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Stage of Estrous Cycle Influences Behavior After Social Stress

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STAGE OF ESTROUS CYCLE INFLUENCES

BEHAVIOR AFTER SOCIAL STRESS

By

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ABSTRACT

STAGE OF ESTROUS CYCLE INFLUENCES
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Since women are almost twice as likely to develop major depression compared to men, research into this and other potentially debilitating mood disorders using behavioral studies should involve females despite hormonal, physiological, and potential behavior changes due to reproductive cyclicity. Until recently, most studies exclusively used males, thus limiting results of previous research to half of the population. Using adult female C57BL/6N mice as model organisms, we exposed them to novel forms of social stress across five days which encompassed the four-day estrous cycle to determine if the stages of the estrous cycle had an impact on behavior when they were exposed to social stress. Our results show that females in an earlier/pre-ovulation stage of the estrous cycle like proestrus display anti-depressive behaviors compared to mice in other stages. In contrast females in a later/post-ovulation stage like metestrus exhibit depressive behavior. Mood disorders can affect both males and females, so it is important to include females in research. However, our results suggest that use of females in behavioral studies, should consider reproductive cycling as an important variable.

KEYWORDS: Mood Disorder, Depression, Reproductive Cycling, Females, Research

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CHAPTER ONE

Introduction

Mood disorders, also known as affective disorders, are a class of psychiatric illnesses that impact emotions, motivation, energy, and overall wellbeing. The two most common mood disorders are major depressive disorder and anxiety disorders with a lifetime prevalence of 16% and 19% respectively[1]. These psychiatric illnesses are associated with increased mortality and a poor quality of life with major depressive disorder being the second leading cause of disability globally[1]. The median onset age for major depressive disorder is 32 and women are almost twice as likely to develop major depression compared with men, even when socioeconomic status and country of origin aren't factors[2]. There are many different factors that can cause a mood disorder or symptoms of a mood disorder such as environmental and social stressors, hormone imbalances and genetics[1]. Studying the differences in sexes and factors contributing to mood disorders will allow us to better understand and how to treat them.

In order to study mood disorders, mice are used as model organisms and are often exposed to social stress to encourage the onset of a mood disorder. Mice can be used as model organisms for female studies of social stress and related affective disorders because their reproductive cycle, the estrous cycle, has similar phases as the human menstrual cycle. Mice are also capable of exhibiting anxious and depressive behaviors, similar to human behavior, as a reaction to social stress which makes them an appropriate model for our purposes.[3] Social stress is a subcategory of environmental stress and results when one member of a social group experiences social rejection or is attacked by other

members of its group. In mice, a key social stressor is social defeat. Social defeat typically occurs when a territorial mouse successfully defends resources from a challenger. The defeated mouse may then develop changes in behavior including proactive coping mechanisms, aggression, and reduced cognitive flexibility[2]. This attack-and-defend behavior is commonly studied in males, but females do not typically have the same purposes for and relationships to social aggression as males. However, female California mice (*Peromyscus californicus*) are territorial and experience social defeat[2]. Female defeated mice exhibit behavioral changes such as reactive coping mechanisms, social avoidance, and low aggression[2]. These natural social stress environments can be replicated or altered in a lab setting to induce desired behavioral effects such as mood disorders. A problem that arises in studying mood disorders is developing a social stress model for female mice.

Attack-and-defend behavior is not typically studied behavior in female mice but has been examined in California mice. Creating a social stress model for females can be complex, so males are primarily used in experiments involving social stress. Female responses may be more variable when it comes to social stress due to societal roles as mothers and hormonal fluctuations due to their relatively quick (4 day) estrous cycle. It is important to study female mice in social stress experiments as the results may provide some translatable information regarding human females, who are twice as likely to suffer from major depression compared to males[2].

The estrous cycle is the type of reproductive cycle found in non-human mammals, including rodents. It lasts for approximately 4-5 days in rats and mice, which is relatively short compared to the human menstrual cycle which lasts approximately 28 days. During

the estrous cycle, the cytology (size, shape, and function of cells) of the uterus, hormone levels, and behavior change as the female mouse's body prepares for reproduction. The cycle can be separated into four stages: proestrus, estrus, metestrus, and diestrus[4]. During proestrus, the uterine cytology shows a predominance of nucleated epithelial cells as clusters or individually with the occasional cornified squamous epithelial cells. Proestrus is the pre-ovulatory stage and corresponds to increases in the hormone estradiol (E_2) during the day along with marked increases in luteinizing hormone (LH) and follicle-stimulating hormone (FSH) at night which cause ovulation. Estrus is characterized by dense clusters of cornified squamous epithelial cells when looking at the uterine cytology. Estradiol levels remain elevated through the morning and returns to basal levels in the afternoon. During Metestrus, the cytology shows a mix of cell types with leukocytes being the most predominant with few nucleated and/or cornified squamous epithelial cells. Plasma E_2 concentration is low in metestrus. Lastly, during diestrus the uterine cytology consists predominantly of leukocytes and E_2 levels begin to increase. During estrus, metestrus, and diestrus the plasma circulation of LH and FSH are low[4]. These hormonal fluctuations can cause behavioral changes consistent with mood disorders and depression. In humans, it has been proposed that swings in ovarian hormone levels, specifically when estrogen and progesterone concentrations are low, contribute to higher incidence of depression[5].

