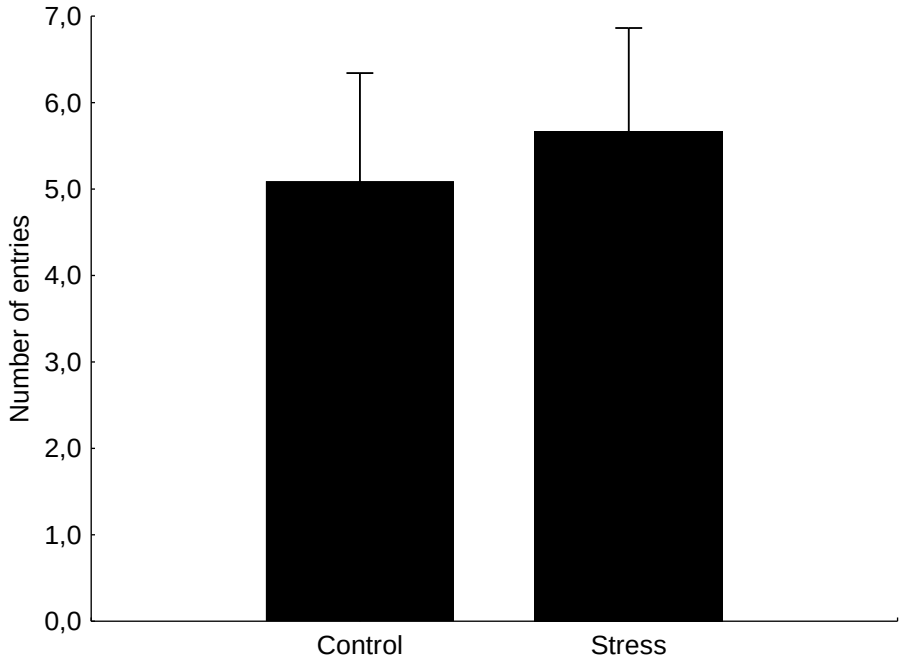


Figure 18: Average entries into maze closed arms for Control and Stress groups. Data displayed as mean ± SD; two-way ANOVA; Control n=11, Stress n=12; p=0.30.

Arm entry attempts

The attempted entries into each arm are recorded as an additional indication of the approach-avoid conflict and hesitancy to act observed in the maze. There was no recorded significant difference between the number of attempted entries between the Control group and the Stress group.

Attempts into open arms

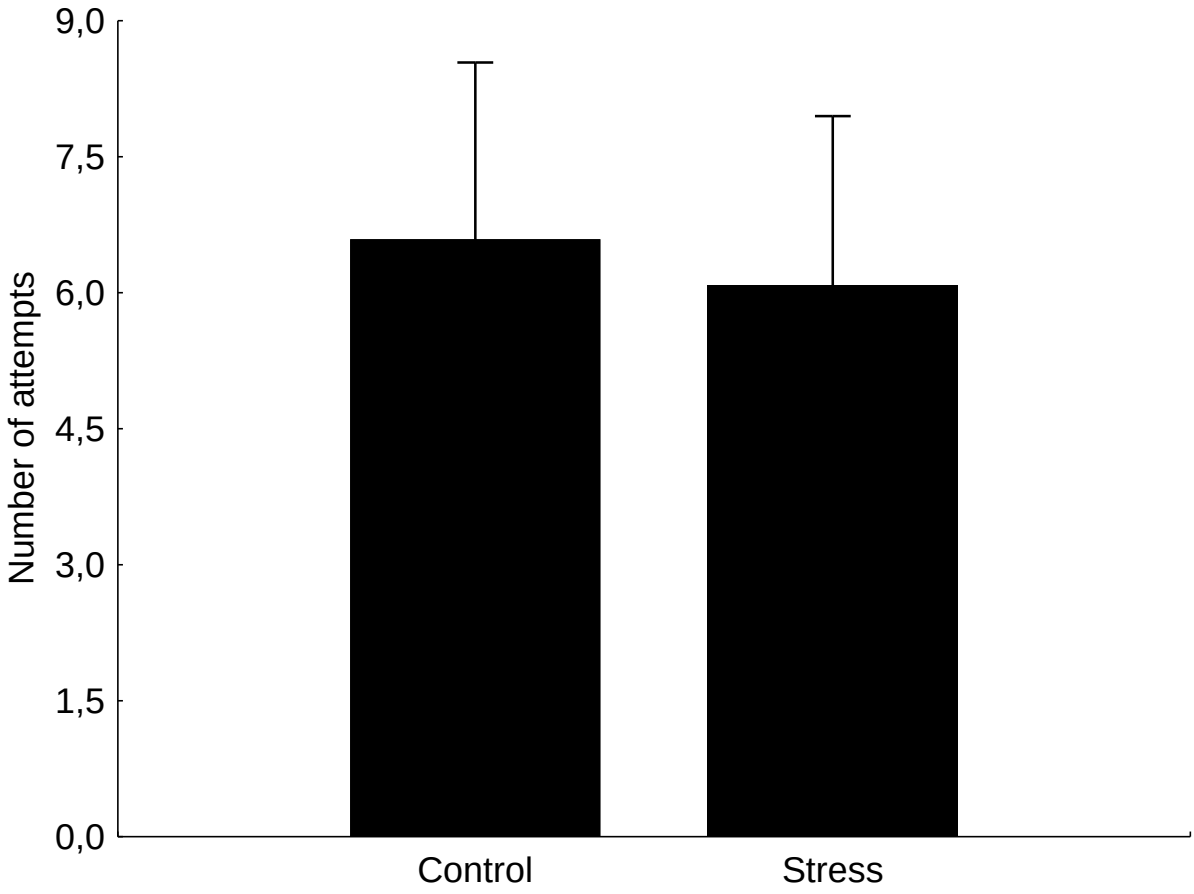


Figure 19: Average number of attempts into maze open arms for Control and Stress groups. Data displayed as mean ± SD; two-way ANOVA; Control n=11, Stress n=12; p=0.58.

Attempts into closed arms

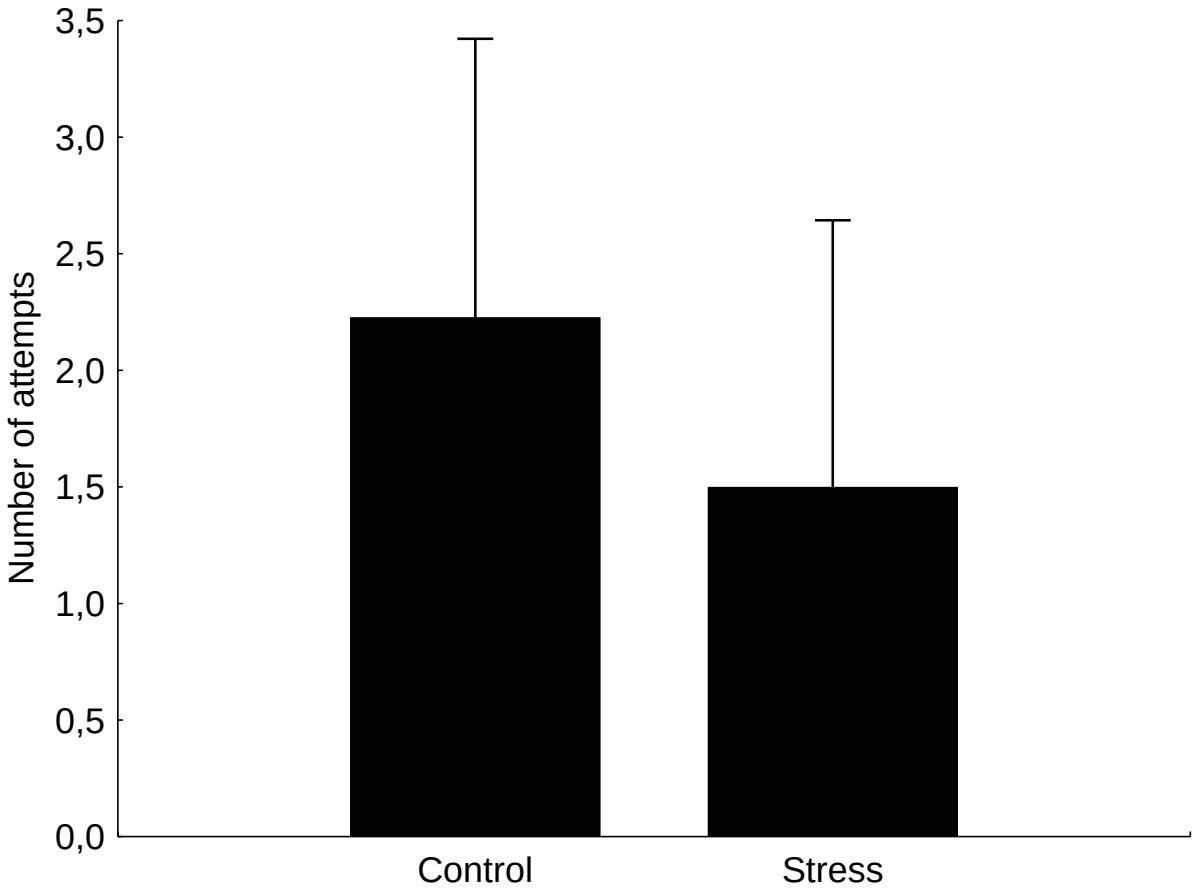


Figure 20: Average number of attempts into maze closed arms for Control and Stress groups. Data displayed as mean ± SD; two-way ANOVA; Control n=11, Stress n=12; p=0.85.

Ethological parameters such as number of rears, head dips and stretch-attend postures were also recorded during the course of the EPM test. There were no significant differences between groups for rears and head-dips. However, the Stress group showed a significant increase in the number of stretch-attend postures compared to the Control group (p=0.05) (Figure 23).

Rears

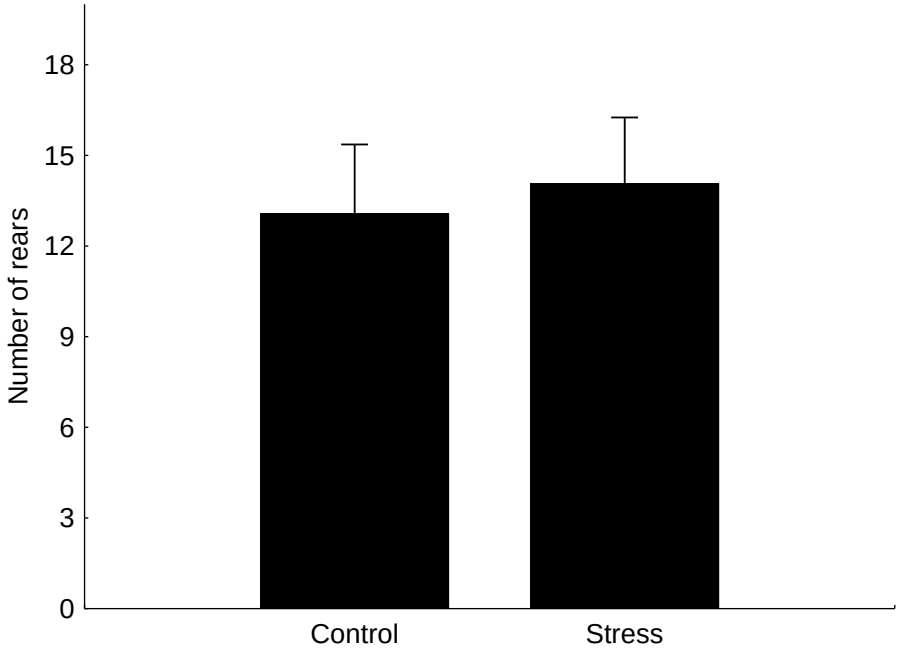


Figure 21: Average number of rears compared for Control and Stress groups. Data displayed as mean ± SD; two-way ANOVA; Control n=11, Stress n=12; p=0.83.

Head dips

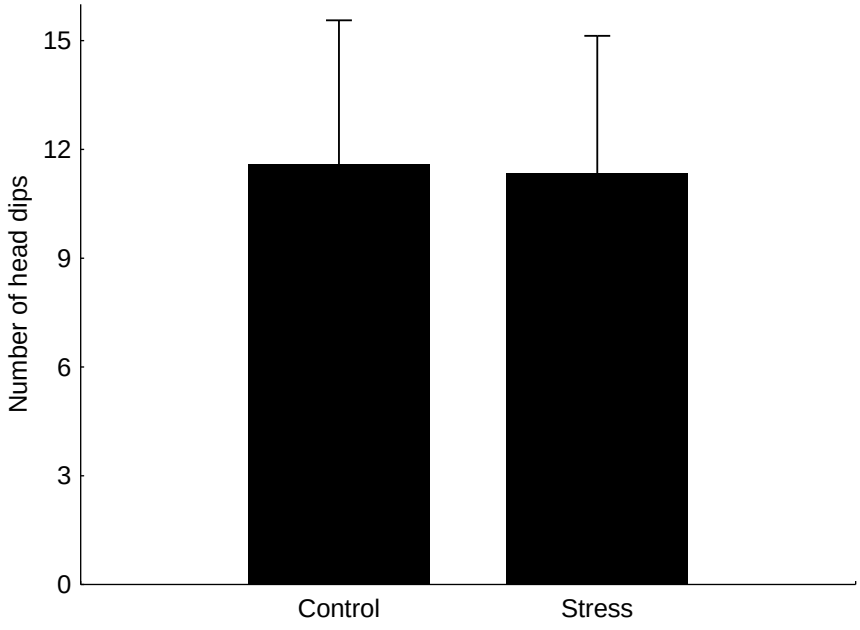


Figure 22: Average number of head dips compared for Control and Stress groups. Data displayed as mean ± SD; two-way ANOVA; Control n=11, Stress n=12; p=0.73.

Stretch-attend postures

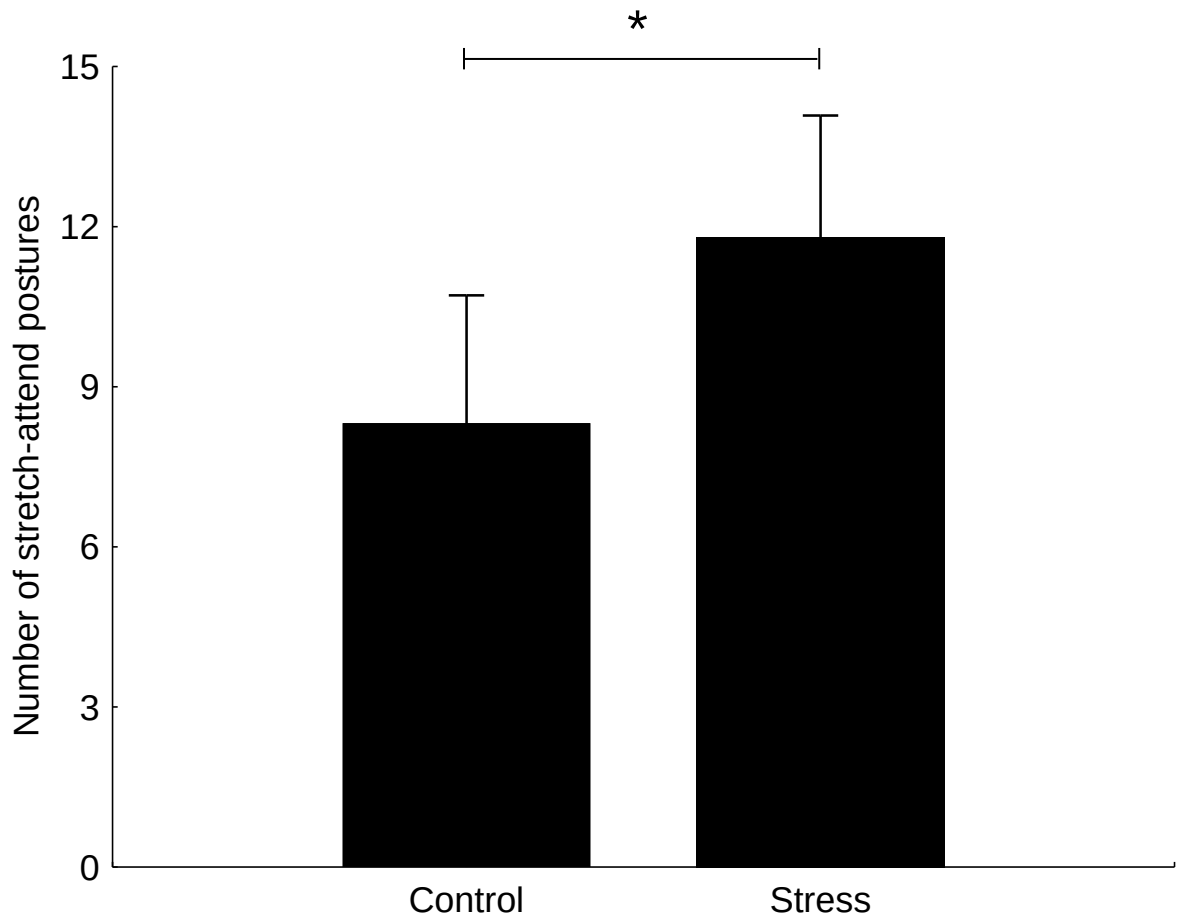


Figure 23: Average number of stretch-attend postures compared for Control and Stress groups. Data displayed as mean ± SD; two-way ANOVA; Control n=11, Stress n=12; p=0.05.

B. Criteria for re-classification of experimental groups

Based on findings from previous rounds of this study, as well as literature that supports individuality in stress responses, the data were next divided into different subgroups. Here the experimental Stressed group was subdivided into ‘’Stress susceptible’’ and ‘’Stress resilient rats’’, based on a set of criteria developed by our research team.

