CHRONIC GUT INFLAMMATION CHANGES ANXIODEPRESSIVE AND COPING BEHAVIOUR IN FEMALE MICE

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Bachelor of Science, University of Lethbridge, 2023

A thesis submitted

in partial fulfilment of the requirements for the degree of

MASTER OF SCIENCE

in

NEUROSCIENCE

Department of Neuroscience

University of Lethbridge

LETHBRIDGE, ALBERTA, CANADA

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Date of Defense: July 8, 2025

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DEDICATION

To my loving, supportive family and friends – thank you for your dedication and for cheering me on all these years, I am forever grateful for your love.

ABSTRACT

Major depressive disorder (MDD) is a complex condition, with increasing evidence supporting the existence of an inflammation-associated subtype. People with inflammatory bowel diseases (IBDs), which cause chronic gut inflammation, experience disproportionately high rates of anxiety and depression - females with IBD appear to be particularly susceptible. It remains unclear why females are at higher risk. This is partly because most preclinical research to date has used male rodents to study how chronic inflammation affects the brain. In this study I investigated the impact of chronic gut inflammation on anxiety-like and threat-coping behaviours in female mice. The treatment group exhibited changes to threat coping behaviours and shock reactivity in the shock probe defensive burying test, and changes in threat coping behaviours in the forced swim test when compared to controls. However, there were no significant differences observed between the groups on tests of anxiety-like behaviour in the elevated plus maze and open field task. The findings suggest that chronic gut inflammation selectively alters coping behaviour without inducing general increases in anxiety-like behaviour. To the author's knowledge, this is the first study to report behavioural changes in the shock probe defensive burying task and forced swim test in female mice exposed to chronic DSS treatment. This work highlights the importance of sex-specific research and the use of nuanced behavioural paradigms in preclinical models to better understand the effects of chronic inflammation on anxiodepressive behaviours. Overall, the novel findings contribute to a growing body of literature linking gut inflammation to alterations in central nervous system function and behaviour in a female murine model.

ETHICS STATEMENT

All testing and animal care was in compliance with Canadian Council on Animal Care guidelines. Ethical approval for all testing and methods was approved by the University of Lethbridge Animal Welfare Committee under protocol #2308.

The work described in this thesis received research ethics approval under the following applications:

Title	Number	Date
Entheogens, Gut Inflammation, and The Brain	AWP#2308	Feb. 8, 2023

USE OF GENERATIVE AI

Artificial intelligence was used sparingly to assist in outlining portions of the results, discussion, and conclusion. All AI generated content was edited.

ACKNOWLEDGEMENTS

Thank you, Dr. Aaron Gruber, for your guidance and support over the years, you have always been supportive of my ideas and limitations, I could not be more grateful to have worked with you. Thank you to Dr. Chelsea Matisz, your support and expertise were a pillar of my education, I cherish your lessons and the time we spent working together. Thank you to Dr. Ian Whishaw, I have learned so much from you, your support in all my different academic endeavours has opened my eyes to new avenues of research. Thank you to my committee members, whose guidance has been much appreciated.

Thank you to my friends, inside and out of the Neuroscience department at the University of Lethbridge, I appreciate you all beyond words. Thank you to all current and previous members of the Gruber lab, especially Dr. Matisz, Dr. Ivan, Ben Livingstone, and Cailin Douglas - this thesis would not have been possible without your help and dedication. Thank you to the University support staff and ACS for keeping the lights on and animals cared for!

Finally, thank you to my family and healthcare team for keeping me going. I would never have made it this far without you.

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List of Abbreviations

DAI Disease Activity Index

DSS Dextran Sodium Sulphate

EPM Elevated Plus Maze

FST Forced Swim Task

IBD Inflammatory Bowel Diseases

OFT Open Field Task

SPDB Shock Probe Defensive Burying

Introduction:

Major depressive disorder is a mood disorder characterized by changes to mood and mentation, with persistent feelings of sadness or emptiness that cannot be attributed to the effects of a substance or medical condition (American Psychiatric Association, 2013).

The 5th edition of the Diagnostic and Statistical Manual of Mental Disorders acknowledges that there are certain inflammatory factors that may be associated with this condition, but do not elaborate beyond mentioning these phenomena. Whereas there is no causal link yet demonstrated between major depressive disorder and inflammation, there exists ample evidence for an inflammatory sub-type of depression (Raison & Miller, 2011). Whether the repeated findings of increased inflammation in a subset of the population with major depressive disorder is a result of certain depressed individuals being particularly vulnerable to experience inflammation, or whether it is evolutionarily adaptive for low levels of inflammation to drive depressive behavioural phenotypes is not known. Whereas both possibilities have been proposed as contributors to elevated inflammatory markers in some people with depression, there are reported cases of inflammation related to changes in the brain.

Human clinical research into the rates of anxiodepressive disorders in people with IBS show such brain changes. The connections between the anterior cingulate cortex and other brain regions (Davey et al., 2012), and neurotransmitter and neuromodulator activity in anterior cingulate cortex (Tripp et al., 2012) are altered in people with depression. Changes to the anterior cingulate cortex are documented in the case of depression associated with inflammatory bowel diseases. People with Crohn's disease, a form of inflammatory bowel disease, show changes to the anterior cingulate cortex structure, metabolism, and functional connectivity (Kong

et al., 2021). Further evidence of the link between chronic gut inflammation and disorders involving anxiety and depression (anxiodepressive disorders) is provided by the disproportionately high rates of depression and anxiety in people with inflammatory bowel diseases. One Canadian study conducted on diagnosed inflammatory bowel disease patients in a gastroenterology outpatient clinic found that those so diagnosed had a 12-month rate of depression ~5.5X higher than the general population, and a 12-month rate of anxiety ~8.2X higher than the general population (Byrne et al., 2017). The rate of depression and anxiety increased dramatically in those with active inflammatory bowel disease, with 52.4% of participants having anxiety and/or depression compared to 20.9% in those with inactive inflammatory bowel disease. Tarar et al. (2022) report that female humans with inflammatory bowel diseases are at higher risk of developing anxiety and depression than males – with rates in females double those in males. Interestingly, acute inflammation has been reported to lead to a greater increase in depressive feelings and perceived social disconnection in females compared to male participants, suggesting unique affective responses to inflammation in females (Moieni et al., 2015).

The reasons for the difference in incidence rates of depression across the sexes are not well understood. There are many contributing socioeconomic and biological factors, with one potential biological factor being the effects of circulating sex hormones. Research done in rodent models have proven useful in exploring these biological factors, with the estrogen 17β-estradiol shown to have an impact on the development and progression of chronic gut inflammation (Bábíčková et al., 2015; Verdú et al., 2002). However, how sex-dependent mechanisms might alter anxiodepressive behaviours resultant from chronic gut inflammation is not yet well understood. This may in part be due to a bias in use of male animal subjects in

research studies. A search of PubMed using the search terms "(anxiodepressive) OR (anxious)) OR (depressive)) AND (rodent)" reveal 2.67 results that include male subjects for every single result that includes female subjects**.**

Inflammatory bowel diseases involve repeated or persistent inflammation of the bowel. The enduring nature of this inflammation is what characterizes it as chronic. Pro-inflammatory cytokines are chemical signals circulating in the blood stream that perpetuate an inflammatory response. In the case of inflammatory bowel diseases their production is triggered by colonic inflammation (colitis), but they then circulate throughout the body via the cardiovascular system. Animal models show that this chronic whole-body exposure to pro-inflammatory cytokines results in injury to the blood-brain barrier, which may increase permeability to the brain (Craig et al., 2022; Hathaway et al., 1999). The severity of the increased permeability, at least in a model of acute inflammation, is debated (Han et al., 2018). However, increased blood-brain barrier permeability has been observed in a chronic dextran sulfate sodium (DSS) murine model of inflammatory bowel disease colitis (Mitchell et al., 2022), suggesting that the increased blood-brain barrier permeability requires repeated or chronic inflammatory insults like those seen in inflammatory bowel diseases. This increased permeability is thought to allow more circulating neuroinflammatory markers to cross from the blood into the brain, eliciting inflammatory responses in the brain (Craig et al., 2022). Pain signaling and changes to gut microbiome composition are thought to contribute to neural remodeling in inflammatory bowel disease as well (Craig et al., 2022; Kong et al., 2021; Matisz & Gruber, 2022). These many mechanisms are illustrated by Matisz & Gruber (2022) (Fig.1), who summarize the potential causal mechanisms of inflammatory bowel disease-induced changes to the anterior cingulate cortex as a complex interplay of nociceptive signals, altered gut-microbiome-host interactions, stress,

Hypothalamic-Pituitary-Adrenal (HPA) axis signaling, neurotransmitter activity, and immune signaling. Through these many mechanisms, chronic inflammation in the gut is proposed to induce heightened threat perception. When this inflammation persists, an organism may adopt a negativity bias and become unable to shift selective attention away from negative stimuli, as posited by the cognitive model of depression (Disner et al., 2011). It has been proposed that through these mechanisms, chronic inflammation may induce hypersensitivity in anterior cingulate cortex threat signaling cells thereby increasing threat-coping behaviours (Matisz & Gruber, 2022).

Depression-like symptoms may be adaptive responses to internal and external pressures. For example, it is adaptive for an ill animal to adopt acute sickness behaviours, like remaining asleep in the den or becoming hypervigilant, to reduce the likelihood of predation or exposure to unnecessary risks while in a vulnerable illness state brought on by significant infection or injury. The threshold of what is perceived to be harmful may become lower during illness (a negativity bias), and the behavioural response to such stimuli may become more extreme (increased coping behaviours). Coping behaviours in rodent studies can be characterized by their valence; either as passive responses including immobility, or as active responses like defensive burying.