Mood disorders such as depression can be debilitating and potentially life altering. Females are twice as likely to suffer from depression compared to men, but research involving females can be challenging. This is primarily because females have a cyclic reproductive function and constantly changing endocrine physiology making them

physiologically and emotionally unique from other individual females. This means that for almost all the preclinical research that has ever been done, approximately one-half of any population has been ignored with respect to health. To overcome this problem, it is necessary to conduct experiments that examine the contribution of reproductive cycling on prevalent clinical and veterinary disorders. In order to study mood/affective disorders in a preclinical setting on female mice, the estrous cycle cannot be ignored. In the study reported here, I investigate how estrous cycle stage impacts stress-induced behavior. Our hypothesis was that mice in an earlier stage of the estrous cycle like proestrus will experience an increase in social preference during the social interaction/ preference test and show reduced contextual and cued fear responses compared to other animals during the fear condition test, while mice in a later, post-ovulation stage like metestrus will exhibit an increase in social avoidance and freezing behavior.

CHAPTER TWO

Materials and Methods

Subjects and Housing

Adult (6–8 weeks) female C57BL/6N mice weighing ~22–26 g (Envigo, Indianapolis, IN; N=58) were group housed (4–5 per cage) for 5 days of acclimation. After acclimation they were housed individually on a 12:12 light-dark cycle (lights off at 6 P.M.) at 22 °C, with ad libitum food and water. A separate cohort of male Hsd:ICR (CD1) retired breeder mice weighing ~50 g (Envigo, Indianapolis, IN; N=10) were similarly housed, and used to provide aggression during social interaction in the Stress Alternatives Model (SAM; Fig. 1) and as a target for the Social Interaction/Preference (SIP) tests. During the dark, active phase mice (C57BL/6N) were exposed to daily handling for 7 days prior to behavioral aggression and testing. All behavioral procedures were performed in a manner that minimized suffering and the number of animals used and were in accordance with the National Institutes of Health's Guide for the Care and Use of Laboratory Animals (NIH Publications No. 80-23) and approved by the Institutional Animal Care and Use Committee of the University of South Dakota.

Stress Alternatives Model (SAM)

The SAM apparatus is constructed of a white rectangular box (91 cm long, 22 cm wide, and 26 cm high) with curved opaque dividers (r=10.25 cm) that partition it into 3 sections: two enclosed areas (10×22×26 cm) at each end of the oval-shaped open field (OF=71×22×26 cm) arena for social interaction (Fig. 1). All behavioral interactions were

conducted during scotophase (dark; active period). The two enclosed areas are accessible from the OF arena through escape routes (one each at the distal ends of the oval) only large enough for a smaller C57BL/6N test mouse to pass through (Fig. 1).

A short time before social interaction began, a novel large, retired breeder CD1 mouse (aggressor) was placed into the SAM OF arena outside of a centered opaque cylindrical divider (15 cm diameter and 20 cm tall). A smaller C57BL/6N mouse (female test mouse) was then placed inside the opaque cylinder (Fig. 1). Our lab has previously shown that fear conditioning influences gene expression of Orx receptors, endocannabinoid receptors (Cb2), and brain-derived neurotropic factor (BDNF) in the amygdala and hippocampus[6,7]; To incorporate a SAM-inclusive fear conditioning paradigm, after a 30 s acclimation period inside the cylindrical divider, a 5 s tone (2500 Hz at 75 dB, CS+) was presented. Following the tone there was a 10 s trace period, after which the divider was lifted allowing test and aggressor (US+) mice to freely interact within the SAM arena for 5 min.

Retired breeder CD1 mice are naturally aggressive toward other male mice, including smaller C57BL/6N, and interactions led to high intensity social aggression and defeat[8], but this is not the case with female mice (see section below). Different from traditional social defeat models[8], test mice in the SAM are provided the option to either exit the arena (Escape) through one of the two escape routes or remain in the arena (Stay) with the larger aggressive mouse. Mice that escape the arena (Escape) have been shown to produce lower physiological and behavioral measurements of stress when compared to their submissive non-escaping (Stay) counterparts[9,10]. It is important to note, that both Escape and Stay mice experience high levels of aggression from the novel CD1 mouse,

and have elevated hormonal, behavioral, and gene expression stress responses, though these measures are significantly higher in the Stay mice[9]. Results from this and previous experiments have demonstrated that test mice generally choose a behavioral phenotype (Escape or Stay) by the second day of SAM interaction[9].