The criteria centered on the idea of considering each rat as possessing a unique “profile” (based on our behavioral and physiological tests) which could then be classed as either stress susceptibility or stress resilience. The criteria first considered molecular analyses, thus their plasma ACTH and corticosterone levels. The ACTH levels were regarded as carrying slightly more weight. This was decided as there was increased confidence in the interpretation of the results of the ACTH ELISA kit compared to that of the corticosterone ELISA kit. This decision rested on the fact that the same kit was used for the plasma corticosterone measurements as was used for the hair corticosterone analysis. As the results of the plasma corticosterone were also difficult to interpret, they were deemed less trustworthy than the ACTH results. The criteria then considered the rat’s behavioral responses to stress, namely their performances in both the EPM test and SPT. Again, the EPM test results carried slightly more weight than the SPT result, as there were fewer confounding variables during the EPM test and higher confidence in the test itself, based on a review of the literature surrounding both tests. As will be discussed in the following section, the EPM is viewed as a more validated test than the SPT.

Where there was uncertainty (e.g., increased biological response with a mismatched behavioral response), broader behavioral characteristics such as ethological EPM measures, and aggressive/escapist behavior were also included. The necessary considerations were made to determine whether the rats’ behavior represented the profile of a stressed rat or a non-stressed rat. The ‘’perfect’’ Stress susceptible rat would therefore be expected to display a) increased corticosterone and ACTH levels, b) more time spent in the closed arms of the EPM than in the open, and c) a general decrease in sucrose preference over the 10 weeks stress protocol. Stressed rats would also be more prone to aggressive behaviors such as piloerection. In contrast, a Stress resilient rat would typically exhibit opposite results.

C. Control vs Stress susceptible vs Stress resilient

3.4 General measurements

a. Food consumption

Although the significant difference between Stress and Control groups is now lost with the new sub-division, it is clear that such significance occurred due to the Stress susceptible group exhibiting increased food consumption versus the other two groups (Figure 24). The p-value indicates that there was no interaction between time and groups.

Figure 24: Average percentage food consumption for Control, Stress resilient and Stress susceptible groups. Data displayed as mean ± SD; repeated measures, mixed model ANOVA in R; n=14; p=0.77.

b. Organ weights

Left adrenal glands

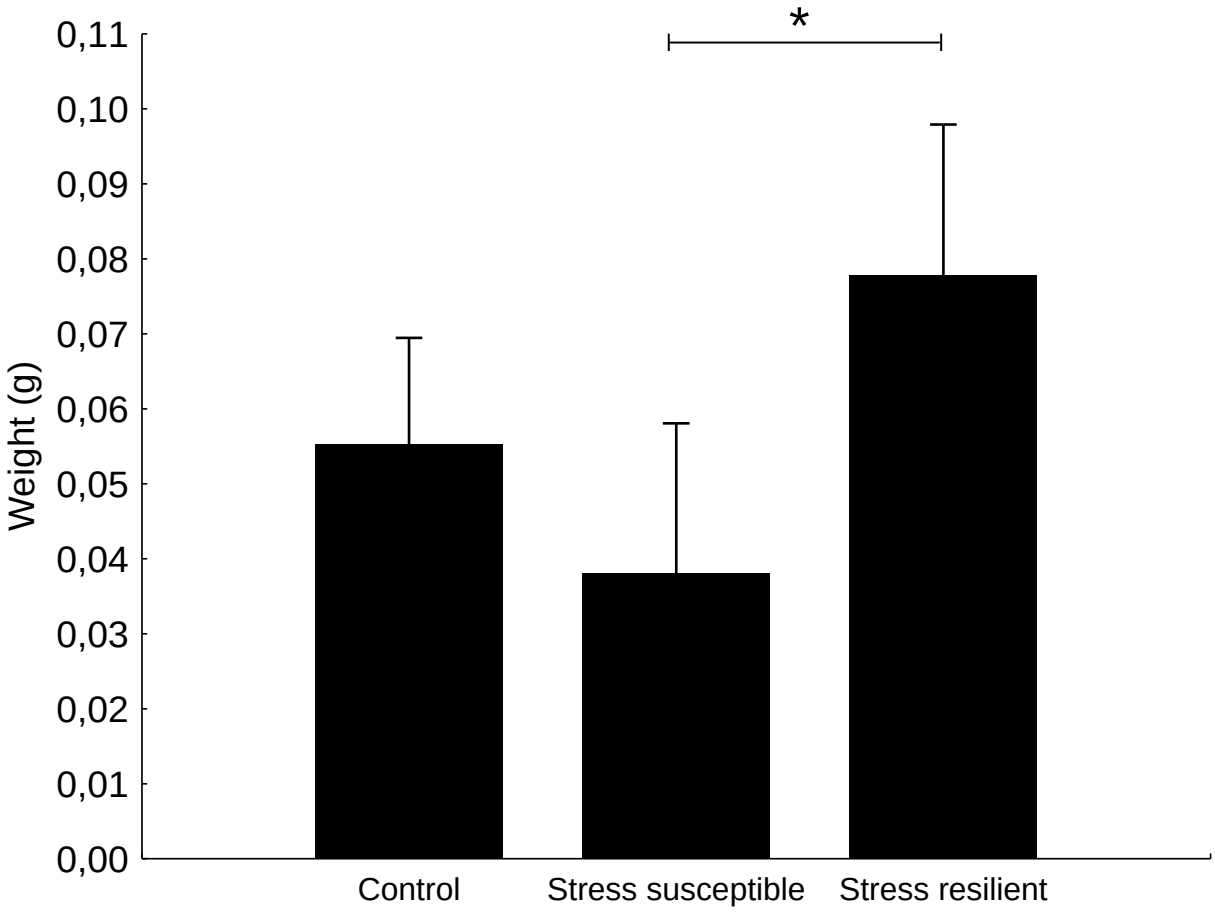


Figure 25: Average left adrenal gland weights compared between Control, Stress susceptible and Stress resilient groups. Data displayed as mean ± SD; two-way ANOVA; Control n=14, Stress susceptible n=7, Stress resilient n=7; *p=0.01.

As observed in Figure 25, the Stress resilient group showed significantly larger left adrenal gland weights than the Stress susceptible group. Although not statistically significant, the Stress susceptible rats also displayed decreased left adrenal gland weights compared to the Control group.

3.5 Molecular analyses

a. ACTH

A significant difference was observed between the ACTH levels of the Stress susceptible and Stress resilient groups, as seen in Figure 26.

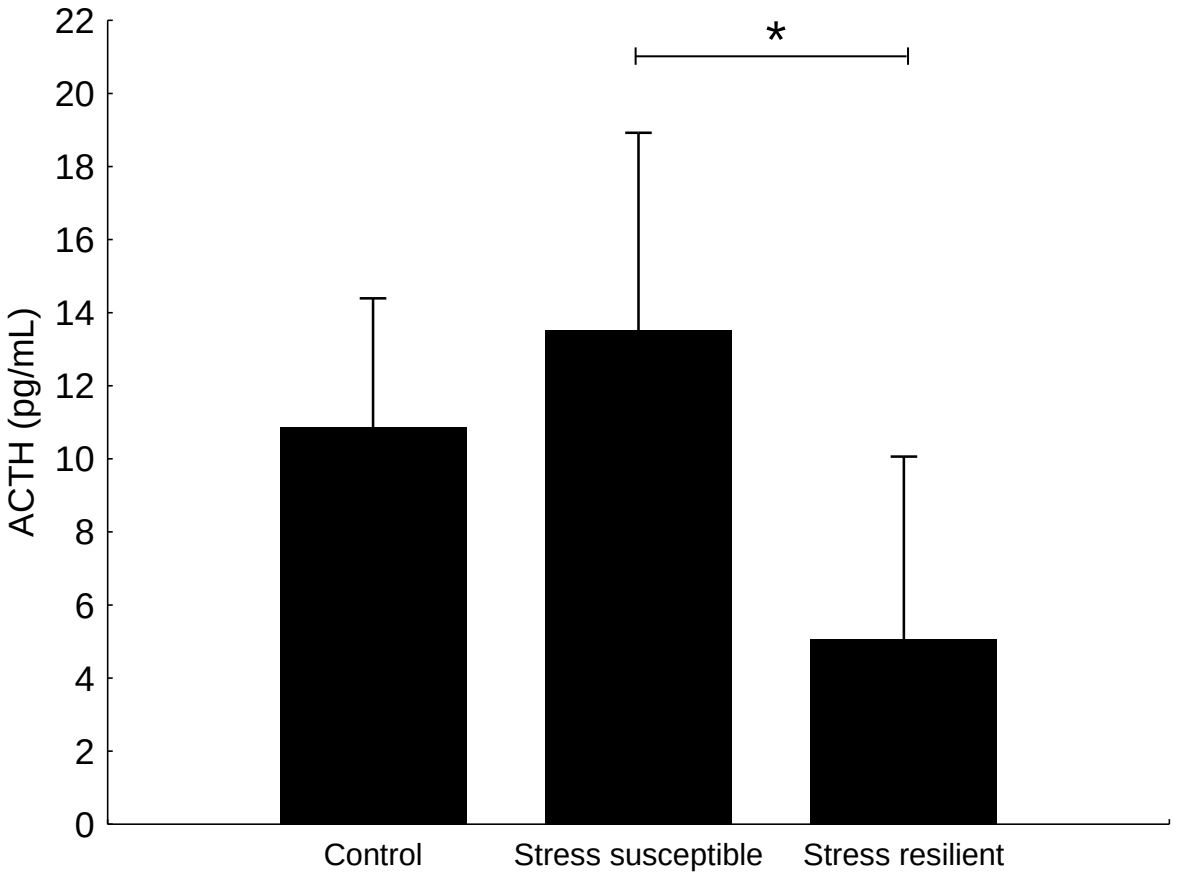


Figure 26: Plasma ACTH levels compared between Control, Stress susceptible and Stress resilient groups. Data displayed as mean ± SD; two-way ANOVA; Control n=14, Stress susceptible n=7, Stress resilient n=7; *p=0.03.

3.6 Behavioral validation methods

a. Behavioral monitoring

Although most behavioral parameters remained the same following the new sub-division, statistical significance emerged in terms of the rearing behaviors of the rats (Figure 27). The significant differences between groups remained for piloerection (Figure 28). In both cases the p-value indicates a significant interaction across time,

meaning that all three groups showed a significant increase in either rearing or piloerection over the course of 10 weeks.

Rearing

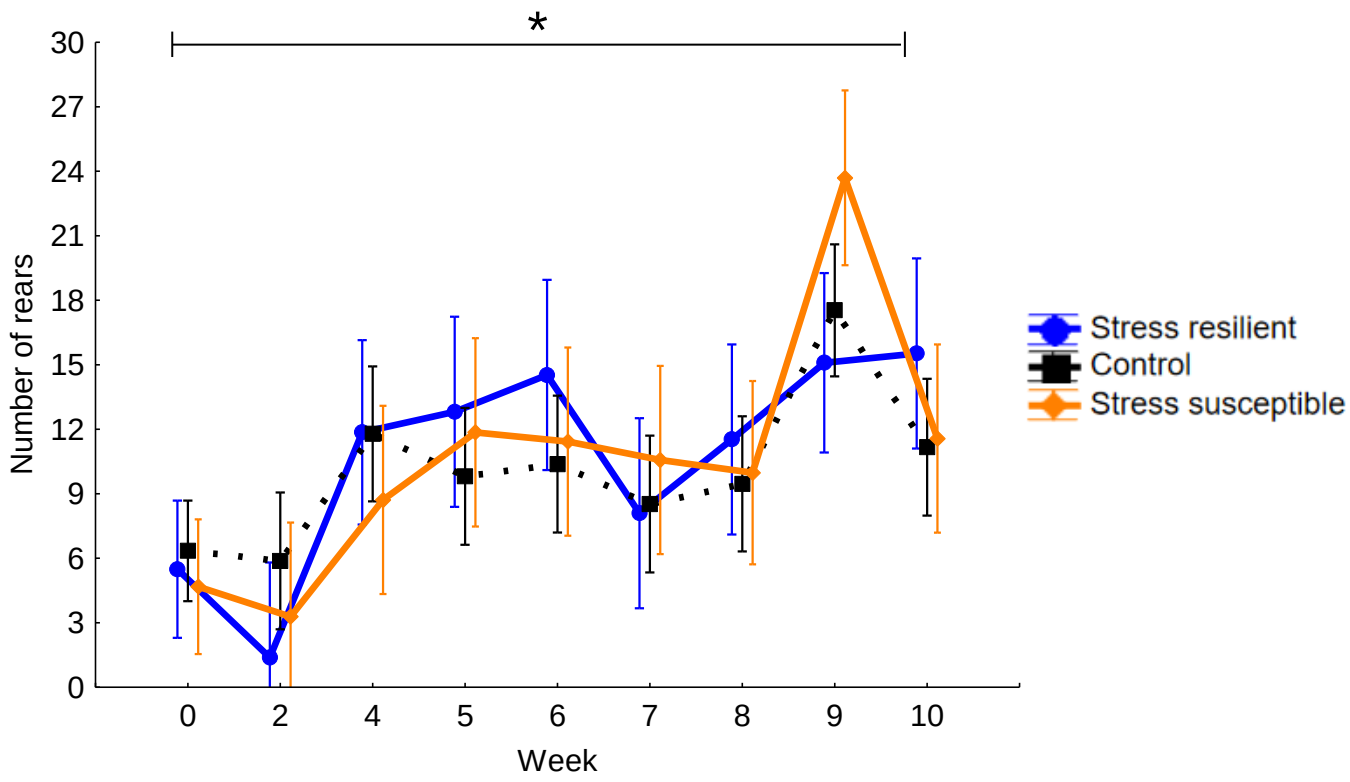


Figure 27: Comparison of average number of rears recorded per session, compared between Control (black), Stress susceptible (orange) and Stress resilient (blue) groups over time. Data displayed as mean ± SD; repeated measures, mixed model ANOVA in R; Control n=14, Stress susceptible n=7, Stress resilient n=7; p=0.02.

Piloerection

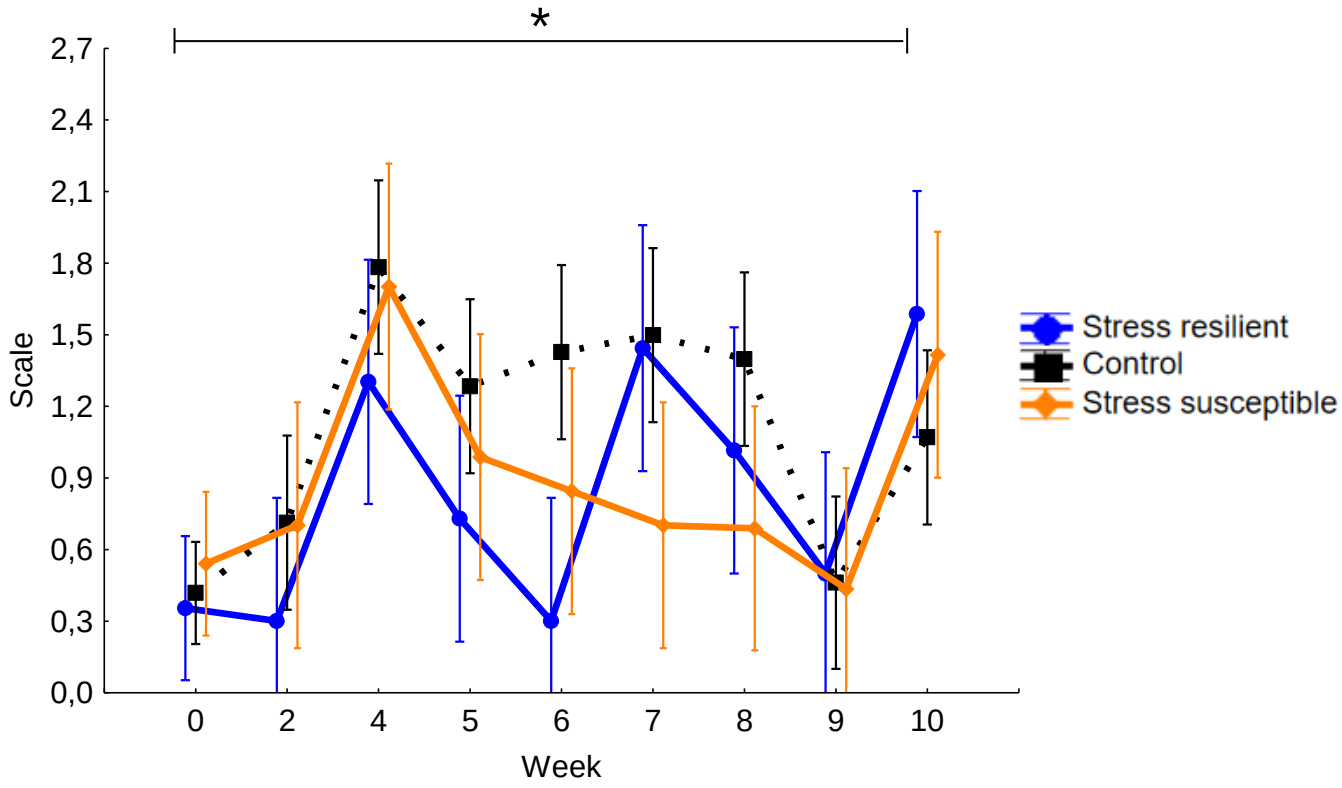


Figure 28: Comparison of average piloerection score recorded per session, compared between Control (black), Stress susceptible (orange) and Stress resilient (blue) groups over time. Data displayed as mean ± SD; repeated measures, mixed model ANOVA in R; Control n=14, Stress susceptible n=7, Stress resilient n=7; $p<0.01$.

b. Sucrose preference test

The statistically significant differences observed between the Control and Stress groups weakened when sub-dividing into Stress susceptible and Stress resilient groups. Here we found a p-value of 0.06 (Figure 29), indicating a near significant interaction between group and time. Therefore, the sucrose preference for the groups showed some increase over time.

The amount of sucrose consumed per gram of body weight did not show any significant changes between groups, or over time for individual groups.