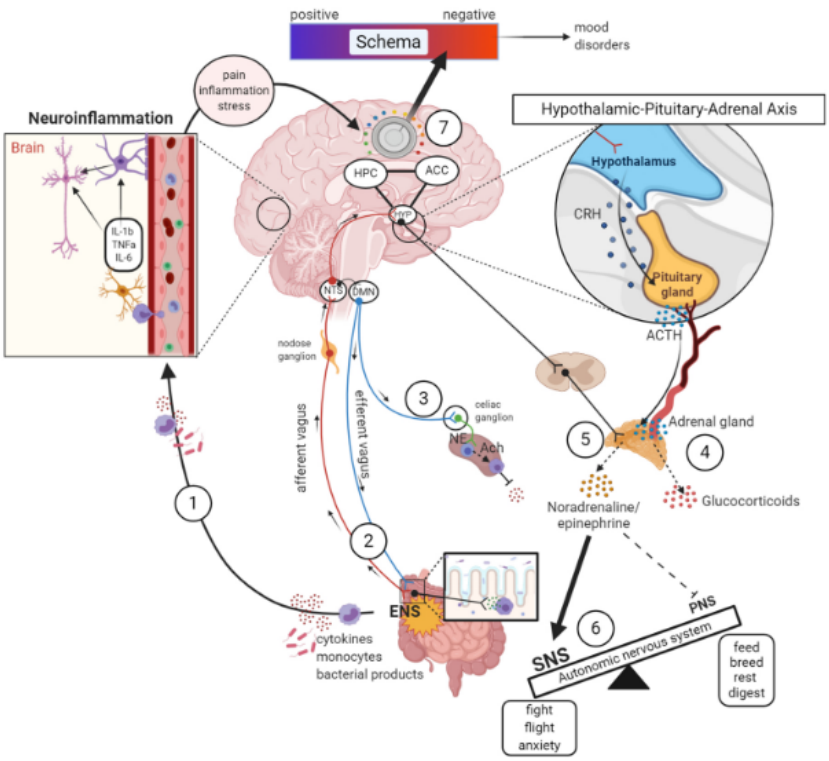

Fig. 3. Inflammation and the gut-brain axis 1) Inflammatory cytokines in the gut are released into the circulation, where they promote neuroinflammation. Microglia exposed to circulating inflammatory mediators transition into a reactive pro-inflammatory phenotype; they secrete chemokines to recruit circulating monocytes into the brain tissue, and induce reactive phenotypes of astrocytes. Reactive microglia and astrocytes influence neuronal function. Other infiltrating immune cells may include t-cells and mast cells. 2) The enteric nervous system (ENS) detects inflammatory mediators, activating afferent vagal fibers These signals are relayed to the nucleus tractus solitarus (NTS), where they can communicate with efferent vagal fibers in the dorsal vagal complex, which includes the dorsal motor nucleus (DMN). Efferent fibers stimulate acetylcholinesterase release from enteric neurons which act on macrophages via the α7-nicotinic receptors to suppress cytokine production. 3) Vagal efferents also communicate with sympathetic nerves in the celiac ganglion that project to the spleen, an organ critically involved in systemic inflammation Stimulation of the splenic sympathetic nerve prompts the release of NE-the dominant neurotransmitter of the SNS- which activates splenic lymphocytes via β2 adrenergic receptors. These activated cells are a source of acetylcholine that attenuate production of the pro-inflammatory cytokines in macrophages. (4) The hypothalamic-pituitary-adrenal axis can be activated by external (e.g. psychosocial stress) and internal stressors. The hippocampus and amygdala play a role in processing experiences, and regulate the activity of the HPA axis. Activated vagal afferents project to the hypothalamus. Stimulation of neurons in the the hypothalamus where they induce release of corticosterone releasing hormone (CRH). This drives the release of adrenocorticotropic hormone from the pituitary, which stimulates the adrenal gland to synthesize and release glucocorticoids (GC) into the circulatory system. While glucocorticoids possess anti-inflammatory properties, chronic activation of the HPA axis can promote an inflammatory effect of glucocorticoids. The Hippocampus is a key region impacted by GC, and is a suggested mechanism of stress-induced depression.5) Brain nuclei (including within the hypothalamus) activate sympathetic fibers that project to the adrenal medulla, driving the release of epinephrine and noradrenaline (NE) into circulation. These catecholamines induce physiological responses (increased heart rate, respiration rate, increased delivery of blood to skeletal muscles) collectively terms the 'fight or fight response'. The SNS innervates the gut, and communicates with the ENS. Generally the SNS inhibits gut function, but also modulates immune function. In certain contexts, chronic SNS activation drives pro-inflammatory responses. 6) Activation of the SNS attenuates parasympathetic responses, including anti-inflammatory vagal reflexes. 7) The ACC appraises environmental and personal stress. It plays a key role in processing emotional states, and emotional aspects of pain. It regulates behavioural and autonomic responses to emotional and stressful stimuli, serving as an interface between appraisal/prospection and allodynamic control. Input from the ACC guides responses to internal and external stimuli. In GIDD, the ACC is associated with reduced cortical thickness, increased activity, and altered functional connectivity. Reduced volume of the ACC is associated with dysregulation of the HPA. These neural changes in the ACC are hypothesized to promote a negative schema.

Figure.1: Matisz & Gruber's (2022) summary of the mechanisms by which anterior cingulate cortex changes occur in people with IBD. Reproduced with permission.

One goal of the present thesis is to test whether exposing female mice to chronic DSS treatment can model some of the changes seen in humans with inflammatory bowel disease and alter anxiodepressive behaviours. Another goal of this thesis is to assess whether female mice

showed changes to their coping behaviours after chronic exposure to DSS. Previous experiments conducted by the Gruber lab showed interesting forced swim task (FST) results in male mice treated with DSS; treatment animals trended towards reduced immobility times compared to controls (Matisz et al., 2022), and males exposed to acute, but not chronic DSS, had significantly reduced immobility times compared to controls (Matisz et al., 2020). One might expect that chronically inflamed animals, not acutely inflamed ones, would have greater behavioural differences compared to non-inflamed controls because of the enduring nature of chronic inflammation. However, current evidence suggests there to be some degree of behavioural adaptation in male mice with chronic gut inflammation. This thesis aims to ascertain whether this pattern holds true for female animals treated with the same gut-inflammation induction protocol.

1.1 Experimental Design and Hypothesis:

The theory behind this experiment posits that chronic gut inflammation induces brain inflammation. The consequences of this process will alter an animal's response to its environment and stimuli in ways that are adaptive and promote survival. Responses to potentially dangerous or threatening stimuli (like an electrified prod) or environments (like an inescapable body of water) will be different in animals with chronic gut inflammation because of inflammation-induced changes to the brain.

I hypothesize that treating female mice with DSS will accurately model key behavioural changes seen in humans with inflammatory bowel disease, particularly altering their threat coping behaviours. DSS treatment is a well-established model of colitis, it causes weight loss,

diarrhea, and gut inflammation similar to what is experienced by people with colitis. I expect DSS-colitis to alter how treatment animals explore and interact with threatening stimuli and environments. Observing such changes to behaviour requires a model system that possesses sufficient sensory and motoric processing to respond to threats, and gut physiology that resembles what is seen in humans. Cell cultures or simple animal models like flat worms are therefore inappropriate. Previous studies using the same DSS protocol used here have shown that mice are a useful model to study the associations between chronic gut inflammation and threat coping (Matisz et al., 2020).

Methods:

2.1 Subjects:

Twenty-four adult C57Bl/6 female mice aged 7 weeks were procured from Jackson lab, Bar Harbor, Maine. Throughout the duration of the experiment, the animals were housed four to a cage, given standard enrichment*, ad libitum* food and water, and housed in a 12-hour light/dark cycle with the lights on between the hours of 7:30 am to 7:30 pm. The animals were split into two groups, twelve in the treatment group exposed to DSS in their drinking water, and twelve in the control group given normal drinking water. One DSS-treated animal was excluded early in the study, resulting in a final n=23. Animals were allowed one week of habituation to their housing cages before handling habituation began. Animals were handled and weighed daily for one week to habituate them to the handling process. The animals were habituated to scruffing for the final three days of that week to help minimize the stress of scruffing during vaginal

lavage sampling. DSS exposure began two weeks after habituation, at 11 weeks of age. A clean, standard water bottle was filled with 200ml of DSS solution and weighed on the morning of the first day of DSS exposure. On the final day of DSS exposure, the DSS solution-filled water bottles were weighed and replaced with clean standard water bottles. The consumption of individual animals could not be accounted for, and as such, each individual animal received a different dose of DSS which may account for some variability. During testing, the animals were weighed and assessed in the mornings every Monday, Wednesday, and Friday except during peak DSS-induced gut illness (cycle days 7-12) wherein they were weighed and assessed daily. All handling and testing were staggered by one week between the control and treatment groups, beginning with the treatment group and followed by the control group undergoing identical procedures the following week (fig. 1.A). All testing and animal care was in compliance with Canadian Council on Animal Care guidelines. All testing and methods were approved by the University of Lethbridge Animal Welfare Committee under protocol #2308.

2.2 Fecal Collection and Analysis:

Baseline testing began with a disease activity index (DAI) fecal analysis and collection. Each animal was weighed and then placed alone in a labelled home cage lined with paper towel on a transport rack in the behavioural testing room. The animals were left for a minimum of one hour before being returned to their home cage. Each animal's feces were analyzed for signs of diarrhea and blood, both indicators of intestinal damage. This information was recorded and, in conjunction with weight loss, was used to titrate the dose of DSS used in the subsequent exposure. Untested feces were then placed in Eppendorf tubes labelled with the animal number and date of collection. All samples were stored in a -20 freezer for later homogenization and

analysis. Animals were returned to their home cage after samples were collected. If individual animals did not defecate during the one hour wait period, that animal was kept for another hour.

2.3 DSS Exposure and Gut- Inflammation Induction:

DSS is a water-soluble chemical used in animal models of chronic gut inflammation and inflammatory bowel diseases (Thermo Scientific Chemicals, Waltham, MA, USA; Cat# J14489.22). It is mixed in a weight/volume solution in the subject's regular drinking water and offered *ad libitum*. The percentage of DSS in the drinking water typically ranges from 3-5%, the DSS solution was made the morning of treatment cycle day 1. During the first and second cycles a 3% (wt./vol) solution was used, however the final cycle was increased to a 3.5% solution. Each animal was weighed before the DSS bottle was placed in each home cage. This weight was considered the baseline weight for that cycle of DSS exposure. Each animal was weighed thrice during the DSS exposure on the Monday (day 1), Wednesday (day 3), and Friday (day 5) that DSS was offered. The bottles of DSS were replaced on day 5 with standard bottles of drinking water. Animals were weighed and monitored daily until the end of their peak disease period on cycle day 12.