During interactions in which an aggressor mouse threatened the life of a test mouse, a clear, perforated divider (15 cm wide and 20 cm high) was used to briefly interrupt (5–10 s) these intensely aggressive bouts. Life threatening attacks included repeated bites to the head or neck of the test mouse. After 5 min of interaction, both mice were removed from the arena and placed back into their home cages. If test mice escaped, they were left in the enclosed area of the SAM until the end of the 5 min interaction period.

Test mice were exposed to 4 consecutive days of the SAM social aggressive interaction, following fear conditioning. A novel aggressor mouse was used on each day. On day 5 the Social Interaction/ Preference test (SIP) was performed (described below), followed by a final SAM exposure: placement in SAM cylinder, 5 s CS+ (tone), and 10 s trace period, testing for a conditioned response (CR). However, on this day the cylinder was not lifted and there was no large mouse present, and therefore, no social aggression. All interactions were recorded manually and digitally using GoPro Hero3+ cameras under red light. Digitally recorded interactions were scored by individuals naive to the treatment of each test mouse.

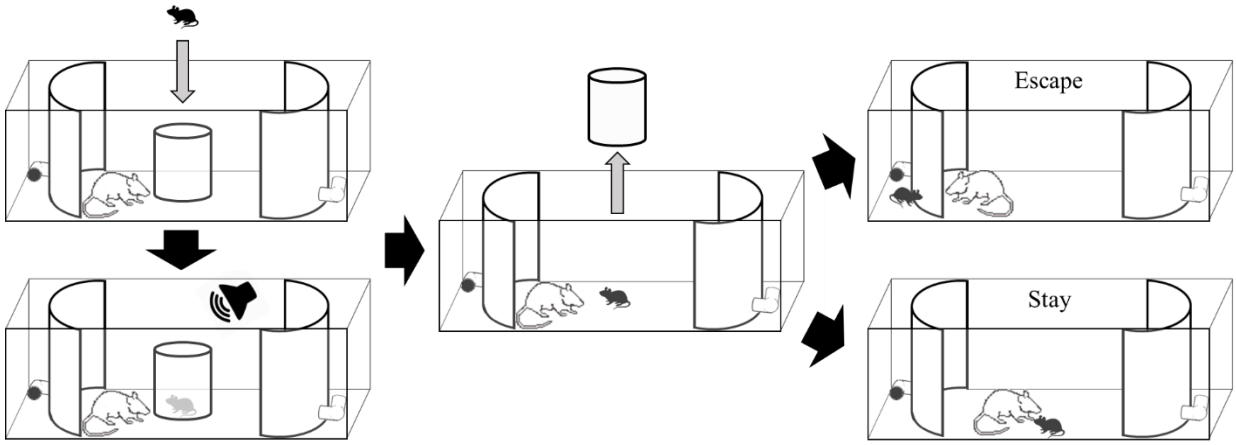


Figure 1: The Stress Alternatives Model (SAM) and its uses detailed above.

Aggression Toward Females

Male CD1 mice were trained to behave aggressively toward female C57BL/6N mice through a classical Pavlovian conditioning technique. Separate female mice (N=5), that were not used in SAM social interactions, were introduced into the home cage of a male CD1 retired breeder. When the male CD1 mouse would investigate the female intruder (sniffing – particularly anogenital sniffing), a mild shock (0.6mA) was applied to the rump of the CD1. In most instances, this resulted in the male mice displaying intense aggression toward the female mouse. Once aggression was displayed by the CD1, mice were separated with the female mouse being returned to her home cage. Typical interactions lasted less than 5 min, as aggression was almost immediately obtained through the pairing of the female with a mild shock. This procedure was repeated for 4 days prior to the use of the male CD1 mice in the SAM paradigm. In the SAM environment, if a CD1 mice did not show aggressive behavior toward the female test mice, a mild shock (0.6mA) was used during the social interaction.

Fear Conditioning Analysis

Freezing behavior, commonly used to assess fear conditioning[11], was measured on the 5th day in the absence of the CD1 aggressor (US). Freezing is the amount of time (> 1 s) that mice remained motionless, except for movements related to respiration. Freezing was measured during the time that a test mouse spent inside the opaque dividing cylinder; which included time prior to the tone (CS-; contextual) and during/after the tone (CS+; cued). Percent time freezing was calculated as freezing time before tone/30 s (as a measure of contextual fear) and freezing time after tone/15 s (as a measure of cued fear).

SAM Behavioral Analysis

Behavioral analyses for SAM socially aggressive interactions begin following the removal of an opaque cylinder and ended when the test mouse escaped, or at the end of the 5 min interaction time. Interaction time was measured as the time from the removal of the cylinder until the end of the interaction (when the test mouse was removed from the arena or escaped). Freezing, time in center, and time attentive to the escape route measurements were normalized by the total time spent interacting with an aggressor. Latency to escape was measured as the time starting from the removal of the cylinder and ending when the torso of the test mouse was through the escape hole.

Freezing behavior during SAM interactions (conflict freezing) was defined in the same way as freezing during assessment of fear conditioning (before the interaction begins). Conflict freezing always occurs during social interaction, and includes freezing in anticipation of aggression, in response to aggression, and contextual freezing in response to the arena in which aggression is experienced (especially on days 2–4 of SAM

interactions). Freezing time was converted into percent freezing time ([time (s) frozen/total interaction time] x 100).