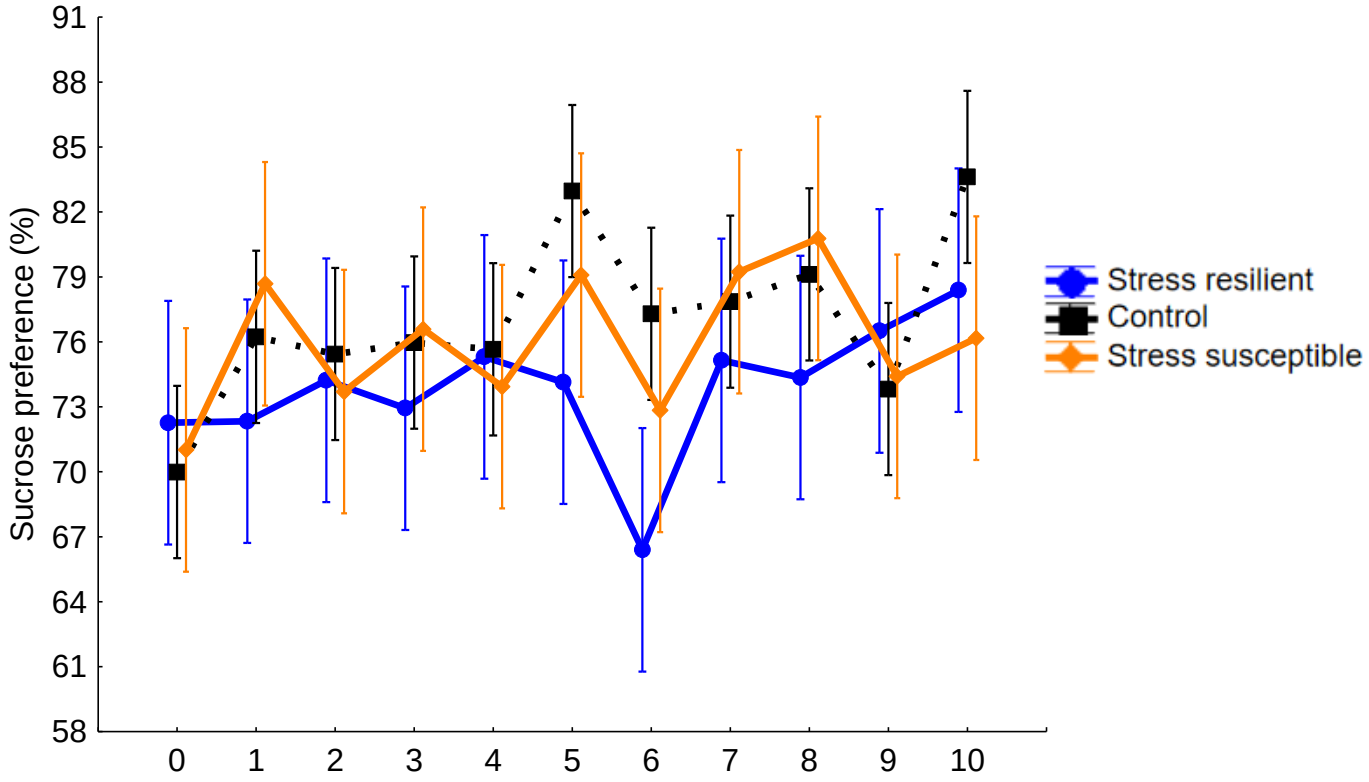


Figure 29: Comparison of average number of rears recorded per session, compared between Control (black), Stress susceptible (orange) and Stress resilient (blue) groups over time. Data displayed as mean ± SD; repeated measures, mixed model ANOVA in R; Control n=14, Stress susceptible n=7, Stress resilient n=7; p=0.06.

c. Elevated plus maze

Significant differences were observed in various EPM parameters following the sub-division. Our results show that the Stress resilient group spent significantly more time

in the open arms than both the control and Stress susceptible groups (Figure 30), whereas the Stress susceptible group spent significantly more time in the closed arms (Figure 31) compared to both the other groups. There were significant differences between all groups regarding entries into open arms (Figure 32), as well as attempted entries into open arms (Figure 33). The Stress susceptible group also displayed significantly more stretch-attend postures than the control group (Figure 34).

Time spent in open arms

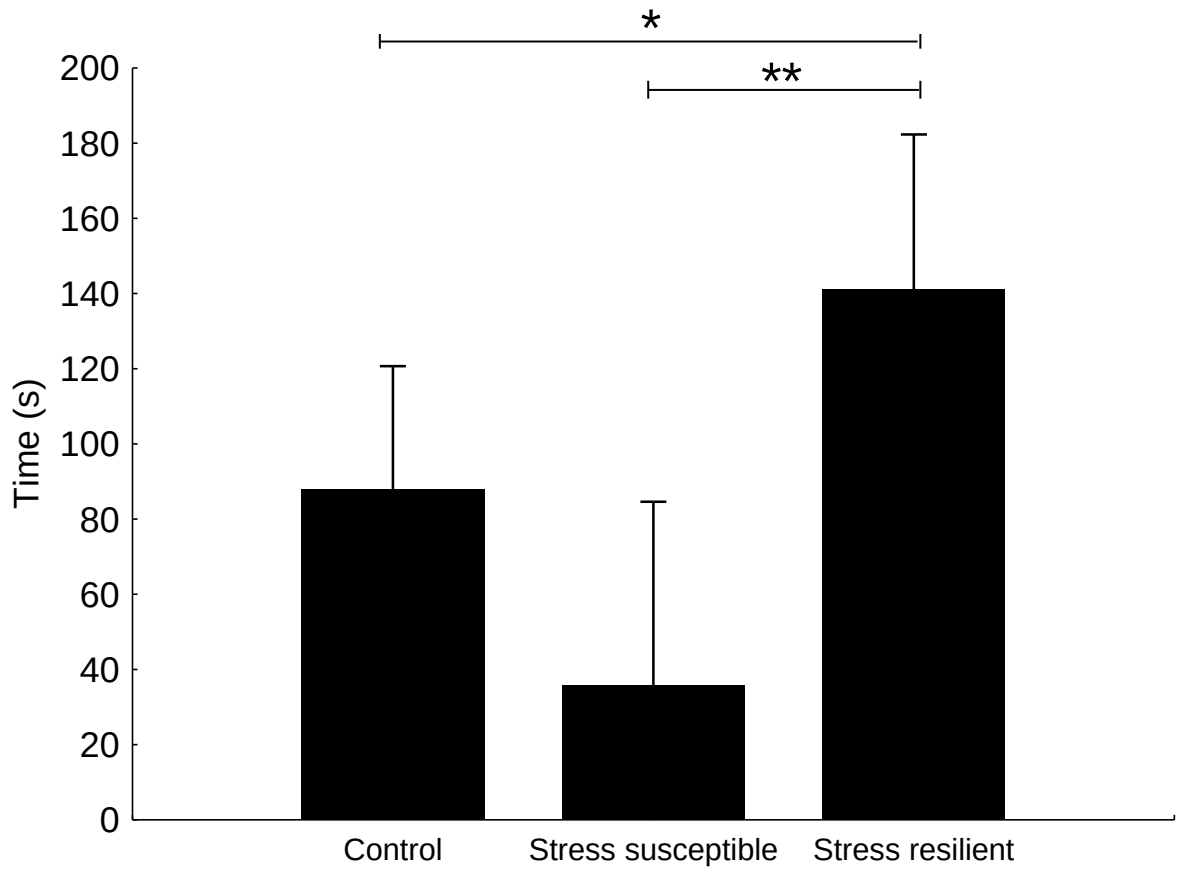


Figure 30: Average time spent in maze open arms compared between Control, Stress susceptible and Stress resilient groups. Data displayed as mean ± SD; two-way ANOVA; Control n=11, Stress resilient n=7, Stress susceptible n=5; *p<0.05, **p<0.01.

Time spent in closed arms

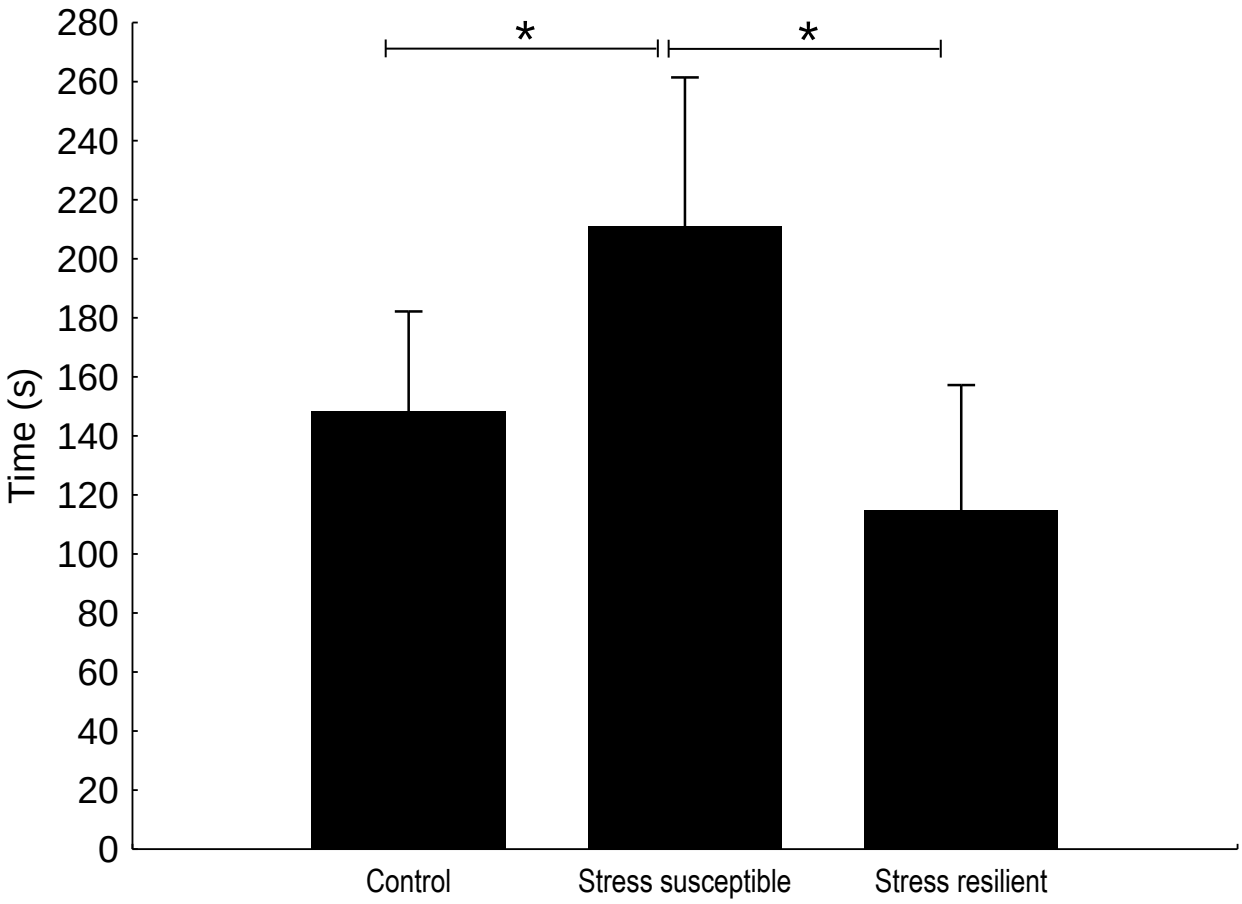


Figure 31: Average time spent in maze closed arms compared between Control, Stress susceptible and Stress resilient groups. Data displayed as mean ± SD; two-way ANOVA; Control n=11, Stress resilient n=7, Stress susceptible n=5; *p<0.05.

Entries into open arms

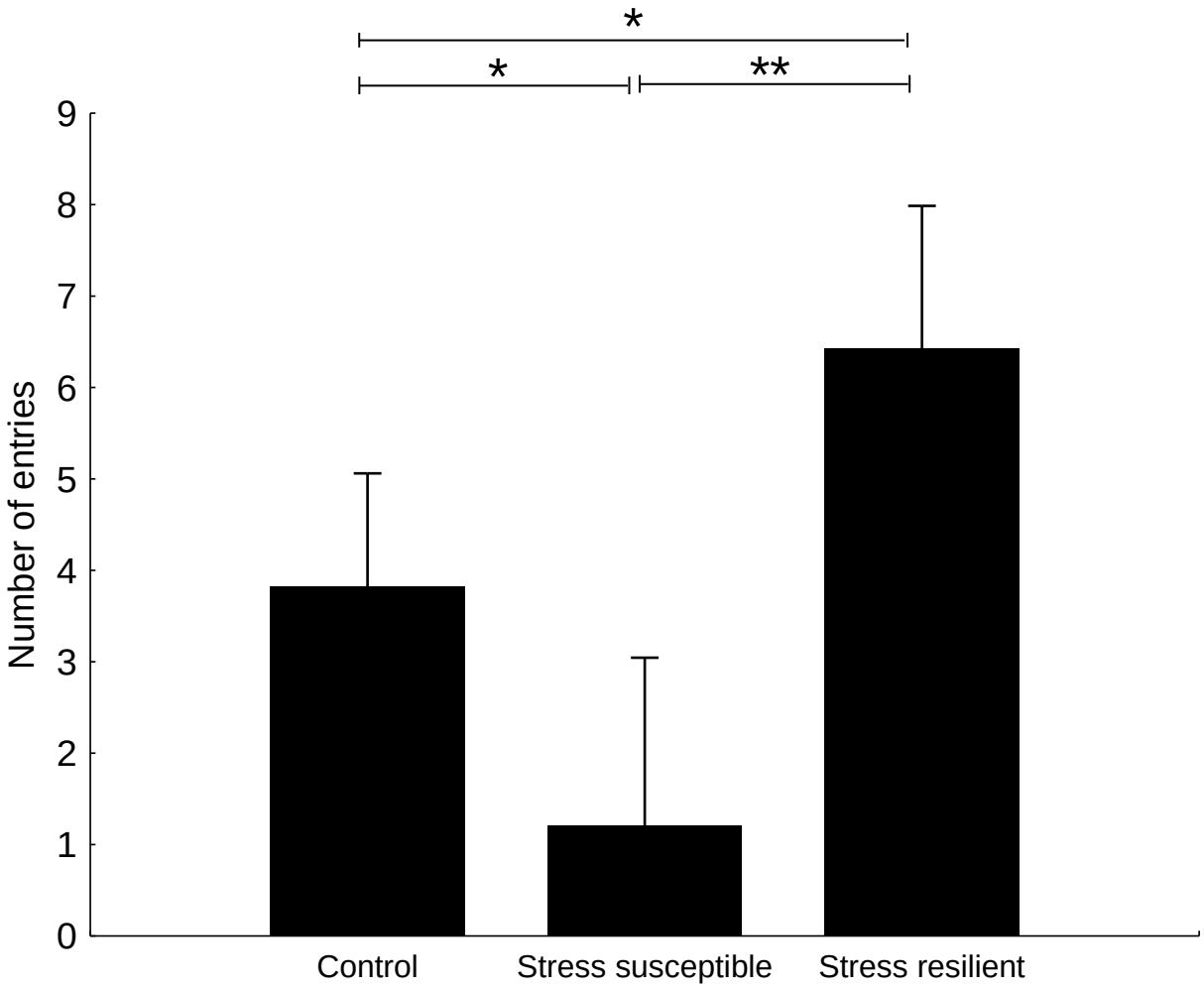


Figure 32: Average number of entries into open arms compared between Control, Stress susceptible and Stress resilient groups. Data displayed as mean ± SD; two-way ANOVA; Control n=11, Stress resilient n=7, Stress susceptible n=5; *p<0.05, **p<0.01.

Attempts into open arms

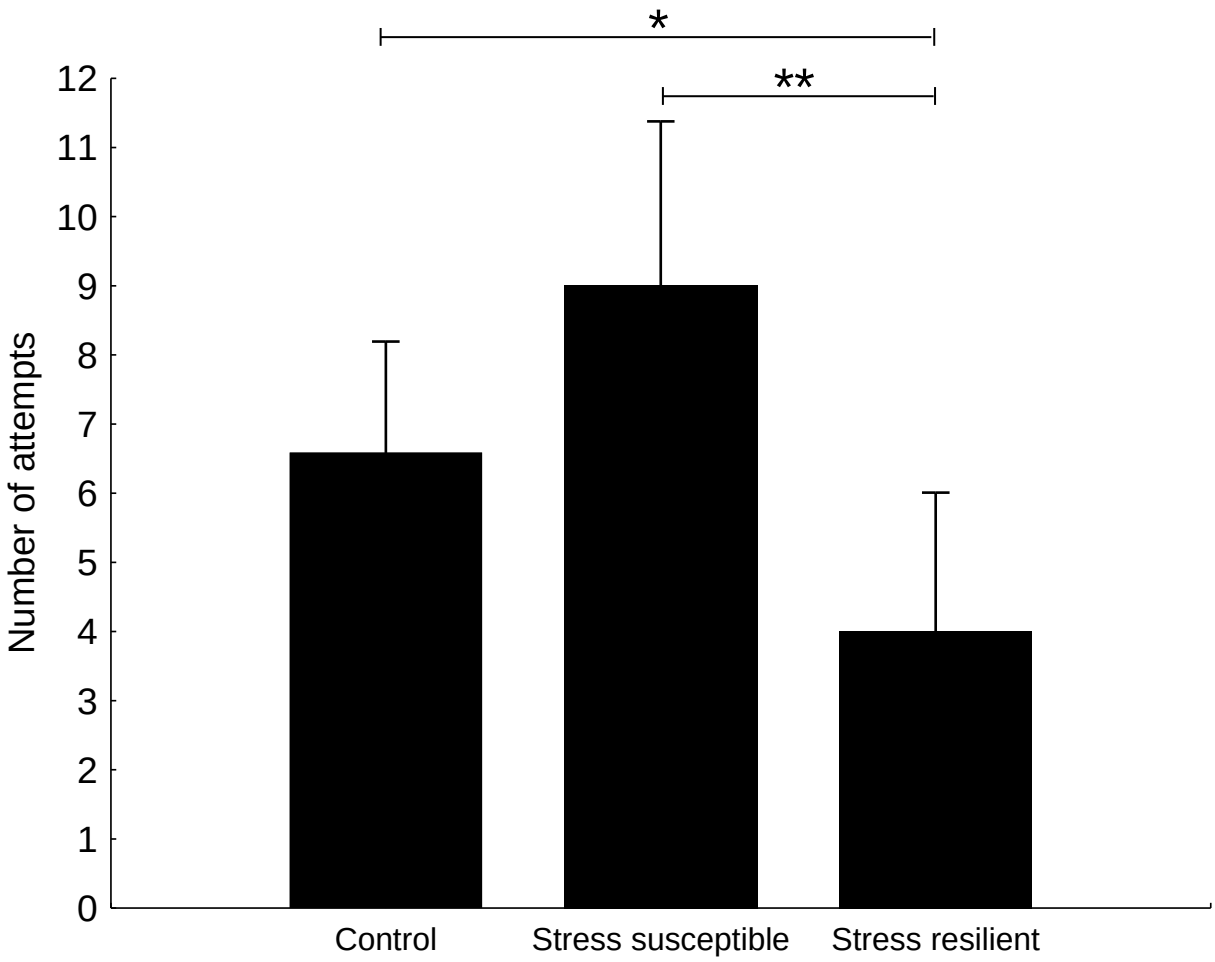


Figure 33: Average number of attempted entries into open arms compared between Control, Stress susceptible and Stress resilient groups. Data displayed as mean ± SD; two-way ANOVA; Control n=11, Stress resilient n=7, Stress susceptible n=5; *p<0.05, **p<0.01.