2.4 Elevated Plus Maze and Open Field Task Testing:

All animals were allowed one hour to habituate to the behavioural testing room, housed in their home cage, on the first day of testing. Subsequent testing sessions allowed the animals to habituate for a minimum of 15 minutes in their home cage. A camera was positioned above the elevated plus maze (EPM) or open field test (OFT) apparatus, and the apparatus was cleaned

with the accelerated hydrogen peroxide disinfectant, Prevail®. The animal subject was placed in the middle of the elevated plus maze or open field test apparatus and filmed for 10 minutes. The experimenter remained in the testing room and recorded notes on the animal's behaviour, noises-produced by the animal or in the environment, or other occurrences during testing. All testing was performed under standard lighting conditions, and the apparatus was disinfected with Prevail® between all subjects. Behavioural videos were subsequently scored by a machine learning algorithm designed to track the center of a moving object. The algorithm tracked the dark edges of the mouse against the white background of the testing apparatuses. The position of the animal was recorded as the center of a box whose sides touched the periphery of the animal's silhouette.

2.5 Shock-Probe Defensive Burying Testing:

The shock-probe defensive burying (SPDB, or shock-probe) test is used as a measure of threat-coping behaviours in rodents. The specific innate rodent threat coping responses measured in this test include freezing (passive threat coping) and burying (active threat coping). SPDB testing was conducted in a separate room from the EPM and OFT dedicated to high-stress behavioural testing. Animals were allowed one hour to habituate to this testing room on the first day, and a minimum of 15 minutes every subsequent day. The testing apparatus consisted of an opaque cylindrical chamber with a diameter of 25cm, a height of 24.75 cm, and a single hole to accommodate the shock-prod, which was located 4cm up from the bottom of the chamber. Above the chamber was a fixed video camera to record the animals' responses. The apparatus was cleaned with Prevail®, then was filled with the animal's standard sanitized bedding 3 cm from the bottom (~ 4 cups). The position of the apparatus and the camera were marked to ensure

that they remained in the same location between subjects. The camera remained above the apparatus every day to habituate the animals to its presence, regardless of whether it was recording or not. The animals were placed in the apparatus for 15 minutes on the habituation days (1-4) to acclimate to the apparatus. Used bedding was discarded in a separate room, and the chamber cleaned with Prevail® before being refilled with fresh new bedding between each animal as to prevent interference from the smell of previous subjects on subsequent subjects' behaviours. On day 5, the shock-prod was inserted and a single 2mA shock was delivered to the animal – the time of shock was noted, and the animal was recorded for 15 minutes after receiving the shock. After the recording stopped, the animal was returned to its home cage. Videos of animal behaviours were scored on their burying, freezing, and shock response behaviours by a single group-blind researcher. Burying behaviour was defined as the active displacement of bedding in any section of the chamber, be it pushing bedding towards the probe or burrowing into the bedding. Freezing was defined as immobility in the back half of the chamber opposite the shock probe. Behaviours that require a degree of stillness such as grooming, rearing, attend-stretches, or sitting with obvious and large head movements were not scored as immobility. The animal's immediate reaction to the shock was scored on a 4 point scale (Fucich & Morilak, 2018; Sluyter et al., 1999; Treit, 1990), as originally outlined by Sluyter et al. (1999). After the mouse is shocked, its reaction to the shock was scored from 1-4; a score of 1 being defined as a head or paw flinch, a score of 2 being defined as a full body flinch without immediately moving away from the probe, a score of 3 being an intense full body flinch and/or jump away before immediately moving away from the probe, and a score of 4 being an intense full body flinch and jump with all 4 paws leaving the ground and immediately running away from the probe.

2.6 Forced Swim Task:

The Forced Swim task is often purported to be a test of depressive behaviours in rodents (Yankelevitch-Yahav et al., 2015), however this assertion is contentious (Armario, 2021; Commons et al., 2017). It involves placing the animal in an inescapable testing apparatus filled with room temperature water then measuring the amount of time spent floating immobile and the amount of time spent actively swimming. This test was conducted in a familiar room, and as such, animals were given a minimum of 15 minutes to acclimate to the testing room before testing began. A clear, cylindrical testing apparatus was used and filled with room-temperature water to a marked point where animals would be unable to feel the bottom of the chamber with their feet or tails. The animal was then gently placed in the water, with care taken not to submerge the animal's head or cause undue stress, and then the subject was filmed for 6 minutes before being retrieved. The animal was towel dried and placed in a recovery cage consisting of a standard paper towel-lined housing cage with a heating pad under half of the cage. This configuration allows the animal to better thermoregulate by providing a warmer and cooler end of the recovery chamber. The temperature of the heating pad was monitored via thermometer to ensure it did not exceed 37 °C. Animals from the same home cage were placed together in the same recovery cage, and given a minimum of 20 minutes to recover before being returned to their home cages once sufficiently dry.

Videos were scored by a single group-blind researcher. The scoring was split into two sections; the 2 minute "pre-test" which is characterized by lots of movement with little immobility, and the following 4 minute "test" (Yankelevitch-Yahav et al., 2015). Only the final 4-minute test section was analyzed, as per typical protocols. Immobility was defined as any time the animal is passively floating and not kicking to propel itself forward. Small kicks the animal

made to keep itself right or keep its head above the water are included in immobility time. Swimming was defined as the time the animal spends paddling its fore- or hindlimbs to propel itself forward. Struggling or climbing behaviours were defined as quick movements of the forelimbs that broke the surface of the water and any attempts to scale the side of the apparatus – struggling behaviours were not analyzed as they occurred almost exclusively in the pretest phase.

2.7 Fecal Homogenization:

Upon completion of all the behavioural tests, the animal's feces were homogenized to be used in the mouse lipocalin-2/NGAL DuoSet ELISA (R&D Systems, Minneapolis, MN, USA; Cat# DY1857). This homogenization process suspends the target proteins in a liquid homogenate separate from the solid feces (Chassaing et al., 2012). To begin, an Eppendorf tube was labelled with the animal ID and collection date of a frozen fecal sample. The tube was weighed, and the scale tared to the tube's weight, then ~50-100g of feces was measured out. The weight amount of feces in each tube was recorded, 10ml of a 1XPBS and 0.1% Tween® 20 solution was added to each gram of feces in each tube, adjusted for the weight of the feces. The tubes were then closed and agitated by hand to begin breaking up the feces, before being placed on a vortex mixer for 20 minutes. The tubes were then placed in a 4 °C centrifuge at 12000RPM for 10 minutes. The fecal homogenization supernatant was pipetted into new labelled Eppendorf tubes, taking care not to pipette any solids when transferring the supernatant. The fecal supernatant was stored in a -20 freezer until analyzed.

2.8 Mouse Fecal Lipocalin-2 ELISA:

ELISAs are a method of measuring the amount of a target protein in a sample by comparing the light absorption of samples treated with antigen-fluorophore conjugates against a standard curve with known absorption values (Alhajj et al., 2025). To begin, 83 µL of the primary antibody was diluted in 10 ml of 1X PBS and mixed thoroughly with a multichannel pipette. 100 µL of this solution was added to each well of the ELISA plate, and the plate was incubated overnight, covered in tin foil and gently agitated on an orbital shaker. Homogenized fecal samples were thawed and diluted to the appropriate working concentration. All baseline and control samples were diluted 1:2 fecal homogenate:reagent diluent, while chronic experimental samples were diluted 1:2000. Once the standard and samples had been diluted, the plate was washed 3 times with washing buffer. These samples and the standard curve were diluted and added to the plate in duplicate as per manufacturers instructions, then left in a 4 °C fridge overnight. The following morning, the plate was washed 3 times with washing buffer before the detection antibody was added and allowed to incubate at room temperature on an orbital shaker for a minimum of 2 hours. The plate was washed 3 times, and HRP was added to the wells as per manufacturer's instructions then left to incubate for no more than 10 minutes. Finally, the stop solution was added, and the plates were immediately imaged with a SpectraMax i3x Multi-Mode Detection Platform plate reader.

2.9 Experimental Timeline

The experimental design was similar to previous experiments done with male animals, however in this study the shock-probe defensive burying (SPDB) task was added as an established

measure of threat coping. Twenty-four mice were divided into control and treatment groups (n=12 each; one DSS-treated mouse was excluded, final n=23). Mice were housed under standard conditions and allowed to acclimate to the facility before undergoing one week of handling habituation by the experimenters. The DSS protocol then began (Fig. 2A), with animals undergoing baseline gut inflammation and anxious behaviour testing in the elevated plus maze and open field test. After baseline measures were collected, the treatment group (n=11) received three cycles of 3–3.5% DSS in their drinking water for five consecutive days, while the control group remained drinking standard water. After the first acute DSS exposure, both groups underwent the same gut inflammation and anxious behaviour testing that was performed at baseline. The treatment animals were then exposed to two subsequent five-day treatments of DSS before all animals underwent the final round of testing. Gut inflammation and anxious behaviour testing was performed for a third time, followed by five days of shock-probe defensive burying testing and one day of the forced swim task testing. Following testing, the animals were humanely euthanized.

2.10 Statistical Analysis:

Statistical analysis was conducted using GraphPad Prism (version 8.0.2) for Windows (GraphPad Software, Boston, Massachusetts USA), and IBM SPSS Statistics for Windows, version 27 (IBM Corp., Armonk, N.Y., USA). Before all ANOVAs and unpaired t-tests, outliers were identified in every dataset using the ROUT (Q=1%) method, and excluded from analysis. While this process was done to all datasets, many datasets contained no outliers and the majority of sets with outliers included no more than two outliers. Data was then tested for normality using the Shapiro-Wilk test. Unpaired t-tests were used to determine group differences between

DSS and control animals on measures/performance in the SPDB, EMP, OFT, FST, DAI scores, LCN-2 levels, and weights. Non-parametric data were assessed with Kolmogorov-Smirnov tests, except in cases with many tied values in which Mann-Whitney tests were used. Parametric data were analyzed using Welch's T tests. Repeated measures ANOVAS were performed in IBM SPSS 27 and GraphPad Prism. ANOVAs and repeated measures were performed to compare measures across timepoints. All graphs were designed in GraphPad Prism.

Results:

3.1 DSS Induced Weight Loss and Gut Inflammation

As expected, the experimental group, but not the control group, lost weight in a cyclical manner throughout the duration of the experiment after each DSS exposure (fig. 2.B). Control and DSS treated animals did not differ in weight during the baseline period (Welch's t test; $t=1.634$; $p = 0.1193$), however the DSS group weighed significantly less than controls after the final cycle of DSS exposure (Welch's t test; $t=3.524$; $p = 0.0020$).