The amount of time the test mouse spent in contact with the escape tunnel is an indicator of stress-sensitive novelty exploration[12,13]. Although the entire SAM arena is novel initially, the escape route is a unique attribute, distinctly different from the rest of the apparatus, as it clearly presents a physically different path for movement. The chamber at the other end of the escape route is also unknown, until the first passage. Thus, this indicator of novelty assessment is also specifically associated with the stress-sensitive act by which the Escape and Stay Phenotypes are demarcated. We defined the amount of time spent attentive to the hole as only including time when the test mouse was actively interested in and investigating the escape hole and tunnel directly (sniffing and placing head in the hole). This measure is a novel indicator of anxious behavior and decision-making unique to this model, which has been effectively used in SAM experiments on mice and rainbow trout[7, 14]. We consider Escape and Stay behavioral outcomes to be the result of decision-making because early responses are variable, become stable with experience, and are modifiable by learning as well as anxiogenic or anxiolytic drugs[7, 10, 14, 15].

Social Interaction/Preference Test (SIP)

The Social Interaction/Preference (SIP) test was performed on day 5 as a reliable indicator of whether mice are resilient or susceptible to social stress following the SAM interactions and drug treatments[16, 17]. SIP test was conducted in a square box (40 cm^3) with a perforated clear container (12 cm diameter and 20 cm high) placed alongside the middle of one wall (Fig. 2). To begin, test mice were placed into the arena at the

opposite end from the empty container and allowed to explore for 2.5 min, before being removed. The empty vessel was then replaced with an identical container holding a novel male CD1 mouse. Test mice were then placed back into the arena for another 2.5 min. Social preference (% time spent around social target), social avoidance (% time spent in corner areas), and freezing behavior were all scored and analyzed from recorded test videos.

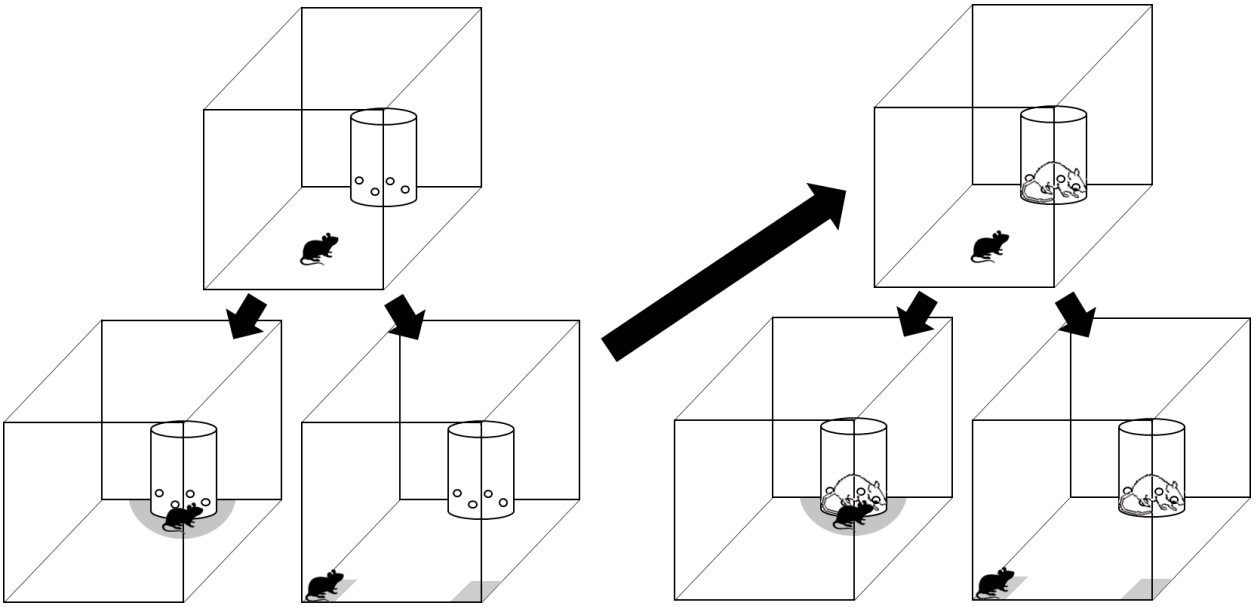


Figure 2: Social Interaction/Preference Test (SIP)

Vaginal Lavage

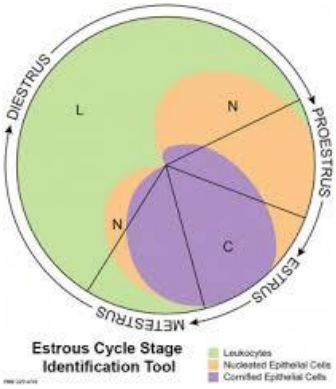
Every day, prior to SAM interaction or testing, vaginal lavage samples were taken from female mice. Mice were allowed to stand on home cage lids while their hind end was propped up exposing the vaginal area. Distilled water (~60 uL) was flushed in and out of the vaginal cavity several (5-10) times. If the female mouse urinated or defecated during sample collection, the sample was discarded, and the process was revisited after the vaginal area was rinsed with distilled water. Collected samples were placed on a slide

and allowed to air dry before being placed in a slide box. Later, slides were stained using crystal violet[18]. Photographs of samples were taken and later assessed to determine the estrous stage of the mouse sampled.