Stretch-attend postures

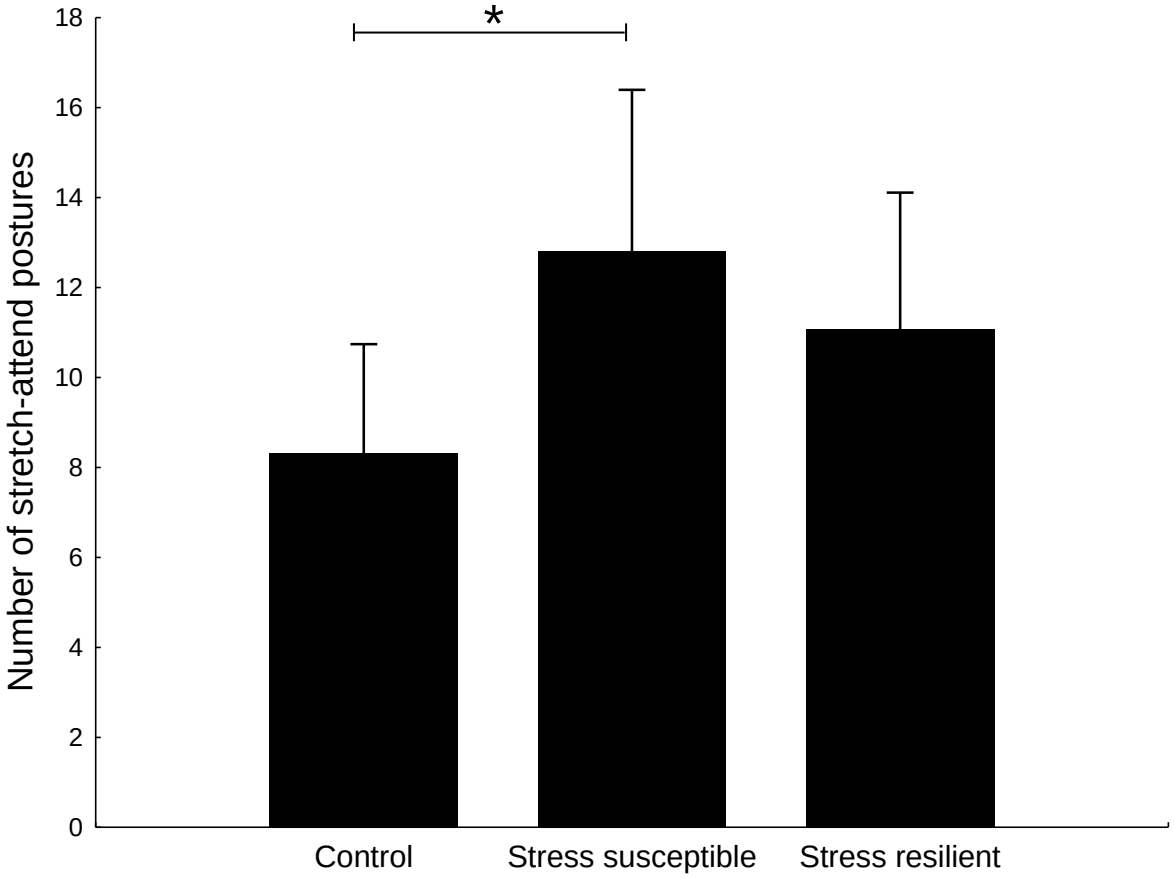


Figure 34: Average number of stretch-attend postures, compared between Control, Stress susceptible and Stress resilient groups. Data displayed as mean ± SD; two-way ANOVA; Control n=11, Stress resilient n=7, Stress susceptible n=5; *p<0.05.

4. DISCUSSION

This study forms part of a larger research project aimed at establishing the rat UCMS model in a South African laboratory, with the eventual aim to investigate underlying mechanisms driving stress-mediated CVD onset and progression. The specific goal of this thesis was to establish the UCMS model and to validate this by various behavioral and physiological tests. Of note, our aim was not to replicate a specific psychiatric disorder such as depression and/or anxiety, but to rather evaluate to what extent a state of chronic stress was induced.

The issue of susceptible and resilient rats

Collectively, the data revealed relatively limited behavioral and physiological changes between the control and stress rats after 10 weeks of chronic exposure to unpredictable mild stressors. However, a major finding was that two distinct groups, i.e. Stress susceptible and Stress resilient, could be distinguished. There is evidence in the literature to support the identification of such groups. For example, some classified experimental groups in both animal and human studies either as responders or non-responders based on cortisol levels (Dimitrov, Demin, Fehlner, Walter, Erk & Veer, 2018; Haeffel & Vargas, 2011; Henningsen, Palmfeldt, Christiansen, Baiges, Bak *et al.*, 2012; Hjemdal, Vogel, Solem, Hagen & Stiles, 2011), or as susceptible or resilient according to the extent of anhedonia (Yang, Fang, Zhan, Huang, Li *et al.*, 2019). Likewise, the CMS model reported the persistent development of stress-sensitive and stress-resistant rats with regard to the development of anhedonia (Bergström *et al.*, 2008).

Mechanisms implicated in stress resilience

The differences between the individual responses to chronic stress exposure can be attributed to resilience/adaptability to stressors. In such cases, Stress susceptible rats can be regarded as lacking the necessary coping mechanisms to adjust to chronic stress, while the resilient rats can successfully adapt and restore whole body homeostasis. Our data revealed a significant decrease in plasma ACTH levels of the Stress resilient group versus the Stress susceptible group ($p=0.03$), with near significance when compared to the controls ($p=0.06$). This supports the notion that the Stress resilient rats indeed experienced stress but possessed or developed a

protective mechanism(s) that was absent in the Stress susceptible rats. This theory is supported by Bergström *et al.* (2008), who indicated that mechanism is likely secondary to HPA axis effects, or entirely unrelated. They also proposed that this phenomenon may be explained by increased brain-derived neurotrophic factor (BDNF) levels as this modulator is known to exert neuroprotective effects (Bergström *et al.*, 2008). Moreover, chronic stress can lead to decreased hippocampal BDNF levels that is associated with depression (Southwick *et al.*, 2005). Vialou *et al.* (2010) also investigated the nucleus accumbens (NAc) (the brain reward region) as a putative molecular basis to help explain the phenomenon of stress resilience. Here they found that increased levels of protein FosB, a NAc transcription factor, are associated with adaption and therefore provided some of the first molecular evidence to help explain the notion of stress resilience (Vialou *et al.*, 2010).

However, the theory that supports BDNF as protective factor has been disputed as there are studies showing that several NAc proteins (including BDNF) are associated with depressive behaviors after exposure to social defeat stress (Berton *et al.*, 2006). For example, a murine study employing a social defeat stress protocol found that increased BDNF levels were only displayed by Stress susceptible mice, with a nearly 90% increase versus controls (Krishnan *et al.*, 2007). These results therefore posit that BDNF induction, and the effects thereof, act as mediators to social defeat-induced stress avoidance instead of fulfilling a protective role in the brain (Krishnan *et al.*, 2007). The theories surrounding BDNF illustrate the complexity of such responses, i.e. that a molecule is neither entirely protective nor damaging and that its functional role depends on the context. Further neurobiological studies into the relationship of chronic stress and BDNF are required to provide more insights regarding the underlying mechanisms.

As discussed, stress-susceptible individuals may lack the necessary adaptation response and may therefore be more affected by chronic stress protocols. In this case, chronic stress would lead to the dysregulation or exhaustion of various systems, impairing the body's ability to appropriately respond to stress (Beckie, 2012). For example, some found that a continually upregulated stress response can interfere with the synthesis and functioning of adrenal hormones and result in GC dysfunction (Hannibal *et al.*, 2014; He *et al.*, 2017). This can happen in various ways as the body either fails to synthesize the necessary GC levels or is unable to appropriately respond

to GC secretion (Hannibal *et al.*, 2014). Such dysfunction can also be caused by GR insensitivity, downregulated GR expression in various systems, or an overactive negative feedback system (McEwen, 2008). This subsequently leads to hypoactivation of the HPA and SAM pathways, potentially explaining the lack of stress hormone upregulation in the susceptible group.

Potential adrenal implications in stress susceptibility

It is our opinion that the changes in left adrenal gland weights together with the ACTH data found in this study support the above-mentioned theories. Here the Stress resilient group displayed increased left adrenal gland weights compared to the Stress susceptible group ($p=0.03$). Moreover, the Stress susceptible group exhibited slightly decreased weights compared to control rats, although not statistically significant. These results are comparable due to the similar growth rates across all groups and can be interpreted as increased HPA axis/SAM pathway activity in the Stress resilient rats. Moreover, it may also indicate a lack of adrenal activation in such rats.

Adrenal fatigue/insufficiency is a well-established occurrence where the adrenal gland is incapable of maintaining the necessary GC secretion, a well-described characteristic of individuals facing “burnout” (Charmandari *et al.*, 2014). Burnout was initially described as the emotional exhaustion and depersonalization of an individual and is linked to disorders such as depression (Grossi *et al.*, 2003). Although the physiological effects of burnout as well as the contributions of chronic stress in this regard are not well-studied, adrenal insufficiency has been implicated in this instance. Here Reber *et al.* (2007) investigated the effects of a chronic psychosocial stress protocol on adrenal function and concluded that adrenal cells became insufficient and failed to produce the necessary GCs to cope with a prolonged stress exposure (Reber *et al.*, 2007). Additionally, a study done on women in the public sector reported lower cortisol levels in patients suffering from burnout as HPA axis insufficiency is frequently associated with this condition (Grossi *et al.*, 2003).

Together our results for the Stress susceptible group (unchanged corticosterone levels, low adrenal weights) support the theories discussed and we propose that these rats likely experienced HPA axis exhaustion due to a lack of stress adaptability. By contrast, we propose that the Stress resilient rats possessed more effective adaptive coping mechanisms and/or have an innate resilience to stress and would therefore

take longer to show the effects caused by chronic stress exposure. Such interesting observations require further laboratory investigations to confirm whether this is indeed the case.

Behavioral considerations and the validity of tests used

A common theme noted in many studies subdividing experimentally stressed groups is the use of a single parameter such as sucrose preference to classify rats as sensitive or resistant to the effects of stress. However, for the present study we adopted a unique approach to divide the experimental group into two subgroups by considering *both* physiological and ethological parameters. We reasoned that a classification not focused on using merely markers of physiologic dysregulation (e.g. increased corticosterone), or symptoms of a specific psychiatric disorder (e.g. anhedonia as a symptom of depression), should provide a better assessment regarding the overall well-being of the rats.

In keeping with this approach, different behavioral studies were conducted over the course of the experiment, most notably the SPT and EPM tests. General rat behavior was also assessed once per week and delivered supporting results, such as increased rearing over the course of 10 weeks ($p=0.02$) when examining the statistical interaction between group and time. This was the case for all groups (control, resilient and susceptible) and could be indicative of a gradual acclimatization effect to the observation procedure and increased explorative effects (Lapiz-Bluhm *et al.*, 2008). The rats could also be more willing to explore their surroundings once the novelty of the environment has worn off. However, the results from the piloerection data show that this particular sign of aggression increased over time for all groups as well ($p<0.01$), which could be more indicative of their general mental state, considering that spiky/unkept coats are also a sign of distress in rats (Mutlu *et al.*, 2012). Moreover, aggressive behaviors in rats are associated with increased levels of stress and anxiety-like behaviors, leading to the assumption that the observation procedure caused some form of stress in itself (Patki *et al.*, 2015). A separate behavioral effect of chronic stress pertains to rodent metabolism, and the metabolic influences of an upregulated stress response are well documented (Nieuwenhuizen *et al.*, 2008). Chronic stress has not only been linked to an increase, but also to a decrease in body weight and food consumption (Dallman *et al.*, 2005; Depke *et al.*, 2008). However, due

to certain inconsistencies regarding food sampling (discussed in the Limitations section) rendering the data somewhat unreliable, no such connections could be made between the decline in food consumption and incline in body weights of the rats.

The SPT is one of the most widely used evaluations of anhedonia in stress studies. Despite the fact that the test remains a very popular model validation, its validity as a measure of anhedonia has been questioned in recent years with a major concern being that the test is not easily reproducible. For example, a review study recently revealed that about a quarter of laboratories had difficulties reproducing the chronic stress-induced decrease in sucrose preference (Willner, 2017a). Of these studies, 13% reported that they experienced some difficulties but that the protocol was usually reliable, 8% stated that they encountered difficulties with the SPT (but not with any other test), while 4% could not replicate the effects at all (Willner, 2017a) .

There are a large number of factors that may impact on the reproducibility of the SPT protocol in the context of a chronic stress model, for example sex, strain, and age (Bekris *et al.*, 2005; Willner, 2017a). It can therefore be argued that the SPT is not reliable enough to serve as the gold standard for establishing anhedonia in stress models. This idea is supported by Sequeira-Cordero *et al.* (2019) who concluded (after much experimentation) that the SPT is the weakest test used to assess the effects of a CUS model. They based their conclusion on the results of two experiments that both showed increased, rather than decreased sucrose consumption following their stress protocol (Sequeira-Cordero *et al.*, 2019).

The findings in our study nearly mirror these results as the SPT failed to confirm anhedonia in rats exposed to the UCMS protocol. Our results reveal that the groups started off with similar baseline preferences for sucrose, showing that they were well matched at the start. Neither the Stress susceptible nor Stress resilient rats showed a significant decrease in preference over the course of the stress protocol. However, the control group showed a highly significant increase in sucrose preference ($p<0.01$) and by the end of the protocol exhibited an increased preference compared to the responders ($p=0.03$). The difference between these groups can unfortunately not be attributed to the responders decreasing their preference, as the significance is due to a large spike in control preference in Week 10. The statistical interaction between groups and time revealed a near significant increase ($p=0.06$) in sucrose preference

over all groups, however when analyzing the SPT as sucrose consumed per gram of body weight, no significant differences were observed between groups by the end of the study.

Where the SPT test failed to indicate anhedonia in the rats, the EPM test results showed more conclusive evidence regarding the anxiety-inducing effects elicited by the UCMS model. The EPM is considered a very robust validation of behavior by various authors as it possesses face, construct and predictive validity (File *et al.*, 1994; Lister, 1990; Pellow *et al.*, 1985; Rodgers *et al.*, 1999; Walf *et al.*, 2007). Moreover, the EPM test is as popular as the SPT with a plethora of publications reporting on its implementation and rarely criticizing its reliability (Carobrez *et al.*, 2005). The findings from the present study show a clear distinction between groups, with Stress resilient rats spending significantly more time in the open arms of the maze than the Stress susceptible ($p<0.01$) and control rats ($p=0.05$). In contrast, the Stress susceptible group spent more time in the closed arms than both the Stress resilient ($p=0.01$) and control ($p=0.04$) groups. The Stress resilient group also made more entries into the open arms than both the Stress susceptible ($p<0.01$) and control ($p=0.01$) groups.

The same analysis showed that the opposite is true for the Stress susceptible group, that made fewer entries into open arms than the control group ($p=0.02$). Further strengthening these findings, the Stress susceptible group made more entries into the closed arms than the Stress resilient group ($p=0.05$). These results clearly show the presence of anxiety-like behaviors in the group most affected by chronic stress, where the Stress resilient group displayed increased exploration in the maze. These findings are supported by a recent study that used a social defeat stress protocol and found nearly identical results in their high-susceptible and low-susceptible murine groups (Nasca *et al.*, 2019).

As with the SPT there are many variables that can influence the results of the test, discussed at length in relation to both tests in the literature review. However, as with most validation tests there is no formula that would describe the perfect use of the test and each variable factor needs to be considered with every application. In order to resolve this, reviewers encourage the use of behavioral and ethological validation instead of solely relying on biological or pharmacological proof that the test is effective (Rodgers *et al.*, 1997b). In an attempt to incorporate more ethological parameters into

the EPM test, our study observed head dips, rears, arm entry attempts and stretch-attend postures, indicative of exploration and risk assessment (Schneider *et al.*, 2011).

Our current findings confirmed that the Stress susceptible group displayed more hesitancy when exploring and frequently chose to retreat rather than to move forward, providing evidence of increased risk assessment behaviors (Rodgers *et al.*, 1997a). This was evident from an increased number of stretch-attend postures in the initial combined stress group versus controls ($p=0.05$). Following classification into Stress resilient and Stress susceptible groups, it became clear that this initial difference was attenuated by the Stress susceptible group that exhibited more stretch-attend postures versus controls ($p=0.04$). Additionally, the Stress susceptible group made a higher number of open arm entry attempts compared to the Stress resilient group ($p<0.01$). In contrast, the Stress resilient rats made a lower number of entries compared to the control group ($p=0.05$). Taken together with the traditional EPM parameter results, these data indicate clear anxiety-like profiles for the Stress susceptible rats while the more resilient rats showed increased exploration, less hesitancy and a lack of anxiety-like behaviors.