Measures of gut inflammation were taken at baseline and after acute and chronic treatment exposure. The disease index assessment of feces via DAI was significantly elevated in DSS-treated mice. A repeated measures ANOVA revealed a significant interaction between testing timepoint and group (Wilks' $\Lambda = .252$, $F(2, 20) = 29.70$, $p < .001$, partial $\eta^2 = .748$). Post hoc tests on DAI scores for fecal consistency (diarrhea) reveal no differences between the groups at baseline (Mann-Whitney Test, $U=66$, $p >0.9999$, two-tailed) and significant group differences

during the acute (Mann-Whitney Test, U=24, p= 0.0013, two-tailed) and chronic (Mann-Whitney Test, U=9, p= <0.0001, two-tailed) timepoints. Despite changes in measures of fecal consistency, there was no blood detected in fecal samples from DSS or control groups at any timepoint, suggesting that although gut inflammation occurred, it did not cause macroscopic intestinal bleeding. As expected, assessment of the fecal protein indicator of inflammation LCN-2 was markedly higher in DSS-treated animals; a repeated measures ANOVA revealed a significant interaction between testing timepoint and group on fecal LCN2 levels (Wilks' Λ = .437, $F(1, 21) = 27.05$, $p < .001$, partial $\eta^2 = .563$). Fecal levels of LCN-2 did not differ significantly between the groups at baseline (Welch's t-test, t=1.528, df=19.70, p=0.1423), however DSS-exposed animals had significantly higher fecal LCN-2 levels at the chronic timepoint (Kolmogorov-Smirnov test, D= 1.000, p <0.0001) (fig. 2.C). These data show that the DSS animals developed gut inflammation as a result of DSS exposure, while the control animals did not.

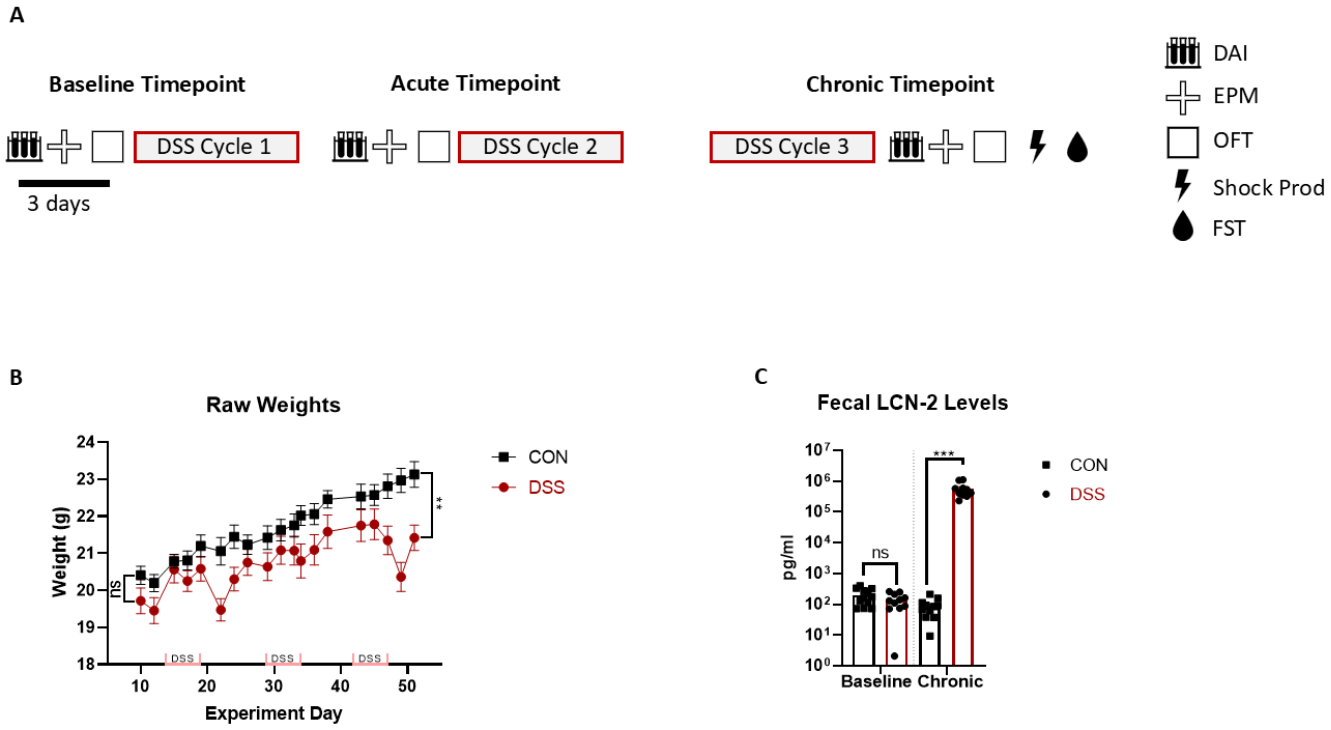

Figure 2: A) Experimental timeline. B) Weights of mice (+/-SEM) from the beginning of weight collection (experimental day 10) to the final day of weight collection (day 51). C) Fecal LCN-2 levels (pg/ml) (+/-SEM). ** = $P \leq 0.01$, *** = $P \leq 0.001$.

Chronic Gut Inflammation did not Affect Behaviour on the Elevated Plus Maze or Open Field Test

The elevated plus maze was conducted at three different timepoints across the study; at the baseline, acute, and chronic treatment exposure timepoints (Fig 2.A). A repeated measures ANOVA revealed an effect of testing timepoint on the time spent in the open arms (Wilks' Λ = .43, $F(2, 20) = 13.50$, $p < .001$, partial $\eta^2 = .57$) consistent with what might occur as the animals became more habituated to the plus maze. However, there was no significant interaction between the treatment group and testing timepoint on the time spent in the open arms at the different timepoints (Wilks' $\Lambda = .79$, $F(2, 20) = 2.60$, $p = .099$, partial $\eta^2 = .21$.) (fig. 3.A). In other words, both groups spent less time in the open arms at progressive testing timepoints, and there was no significant difference between the groups. DSS treatment, therefore, did not affect anxious behaviour as tested by the EPM.

There was also no effect of DSS on behaviour in the open field test. A repeated measures ANOVA revealed no effect of testing timepoint on the time spent in the periphery of the open field test apparatus (Wilks' $\Lambda = .76$, $F(2, 18) = 2.81$, $p = .087$, partial $\eta^2 = .24$), and there was no interaction between treatment and testing timepoint on time spent in the periphery (Wilks' Λ = .84, $F(2, 18) = 1.66$, $p = .218$, partial $\eta^2 = .16$) (fig. 3.B). As in the EPM, DSS treatment did not affect anxious behaviour as measured by the OFT.

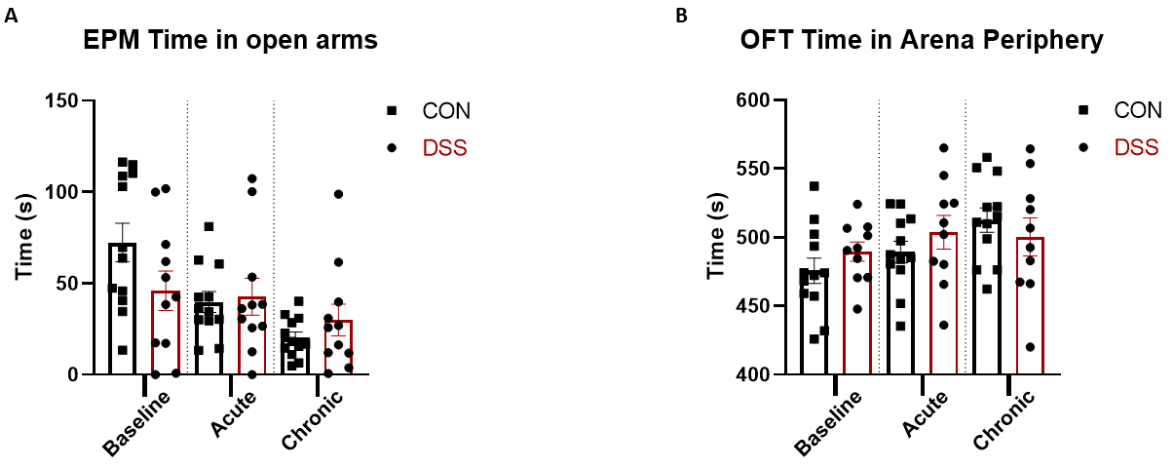

Figure. 3: Effects of DSS treatment on classic behavioural tests of anxiety-like murine behaviour. A) Both DSS-treated mice and controls reduced time (s) spent in the open arms of the elevated plus maze (EPM) for subsequent testing epochs. B) The time (s) animals spent in the periphery of the open field apparatus (+/-SEM). No significant effect of testing timepoint nor interaction between timepoint and treatment group was found.

3.3 DSS Evoked Increased Shock Reactivity and Dichotomous Threat Response in Shock-Probe and Forced Swim Tasks

DSS treatment led to increased active coping in the SPDB test. The two primary behaviours quantified in the SPDB task are burying and immobility. Burying is defined as the active displacement of bedding anywhere in the test apparatus (Fucich & Morilak, 2018). Immobility is defined as full-body immobility with 4 paws on the ground, with the exception of minor postural, head, ear, or breathing-related movements. All animals will engage in a mix of

these behaviours, however, when an animal engages in one of the behaviours more frequently than the other, this is indicative of a preference in coping strategy. The preferred threat coping style of an animal can be quantified by the 'bury ratio', which is the ratio of time spent active (burying) vs passive (immobility) behaviours. The ratio ranges from 0-1, with any score above a 0.5 indicating adoption of an active coping response with more time spent burying than immobile. Each animal adopted a different threat coping strategy – either burying or remaining immobile more frequently. There was a statistically significant difference between the DDS and control animals on mean bury ratio scores (Welch's t test, t=2.828, df=13.38 p= 0.0139), with DSS animals displaying a more active threat coping style compared to controls (fig. 4.A).

DSS treatment led to increased passive coping in the FST. The response to water threat was similarly quantified by the ratio of time spent actively swimming versus passively floating in the FST. There was a statistically significant difference in this swim ratio between DSS-treated animals and controls (Welch's t test, t=2.183, df=20.45, p= 0.0383); DSS-treated animals had lower mean swim ratio scores than controls did (fig. 4.B).