Lavage Sample Cytology

Photographs from the vaginal lavages were analyzed to determine the stage of the estrous cycle. Multiple photos, taken of each sample at varying magnifications (4x, 10x, 40x), were uploaded to a computer. The stages were determined by the relative abundance of three cell types when looking at the cytology photographs (Fig. 3). During proestrus, the uterine cytology shows a predominance of nucleated epithelial cells as clusters or individually with the occasional cornified squamous epithelial cells. Estrus is characterized by dense clusters of cornified squamous epithelial cells. During Metestrus, the cytology shows a mix of cell types with leukocytes being the most predominate with few nucleated and/or cornified squamous epithelial cells. Lastly, during diestrus the uterine cytology consists predominantly of leukocytes.

Figures 3: Shows proportional amounts of each cell type during different stages of the estrous cycle.



Data Organization

Data collected for the first four days of the SAM paradigm consisted of numerical values which tracked the position of the mouse's head and body, the amount of time moving vs freezing, as well as its relative location in the SAM apparatus. Using Microsoft Excel, the data were compiled and organized based on the desired behavior, the day the data were collected, and the stage of the estrous cycle. The three behaviors analyzed in the SAM paradigm were "time spent in center", "attention to escape route", and "conflict freezing time". On the fifth day, the SIP test collected data similar to the SAM test, but with different desired behaviors such as "social preference", "social avoidance", and "freezing behavior". These data were once again organized and categorized based on desired behavior, the day the data were collected, and the stage of the estrous cycle. Lastly, the fear conditioning test was conducted in which only freezing time both pre- and post- tonal were recorded. These data were organized in Excel based on the desired behavior, the day the data were collected, and the stage of the estrous cycle. Once all the data were organized, statistical analyses were performed to establish significance.

Statistical analysis

All experimental designs and statistical analyses were based on a priori hypotheses. For conditions that changed over 4 days of SAM social interaction, we compared outcomes using a two-way repeated measures ANOVA (Estrous Cycle Stages

x day of SAM interaction design), where Estrous Cycle Stage was Proestrus, Estrus, Metestrus, or Diestrus. To compare changes occurring within estrous cycle group across SAM interaction days, a one-way repeated measures ANOVA (Estrous Cycle Stage x day of SAM interaction design) was performed. Comparisons between two stages (Proestrus, Estrus, Metestrus, or Diestrus) were analyzed by Student's t-tests.

Each animal provided only a singular datum for all analyses. Five assumptions of parametric statistics were applied to the data, which were transformed when necessary, but graphed in their raw form. Analyses with both non-parametric and parametric statistics were performed along with examination for multiple comparisons using the Holm-Sidak method, and when the statistical analyses match, as they do for the data herein, we report the parametric results without α adjustment. Significant effects between groups for one-way analyses were examined with Student–Newman–Keuls post hoc analyses (to minimize Type I error) and Duncan's Multiple Range Test (to minimize Type II error).

CHAPTER THREE

RESULTS

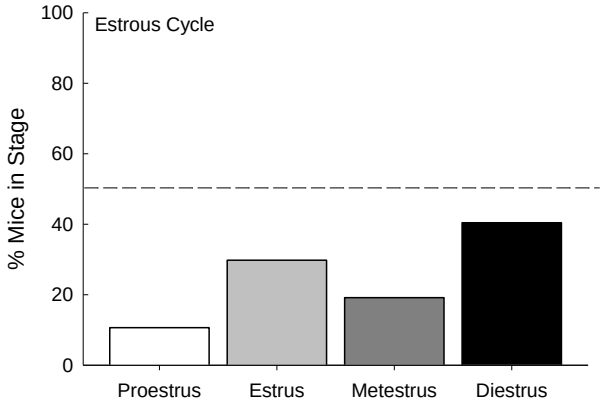


Figure 4: Mice were found to be in all four stages of estrus cycle
Proestrus – 10.6%; Estrus – 29.8%; Metestrus – 19.1%; Diestrus – 40.4%

Estrous cycle stage does not influence the time mice spend in the center of the SAM

The amount of time in the center the mice spent in the SAM was measured for 4 days in the to assess whether the time in the center is dependent on estrous cycle stage (Fig. 5). Generally, mice that spend more time in the center, rather than the edges, experience more depression/anxiety - and don't escape[9]. There were no significant differences for proestrus, estrus, metestrus, or diestrus mice suggesting that time in center is not dependent on estrous cycle stage (Two-Way Repeated Measures ANOVA: Estrous Stage effect: $F_{3,81} = 0.467$, $p \geq 0.708$; Time effect: $F_{3,81} = 0.0981$; $p \geq 0.961$; Interaction effect: $F_{9,81} = 0.226$; $p \geq 0.990$).