Of the two well-defined behavioral rests that was performed, we considered the EPM as more reliable to evaluate the effects of a chronic stress model. In support, the literature supports the EPM test as the more reliable test while noting inconsistencies that arise when employing the SPT. However, Liu *et al.* (2018) argued strongly in favor regarding the utility of the SPT and indicated that it is useful for both the CMS and social defeat models (Liu *et al.*, 2018). In contrast, increased criticism led Sequeira-Cordero *et al.* (2019) to posit that this test is not robust enough to be employed as a gold standard to assess the implications of a chronic stress protocol. It is our opinion that the SPT still remains a cost-effective and relatively simple test for anhedonia and should therefore be considered, but only as part of a larger battery of tests to be conducted in order to validate a stress model.

Although the validity of the EPM test is less disputed, it is also not without criticism. For example, some concluded that this test is not reliable in a CUS model as it failed to detect anxiety-like behaviors in rats (Mitra *et al.*, 2005). However, this is not a commonly reported finding and stands in sharp contrast to our own results that showcased the ability of the EPM test to highlight anxiety-like behaviors in Stress

susceptible rats. The popularity of EPM can also easily be explained by the affordability, convenience and simplicity thereof, and that it does not involve harmful or harsh practices (Pellow *et al.*, 1985; Rodgers *et al.*, 1997b). However, there are concerns that the increased usage of this test is due to such practical factors rather than focusing on theoretical considerations (Carobrez *et al.*, 2005). It is for this reason that researchers are encouraged to include various ethological parameters as the use of more complex approaches should increase the sensitivity of the test and its reliability in terms of the overall data generated (Ohl, 2003).

The EPM test has the potential for greatly diverse applications such as the screening of anxiolytic drugs and understanding the biological characteristics behind learning, pain, memory, post-traumatic stress and other similar factors (Carobrez *et al.*, 2005; Walf *et al.*, 2007). Factors such as these make it well-suited to be employed as a validation test for chronic stress models.

UCMS stress model

The findings reported here are the result of a continuation of a study aimed at establishing the UCMS model in our laboratory at Stellenbosch University (Table 4). The goal of the current study was to learn from the previous rounds of experimentation and implement necessary changes to ensure the model yields positive results. This in turn would be expected to lead to mechanistic-type research to further assess links between chronic stress and CVD-related pathology. The previous two attempts of the experiment were conducted by Lukas Olivier and Lucien Sher and relied mainly on biological methods like plasma ACTH and corticosterone measurements to validate the model (Sher, 2019). They performed non-specific behavioral monitoring and assessed rat coat cleanliness on a weekly basis. However, the lack of more specific behavioral testing meant that there was still uncertainty whether the UCMS protocol was effective in creating a state of chronic stress.

Table 4: Comparisons between the first, second and third attempts of the experiment, indicating adjustments made to each protocol.

	Run #1	Run #2	Current attempt
General protocol			
Size of sample groups	n=9	n=12	n=14
Individual housing (all rats)	✓		✓
2 rats per cage for controls		✓	
Housed in same room	✓		✓
Housed in different rooms		✓	
Group division	Matched for age and weight	Age-matched	Matched for age, weight, sucrose preference
Stress protocol			
Number of stressors	6	6	9
Duration of stressors	4-8 hours	4-8 hours	2-8 hours
During of UCMS protocol	8 weeks	8 weeks	10 weeks
Single/multiple stressor(s) per day	Single	Single	Multiple
Stressed during light phase	✓		✓
Stressed during dark phase		✓	
Biological validation			
Plasma corticosterone	✓	✓	✓
Plasma ACTH		✓	✓
Oxidative stress (SOD/NOX)	✓	✓	
Plasma epinephrine		✓	
Behavioral validations			
Monitoring	Coat condition	Coat condition	Detailed monitoring
SPT			✓
EPM			✓

The present study therefore incorporated various behavioral assessments that included the well-established SPT and EPM tests. Here the rationale was that such tests, together with various physiological validation tests, would be sufficient to determine whether the UCMS was successfully established in our laboratory. Furthermore, we now housed the rats individually in cages, where previously the control rats were housed in pairs. While social isolation is considered a stressor in itself (due to tests employed), individual housing was deemed necessary to obtain the results of this study, as it was part of a larger study that originally required individual fecal sampling (Boggiano *et al.*, 2008).

Thus, greater precaution was taken to ensure that the effects of the stress exposure in the experimental group were not transferred to the control group (Castelhano-Carlos *et al.*, 2009). The experimental group was therefore isolated in a separate room for 2-4 hours following the stress exposure.

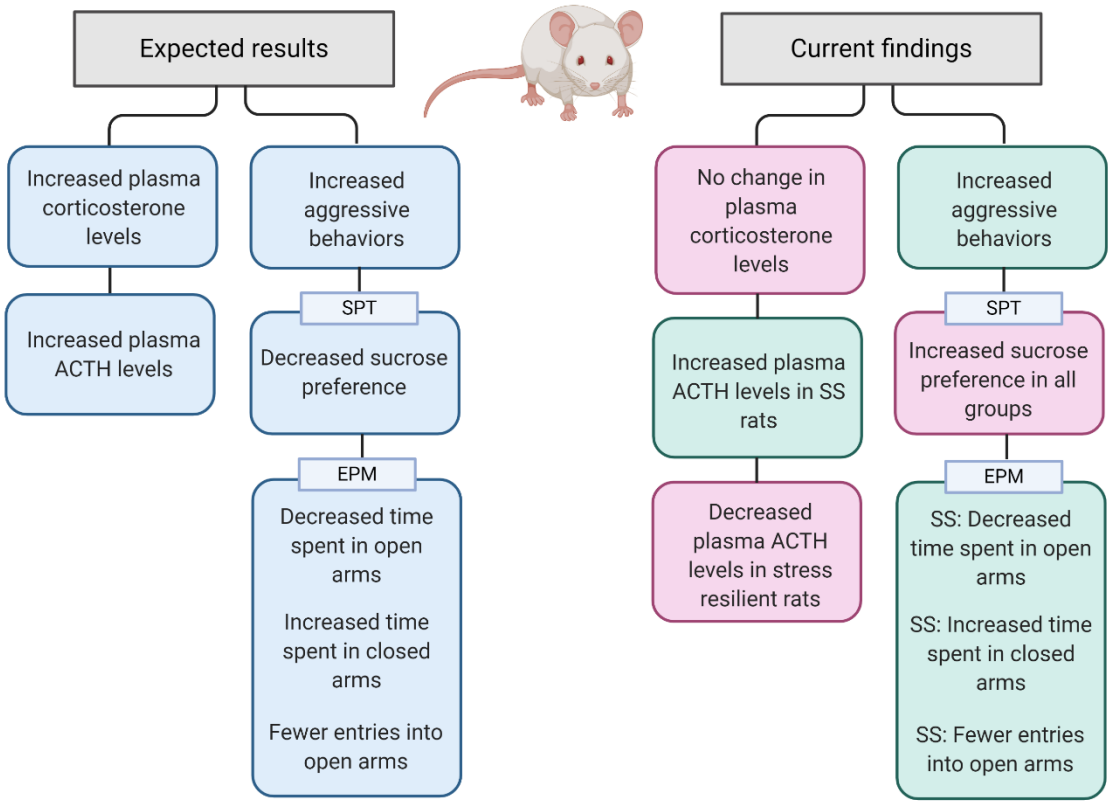


Figure 35: Expected results from the UCMS model, compared to results of the present study. Blocks in green indicate expected results, while blocks in red indicate unexpected findings. SPT - Sucrose Preference Test; EPM - Elevated Plus Maze; SS - Stress susceptible rats; SR – Stress resilient rats. Figure made in BioRender.

Despite the various precautions and adjustments, the current data do not unequivocally demonstrate that the UCMS model was indeed successfully established in our laboratory (Figure 35). The model is described as an excellent one for studying depression as it can induce anhedonia following the stress protocol (Scheggi *et al.*, 2018). However, this was not the case in our study as neither the Stress susceptible nor the Stress resilient groups displayed the decrease in sucrose preference that is necessary to confirm anhedonia. However, the results of the EPM test showed that the model excelled in inducing a state of anxiety in the Stress susceptible rats. It is our and others' opinion that such results raise the question whether or not the UCMS protocol is more successful in replicating a state of anxiety caused by chronic stress

than inducing anhedonia (Antoniuk *et al.*, 2019; Mineur *et al.*, 2006; Scheggi *et al.*, 2018; Willner *et al.*, 1987). Despite such an unclear focus, the lack of significant corticosterone or ACTH results further add to concerns that the UCMS model was not effective in recreating a state of chronic stress as desired.

In the years since the UCMS model was first developed there has been reports that it can be difficult to replicate across laboratories (Lezak *et al.*, 2017). While there are numerous research facilities that managed to successfully replicate the original findings, there are also several contrarian reports. Here a 10-year review revealed that the majority of findings relating to the model only originated from the laboratory in which the model was originally established (Willner, 1997). The question was subsequently raised whether the model can be reliably established elsewhere, and hence a cross-country move of the experiment was attempted by the original research team (Willner, 1997). Here behavioral changes such as decreased sucrose intake following stress were not consistently observed, and rats exhibited a rapid habituation to the effects of the stress model (Willner, 1997).

The reasons for such varying observations were not clearly understood at the time. However, further insights were revealed by subsequent analyses, for example that the reliability of the model may be influenced by factors such as the specific stressors employed and the use of good laboratory practices (Willner, 2017b). Thus the daily timing of the stressors can cause variability in results, e.g. diurnal sensitivity was reported in Wistar rats by D'Aquila, Newton & Willner (1997) who discovered that they showed a limited response to stressors employed during the light phase. Accordingly, decreases in sucrose consumption were observed when the rats were stressed at the start of the dark phase (D'Aquila *et al.*, 1997). The rodents themselves also contribute to variability through individual differences in terms of stress susceptibility (Willner, 2017b). Furthermore, differences between strains are known to influence the efficacy of the UCMS model, particularly in Wistar rats where different susceptibilities to stress were reported (Bekris *et al.*, 2005; Scheggi *et al.*, 2018).

Study limitations

The present study is not without flaws, despite significant improvements from previous experiments conducted in our laboratory. The shelves on which the rat cages were

housed occasionally relayed slight vibrations from a nearby air conditioning system, which could have had a negative effect on the rats (Reynolds *et al.*, 2018). Although measures were taken to prevent this (i.e., placing cages on thick sponge and rubber mats), the vibrations could not be entirely eliminated. There were also incidences where individual rats escaped from their cages and stole food from neighboring cages, albeit that the time spent outside of their cages were brief. While these occurrences were well documented, it influenced the accuracy of food consumption values. There are also some concerns regarding the accuracy of the SPT test results as some bottles leaked slightly when placed into the cages. However, it is likely that the same amount of fluid was lost with each test and therefore consistent amounts lost.

During plasma collection the whole blood samples were centrifuged at an incorrect speed, and this might have caused some samples to hemolyze, however the hemolysis could also have been result of a delay following the decapitation. This may have influenced the accuracy of some of the biological validations used (corticosterone and ACTH levels). Although hair samples were collected to determine the corticosterone levels, the results generated (ELISA kit) showed that all the data points fell outside the standard curve despite multiple concentration runs. For this reason, the data from hair samples was not included here.

Future recommendations

It is strongly recommended that future studies employ an entirely isolated environment to house the rats. This can be achieved by using soundproof rooms, as this prevents any external stimuli from affecting the control rats. It also prevents vocalization from the experimental group to reach the control group during the stressor periods. Cages should also have a secure clip to ensure that rats cannot escape – as we found at times! We also recommend that an automated system be used in the SPT to determine fluid consumption, by measuring the number of licks at each bottle. The same suggestion is made for the EPM test, as using automated software to analyze rat behavior would aid in eliminating bias and lead to more accurate results."

Another aspect strongly recommended for future studies is re-attempting the measure of hair corticosterone, as it remains a highly valuable indicator of how stress affects the chronic secretion of corticosterone (Scorrano *et al.*, 2015). To date, corticosterone levels have been analyzed mainly in the blood plasma and saliva, and while these

methods are still effective, they reflect transient changes in corticosterone levels. They're also very subject to environmental influences and changes due to circadian rhythms. In contrast, hair can accumulate corticosterone over the course of weeks to months, and therefore can be more indicative of the individual's chronic levels (Scorrano *et al.*, 2015).

5. CONCLUSION

Despite the inconsistencies of the UCMS model, it remains a validated and popular model in chronic stress research. The model itself opens a new opportunity to study the complexity of the stress response as the results are highly comparable to the human context. When correctly employed, rodent models of chronic stress possess the ability to shed light on the links between chronic stress exposure and the onset of psychological disorders, such as depression and anxiety. However, for the model to be effective it needs to be properly established and validated. Although certain model validation tests are cast in a more favorable light than others, no single test will be sufficient to determine successful implementation. It is therefore highly recommended that rodent models of chronic stress be validated by multiple tests that focus on evaluating the animal as a whole and not just a single parameter as is sometimes the case.

We propose that future studies aim to establish a consistent protocol for the UCMS model, specifically regarding which stressors to use, as well as the timing and duration thereof. It is our opinion that this should help to establish a universal protocol than can then successfully be replicated on a global scale. Taken together with multiple validation tests, individual behavioral and molecular “profiles” for the rodents can be established, providing a broader assessment of their well-being and the differential effects of chronic stress.

REFERENCES

Agid, O., Kohn, Y. & Lerer, B. 2000. Environmental stress and psychiatric illness. *Biomedicine and Pharmacotherapy*. 54(3):135–141.

Akiskal, H.S. 1986. A developmental perspective on recurrent mood disorders: a review of studies in man. *Psychopharmacology Bulletin*. 22(3):579–86.

Albani, S.H., Andrawis, M.M., Abella, R.J.H., Fulghum, J.T., Vafamand, N. & Dumas, T.C. 2015. Behavior in the elevated plus maze is differentially affected by testing conditions in rats under and over three weeks of age. *Frontiers in Behavioral Neuroscience*. 9:1–10.

Alkhlaif, Y., Bagdas, D., Jackson, A., Park, A.J. & Damaj, I.M. 2017. Assessment of nicotine withdrawal-induced changes in sucrose preference in mice. *Pharmacology Biochemistry and Behavior*. 161:47–52.

Allen, A.P., Kennedy, P.J., Cryan, J.F., Dinan, T.G. & Clarke, G. 2014. Biological and psychological markers of stress in humans: Focus on the Trier Social Stress Test. *Neuroscience and Biobehavioral Reviews*. 38:94–124.

Andrade, M.M.M., Tomé, M.F., Santiago, E.S., Lúcia-Santos, A. & De Andrade, T.G.C.S. 2003. Longitudinal study of daily variation of rats' behavior in the elevated plus-maze. *Physiology and Behavior*. 78(1):125–133.

Antoniuk, S., Bijata, M., Ponimaskin, E. & Wlodarczyk, J. 2019. Chronic unpredictable mild stress for modeling depression in rodents: Meta-analysis of model reliability. *Neuroscience and Biobehavioral Reviews*. 99:101–116.

Arnsten, A.F.T., Raskind, M.A., Taylor, F.B. & Connor, D.F. 2015. The effects of stress exposure on prefrontal cortex: Translating basic research into successful treatments for post-traumatic stress disorder. *Neurobiology of Stress*. 1(1):89–99.

Bannerman, D.M., Rawlins, J.N.P., Mchugh, S.B., Deacon, R.M.J., Yee, B.K., Bast, T., Zhang, W., Pothuizen, H.H.J. & Feldon, J. 2004. Regional dissociations within the hippocampus — memory and anxiety. *Neuroscience and Biobehavioral Reviews*. 28:273–283.

Becker, A. & Grecksch, G. 1996. Illumination has no effect on rats' behavior in the

elevated plus-maze. *Physiology and Behavior*. 59(6):1175–1177.

Beckie, T.M. 2012. *A Systematic Review of Allostatic Load, Health, and Health Disparities*. Vol. 14.

Bekris, S., Antoniou, K., Daskas, S. & Papadopoulou-Daifoti, Z. 2005. Behavioural and neurochemical effects induced by chronic mild stress applied to two different rat strains. *Behavioural Brain Research*. 161(1):45–59.

Belzung, C. & Griebel, G. 2001. Measuring normal and pathological anxiety-like behaviour in mice: A review. *Behavioural Brain Research*. 125(1–2):141–149.

Bergström, A., Jayatissa, M.N., Mørk, A. & Wiborg, O. 2008. Stress sensitivity and resilience in the chronic mild stress rat model of depression; an in situ hybridization study. *Brain Research*. 1196:41–52.

Bertoglio, L.J. & Carobrez, A.P. 2002. Behavioral profile of rats submitted to session 1-session 2 in the elevated plus-maze during diurnal/nocturnal phases and under different illumination conditions. *Behavioural Brain Research*. 132(2):135–143.

Berton, O., McClung, C.A., DiLeone, R.J., Krishnan, V., Renthal, W., Russo, S.J., Graham, D., Tsankova, N.M., Bolanos, C.A., Rios, M., Monteggia, L.M., Self, D.W. & Nestler, E.J. 2006. Essential role of BDNF in the mesolimbic dopamine pathway in social defeat stress. *Science*. 311(5762):864–868.

Bhat, S.A., Wani, A.L. & Ara, A. 2014. Animal models of depression and their criteria of validation. *Journal of Chemical and Pharmaceutical Research*. 6(10):123–130.

Bobak, M. & Marmot, M. 1996. East-West mortality divide and its potential explanations: Proposed research agenda. *British Medical Journal*. 312(7028):421–425.

Boggiano, M.M., Cavigelli, S.A., Dorsey, J.R., Kelley, C.E.P., Ragan, C.M. & Chandler-Laney, P.C. 2008. Effect of a cage divider permitting social stimuli on stress and food intake in rats. *Physiology and Behavior*. 95(1–2):222–228.

Bondi, C.O., Rodriguez, G., Gould, G.G., Frazer, A. & Morilak, D.A. 2008. Chronic unpredictable stress induces a cognitive deficit and anxiety-like behavior in rats that is prevented by chronic antidepressant drug treatment. *Neuropsychopharmacology*. 33(2):320–331.

Brenes, J.C. & Fornaguera, J. 2008. Effects of environmental enrichment and social isolation on sucrose consumption and preference: Associations with depressive-like behavior and ventral striatum dopamine. *Neuroscience Letters*. 436(2):278–282.