DSS-treatment elevated active threat-coping (digging) in the SPDB test but decreased active threat-coping (swimming) in the FST. In contrast, controls had similar levels of active threat coping in both tests (fig. 4.C). Pearson correlations were conducted to examine the relationship between the swim ratio and bury ratio scores for DSS and control animals. A non-significant positive correlation, ($r(10) = .36$, $p = .250$, 95% CI [–.27, .77]) was found in the control group (n=12), and a moderate negative correlation ($r(8) = –.50$, $p = .138$, 95% CI [–.86, .18]) was found in the DSS group (n = 10). Approximately 25% of the variance in one of the ratios was explained by the other ($R^2 = .25$), though this effect was not reliably different from

zero in this sample. While not significant, this finding is underpowered, and a larger sample size may show significant results.

Shock reactivity in the SPDB test was scored on a scale from 1-4 (Sluyter et al., 1999). DSS treatment in the SPDB test elicited a near-significant group difference in the intensity of subject reactivity to receiving the shock. DSS treated animals had a higher mean score on shock reactivity than control animals did (Mann–Whitney U = 36, p = .052, two-tailed) (fig. 4.D). While not significant by 0.002, this finding is underpowered, and a larger sample size may show significant results.

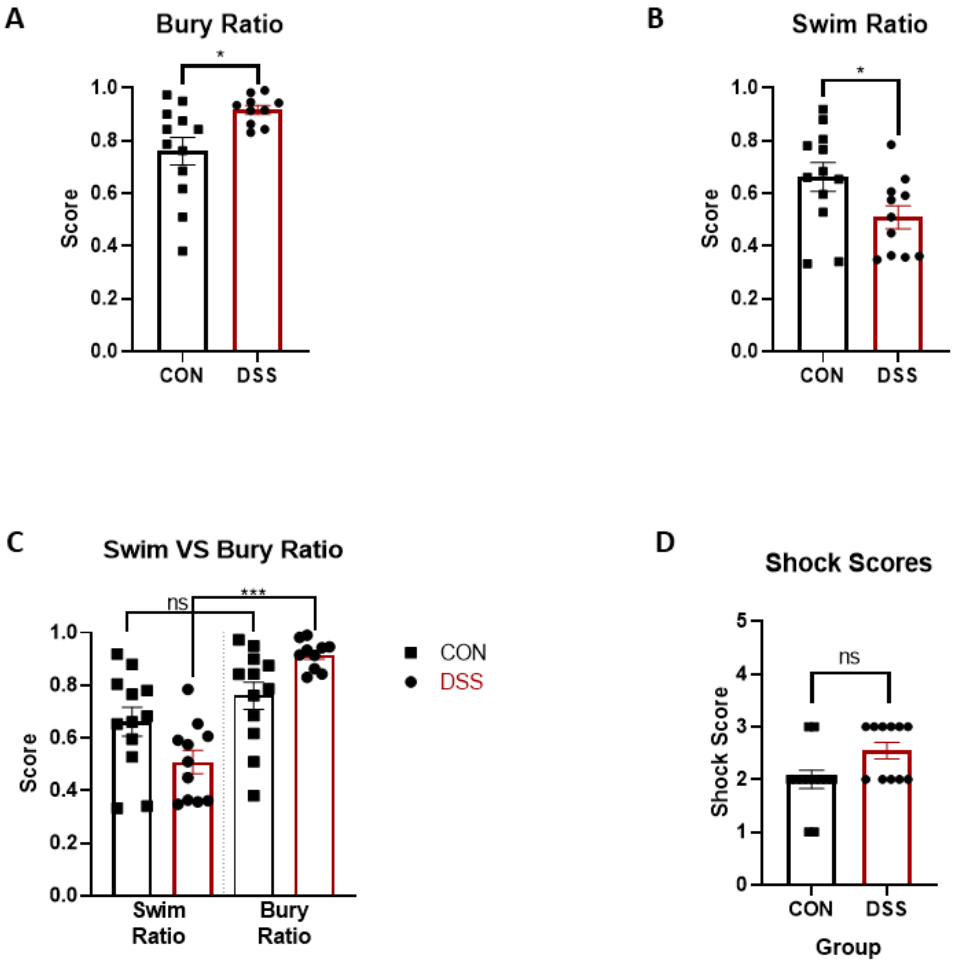

Figure. 4: Behavioural scores in the SPDB test and FST. A) Mean bury ratio (+/-SEM), a measurement of threat coping style represented as the ratio of time spent burying vs immobile. The DSS animals adopted a significantly higher active threat coping style compared to the control group. Any score over 0.5 is considered an indicator of active threat coping. B) Mean swim ratio (+/-SEM), a comparison between active swimming and immobility time. The DSS animals had a significantly lower mean score compared to the control group. Any score below 0.5 indicates that the animal spent more time immobile than mobile, which can be interpreted as passive threat coping. C) Mean swim ratio compared to the mean bury ratio (+/-SEM) for both groups. Whereas control animals show a similar balance of active versus passive threat coping between the two tests, the DSS-treated animals show elevated active coping in the SPDB but reduced active coping in the FST. D) Mean shock reactions (+/-SEM) for both groups in the SPDB test. *= $P \leq 0.05$, *** = $P \leq 0.001$.

Discussion

4.1 Summary of Results

As expected, DSS exposure induced cyclical weight loss and gut inflammation in DSS treated animals, significant group differences were observed in weight and inflammatory markers after the three DSS exposures. These results provide evidence for the hypothesis that DSS treatment would result in gut inflammation and weight loss, which are phenomenologically similar to the effects of inflammatory bowel disease in humans. Unexpectedly, DSS exposure did not alter anxiety-like behaviour as measured by the EPM or OFT. Both DSS and control

animals showed similar habituation patterns in the EPM across timepoints with no group differences. In line with the hypothesis, DSS exposure appears to have affected threat coping behaviours in a context-dependent manner. DSS-treated animals showed increased active coping in the SPDB test but reduced active coping in the FST, whereas control animals maintained consistent coping strategies across both tasks. DSS animals had a more intense response to receiving a shock from the probe, which may represent depressive hyperalgesia.

Ultimately, the data provide evidence for the theory that gut inflammation alters coping behaviours, but did not provide evidence for gut inflammation increasing anxious behaviours in the absence of a threatening stimulus (e.g. shock prod or inescapable water). The findings of this study support the hypothesis that treating female mice with DSS will alter threat coping behaviour. However, the findings did not support the hypothesis that DSS treatment would increase anxious behaviours. To the author's knowledge, this is the first study to document the behavioural changes in the SPDB test and FST in a female murine chronic DSS model of inflammatory bowel disease.

4.2 The Forced Swim Task as a Threat Coping Task

The forced swim task was originally developed for testing the efficacy of antidepressant drug treatments. This test involves placing a rat or mouse in a clear cylindrical testing apparatus filled with water. It is often characterized as measuring the "depression-like" behaviours observed in an inescapable stressful situation wherein the animal is unable to climb out of the water or touch the bottom of the apparatus. Immobility behaviours were originally characterized as a despair the animal develops once it has learned and accepted that the stressful testing

conditions are completely inescapable (Porsolt et al., 1977). It was initially introduced as a measure of antidepressant activity in rats and mice because antidepressant drugs reduce the time spent immobile in the task, but recent literature often erroneously conflates immobility time with mental states of depression and despair (Armario, 2021).

More recent publications have questioned the traditional interpretation of immobility in the FST and instead argue that it is better validated as a measurement of coping with or adapting to a stressful situation. In support of this interpretation, some authors have argued that antidepressants potentiate active threat coping responses, and that this effect of the antidepressant treatment accounts for the decrease in passive immobility behaviours observed with antidepressant treatment in the FST (Armario, 2021). Others argue that the anxiogenic effects of antidepressants increase escape-directed behaviours instead (Anyan & Amir, 2018). Regardless of whether the FST is a more accurate test of the valence of a coping response versus anxious states driving escape attempts, literature suggests that the FST is more accurately interpreted as a test of responses to an acutely stressful situation rather than a test of depressive cognitive states in rodents.

While subtle differences exist, evidence suggests there are no significant sex differences in swimming and immobility time in untreated male and female mice (Tsao et al., 2023; Võikar et al., 2001). However, sex differences in FST behaviour appear to exist in DSS-treated mice. The findings of this study align with the two previous studies utilizing FST in an acute female DSS model, both of which found significant increases in immobility time in DSS-treated female mice compared to controls (Gadotti et al., 2019; Painsipp et al., 2011). The published effects of acute DSS treatment in male mice on FST immobility is less unified, however this may be because more publications use this model. Decreases in immobility (Matisz et al., 2020, 2022),

increases in immobility (Gadotti et al., 2019), and no changes to immobility (Painsipp et al., 2011) have all been reported after acute DSS treatment in male mice. Previous studies have found no significant difference in immobility time in male mice treated with chronic DSS (Matisz et al., 2020), contrasting the results observed in the female subjects of this experiment. Should future research find sustained increases in passive threat coping behaviours in chronically inflamed female - but not male - animals, chronic DSS treatment may be an effective model to research the increased rate of anxiety and depression in females with chronic gut inflammation.

4.3 Shock Sensitivity and Dichotomous Threat Coping Behaviours in Forced Swim Task and Shock Probe Defensive Burying Test

The two threat coping behaviours analyzed in the SPDB test are burying and immobility behaviours. Burying is considered an active coping strategy wherein the animal is engaging with the environmental threat (in this experiment the shock probe) by burying it with bedding or burrowing into the bedding. Meanwhile immobility is considered to be a passive coping strategy, wherein the animal remains as still as possible, likely a selected for behaviour stemming from the pressure to remain undetected by predators (De Boer & Koolhaas, 2003). A literature review by De Boer & Koolhaas (2003) report no effect of sex on burying behaviours in rodents, but do report an effect of estrous cycle phase on burying behaviours. Estrus effects specific to the SPDB test show that proestrus rats freeze significantly less than estrus, diestrus, or male rats (Frye et al., 2000). Proestrus is when estradiol is highest and progesterone is lowest in the rodent estrous cycle (Nilsson et al., 2015), implicating sex hormones in these cycle phase differences. Further evidence can be found in studies chronically treating rats with hormones; withdrawals from hormone treatment of 500 µg progesterone and hormone treatment of 50 µg progesterone +

2 µg estradiol significantly increase the time spent burying in treated female rats compared to non-treated controls in a modified SPDB test (Gallo & Smith, 1993). However, priming the 50µg progesterone treatment withdrawal with 2 days of 2µg estradiol treatment (which approximates endogenous progesterone and estradiol temporal activity and levels during the estrous cycle) prevented the anxiogenic effects of progesterone withdrawal. Such effects likely do not occur in females with normal estradiol and progesterone levels/cycles, but alterations to these hormones have an impact on threat coping behaviours as measured by the SPDB test. As such, rodent models suggest that sex hormones may represent one biological factor that may account for sex differences in threat coping.