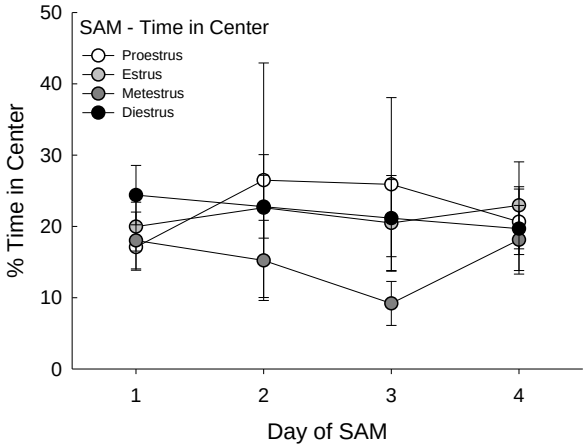


Figure 5: Stage of estrous cycle does not affect time in center ($p \geq 0.708$) across the 4 days in the SAM.

Diestrus mice exhibit more attention toward escape route later in SAM paradigm

Attention to escape route was measured for 4 days in the behavioral paradigm to assess whether attention to escape route was dependent on estrous cycle stage (Fig. 6). Normally, mice that end up escaping tend to spend more time near/looking through the escape holes, rather than the center of the arena. There were no significant differences comparing for proestrus, estrus, or metestrus stages. However, for diestrus, the animals spent more time attentive to the escape route on day 4. This was significant when comparing Day 4 to days 1,2, and 3 (One-Way Repeated Measures ANOVA: Diestrus mice, $F_{11,33} = 5.829$, $p \leq 0.003$; Day 1 vs Day 4: $t_{11} = 3.779$, $p < 0.001$; Day 2 vs Day 4: $t_{11} = 3.317$, $p \leq 0.002$; Day 3 vs Day 4: $t_{11} = 2.935$, $p \leq 0.006$).

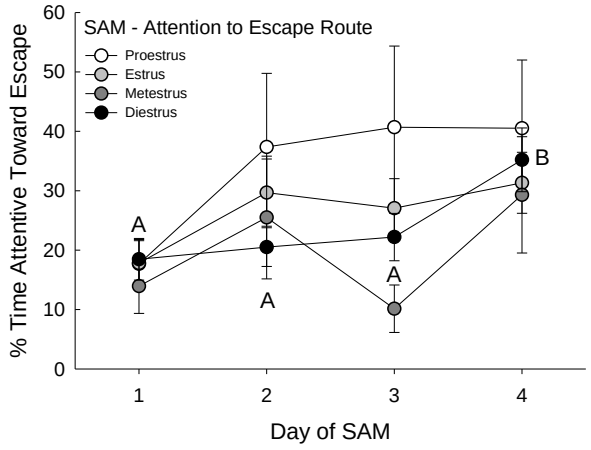


Figure 6: Stage of estrous cycle did not affect Escape Motivation (measured as attention to escape route), when comparing all phases proestrus, estrus, metestrus and diestrus mice. However, diestrus mice show do show more attention to escape route on the 4th day, when comparing Day 4 to days 1($p < 0.001$), 2($p \leq 0.002$), and 3($p \leq 0.006$), within this one group.

Estrous cycle stage does not influence conflict freezing behavior in the SAM

Conflict freezing behavior was measured for 4 days in the behavioral paradigm to assess whether conflict freezing is dependent on estrous cycle stage (Fig. 7). Freezing time is the amount of time the mice stopped moving in the presence of an aggressor (CD1). There were no significant differences in this measurement (Two-Way Repeated Measures ANOVA: Estrous Stage effect: $F_{3,81} = 0.0851$, $p \geq 0.968$; Time effect: $F_{3,81} = 1.924$; $p \geq 0.132$; Interaction effect: $F_{9,81} = 0.919$; $p \geq 0.513$)

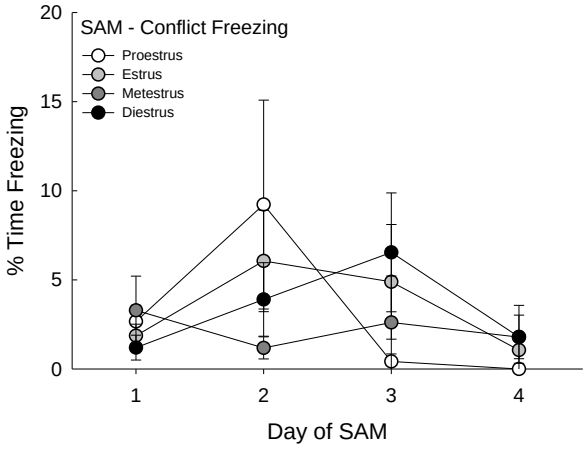


Figure 7: Stage of estrous cycle did not affect freezing time ($p \geq 0.968$) across the 4 days in the SAM.