Brenes Sáenz, J.C., Villagra, O.R. & Fornaguera Trías, J. 2006. Factor analysis of Forced Swimming test, Sucrose Preference test and Open Field test on enriched, social and isolated reared rats. *Behavioural Brain Research*. 169(1):57–65.

Brown, G.R. & Nemes, C. 2008. The exploratory behaviour of rats in the hole-board apparatus: Is head-dipping a valid measure of neophilia? *Behavioural Processes*. 78(3):442–448.

Campos, A.C., Fogaça, M. V., Aguiar, D.C. & Guimarães, F.S. 2013. Animal models of anxiety disorders and stress. *Revista Brasileira de Psiquiatria*. 35(SUPPL.2):101–111.

Cannon, B. 1929. Organization for Physiological Homeostasis. *Physiological Reviews*. 9(3):399–431. [Online], Available: https://doi.org/10.1152/physrev.1929.9.3.399.

Capdevila, S., Giral, M., Ruiz De La Torre, J.L., Russell, R.J. & Kramer, K. 2007. Acclimatization of rats after ground transportation to a new animal facility. *Laboratory Animals*. 41(2):2551–261.

Carnevali, L., Montano, N., Statello, R., Coudé, G., Vacondio, F., Rivara, S., Ferrari, P.F. & Sgoifo, A. 2017. Social stress contagion in rats: Behavioural, autonomic and neuroendocrine correlates. *Psychoneuroendocrinology*. 82(January):155–163.

Carobrez, A.P. & Bertoglio, L.J. 2005. Ethological and temporal analyses of anxiety-like behavior: The elevated plus-maze model 20 years on. *Neuroscience and Biobehavioral Reviews*. 29(8):1193–1205.

Castelhano-Carlos, M.J. & Baumans, V. 2009. The impact of light, noise, cage cleaning and in-house transport on welfare and stress of laboratory rats. *Laboratory Animals*. 43(4):311–327.

Chadman, K.K., Yang, M. & Crawley, J.N. 2009. Criteria for Validating Mouse Models of Psychiatric Diseases. *American Journal of Medical Genetics Part B: Neuropsychiatric Genetics*. 150B(1):1–11.

Chandola, T., Brunner, E. & Marmot, M. 2006. Chronic stress at work and the

metabolic syndrome: Prospective study. *British Medical Journal*. 332(7540):521–524.

Charmandari, E., Tsigos, C. & Chrousos, G. 2005. Endocrinology of the stress response. *Annual Review of Physiology*. 67:259–284.

Charmandari, E., Nicolaides, N.C. & Chrousos, G.P. 2014. Adrenal insufficiency. *The Lancet*. 383(9935):2152–2167.

Chiba, S., Numakawa, T., Ninomiya, M., Richards, M.C., Wakabayashi, C. & Kunugi, H. 2012. Chronic restraint stress causes anxiety- and depression-like behaviors, downregulates glucocorticoid receptor expression, and attenuates glutamate release induced by brain-derived neurotrophic factor in the prefrontal cortex. *Progress in Neuro-Psychopharmacology and Biological Psychiatry*. 39(1):112–119.

Christiansen, S., Bouzinova, E. V., Palme, R. & Wiborg, O. 2012. Circadian activity of the hypothalamic–pituitary–adrenal axis is differentially affected in the rat chronic mild stress model of depression. *Stress*. 15(6):647–657.

Claassen, V. 1994. Food and Water Intake. in *Handbook of Behavioral Neuroscience* Vol. 12. Elsevier. 267–287.

Cohen, S., Janicki-Deverts, D. & Miller, G.E. 2007. Psychological Stress and Disease. *JAMA*. 298(14):1685.

Cox, B.M., Alsawah, F., McNeill, P.C., Galloway, M.P. & Perrine, S.A. 2011. Neurochemical, hormonal, and behavioral effects of chronic unpredictable stress in the rat. *Behavioural Brain Research*. 220(1):106–111.

Cruz, A.P.M., Frei, F. & Graeff, F.G. 1994. Ethopharmacological Analysis of Rat Behavior on the Elevated Plus-Maze. *Pharmacology Biochemistry and Behavior*. 49(1):171–176.

Cryan, J.F. & Mombereau, C. 2004. In search of a depressed mouse : utility of models for studying depression-related behavior in genetically modified mice. *Molecular Psychiatry*. 9:326–357.

D'Aquila, P.S., Newton, J. & Willner, P. 1997. Diurnal variation in the effect of chronic mild stress on sucrose intake and preference. *Physiology and Behavior*. 62(2):421–426.

Dallman, M.F., Pecoraro, N.C. & La Fleur, S.E. 2005. Chronic stress and comfort foods: Self-medication and abdominal obesity. *Brain, Behavior, and Immunity*. 19(4):275–280.

Daubner, S.C., Le, T. & Wang, S. 2012. Tyrosine Hydroxylase and Regulation of Dopamine Synthesis. *Archives of Biochemistry and Biophysics*. 508(1):1–12.

Depke, M., Fusch, G., Domanska, G., Geffers, R., Völker, U., Schuett, C. & Kiank, C. 2008. Hypermetabolic syndrome as a consequence of repeated psychological stress in mice. *Endocrinology*. 149(6):2714–2723.

Dimitrov, A., Demin, K., Fehlner, P., Walter, H., Erk, S. & Veer, I.M. 2018. Differences in Neural Recovery From Acute Stress Between Cortisol Responders and Non-responders. *Frontiers in Psychiatry*. 9(November):1–13.

Ducottet, C. & Belzung, C. 2005. Correlations between behaviours in the elevated plus-maze and sensitivity to unpredictable subchronic mild stress: Evidence from inbred strains of mice. *Behavioural Brain Research*. 156(1):153–162.

Everly Jr., G.S. & Lating, J.M. 2019. The Anatomy and Physiology of the Human Stress Response. in *A Clinical Guide to the Treatment of the Human Stress Response* Springer, New York. 19–56.

Everson, S.A., Lynch, J.W., Chesney, M.A., Kaplan, G.A., Goldberg, D.E., Shade, S.B., Cohen, R.D., Salonen, R. & Salonen, J.T. 1997. Interaction of workplace demands and cardiovascular reactivity in progression of carotid atherosclerosis: population based study. *BMJ*. 314:553–558.

File, S.E. & Wardill, A.G. 1975. Validity of head-dipping as a measure of exploration in a modified hole-board. *Psychopharmacologia*. 44(1):53–59.

File, S.E., Zangrossi, H., Sanders, F.L. & Mabbutt, P.S. 1994. Raised corticosterone in the rat after exposure to the elevated plus-maze. *Psychopharmacology*. 113(3–4):543–546.

Ford, J.D., Grasso, D.J., Elhai, J.D. & Courtois, C.A. 2015. Neurobiology of traumatic stress disorders and their impact on physical health. in *Posttraumatic Stress Disorder*. 183–232.

Franco, A.J., Chen, C., Scullen, T., Zsombok, A., Salahudeen, A.A., Di, S., Herman,

J.P. & Tasker, J.G. 2016. Sensitization of the Hypothalamic-Pituitary-Adrenal Axis in a Male Rat Chronic Stress Model. *Endocrinology*. 157(6):2346–2355.

Frisbee, J.C., Brooks, S.D., Stanley, S.C. & Audiffret, A.C. 2015. An Unpredictable Chronic Mild Stress Protocol for Instigating Depressive Symptoms, Behavioral Changes and Negative Health Outcomes in Rodents. *Journal of Visualized Experiments*. (December):1–8.

Gadek-Michalska, A. & Bugajski, J. 2003. Repeated handling, restraint, or chronic crowding impair the hypothalamic-pituitary-adrenocortical response to acute restraint stress. *Journal of Physiology and Pharmacology*. 54(3):449–459.

Godoy, L.D., Rossignoli, M.T., Delfino-Pereira, P., Garcia-Cairasco, N. & Umeoka, E.H. de L. 2018. A comprehensive overview on stress neurobiology: Basic concepts and clinical implications. *Frontiers in Behavioral Neuroscience*. 12(July):1–23.

Golbidi, S., Frisbee, J.C. & Laher, I. 2015. Chronic stress impacts the cardiovascular system: animal models and clinical outcomes. *American Journal of Physiology-Heart and Circulatory Physiology*. 308(12):H1476–H1498.

Golombek, D.A., Rosenstein, R.E., Yannielli, P.C., Keller Sarmiento, M.I. & Cardinali, D.P. 1997. Aging attenuates diurnal variation in hamster locomotion, anxiolysis and GABA turnover. *Neuroscience Letters*. 233(1):9–12.

Gomez-Sanchez, E. & Gomez-Sanchez, C.E. 2014. The multifaceted mineralocorticoid receptor. *Comprehensive Physiology*. 4(3):965–994.

Griebel, G., Moreau, J., Jenck, F. & Martin, J.R. 1993. Some critical determinants in the elevated of rats. *Behavioural Processes*. 29(1):37–47.

Grossi, G., Perski, A., Evengård, B., Blomkvist, V. & Orth-Gomér, K. 2003. Physiological correlates of burnout among women. *Journal of Psychosomatic Research*. 55(4):309–316.

Haeffel, G.J. & Vargas, I. 2011. Resilience to depressive symptoms: The buffering effects of enhancing cognitive style and positive life events. *Journal of Behavior Therapy and Experimental Psychiatry*. 42(1):13–18.

Handley, S.L. & Mithani, S. 1984. Effects of alpha-adrenoceptor agonists and antagonists in a maze-exploration model of 'fear'-motivated behaviour. *Naunyn-*

Schmiedeberg's Archives of Pharmacology. 327(1):1–5.

Handley, S.L., McBlane, J.W., Critchley, M.A.E. & Njung'e, K. 1993. Multiple serotonin mechanisms in animal models of anxiety. Environmental, emotional and cognitive factors. *Behavioural Brain Research*. 58(1–2):203–210.

Hannibal, K.E. & Bishop, M.D. 2014. Chronic stress, cortisol dysfunction, and pain: A psychoneuroendocrine rationale for stress management in pain rehabilitation. *Physical Therapy*. 94(12):1816–1825.

Hasler, G., Drevets, W.C., Manji, H.K. & Charney, D.S. 2004. Discovering endophenotypes for major depression. *Neuropsychopharmacology*. 29(10):1765–1781.

He, L., Zeng, L., Tian, N., Li, Y., He, T., Tan, D., Zhang, Q. & Tan, Y. 2020. Optimization of food deprivation and sucrose preference test in SD rat model undergoing chronic unpredictable mild stress. *Animal Models and Experimental Medicine*. 3(1):69–78.

He, Z., Lv, F., Ding, Y., Huang, H., Liu, L., Zhu, C., Lei, Y., Zhang, L., Si, C. & Wang, H. 2017. High-fat diet and chronic stress aggravate adrenal function abnormality induced by prenatal caffeine exposure in male offspring rats. *Scientific Reports*. 7(1):1–9.

Henn, F.A. & Vollmayr, B. 2005. Stress models of depression: Forming genetically vulnerable strains. *Neuroscience and Biobehavioral Reviews*. 29(4–5):799–804.

Henningsen, K., Palmfeldt, J., Christiansen, S., Baiges, I., Bak, S., Jensen, O.N., Gregersen, N. & Wiborg, O. 2012. Candidate hippocampal biomarkers of susceptibility and resilience to stress in a rat model of depression. *Molecular and Cellular Proteomics*. 11(7):1–12.

Herman, J.P. 2013. Neural control of chronic stress adaptation. *Frontiers in Behavioral Neuroscience*. 7(MAY):1–12.

Hirsch, D. & Zukowska, Z. 2011. NPY and Stress 30 Years Later: The Peripheral View Dalay. *Cellular and Molecular Neurobiology*. 23(1):1–7.

Hjemdal, O., Vogel, P.A., Solem, S., Hagen, K. & Stiles, T.C. 2011. The relationship between resilience and levels of anxiety, depression, and obsessive-compulsive

symptoms in adolescents. *Clinical Psychology and Psychotherapy*. 18(4):314–321.

Hoffman, K.L. 2016. What can animal models tell us about depressive disorders? in *Modeling Neuropsychiatric Disorders in Laboratory Animals*. 35–86.

Hogg, S. 1996. A review of the validity and variability of the elevated plus-maze as an animal model of anxiety. *Pharmacology Biochemistry and Behavior*. 54(1):21–30.

Huhman, K.L. 2006. Social conflict models: Can they inform us about human psychopathology? *Hormones and Behavior*. 50(4):640–646.

Isaacson, R.L. 2001. Limbic System. in *International Encyclopedia of the Social & Behavioral Sciences* 1st ed. 8858–8862.

Jaggi, A.S., Bhatia, N., Kumar, N., Singh, N., Anand, P. & Dhawan, R. 2011. A review on animal models for screening potential anti-stress agents. *Neurological Sciences*. 32(6):993–1005.

Jankord, R. & Herman, J.P. 2008. Limbic regulation of hypothalamo-pituitary-adrenocortical function during acute and chronic stress. *Annals of the New York Academy of Sciences*. 1148(1):64–73.

Jimenez, V.A., Herman, M.A., Cuzon Carlson, V.C., Walter, N.A., Grant, K.A. & Roberto, M. 2019. Synaptic adaptations in the central amygdala and hypothalamic paraventricular nucleus associated with protracted ethanol abstinence in male rhesus monkeys. *Neuropsychopharmacology*. 44(5):982–993.

Joëls, M. & Baram, T.Z. 2010. The neuro-symphony of stress. *Nat Rev Neurosci.* 10(6):459–466.

Johnson, E.O., Kamilaris, T.C., Chrousos, G.P. & Gold, P.W. 1992. Mechanisms of stress: A dynamic overview of hormonal and behavioral homeostasis. *Neuroscience and Biobehavioral Reviews*. 16(2):115–130.

Jones, N. & King, S.M. 2001. Influence of circadian phase and test illumination on pre-clinical models of anxiety. *Physiology and Behavior*. 72(1–2):99–106.

Jones, J.., Huxtable, C.. & Hodgson, J.. 2003. *Self-reported work-related illness in 2003/2004 : Results from the Labour Force Survey*.

José Jaime, H.-P., Venus, B.-C., Graciela, J.-R., Hernández-Hernández, O.T. &

Martínez-Mota, L. 2016. Young-Adult Male Rats' Vulnerability to Chronic Mild Stress Is Reflected by Anxious-Like instead of Depressive-Like Behaviors. *Neuroscience Journal*. 2016:1–12.

Juczewski, K., Koussa, J.A., Kesner, A.J., Lee, J.O. & Lovinger, D.M. 2020. Stress and behavioral correlates in the head-fixed method: stress measurements, habituation dynamics, locomotion, and motor-skill learning in mice. *Scientific Reports*. 10(1):1–19.

Juster, R.-P., McEwen, B.S. & Lupien, S.J. 2010. Allostatic load biomarkers of chronic stress and impact on health and cognition. *Neuroscience and Biobehavioral Reviews*. 35(1):2–16.

Kabbaj, M., Norton, C.S., Kollack-Walker, S., Watson, S.J., Robinson, T. & Akil, H. 2001. Social defeat alters the acquisition of cocaine self-administration in rats: Role of individual differences in cocaine-taking behavior. *Psychopharmacology*. 158(4):382–387.

Katz, R.J. 1982. Animal model of depression: Pharmacological sensitivity of a hedonic deficit. *Pharmacology, Biochemistry and Behavior*. 16(6):965–968.

Kendler, K.S., Gardner, C.O. & Prescott, C.A. 2002. Toward a comprehensive developmental model for major depression in women. *American Journal of Psychiatry*. 159(7):1133–1145.

Kirschbaum, C., Wolf, O.T., May, M., Wippich, W. & Hellhammer, D.. 1996. Stress- and treatment-induced elevations of cortisol levels associated with impaired declarative memory in healthy adults. *Life Sciences*. 58(17):1475–1483.

Krishnan, V. & Nestler, E.J. 2011. Animal Models of Depression: Molecular Perspectives. *Curr Top Behav Neurosci.* 7:121–147.

Krishnan, V., Han, M.H., Graham, D.L., Berton, O., Renthal, W., Russo, S.J., LaPlant, Q., Graham, A., Lutter, M., Lagace, D.C., Ghose, S., Reister, R., Tannous, P., Green, T.A., Neve, R.L., Chakravarty, S., Kumar, A., Eisch, A.J., Self, D.W., et al. 2007. Molecular Adaptations Underlying Susceptibility and Resistance to Social Defeat in Brain Reward Regions. *Cell*. 131(2):391–404.

Kumar, B., Kuhad, A. & Chopra, K. 2011. Neuropsychopharmacological effect of sesamol in unpredictable chronic mild stress model of depression: Behavioral and

biochemical evidences. *Psychopharmacology*. 214(4):819–828.

Kvetnansky, R., Sabban, E.L. & Palkovits, M. 2009. Catecholaminergic systems in stress: Structural and molecular genetic approaches. *Physiological Reviews*. 89(2):535–606.

Kyrou, I. & Tsigos, C. 2009. Stress hormones: physiological stress and regulation of metabolism. *Current Opinion in Pharmacology*. (9):787–793.

La-Vu, M., Tobias, B.C., Schuette, P.J. & Adhikari, A. 2020. To Approach or Avoid : An Introductory Overview of the Study of Anxiety Using Rodent Assays. *Frontiers in Behavioral Neuroscience*. 14(August):1–7.

Labots, M., Lith, H.A. Van, Ohl, F. & Arndt, S.S. 2015. The Modified Hole Board - Measuring Behavior, Cognition and Social Interaction in Mice and Rats. *Journal of Visualized Experiments*. 98:1–7.

Lagraauw, H.M., Kuiper, J. & Bot, I. 2015. Acute and chronic psychological stress as risk factors for cardiovascular disease: Insights gained from epidemiological, clinical and experimental studies. *Brain, Behavior, and Immunity*. 50:18–30.

Lapiz-Bluhm, M.D.S., Bondi, C.O., Doyen, J., Rodriguez, G.A., Bédard-Arana, T. & Morilak, D.A. 2008. Behavioural assays to model cognitive and affective dimensions of depression and anxiety in rats. *Journal of Neuroendocrinology*. 20(10):1115–1137.

Lezak, K.R., Missig, G. & Jr, W.A.C. 2017. Behavioral methods to study anxiety in rodents. *Dialogues in Clinical Neuroscience*. 19(2):181–191.