Several factors, including inflammation, have been shown to affect anxiety-like behaviours in the SPDB test. In male mice bred to display polarized aggression via short (SAL) and long (LAL) latency to attack, mice injected with saline or the inflammatory endotoxin lipopolysaccharide (LPS) 3.5 hours before SPDB testing buried less than un-injected control animals (Gasparotto et al., 2007). This suggests that both the psychological stress of a saline injection, and the separate inflammatory insult of an LPS injection both alter coping behaviours. Only LAL mice showed significant increases in immobility behaviours after both the saline and LPS injection, again with the LPS inducing significantly longer immobility times. The effect of the injections on increases in immobility and decreases in burying were stronger in the more passive LAL mice than the more aggressive SAL mice – meaning there are inter-subject reactivity factors that impact coping style in the SPDB test after an inflammatory insult. Both socially aggressive behaviours and increased burying in the SPDB task have been associated with higher levels of noradrenaline and adrenaline than in less socially aggressive and more immobile mice (Sgoifo et al., 1996). Other internal factors impacting SPDB performance

include neuromodulator levels; 5HT1A modulation impacts active, but not passive coping behaviours (Korte et al., 1992), whereas high corticosterone levels have been observed during immobility behaviours, and relatively higher norepinephrine levels have been observed during burying behaviours. While the effects of some internal and external factors on this test have been described, there have been no documented effects of chronic gut inflammation in female mice in the SPDB test until now.

Should the FST be interpreted as a threat coping task like the SPDB test, then it is logical to apply the coping style ratio central to the SPDB task by comparing active coping behaviours (swimming) with passive ones (immobility). To my best knowledge, this exact comparison has never used in the FST literature, however, it follows the same logic behind the literature-backed SPDB bury ratio. The DSS treated group had a significantly lower swim ratio ($p = 0.0409$) compared to the control group when an analog of the SPDB "bury ratio" equation is applied to FST results (fig. 4.B). If this is interpreted through the lens of threat coping, the DSS group adopts a less active/more passive threat coping style in the FST when compared to control animals. Interestingly, increased passive threat coping in the DSS group is opposite to the SPDB findings. When comparing the same treatment group across the two tests, only the DSS treated animals show significantly different coping strategies. The DSS animals scored significantly lower in the FST, and significantly higher in the SPDB test. Meanwhile, control animals scored within an extremely similar range across both tests with no significant difference in mean score, suggesting that their threat coping styles largely did not differ between the two tasks. The swim:bury ratio correlation in the DSS-treated group ($n=10$) was found to have a p-value of 0.1376, which is suggestive of a potential trend but not a significant finding. However, because this finding is underpowered, further research should be done with larger group sizes to support

these findings. Regardless, these strongly dissociated results across the two tests posit an interesting question; why did the DSS animals differ so much in coping strategy when controls did not?

It is known that external and environmental factors have an impact on behaviours displayed in the SPDB test because certain coping behaviours require certain environmental conditions to be met; if there is no bedding in the SPDB test, animals will not display any burying behaviour and will only adopt immobile behaviours in order to cope (Korte et al., 1992). Of note, specific environmental conditions seem to impact threat responses; rats that were given no bedding on test day (regardless of whether they had bedding during training days) had elevated plasma corticosterone and lower norepinephrine levels than animals that did have bedding during the test and habituation days (Korte et al., 1992). Such environmental factors also influence FST performance; male mice swimming in either 20◦C, 25◦C, or 35 ◦C water showed strain-specific differences in floating and swimming times across the different temperatures (Bächli et al., 2008). Both behaviours seem to share some neural substrates, as ketamine treatment seems to reduce passive threat coping in both tests (Jett et al., 2015). Passive threat coping in the SPDB test is increased in a chronic unpredictable stress (CUS) model using male rats, and treatment with ketamine 24hrs prior to SPDB testing results in less immobility and more burying when compared to vehicle-treated CUS rats. Similarly, ketamine treatment one week before FST testing in male rats was found to reduce immobility behaviours compared to vehicle-treated controls. Ketamine shows great potential in the treatment of anxiodepressive behaviours associated with chronic inflammation due to its immune-modulating activities and diverse effects on neurotransmitters, synapses, and neural pathways (Nikkheslat, 2021).

The finding of increased shock reactivity in the SPBD test may be indicative of depression-like hyperalgesia. Depression and pain are frequently comorbid; an average of 65% of people with depression also report pain, however rates as high as 85% have been reported in the literature (Bair et al., 2003). People with major depressive disorder have increased sensitivity to pain compared with people who have never been depressed, with increased sensitivity to experimentally induced mechanical finger pain being associated with increased anxiety symptoms (Hermesdorf et al., 2016). In this study, the DSS-treated female mice had more intense reactions to receiving a mild shock in the SPDB test, possibly indicating that DSS-treated animals displayed depression-like hyperalgesia. However, more research must be done to confirm this finding, ideally with the inclusion of an established pain sensitivity test such as the Von Frey test.

4.4 EPM and OFT are not Indicative of Threat Coping

Despite these differences in coping behaviours across the FST and SPDB tests, there were no group differences in the OFT and EPM tests. This matches previous studies in rats that have shown no correlation between time spent burying, percent time in the open arms of the EPM maze (De Boer & Koolhaas, 2003), and latency and segments entered in the OFT (Paré, 1994). The anxiety-like behaviours displayed in the SPDB test and OFT appear to be dissociated by region; with amygdala lesions increasing the time spent avoiding a shock prod, and septum lesions decreasing the time spent burying in the SPDB test and open-arm avoidance in the EPM in male rats (Treit et al., 1993). These results have largely been reported in male animals, with this study supporting these findings in female mice.

The EPM and OFT tests were repeated three times with the same apparatuses in the same orientation in the same room. As such, it is highly likely that the animals became habituated to the testing apparatuses, which may have altered results. Through the lens of passive vs active threat coping applied to the SPDB and FST tests, increased immobility time in the closed arms of the EPM would be interpreted as an increase in anxiety-like behaviours, however that may not accurately reflect the subject's internal state. Instead, as the subjects became more accustomed to the testing environment, they may have been less inclined to explore – which could result in increased time spent waiting in the comparatively comfortable closed arms of the EPM. This issue of habituation that occurs with repeated testing in the same context may be mitigated by changing the context; changing which rooms the testing is conducted in at the different testing timepoints may be sufficient. However, because the apparatuses will remain the same, there is a chance there will always be some degree of habituation. Limiting testing to baseline and chronic timepoints in different rooms may be an ideal solution to this issue as it would still allow researchers to compare the behaviour of treatment animals both before and after chronic DSS exposure while limiting habituation.

4.5 Sex Differences in Colitis

It has been reported that in an acute murine DSS-induced colitis model, sex has no significant effect on mortality rate (Bábíčková et al., 2015), however female mice treated with 2% DSS experienced less colon shortening, recovered their weight faster, showed fewer histological markers of gut inflammation, and had better stool consistency - suggesting the impact of DSS was less severe in female mice. The study also found that hormonal manipulation via ovariectomy and the replacement of 17-β-estradiol in female mice improved the

inflammatory response to DSS and subsequent tissue regeneration rates compared to all other DSS-treated groups. It must be noted that contradicting findings have been reported using different IBD-induction methods and when supplementing estrogen at supraphysiological doses that mimic pregnancy (Verdú et al., 2002). As such, blanket statements about different forms and doses of estrogens should not be made, however it appears clear that estrogens modulate the effects DSS has on colitis induction. The observed increased passive coping in the FST may differ from previous results (Matisz et al., 2020, 2022; Painsipp et al., 2011) because of the protective effects estradiol has on gut inflammation, however this appears to be a paradoxical explanation. Estradiol protects against the negative effects of DSS-induced colitis (Bábíčková et al., 2015), and the high-estradiol proestrus phase is associated with decreased immobility time in the SPDB test (Frye et al., 2000). Yet the literature that has utilized the FST in DSS-treated female mice has observed increases in immobility time compared to controls, when DSS treatment shows conflicting results in male mice (Gadotti et al., 2019; Matisz et al., 2020, 2022; Painsipp et al., 2011). It is clear that the increased incidence rate of anxiety in women with IBD, and the rodent models used to study the negative psychological effects of IBD in females both require further research.

Similar to the Hypothalamic-Pituitary-Adrenal (HPA) axis, there also exists the Hypothalamic-Pituitary-Gonadal (HPG) axis. This axis is likewise sensitive to stressors; pre-pubertal female murine models have shown that chronic inflammation from LPS injections alters Hypothalamic-Pituitary-Ovarian signalling, resulting in increases to luteinizing and follicle-stimulating hormone levels (Garcia et al., 2024). Stressors may reduce the anti-anxiety effects of estrogen in females, which may result in sex-specific alterations to behaviour following said stressor (Kundakovic & Rocks, 2022). The mechanisms of these sexually-dimorphic

behavioural changes can occur at the cellular scale; with gene activity (Seney et al., 2018) and mitochondrial activity and signalling being implicated (Ruszkiewicz et al., 2019). One such possible sexually dimorphic behaviour that may be impacted by HPG axis signalling disruption is the adoption of more extreme environment-specific coping behaviours post-chronic gut inflammation in females. However, this idea is speculative and to date the author is aware of no scientific evidence to support this concept beyond the observed dichotomous response to the FST and SPDB test results from this study. Building on the findings of this study may represent an opportunity for interesting future research, should the findings of this study be largely replicated, the chronic DSS model may present itself as a useful model to study the differing rates of IBD-associated anxiodepressive disorders across the sexes.

It is important to note that in humans there is a great variety of people with different gender identities, sex hormone levels, and undergoing different hormone treatments. As such, care must be taken when discussing the topics of sex and sex hormones in humans, as complex social, temporal, and identity factors apply when using such terms. For instance, female sex hormone levels will naturally change (within the menstrual cycle, menarche and menopause) in almost all people assigned female at birth across their lifetimes. As such specific terminology must be used in clinical studies to ensure clinicians know who the findings apply to and why. This is not as pertinent in the current study, however it always bears mentioning when engaging in discussions of sexual dimorphism.