Contextual and cued fear responses are reduced in proestrus mice

Fear freezing (conditioned response) was measured on Day 5 of the behavioral paradigm to assess whether fear learning is dependent on estrous cycle stage (Fig. 8). While all animals exhibited increased freezing post-tone (cued; CS^+) compared to pre-tone (contextual; CS^-; Two-Way Repeated Measures ANOVA, Estrous Stage effect: $F_{3,43} = 1.4$, $p \geq 0.262$; CS effect: $F_{1,43} = 19.7$; $^*p < 0.001$; Interaction effect: $F_{3,43} = 0.523$; $p \geq 0.669$), proestrus mice exhibited a reduced contextual freezing response compared to estrus animals ($t_{17} = 2.144$; $^\wedge p \leq 0.047$) and a decreased cued freezing response compared to metestrus mice ($t_{12} = 2.114$; $^+p \leq 0.05$). While no significant differences were observed between estrus, metestrus, and diestrus, there were mild variations in cued fear expression with metestrus mice having the highest freezing time.

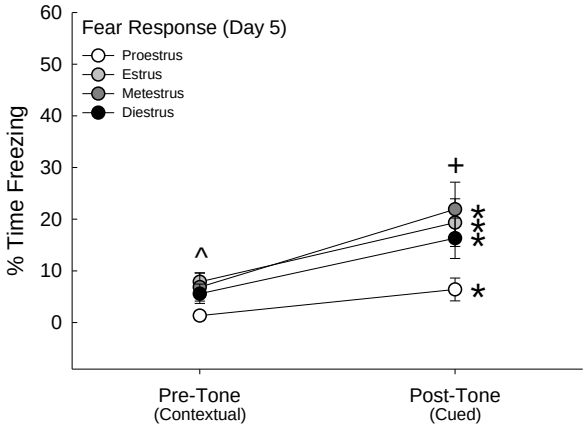


Figure 8: Stage of estrous cycle did not affect fear response to tone; mice in all phases exhibit enhanced freezing to the tone. However, proestrus mice exhibit reduced contextual freezing (compared to estrus animals) and diminished cued freezing (compared to metestrus mice). *Pre-tone (contextual) freezing is significantly higher than post-tone (cued) freezing in proestrus mice; $p \leq 0.05$. ^Estrus is significantly different from proestrus; $p \leq 0.05$. +Metestrus is statistically different from proestrus; $p \leq 0.05$.

Mice in proestrus show increased social preference in SIP test

The Social Interaction/Preference (SIP) test was performed on day 5 as a reliable indicator of whether mice are resilient or susceptible to social stress following the SAM interactions. Social preference (% time spent around social target) was scored and analyzed from recorded test videos to determine if it is dependent on estrous cycle stage. Only proestrus showed increase time near social target compared to novel target (Two-Way Repeated Measures ANOVA: Estrous Cycle Stage effect: $F_{3,43} = 3.24$, $p \leq 0.031$; Target effect: $F_{1,43} = 20.724$, $^{*}p < 0.001$; Interaction effect: $F_{3,43} = 5.795$, $p \leq 0.002$; Proestrus, Novel Target vs Social Target: $t_4 = 7.5$, $^{*}p \leq 0.002$).

Comparing time near the social target, proestrus mice displayed higher time compared to estrus ($t_{17} = 2.49$, $^{\wedge}p \leq 0.023$), metestrus ($t_{12} = 4.631$, $^{+}p < 0.001$), and diestrus ($t_{22} = 2.852$, $^{\#}p \leq 0.009$) animals. Also, metestrus was lower than diestrus ($t_{26} = 1.997$, $^{!}p \leq 0.05$).

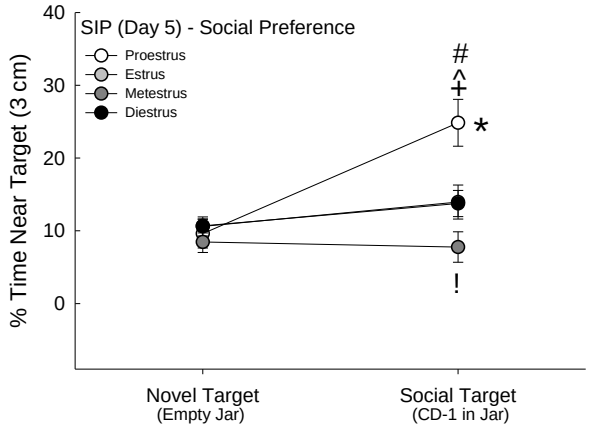


Figure 9: The stage of the estrous cycle stage did not have an effect on social preference, specifically comparing the novel target with the social target, except for proestrus mice ($^{*}p \leq 0.002$). Comparing time near the social target, proestrus mice displayed higher time compared to estrus ($^{\wedge}p \leq 0.023$), metestrus ($^{+}p < 0.001$), and diestrus ($^{\#}p \leq 0.009$) animals. Though their degree of preference between non-social and social target was not different metestrus mice did exhibit reduced social approach, compared to other stages, including diestrus mice ($^{!}p \leq 0.05$).