Lister, R.G. 1987. The use of a plus-maze to measure anxiety in the mouse. *Psychopharmacology*. 92(t 987):180–185.

Lister, R.G. 1990. Ethologically-Based Animal Models Of Anxiety Disorders. *Pharmacology & Therapeutics*. 46(3):321–340.

Liu, M.Y., Yin, C.Y., Zhu, L.J., Zhu, X.H., Xu, C., Luo, C.X., Chen, H., Zhu, D.Y. & Zhou, Q.G. 2018. Sucrose preference test for measurement of stress-induced anhedonia in mice. *Nature Protocols*. 13(7):1686–1698.

Low, C.A., Salomon, K. & Matthews, K.A. 2009. Chronic life stress, cardiovascular reactivity, and subclinical cardiovascular disease in adolescents. *Psychosomatic*

Medicine. 71(9):927–31.

Magarinos, A.M. & McEwen, B.S. 1995. Stress-induced atrophy of apical dendrites of hippocampal CA3c neurons: comparison of stressors. *Neuroscience*. 69(1):83–88. [Online], Available: http://dx.doi.org/10.1038/nrn2935.

Marais, L., van Rensburg, S.J., van Zyl, J.M., Stein, D.J. & Daniels, W.M.U. 2008. Maternal separation of rat pups increases the risk of developing depressive-like behavior after subsequent chronic stress by altering corticosterone and neurotrophin levels in the hippocampus. *Neuroscience Research*. 61(1):106–112.

Markowe, H.L.J., Marmot, M.G., Shipley, M.J., Bulpitt, C.J., Meade, T.W., Stirling, Y., Vickers, M.V. & Semmence, A. 1985. Fibrinogen: A possible link between social class and coronary heart disease. *BMJ*. 291:1312–1314.

Martínez, J.C., Cardenas, F., Lamprea, M. & Morato, S. 2002. The role of vision and proprioception in the aversion of rats to the open arms of an elevated plus-maze. *Behavioural Processes*. 60(1):15–26.

Matthews, K., Forbes, N. & Reid, I.C. 1995. Sucrose consumption as an hedonic measure following chronic unpredictable mild stress. *Physiology and Behavior*. 57(2):241–248.

McAlonan, K., Brown, V.J. & Bowman, E.M. 2000. Thalamic Reticular Nucleus Activation Reflects Attentional Gating during Classical Conditioning. *The Journal of Neuroscience*. 20(23):8897–8901.

McEwen, B.S. 1998. Protective and Damaging Effects of Stress Mediators. *The New England Journal of Medicine*. 338(3):171–179.

McEwen, B.S. 2006. Protective and damaging effects of stress mediators: Central role of the brain. *Dialogues in Clinical Neuroscience*. 8(4):367–381.

McEwen, B.S. 2007. Physiology and neurobiology of stress and adaptation: Central role of the brain. *Physiological Reviews*. 87(3):873–904.

McEwen, B.S. 2008. Central effects of stress hormones in health and disease: Understanding the protective and damaging effects of stress and stress mediators. *European Journal of Pharmacology*. 583(2–3):174–185.

McEwen, B.S. 2017. Neurobiological and Systemic Effects of Chronic Stress. *Chronic Stress*. 1:2470547017692328.

McEwen, B.S. & Gianaros, P.J. 2010. Central role of the brain in stress and adaptation: Links to socioeconomic status, health, and disease. *Annals of the New York Academy of Sciences*. 1186:190–222.

McEwen, B.S. & Magarinos, A.M. 1997. Stress effects on morphology and function of the hippocampus. *Annals of the New York Academy of Sciences*. 821:271–284.

McEwen, B.S. & Sapolsky, R.M. 1995. Stress and cognitive function. *Current Opinion in Neurobiology*. 5(2):205–216.

McEwen, B.S. & Stellar, E. 1993. Stress and individual. *Arch Intern Med.* 153:2093–2101.

McEwen, B.S. & Wingfield, J.C. 2003. The concept of allostasis in biology and biomedicine. *Hormones and Behavior*. 43(1):2–15.

McEwen, B.S., De Kloet, E.R. & Rostene, W. 1986. Adrenal steroid receptors and actions in the nervous system. *Physiological Reviews*. 66(4):1121–1188.

Meerlo, P., Overkamp, G.J.F., Daan, S., Van Den Hoofdakker, R.H. & Koolhaas, J.M. 1996. Changes in behaviour and body weight following a single or double social defeat in rats. *Stress*. 1(1):21–32.

Merkulov, V.M., Merkulova, T.I. & Bondar, N.P. 2017. Mechanisms of brain glucocorticoid resistance in stress-induced psychopathologies. *Biochemistry (Moscow)*. 82(3):351–365.

Milena, A., Garcia, B., Cardenas, F.P. & Morato, S. 2005. Effect of different illumination levels on rat behavior in the elevated plus-maze. *Physiology & Behavior*. 85:265–270.

Miller, D.B. & O'Callaghan, J.P. 2002. Neuroendocrine aspects of the response to stress. *Metabolism: Clinical and Experimental*. 51(6 SUPPL. 1):5–10.

Mineur, Y.S., Belzung, C. & Crusio, W.E. 2006. Effects of unpredictable chronic mild stress on anxiety and depression-like behavior in mice. *Behavioural Brain Research*. 175(1):43–50.

Mitra, R., Vyas, A., Chatterjee, G. & Chattarji, S. 2005. Chronic-stress induced

modulation of different states of anxiety-like behavior in female rats. *Neuroscience Letters*. 383(3):278–283.

Molinoff, P.B. & Axelrod, J. 1971. Biochemistry of catecholamines. *Annual Review of Biochemistry*. 40(1):465–499.

Montgomery, K.C. 1955. The relation between fear induced by novel stimulation and exploratory drive. *Journal of Comparative and Physiological Psychology*. 48(4):254–260.

Morgan, C., Kirkbridge, J., Leff, J., Craig, T., Hutchinson, G., Mckenzie, K., Morgan, K., Dazzan, P., Doody, G.A., Jones, P., Murray, R. & Fearon, P. 2007. Parental separation, loss and psychosis in different ethnic groups: A case-control study. *Psychological Medicine*. 37(4):495–503.

Murison, R. 2016. The Neurobiology of Stress. in *Neuroscience of Pain, Stress, and Emotion* Elsevier Inc. 29–49.

Mutlu, O., Gumuslu, E., Ulak, G., Celikyurt, I.K., Kokturk, S., Kir, H.M., Akar, F. & Erden, F. 2012. Effects of fluoxetine, tianeptine and olanzapine on unpredictable chronic mild stress-induced depression-like behavior in mice. *Life Sciences*. 91(25–26):1252–1262.

Myers, B., Scheimann, J.R., Franco-Villanueva, A. & Herman, J.P. 2017. Ascending mechanisms of stress integration: Implications for brainstem regulation of neuroendocrine and behavioral stress responses. *Neuroscience and Biobehavioral Reviews*. 74:366–375.

Nasca, C., Menard, C., Hodes, G., Bigio, B., Pena, C., Lorsch, Z., Zelli, D., Ferris, A., Kana, V., Purushothaman, I., Dobbin, J., Nassim, M., DeAngelis, P., Merad, M., Rasgon, N., Meaney, M., Nestler, E.J., McEwen, B.S. & Russo, S.J. 2019. Multidimensional Predictors of Susceptibility and Resilience to Social Defeat Stress. *Biological Psychiatry*. 86(6):483–491.

Nieuwenhuizen, A.G. & Rutters, F. 2008. The hypothalamic-pituitary-adrenal-axis in the regulation of energy balance. *Physiology and Behavior*. 94(2):169–177.

Nojilana, B., Bradshaw, D., Pillay-van Wyk, V., Msemburi, W., Somdyala, N., Joubert, J.D., Groenewald, P., Laubscher, R. & Dorrington, R.E. 2016. Persistent burden from

non-communicable diseases in South Africa needs strong action. *South African Medical Journal*. 106(5):436.

Nylander, I. & Roman, E. 2013. Is the rodent maternal separation model a valid and effective model for studies on the early-life impact on ethanol consumption? *Psychopharmacology*. 229(4):555–569.

O'Leary, O.F. & Cryan, J.F. 2013. Towards translational rodent models of depression. *Cell and Tissue Research*. 354(1):141–153.

Ohl, F. 2003. Testing for anxiety. *Clinical Neuroscience Research*. 3:233–238.

Ohl, F., Holsboer, F. & Landgraf, R. 2001. The modified hole board as a differential screen for behavior in rodents. *Behavior Research Methods, Instruments, and Computers*. 33(3):392–397.

Oken, B.S., Chamine, I. & Wakeland, W. 2015. A systems approach to stress, stressors and resilience in humans. *Behavioural Brain Research*. 282:144–154.

Van Oort, J., Tendolkar, I., Hermans, E.J., Mulders, P.C., Beckmann, C.F., Schene, A.H., Fernández, G. & van Eijndhoven, P.F. 2017. How the brain connects in response to acute stress: A review at the human brain systems level. *Neuroscience and Biobehavioral Reviews*. 83(October):281–297.

Ostrander, M.M., Ulrich-lai, Y.M., Choi, D.C., Richtand, N.M. & Herman, J.P. 2006. Hypoactivity of the Hypothalamo-Pituitary-Adrenocortical Axis during Recovery from Chronic Variable Stress. *Endocrinology*. 147(4):2008–2017.

Papp, M. 2012. Models of affective illness: Chronic mild stress in the rat. *Current Protocols in Pharmacology*. (SUPPL.57):1–11.

Paravati, S. & Warrington, S.J. 2019. *Physiology, Catecholamines*. StatPearls Publishing. [Online], Available: http://www.ncbi.nlm.nih.gov/pubmed/29939538 [2020, November 08].

Patchev, V.K. & Patchev, A. V. 2006. Experimental models of stress. *Dialogues in Clinical Neuroscience*. 8(5):417–432.

Patki, G., Atrooz, F., Solanki, N. & Salim, S. 2015. High aggression in rats is associated with elevated stress, anxiety-like behavior, and altered catecholamine

content in the brain. *Neuroscience Letters*. 584(1):308–313.

Pellow, S., Chopin, P., File, S.E. & Briley, M. 1985. Validation of open : closed arm entries in an elevated plus-maze as a measure of anxiety in the rat. *Journal of Neuroscience Methods*. 14(3):149–167.

Planchez, B., Surget, A. & Belzung, C. 2019. Animal models of major depression: drawbacks and challenges. *Journal of Neural Transmission*. 126(11):1383–1408.

Pothion, S., Bizot, J.C., Trovero, F. & Belzung, C. 2004. Strain differences in sucrose preference and in the consequences of unpredictable chronic mild stress. *Behavioural Brain Research*. 155(1):135–146.

Pucilowski, O., Overstreet, D.H., Rezvani, A.H. & Janowsky, D.S. 1993. Chronic mild stress-induced anhedonia: Greater effect in a genetic rat model of depression. *Physiology and Behavior*. 54(6):1215–1220.

Räikkönen, K., Lassila, R., Keltikangas-Järvinen, L. & Hautanen, A. 1996. Association of chronic stress with plasminogen activator inhibitor-1 in healthy middle-aged men. *Arteriosclerosis, Thrombosis, and Vascular Biology*. 16(3):363–367.

Rajmohan, V. & Mohandas, E. 2007. The limbic system. *Indian Journal of Psychiatry*. 49(2):132–139.

Rasmussen, D.D., Mitton, D.R., Green, J. & Puchalski, S. 2001. Chronic Daily Ethanol and Withdrawal: Behavioral Changes During Prolonged Abstinence. *Alcoholism: Clinical and Experimental Research*. 25(7):999–1005.

Reber, S.O., Birkeneder, L., Veenema, A.H., Obermeier, F., Falk, W., Straub, R.H. & Neumann, I.D. 2007. Adrenal insufficiency and colonic inflammation after a novel chronic psycho-social stress paradigm in mice: Implications and mechanisms. *Endocrinology*. 148(2):670–682.

Reynolds, R.P., Li, Y., Garner, A. & Norton, J.N. 2018. Vibration in mice: A review of comparative effects and use in translational research. *Animal Models and Experimental Medicine*. 1(2):116–124.

Rodgers, R.J. & Dalvi, A. 1997a. Anxiety, defence and the elevated plus-maze. *Neuroscience and Biobehavioral Reviews*. 21(6):801–810.

Rodgers, R.J., Cao, B.J., Dalvi, A. & Holmes, A. 1997b. Animal models of anxiety: An ethological perspective. *Brazilian Journal of Medical and Biological Research*. 30(3):289–304.

Rodgers, R.J., Haller, J., Holmes, A., Halasz, J., Walton, T.J. & Brain, P.F. 1999. Corticosterone response to the plus-maze: High correlation with risk assessment in rats and mice. *Physiology and Behavior*. 68(1–2):47–53.

Russo, S.J. & Nestler, E.J. 2013. The Brain Reward Circuitry in Mood Disorders. *Nature Reviews Neuroscience*. 14(9):1–34.

Salgado, J.V. & Sandner, G. 2013. A critical overview of animal models of psychiatric disorders: Challenges and perspectives. *Revista Brasileira de Psiquiatria*. 35(SUPPL.2):77–81.

Salleh, M.R. 2008. Life event, stress and illness. *Malaysian Journal of Medical Sciences*. 15(4):9–18.

Sandbak, T. & Murison, R. 2001. Behavioural Responses To Elevated Plus-Maze And Defensive Burying Testing: Effects On Subsequent Ethanol Intake And Effect Of Ethanol On Retention Of The Burying Response. *Alcohol & Alcoholism*. 36(1):48–58.

Sapolsky, R.M. 1990. Stress in the Wild. *Scientific American*. 262(1):116–123.

Sapolsky, R.M., Leung, S., Li, X. & Stark, G.R. 1996. Why Stress Is Bad for Your Brain Find That Hanging Together Can Be Stimulating. *Science*. 273(August):9–10.

Sapolsky, R.M., Romero, L.M. & Munck, A.U. 2000. How do glucocorticoids influence stress responses? Integrating permissive, suppressive, stimulatory, and preparative actions. *Endocrine Reviews*. 21(1):55–89.

Scheggi, S., De Montis, M.G. & Gambarana, C. 2018. Making sense of rodent models of anhedonia. *International Journal of Neuropsychopharmacology*. 21(11):1049–1065.

Schnall, P.L., Schwartz, J.E., Landsbergis, P.A., Warren, K. & Pickering, T.G. 1992. Relation between job strain, alcohol, and ambulatory blood pressure. *Hypertension*. 19(5):488–494.

Schneider, P., Ho, Y.-J., Spanagel, R. & Pawlak, C.R. 2011. A novel elevated plus-maze procedure to avoid the one-trial tolerance problem. *Frontiers in Behavioral*

Neuroscience. 5(July):1–8.

Schneiderman, N., Ironson, G. & Siegel, S.D. 2005. Stress and health: Psychological, behavioral, and biological determinants. *Annual Review of Clinical Psychology*. 1(Lacey 1967):607–628.

Schrader, A.J., Taylor, R.M., Lowery-Gionta, E.G. & Moore, N.L.T. 2018. Repeated elevated plus maze trials as a measure for tracking within-subjects behavioral performance in rats (Rattus norvegicus). *PLoS ONE*. 13(11):1–15.

Schulkin, J., McEwen, B.S. & Gold, P.W. 1994. Allostasis, Amygdala, and Anticipatory Angst. *Neuroscience and Biobehavioral Review*. 18(3):385–396.

Scorrano, F., Carrasco, J., Pastor-Ciurana, J., Belda, X., Rami-Bastante, A., Bacci, M.L. & Armario, A. 2015. Validation of the long-term assessment of hypothalamic-pituitary-adrenal activity in rats using hair corticosterone as a biomarker. *FASEB Journal*. 29(3):859–867.

Scotton, E., Colombo, R., Reis, J.C., Possebon, G.M.P., Hizo, G.H., Valiati, F.E., Géa, L.P., Bristot, G., Salvador, M., Silva, T.M., Guerra, A.E., Lopez, T.F., Rosa, A.R. & Kunz, M. 2019. BDNF prevents central oxidative damage in a chronic unpredictable mild stress model: the possible role of PRDX-1 in anhedonic behavior. *Behavioural Brain Research*. 378(March 2019):112245.

Seewoo, B.J., Hennessy, L.A., Feindel, K.W., Etherington, S.J., Croarkin, P.E. & Rodger, J. 2020. Validation of Chronic Restraint Stress Model in Young Adult Rats for the Study of Depression Using Longitudinal Multimodal MR Imaging. *eNeuro*. 7(4):1–48.

Seligman, M.E. & Beagley, G. 1975. Learned helplessness in the rat. *Journal of Comparative and Physiological Psychology*. 88(2):534–541.

Selye, H. 1998. A Syndrome produced by Diverse Nocuous Agents. *The Journal of Neuropsychiatry and Clinical Neurosciences*. 10(2):230–231.

Sengupta, P. 2013. The laboratory rat: Relating its age with human's. *International Journal of Preventive Medicine*. 4(6):624–630.

Sequeira-Cordero, A., Salas-Bastos, A., Fornaguera, J. & Brenes, J.C. 2019. Behavioural characterisation of chronic unpredictable stress based on ethologically

relevant paradigms in rats. *Scientific Reports*. 9(1):1–21.

Sher, L.D. 2019. The Endothelium : An Essential Barrier Between Poor Mental Health and Cardiovascular Disease. Stellenbosch University.

Sher, L.D., Geddie, H., Olivier, L., Cairns, M., Truter, N., Beselaar, L. & Essop, M.F. 2020. Chronic stress and endothelial dysfunction: mechanisms, experimental challenges, and the way ahead. *American Journal of Physiology: Heart and Circulatory Physiology*. 319(2):H488–H506.

Smith, C. 2012. Using Rodent Models to Simulate Stress of Physiologically Relevant Severity: When, Why and How. in *Glucocorticoids - New Recognition of Our Familiar Friend*. 211–230.

Southwick, S.M., Vythilingam, M. & Charney, D.S. 2005. The psychobiology of depression and resilience to stress: Implications for prevention and treatment. *Annual Review of Clinical Psychology*. 1:255–291.