Caveats

This study has five main limitations. Primarily, rodent models of anxiodepressive behaviours are not perfect correlates to anxiety and depression in humans; the literature often measures observable behaviour in rodent models of anxiodepressive behaviour while measuring subjective affective symptoms in humans through questionnaires like the Beck Depression Inventory. Further limitations include the use of mice as an animal model; the majority of research done with the FST and SPDB tasks has been performed in rats. Rats possess different biology and adopt different behaviours than mice. Protocols for conducting the SPDB test and FST in these animals differ, and as such results found in a mouse model are not perfectly comparable to those found in a rat model.

This study included no male animals, meaning no comparisons between female and male animals that have undergone identical DSS exposure and testing can be made. It can be argued that no meaningful comparisons between male and female rodent models of DSS-induced colitis can be made in general because of the differential response to DSS between the sexes. However, comparative studies between male and female animal models of chronic gut inflammation may be beneficial in identifying biological factors that impact the differential rates of depression and anxiety between the sexes.

Another caveat pertaining to issues of biological sex include the stress of vaginal lavage. Vaginal lavage is only performed on female mice, and as such, the stress of tracking estrous cycles will impose a stressor on female, but not male, animals. Alternative methods of cycle tracking include visual inspection of the external genitalia (Champlin et al., 1973). This process is less stressful for subjects, is less time consuming than lavage sampling and slide scoring, and the examination process could be exactly replicated in male animals to ensure identical

procedures were performed on both sexes. However, because this method relies on subjective researcher scoring, it is highly subject to inter-researcher variability. The cycle staging process could be streamlined and human error mitigated if a deep learning tool similar to EstrousNet (Wolcott et al., 2022) existed to classify the cycle stage of rodent subjects via images of the external genitals. To the best of my knowledge, no such tool exists, and I am unable to comment on the feasibility of developing such a tool. However, this represents a very promising opportunity; the creation of this tool would benefit rodent research greatly and make cycle staging in female rodents more viable and accessible to a variety of researchers.

Finally, unlike previous studies using male animals (Matisz et al., 2022), no histology was performed on brain or intestinal tissue from these female mice, and as such there is no confirmed histological evidence of gut-inflammation-induced neuroinflammation in this study. Future research should include such measures, that would allow for the degree and localization of gut-induced neuroinflammation to be compared between groups and sexes. Furthermore, measures of neuroinflammation can be correlated to behavioural changes, which may also show important sex differences.

Conclusions and Future Directions:

Major depressive disorder is a complex and heterogeneous condition with growing evidence suggesting that an inflammatory subtype exists. While not all individuals with major depressive disorder present with elevated inflammatory markers, people with chronic inflammatory conditions, such as inflammatory bowel diseases, show disproportionately high

rates of anxiety and depression. Research also shows that there is particularly elevated risk for females with IBDs. The mechanisms linking chronic gut inflammation to anxiodepressive behaviours appear to involve the anterior cingulate cortex. Such mechanisms are driven by circulating inflammatory cytokines, altered gut-brain signaling, and increased blood-brain barrier permeability among others. Given these associations, this study investigated the effects of chronic DSS-induced gut inflammation on threat-coping and anxiodepressive behaviours in female mice - a model that remains understudied.

DSS exposure induced weight loss, intestinal inflammation, and altered coping responses in a context-dependent manner in the SPDB test and FST. DSS-treated animals displayed increased active coping in the SPDB and decreased active coping in the FST, while control animals showed similar coping across both tasks. This suggests that DSS treatment altered stress coping in a context-dependent manner. Shock score reactivity was higher in DSS treated animals compared to controls, which may be due to depression-like hyperalgesia. Despite the significant group differences in measures of gut inflammation and coping behaviours, there were no group differences in the EPM and OFT, both are traditionally considered tests of anxiety-like behaviour and are used frequently in anxiodepressive research. To the authors' knowledge, this is the first study to document how chronic DSS exposure changes female mouse behaviour in the SPDB test and FST. This work highlights the importance of sex-specific research and nuanced behavioural paradigms in uncovering the links between chronic inflammation and anxiodepressive disorders, particularly in females who may exhibit distinct affective and coping responses to inflammatory stressors.

One potential future direction is to perform an identical study with male subjects. Previous research in male mice with chronic gut inflammation has found that male mouse threat

coping behaviours in the FST are not significantly different from controls (Matisz et al., 2020). An experiment comparing the threat coping behaviours of male and female mice with and without chronic gut inflammation may disambiguate the potential sex difference reported in this study. Furthermore, whether the dissociated results in the FST swim ratio and SPDB bury ratio exists in male animals should be explored as well. Finally, the present study should be replicated with measures of brain inflammation included. These behavioural changes may then be correlated to the severity and localization of brain inflammation induced by the chronic gut inflammation. Should the findings of this study hold up to future scrutiny, the chronic DSS model may prove to be an effective tool in researching sex differences in anxiodepressive disorders and threat coping in IBD.

References:

Alhajj, M., Zubair, M., & Farhana, A. (2025). Enzyme linked immunosorbent assay. In *StatPearls*. StatPearls Publishing. http://www.ncbi.nlm.nih.gov/books/NBK555922/

American Psychiatric Association. (2013). Depressive disorders. In *Diagnostic and statistical manual of mental disorders* (5th ed., pp. 155–188).

Anyan, J., & Amir, S. (2018). Too depressed to swim or too afraid to stop? A reinterpretation of the forced swim test as a measure of anxiety-like behavior. *Neuropsychopharmacology*, *43*(5), 931–933. https://doi.org/10.1038/npp.2017.260

Armario, A. (2021). The forced swim test: Historical, conceptual and methodological considerations and its relationship with individual behavioral traits. *Neuroscience & Biobehavioral Reviews*, *128*, 74–86. https://doi.org/10.1016/j.neubiorev.2021.06.014

Bábíčková, J., Tóthová, Ľ., Lengyelová, E., Bartoňová, A., Hodosy, J., Gardlík, R., & Celec, P. (2015). Sex differences in experimentally induced colitis in mice: A role for estrogens. *Inflammation*, *38*(5), 1996–2006. https://doi.org/10.1007/s10753-015-0180-7

Bächli, H., Steiner, M. A., Habersetzer, U., & Wotjak, C. T. (2008). Increased water temperature renders single-housed C57BL/6J mice susceptible to antidepressant treatment in the forced swim test. *Behavioural Brain Research*, *187*(1), 67–71. https://doi.org/10.1016/j.bbr.2007.08.029

Bair, M. J., Robinson, R. L., Katon, W., & Kroenke, K. (2003). Depression and pain comorbidity: A literature review. *Archives of Internal Medicine*, *163*(20), 2433–2445. https://doi.org/10.1001/archinte.163.20.2433

Byrne, G., Rosenfeld, G., Leung, Y., Qian, H., Raudzus, J., Nunez, C., & Bressler, B. (2017). Prevalence of anxiety and depression in patients with inflammatory bowel disease. *Canadian Journal of Gastroenterology and Hepatology*, *2017*(1), 6496727. https://doi.org/10.1155/2017/6496727

Champlin, A. K., Dorr, D. L., & Gates, A. H. (1973). Determining the stage of the estrous cycle in the mouse by the appearance of the vagina. *Biology of Reproduction*, *8*(4), 491–494. https://doi.org/10.1093/biolreprod/8.4.491

Chassaing, B., Srinivasan, G., Delgado, M. A., Young, A. N., Gewirtz, A. T., & Vijay-Kumar, M. (2012). Fecal lipocalin 2, a sensitive and broadly dynamic non-invasive biomarker for intestinal inflammation. *PLoS ONE*, *7*(9), e44328. https://doi.org/10.1371/journal.pone.0044328

Commons, K. G., Cholanians, A. B., Babb, J. A., & Ehlinger, D. G. (2017). The rodent forced swim test measures stress-coping strategy, not depression-like behavior. *ACS Chemical Neuroscience*, *8*(5), 955–960. https://doi.org/10.1021/acschemneuro.7b00042

Craig, C. F., Filippone, R. T., Stavely, R., Bornstein, J. C., Apostolopoulos, V., & Nurgali, K. (2022). Neuroinflammation as an etiological trigger for depression comorbid with inflammatory bowel disease. *Journal of Neuroinflammation*, *19*(1), 4. https://doi.org/10.1186/s12974-021-02354-1

Davey, C. G., Harrison, B. J., Yücel, M., & Allen, N. B. (2012). Regionally specific alterations in functional connectivity of the anterior cingulate cortex in major depressive disorder. *Psychological Medicine*, *42*(10), 2071–2081. https://doi.org/10.1017/S0033291712000323

De Boer, S. F., & Koolhaas, J. M. (2003). Defensive burying in rodents: Ethology, neurobiology and psychopharmacology. *European Journal of Pharmacology*, *463*(1), 145–161. https://doi.org/10.1016/S0014-2999(03)01278-0

Disner, S. G., Beevers, C. G., Haigh, E. A. P., & Beck, A. T. (2011). Neural mechanisms of the cognitive model of depression. *Nature Reviews Neuroscience*, *12*(8), 467–477. https://doi.org/10.1038/nrn3027

Frye, C. A., Petralia, S. M., & Rhodes, M. E. (2000). Estrous cycle and sex differences in performance on anxiety tasks coincide with increases in hippocampal progesterone and 3α,5α-THP. *Pharmacology Biochemistry and Behavior*, *67*(3), 587–596. https://doi.org/10.1016/S0091-3057(00)00392-0

Fucich, E., & Morilak, D. (2018). Shock-probe defensive burying test to measure active versus passive coping style in response to an aversive stimulus in rats. *BIO-PROTOCOL*, *8*(17). https://doi.org/10.21769/BioProtoc.2998

Gadotti, V. M., Andonegui, G., Zhang, Z., M'Dahoma, S., Baggio, C. H., Chen, L., Basso, L., Altier, C., MacNaughton, W. K., Kubes, P., & Zamponi, G. W. (2019). Neuroimmune responses mediate depression-related behaviors following acute colitis. *iScience*, *16*, 12–21. https://doi.org/10.1016/j.isci.2019.05.012

Gallo, M. A., & Smith, S. S. (1993). Progesterone withdrawal decreases latency to and increases duration of electrified prod burial: A possible rat model of PMS anxiety. *Pharmacology Biochemistry and Behavior*, *46*(4), 897–904. https://doi.org/10.1016/0091-3057(93)90219-J