Steimer, T. 2011. Animal models of anxiety disorders in rats and mice: Some conceptual issues. *Dialogues in Clinical Neuroscience*. 13(4):495–506.

Sterling, P. & Eyer, J. 1988. Allostasis: a new paradigm to explain arousal pathology. in *Handbook of Life Stress, Cognition and Health*. 629–649.

Strekalova, T., Spanagel, R., Bartsch, D., Henn, F.A. & Gass, P. 2004. Stress-induced anhedonia in mice is associated with deficits in forced swimming and exploration. *Neuropsychopharmacology*. 29(11):2007–2017.

Strekalova, T., Couch, Y., Kholod, N., Boyks, M., Malin, D., Leprince, P. & Steinbusch, H.M.W. 2011. Update in the methodology of the chronic stress paradigm: Internal control matters. *Behavioral and Brain Functions*. 7(1):9.

Swenson, R. 2006. *Chapter 9 - Limbic System*. Dartmouth Medical School. [Online], Available: https://www.dartmouth.edu/~rswenson/NeuroSci/chapter_9.html.

Tian, J.S., Shi, B.Y., Xiang, H., Gao, S., Qin, X.M. & Du, G.H. 2013. 1H-NMR-Based Metabonomic Studies on the Anti-Depressant Effect of Genipin in the Chronic Unpredictable Mild Stress Rat Model. *PLoS ONE*. 8(9):1–11.

Tsigos, C. & Chrousos, G.P. 2002. Hypothalamic – pituitary – adrenal axis,

neuroendocrine factors and stress. *Journal of Psychosomatic Research*. 53:865–871.

Ulrich-Lai, Y.M. & Herman, J.P. 2009. Neural Regulation of Endocrine and Autonomic Stress Responses. *Nature Reviews Neuroscience*. 10(6):397–409.

Ulrich-Lai, Y.M., Figueiredo, H.F., Ostrander, M.M., Choi, D.C., Engeland, W.C. & Herman, J.P. 2006. Chronic stress induces adrenal hyperplasia and hypertrophy in a subregion-specific manner. *American Journal of Physiology-Endocrinology and Metabolism*. 291(5):E965–E973.

Vale, S. 2005. Psychosocial stress and cardiovascular diseases. *Postgraduate Medical Journal*. 81(957):429–435.

Vialou, V., Robison, A.J., Laplant, Q.C., Covington, H.E., Dietz, D.M., Ohnishi, Y.N., Mouzon, E., Rush, A.J., Watts, E.L., Wallace, D.L., Ĩiguez, S.D., Ohnishi, Y.H., Steiner, M.A., Warren, B.L., Krishnan, V., Bolãos, C.A., Neve, R.L., Ghose, S., Berton, O., et al. 2010. ΔfosB in brain reward circuits mediates resilience to stress and antidepressant responses. *Nature Neuroscience*. 13(6):745–752.

Violle, N., Balandras, F., Le Roux, Y., Desor, D. & Schroeder, H. 2009. Variations in illumination, closed wall transparency and/or extramaze space influence both baseline anxiety and response to diazepam in the rat elevated plus-maze. *Behavioural Brain Research*. 203(1):35–42.

Walf, A.A. & Frye, C.A. 2007. The use of the elevated plus maze as an assay of anxiety-related behavior in rodents. *Nature Protocols*. 2(2):322–328.

Wang, D., Levine, J.L.S., Avila-Quintero, V., Bloch, M. & Kaffman, A. 2020. Systematic review and meta-analysis : effects of maternal separation on anxiety-like behavior in rodents. *Translational Psychiatry*. 10:1–12.

Wang, Q., Timberlake, M.A., Prall, K. & Dwivedi, Y. 2017. The recent progress in animal models of depression. *Progress in Neuro-Psychopharmacology and Biological Psychiatry*. 77(March):99–109.

Weiss, S.M., Wadsworth, G., Fletcher, A. & Dourish, C.T. 1998. Utility of ethological analysis to overcome locomotor confounds in elevated maze models of anxiety. *Neuroscience and Biobehavioral Reviews*. 23(2):265–271.

Wiborg, O. 2013. Chronic mild stress for modeling anhedonia. *Cell and Tissue*

Research. 354(1):155–169.

Wilkinson, C.W., Peskind, E.R. & Raskind, M.A. 1997. Decreased Hypothalamic-Pituitary-Adrenal Axis Sensitivity to Cortisol Feedback Inhibition in Human Aging. *Neuroendocrinology*. 65:79–90.

Willner, P. 1984. The validity of animal models of depression. *Psychopharmacology*. 83(1):1–16.

Willner, P. 1997. Validity, reliability and utility of the chronic mild stress model of depression: A 10-year review and evaluation. *Psychopharmacology*. 134(4):319–329.

Willner, P. 2017a. The chronic mild stress (CMS) model of depression: History, evaluation and usage. *Neurobiology of Stress*. 6:78–93.

Willner, P. 2017b. Reliability of the chronic mild stress model of depression: A user survey. *Neurobiology of Stress*. 6:68–77.

Willner, P. & Mitchell, P.J. 2002. The validity of animal models of predisposition to depression. *Behavioural Pharmacology*. 13(3):169–188.

Willner, P., Towell, A., Sampson, D., Sophokleous, S. & Muscat, R. 1987. Reduction of sucrose preference by chronic unpredictable mild stress, and its restoration by a tricyclic antidepressant. *Psychopharmacology*. 93(3):358–364.

Willner, P., Gruca, P., Lason, M., Tota-Glowczyk, K., Litwa, E., Niemczyk, M. & Papp, M. 2019. Validation of chronic mild stress in the Wistar-Kyoto rat as an animal model of treatment-resistant depression. *Behavioural Pharmacology*. 30(2and3-SpecialIssue):239–250.

Wood, G.E., Young, L.T., Reagan, L.P. & McEwen, B.S. 2003. Acute and chronic restraint stress alter the incidence of social conflict in male rats. *Hormones and Behavior*. 43(1):205–213.

World Health Organization. 2013. *Noncommunicable diseases: Fact sheet*. [Online], Available: https://www.who.int/en/news-room/fact-sheets/detail/noncommunicable-diseases [2019, July 22].

Yan, H.C., Cao, X., Das, M., Zhu, X.H. & Gao, T.M. 2010. Behavioral animal models of depression. *Neuroscience Bulletin*. 26(4):327–337.

Yang, C., Fang, X., Zhan, G., Huang, N., Li, S., Bi, J., Jiang, R., Yang, L., Miao, L., Zhu, B., Luo, A. & Hashimoto, K. 2019a. Key role of gut microbiota in anhedonia-like phenotype in rodents with neuropathic pain. *Translational Psychiatry*. 9(1):1–11.

Yang, S., Song, Z., Wang, X., Zhang, Z., Wu, S. & Zhu, G. 2019b. Curculigoside facilitates fear extinction and prevents depression-like behaviors in a mouse learned helplessness model through increasing hippocampal BDNF. *Acta Pharmacologica Sinica*. 40(10):1269–1278.

Yannielli, P.C., Kanterewicz, B.I. & Cardinali, D.P. 1995. Daily rhythms in spontaneous and diazepam-induced anxiolysis in Syrian hamsters. *Pharmacology Biochemistry and Behavior*. 54(4):651–656.

Yannielli, P.C., Kanterewicz, B.I. & Cardinali, D.P. 1996. Circadian changes in anxiolysis-related behavior of Syrian hamsters. Correlation with hypothalamic GABA release. *Biological Rhythm Research*. 27(3):365–373.

Yao, B.C., Meng, L.B., Hao, M.L., Zhang, Y.M., Gong, T. & Guo, Z.G. 2019. Chronic stress: a critical risk factor for atherosclerosis. *Journal of International Medical Research*. 47(4):1429–1440.

Zacharko, R.M. & Anisman, H. 1991. Stressor-induced anhedonia in the mesocorticolimbic system. *Neuroscience and Biobehavioral Reviews*. 15(3):391–405.

APPENDICES

APPENDIX A: PLASMA COLLECTION PROTOCOL

1. Collect trunk blood in EDTA blood tubes following decapitation.

2. Centrifuge blood tubes immediately at 1500 x g for 10 minutes, at 4°C.

3. Remove plasma supernatant from blood collection tube.

4. Divide plasma into 200ul aliquots and store at -80°C until analysis.

APPENDIX B: ACTH ELISA PROTOCOL

1. Take out samples for concentration test and kit reagents to bring to room temperature.

REAGENT PREP (volumes from booklet for 48 wells & converted for 96 wells):

1. 1X Cell extraction buffer
    - Dilute 5X cell extraction buffer PTR 5X to 1X with deionized water.
    - To make 10ml:
    - 8ml deionized water +2ml cell extraction buffer PTR
    - Mix thoroughly and gently.
    - To make 20ml:
    - 16ml deionized water + 4ml extraction buffer
    - Mix thoroughly and gently.

2. 1X wash buffer PT
    - Dilute 10X wash buffer PT to 1X with deionized water.
    - To make 50ml:
    - 5ml wash buffer + 45ml deionized water.
    - Mix thoroughly and gently.
    - To make 100ml:
    - 10ml wash buffer + 90ml deionized water.
    - Mix thoroughly and gently.

3. Antibody cocktail
    - Dilute capture and detector antibodies in antibody diluent 4Bl.
    - To make 3ml:
    - 300ul 10X capture antibody + 300ul 10X detector antibody + 2.4ml antibody diluent 4Bl.
    - To make 6ml:
    - 600ul 10X capture antibody + 600ul 10X detector antibody + 4.8ml antibody diluent 4Bl.
    - Mix thoroughly and gently.

STANDARD PREP (for duplicate):

1. Reconstitute ACTH standard:

- Add 500ul of 1X cell extraction buffer PTR.
- Mix thoroughly and gently
- Hold at room temp for 10 mins
- Mix gently (4000pg/ml stock solution).

2. Label 8 tubes (ST 1-STD8).
3. Add 350ul 1X cell extraction buffer into tube 1.
4. Add 150ul cell extraction buffer to tubes 2-8.
5. Add 50ul of stock to tube 1.
6. Add 150ul from tube 1 to tube 2.
7. Add 150 ul from tube 2 to tube 3 and so on.

SAMPLE PREP:

Dilute if necessary, using 1X cell extraction buffer PTR.

ELISA (exact same process for concentration test and full run):

1. Add 50ul of standards or samples to appropriate wells (see plate layout).
2. Add 50ul of the antibody cocktail to each well.
3. Seal plate and incubate for 1hr at room temp on plate shaker set at 400rpm.
4. Wash each well with 350ul wash buffer (X3) aspirate/decant fully between each wash. On final wash, invert over paper towel to ensure all wash buffer removed.
5. Add 100ul TMB development solution to each well and incubate for 20min in the dark on a plate shaker set to 400rpm.
6. Add 100ul stop solution to each well.
7. Shake plate on plate shaker for 1min.
8. Record OD at 450nm.

APPENDIX C: CORTICOSTERONE ELISA PROTOCOL

Determine the number of wells to be used and return unused wells to the foil pouch with desiccant. Seal the Ziploc plate bag and store at 4°C.

SAMPLE PREP:

- Allow the Dissociation Reagent to warm completely to Room Temperature before use.
- Pipet 5 uL of Dissociation Reagent into 1 mL Eppendorf tubes.
- Add 5 uL of serum or plasma to the Dissociation Reagent in the tube, vortex gently and incubate at room temperature for 5 minutes or longer.
- Dilute with 490 uL of diluted Assay Buffer. This 1:100 dilution can be diluted further with diluted Assay Buffer. Final serum and plasma dilutions should be ≥ 1:100.
- Use all Samples within 2 hours of preparation or store at ≤ - 20°C until assaying.

REAGENT PREP

Assay Buffer

- Dilute Assay Buffer Concentrate 1:5 by adding one part of the concentrate to four parts of deionized water.
- Once diluted this is stable for 3 months at 4°C.

Wash Buffer

- Dilute Wash Buffer Concentrate 1:20 by adding one part of the concentrate to nineteen parts of deionized water.
- Once diluted this is stable for 3 months at room temperature.

Standard Preparation - 50 uL Assay Format

- Label test tubes as #1 through #9.
- Pipet 450 uL of Assay Buffer into tube #1 and 250 uL into tubes #2 to #9. The corticosterone stock solution contains an organic solvent.
- Prerinse the pipet tip several times to ensure accurate delivery.

- Carefully add 50 uL of the corticosterone stock solution to tube #1 and vortex completely.
- Take 250 uL of the corticosterone solution in tube #1 and add it to tube #2 and vortex
- completely.
- Repeat the serial dilutions for tubes #3 through #9.
- The concentration of corticosterone in tubes 1 through 9 will be 10,000, 5,000, 2,500, 1,250, 625, 312.5, 156.25, 78.125 and 39.063 pg/mL.

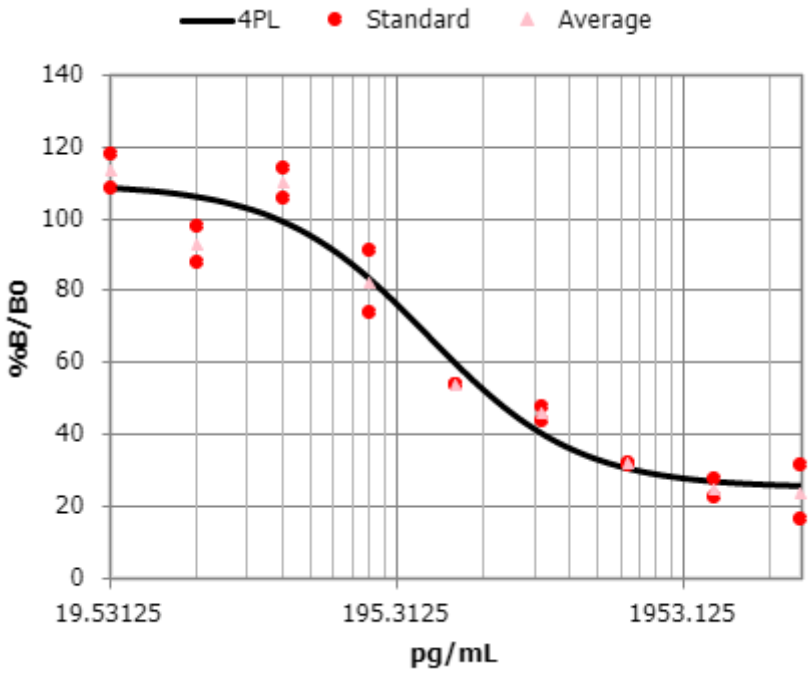
ASSAY PROTOCOL

- Determine the number of wells to be used and return unused wells to the foil pouch with desiccant. Seal the Ziploc plate bag and store at 4°C.
- Pipet 50 uL (100 uL for alternative format) of samples or standards into wells in the plate.
- Pipet 75 uL (125 uL for alternative format) of Assay Buffer into the non-specific binding (NSB) wells.
- Pipet 50 uL (100 μL for alternative format) of Assay Buffer into the maximum binding (B0 or Zero standard) wells.
- Add 25 uL of the DetectX® Corticosterone Conjugate to each well using a repeater pipet.
- Add 25 uL of the DetectX® Corticosterone Antibody to each well, except the NSB wells, using a repeater pipet.
- Gently tap the sides of the plate to ensure adequate mixing of the reagents. Cover the plate with the plate sealer and shake at room temperature for 1 hour. If the plate is not shaken signals bound will be approximately 45% lower.
- Aspirate the plate and wash each well 4 times with 300 uL wash buffer. Tap the plate dry on clean absorbent towels.
- Add 100 uL of the TMB Substrate to each well, using a repeater pipet.
- Incubate the plate at room temperature for 30 minutes without shaking.
- Add 50 uL of the Stop Solution to each well, using a repeater or a multichannel pipet.

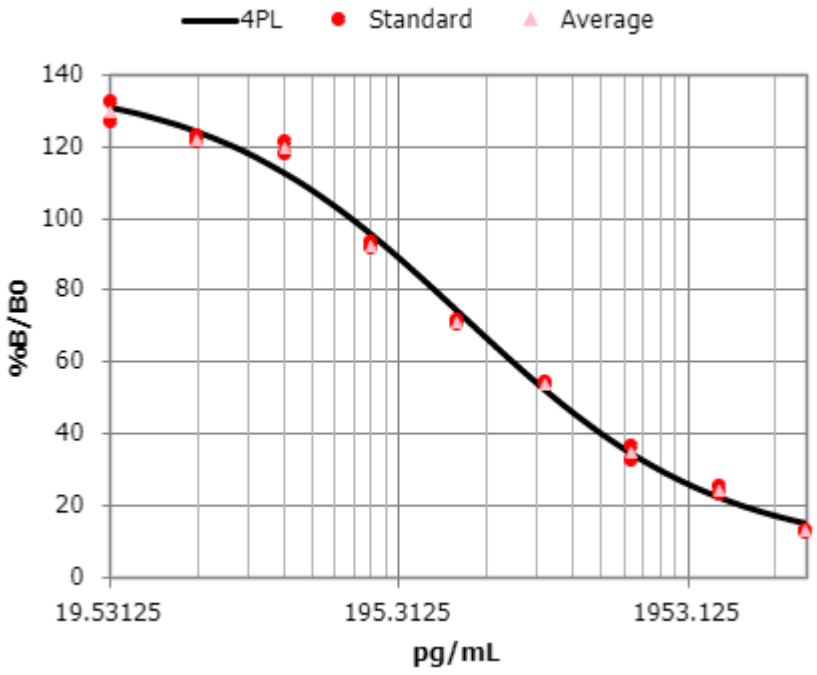
- Read the optical density generated from each well in a plate reader capable of reading at 450 nm.
- Use the plate reader’s built-in 4PLC software capabilities to calculate corticosterone concentration for each sample.

APPENDIX D: STANDARD CURVES FOR CORTICOSTERONE

Plasma Corticosterone



Hair Corticosterone



APPENDIX E: PLAGIARISM REPORT

This appendix includes the plagiarism report generated by Turnitin.

LC Beselaar 18956386 MSc Thesis

ORIGINALITY REPORT

10%	6%	7%	3%
SIMILARITY INDEX	INTERNET SOURCES	PUBLICATIONS	STUDENT PAPERS