Garcia, C., Velez, L. M., Ujagar, N., Del Mundo, Z., Nguyen, T., Fox, C., Mark, A., Fisch, K. M., Lawson, M. A., Duleba, A. J., Seldin, M. M., & Nicholas, D. A. (2024). Lipopolysaccharide-induced chronic inflammation increases female serum gonadotropins and shifts the pituitary transcriptomic landscape. *Frontiers in Endocrinology*, *14*. https://doi.org/10.3389/fendo.2023.1279878

Gasparotto, O. C., Carobrez, S. G., & Bohus, B. G. J. (2007). Effects of LPS on the behavioural stress response of genetically selected aggressive and nonaggressive wild house mice. *Behavioural Brain Research*, *183*(1), 52–59. https://doi.org/10.1016/j.bbr.2007.05.030

Han, Y., Zhao, T., Cheng, X., Zhao, M., Gong, S.-H., Zhao, Y.-Q., Wu, H.-T., Fan, M., & Zhu, L.-L. (2018). Cortical inflammation is increased in a DSS-induced colitis mouse model. *Neuroscience Bulletin*, *34*(6), 1058–1066. https://doi.org/10.1007/s12264-018-0288-5

Hathaway, C. A., Appleyard, C. B., Percy, W. H., & Williams, J. L. (1999). Experimental colitis increases blood-brain barrier permeability in rabbits. *The American Journal of Physiology*, *276*(5), G1174-1180. https://doi.org/10.1152/ajpgi.1999.276.5.G1174

Hermesdorf, M., Berger, K., Baune, B. T., Wellmann, J., Ruscheweyh, R., & Wersching, H. (2016). Pain sensitivity in patients with major depression: Differential effect of pain

sensitivity measures, somatic cofactors, and disease characteristics. *The Journal of Pain*, *17*(5), 606–616. https://doi.org/10.1016/j.jpain.2016.01.474

Jett, J. D., Boley, A. M., Girotti, M., Shah, A., Lodge, D. J., & Morilak, D. A. (2015). Antidepressant-like cognitive and behavioral effects of acute ketamine administration associated with plasticity in the ventral hippocampus to medial prefrontal cortex pathway. *Psychopharmacology*, *232*(17), 3123–3133. https://doi.org/10.1007/s00213-015-3957-3

Kong, N., Gao, C., Xu, M., & Gao, X. (2021). Changes in the anterior cingulate cortex in Crohn's disease: A neuroimaging perspective. *Brain and Behavior*, *11*(3), e02003. https://doi.org/10.1002/brb3.2003

Korte, S. M., Bouws, G. A. H., Koolhaas, J. M., & Bohus, B. (1992). Neuroendocrine and behavioral responses during conditioned active and passive behavior in the defensive burying/probe avoidance paradigm: Effects of ipsapirone. *Physiology & Behavior*, *52*(2), 355–361. https://doi.org/10.1016/0031-9384(92)90284-9

Kundakovic, M., & Rocks, D. (2022). Sex hormone fluctuation and increased female risk for depression and anxiety disorders: From clinical evidence to molecular mechanisms. *Frontiers in Neuroendocrinology*, *66*, 101010. https://doi.org/10.1016/j.yfrne.2022.101010

Matisz, C. E., & Gruber, A. J. (2022). Neuroinflammatory remodeling of the anterior cingulate cortex as a key driver of mood disorders in gastrointestinal disease and disorders. *Neuroscience & Biobehavioral Reviews*, *133*, 104497. https://doi.org/10.1016/j.neubiorev.2021.12.020

Matisz, C. E., Semenoff, N., Ahmed, A.-S. F., Griffin, L., Wallace, L. E., McNabb, P., Gibb, R., Sharkey, K. A., & Gruber, A. J. (2022). Acute gut inflammation reduces neural activity and spine maturity in hippocampus but not basolateral amygdala. *Scientific Reports*, *12*, 20169. https://doi.org/10.1038/s41598-022-24245-y

Matisz, C. E., Vicentini, F. A., Hirota, S. A., Sharkey, K. A., & Gruber, A. J. (2020). Behavioral adaptations in a relapsing mouse model of colitis. *Physiology & Behavior*, *216*, 112802. https://doi.org/10.1016/j.physbeh.2020.112802

Mitchell, J., Kim, S. J., Howe, C., Lee, S., Her, J. Y., Patel, M., Kim, G., Lee, J., Im, E., & Rhee, S. H. (2022). Chronic intestinal inflammation suppresses brain activity by inducing neuroinflammation in mice. *The American Journal of Pathology*, *192*(1), 72–86. https://doi.org/10.1016/j.ajpath.2021.09.006

Moieni, M., Irwin, M. R., Jevtic, I., Olmstead, R., Breen, E. C., & Eisenberger, N. I. (2015). Sex differences in depressive and socioemotional responses to an inflammatory challenge: Implications for sex differences in depression. *Neuropsychopharmacology*, *40*(7), 1709–1716. https://doi.org/10.1038/npp.2015.17

Nikkheslat, N. (2021). Targeting inflammation in depression: Ketamine as an anti-inflammatory antidepressant in psychiatric emergency. *Brain, Behavior, & Immunity - Health*, *18*, 100383. https://doi.org/10.1016/j.bbih.2021.100383

Nilsson, M. E., Vandenput, L., Tivesten, Å., Norlén, A.-K., Lagerquist, M. K., Windahl, S. H., Börjesson, A. E., Farman, H. H., Poutanen, M., Benrick, A., Maliqueo, M., Stener-Victorin, E., Ryberg, H., & Ohlsson, C. (2015). Measurement of a comprehensive sex steroid profile in rodent serum by high-sensitive gas chromatography-tandem mass

spectrometry. *Endocrinology*, *156*(7), 2492–2502. https://doi.org/10.1210/en.2014-1890

Painsipp, E., Herzog, H., Sperk, G., & Holzer, P. (2011). Sex-dependent control of murine emotional-affective behaviour in health and colitis by peptide YY and neuropeptide Y. *British Journal of Pharmacology*, *163*(6), 1302–1314. https://doi.org/10.1111/j.1476-5381.2011.01326.x

Paré, W. P. (1994). Open field, learned helplessness, conditioned defensive burying, and forced-swim tests in WKY rats. *Physiology & Behavior*, *55*(3), 433–439. https://doi.org/10.1016/0031-9384(94)90097-3

Porsolt, R. D., Le Pichon, M., & Jalfre, M. (1977). Depression: A new animal model sensitive to antidepressant treatments. *Nature*, *266*(5604), 730–732. https://doi.org/10.1038/266730a0

Raison, C. L., & Miller, A. H. (2011). Is depression an inflammatory disorder? *Current Psychiatry Reports*, *13*(6), 467–475. https://doi.org/10.1007/s11920-011-0232-0

Ruszkiewicz, J. A., Miranda-Vizuete, A., Tinkov, A. A., Skalnaya, M. G., Skalny, A. V., Tsatsakis, A., & Aschner, M. (2019). Sex-specific differences in redox homeostasis in brain norm and disease. *Journal of Molecular Neuroscience*, *67*(2), 312–342. https://doi.org/10.1007/s12031-018-1241-9

Seney, M. L., Huo, Z., Cahill, K., French, L., Puralewski, R., Zhang, J., Logan, R. W., Tseng, G., Lewis, D. A., & Sibille, E. (2018). Opposite molecular signatures of depression in men and women. *Biological Psychiatry*, *84*(1), 18–27. https://doi.org/10.1016/j.biopsych.2018.01.017

Sgoifo, A., De boer, S. F., Haller, J., & Koolhaas, J. M. (1996). Individual differences in plasma catecholamine and corticosterone stress responses of wild-type rats: Relationship with aggression. *Physiology & Behavior*, *60*(6), 1403–1407. https://doi.org/10.1016/S0031-9384(96)00229-6

Sluyter, F., Korte, S. M., Van Baal, G. C., De Ruiter, A. J., & Van Oortmerssen, G. A. (1999). Y chromosomal and sex effects on the behavioral stress response in the defensive burying test in wild house mice. *Physiology & Behavior*, *67*(4), 579–585. https://doi.org/10.1016/s0031-9384(99)00101-8

Treit, D. (1990). A comparison of anxiolytic and nonanxiolytic agents in the shock-probe/burying test for anxiolytics. *Pharmacology Biochemistry and Behavior*, *36*(1), 203–205. https://doi.org/10.1016/0091-3057(90)90151-7

Treit, D., Pesold, C., & Rotzinger, S. (1993). Dissociating the anti-fear effects of septal and amygdaloid lesions using two pharmacologically validated models of rat anxiety. *Behavioral Neuroscience*, *107*(5), 770–785. https://doi.org/10.1037//0735-7044.107.5.770

Tripp, A., Oh, H., Guilloux, J.-P., Martinowich, K., Lewis, D. A., & Sibille, E. (2012). Brain-derived neurotrophic factor signaling and subgenual anterior cingulate cortex dysfunction in major depressive disorder. *American Journal of Psychiatry*, *169*(11), 1194–1202. https://doi.org/10.1176/appi.ajp.2012.12020248

Tsao, C.-H., Wu, K.-Y., Su, N. C., Edwards, A., & Huang, G.-J. (2023). The influence of sex difference on behavior and adult hippocampal neurogenesis in C57BL/6 mice. *Scientific Reports*, *13*(1), 17297. https://doi.org/10.1038/s41598-023-44360-8

Verdú, E. F., Deng, Y., Bercik, P., & Collins, S. M. (2002). Modulatory effects of estrogen in two murine models of experimental colitis. *American Journal of Physiology. Gastrointestinal and Liver Physiology*, *283*(1), G27-36. https://doi.org/10.1152/ajpgi.00460.2001

Võikar, V., Kõks, S., Vasar, E., & Rauvala, H. (2001). Strain and gender differences in the behavior of mouse lines commonly used in transgenic studies. *Physiology & Behavior*, *72*(1–2), 271–281. https://doi.org/10.1016/S0031-9384(00)00405-4

Wolcott, N. S., Sit, K. K., Raimondi, G., Hodges, T., Shansky, R. M., Galea, L. A. M., Ostroff, L. E., & Goard, M. J. (2022). Automated classification of estrous stage in rodents using deep learning. *Scientific Reports*, *12*, 17685. https://doi.org/10.1038/s41598-022-22392-w

Yankelevitch-Yahav, R., Franko, M., Huly, A., & Doron, R. (2015). The forced swim test as a model of depressive-like behavior. *Journal of Visualized Experiments : JoVE*, *97*, 52587. https://doi.org/10.3791/52587