Mice in metestrus show increased social avoidance in SIP test

The Social Interaction/Preference (SIP) test was performed on day 5 as a reliable indicator of whether mice are resilient or susceptible to social stress following the SAM interactions. Social avoidance (% time spent in corner areas) was scored and analyzed from recorded test videos to determine if it is dependent on estrous cycle stage. Only Metestrus mice showed increase time in corners with social target compared to novel target (Two-Way Repeated Measures ANOVA: Estrous Cycle Stage effect: $F_{3,43} = 4.383$, $p \leq 0.009$; Target effect: $F_{1,43} = 3.212$, $p \geq 0.080$; Interaction effect: $F_{3,43} = 1.489$, $p \geq 0.231$; Metestrus, Novel Target vs Social Target: $t_8 = 2.484$, $^{*}p \leq 0.017$).

Comparing time in corners with the social target, metestrus mice displayed higher time compared to proestrus ($t_{12} = 3.673$, $^{+}p < 0.001$), and diestrus ($t_{22} = 3.305$, $^{!}p < 0.001$) animals.

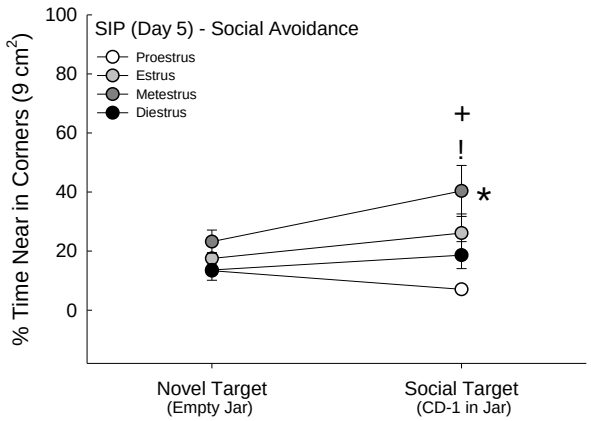


Figure 10: The stage of the estrous cycle stage did not have an effect on social avoidance, except for metestrus mice ($^{*}p \leq 0.017$), who spent more time in the corners when in the presence of a social target. Comparing time in corners with the social target, metestrus mice displayed higher time compared to proestrus ($^{+}p < 0.001$), and diestrus ($^{!}p < 0.001$) animals.

Mice in metestrus show enhanced freezing behavior in SIP test

The Social Interaction/Preference (SIP) test was performed on day 5 as a reliable indicator of whether mice are resilient or susceptible to social stress following the SAM interactions. Freezing behavior was scored and analyzed from recorded test videos to determine if it is dependent on estrous cycle stage. Mice in estrus, metestrus, and diestrus (but not proestrus) showed increased freezing when social target was present (Two-Way Repeated Measures ANOVA: Estrous Cycle Stage effect: $F_{3,43} = 3.129$, $p \leq 0.035$; Target effect: $F_{1,43} = 21.862$, $p < 0.001$; Interaction effect: $F_{3,43} = 2.153$, $p \geq 0.107$; Estrus, Novel vs Social: $t_{13} = 4.095$, $^{*}p < 0.001$; Metestrus, Novel Target vs Social Target: $t_{8} = 2.46$, $^{*}p \leq 0.039$; Diestrus, Novel Target vs Social Target: $t_{18} = 2.697$, $^{*}p \leq 0.015$). Comparing time freezing with the social target, metestrus mice displayed higher time compared to proestrus ($t_{12} = 3.157$, $^{+}p \leq 0.002$), and diestrus ($t_{22} = 3.353$, $^{!}p < 0.001$) animals.

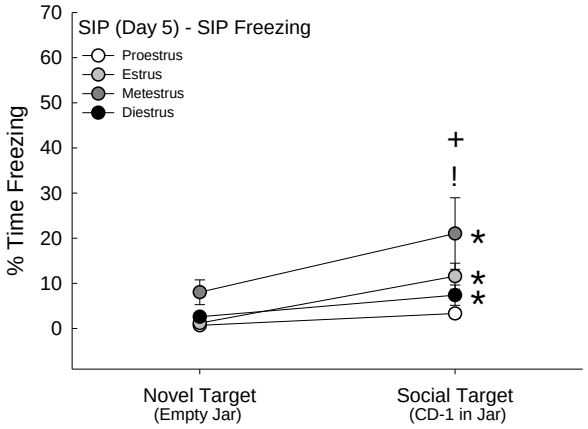


Figure 11: The stage of the estrous cycle stage did have an effect on social freezing relative to the social target for all animals except proestrus mice. Mice in estrus, metestrus, and diestrus showed increased freezing when social target was present (Estrus, $^{*}p < 0.001$; Metestrus, $^{*}p \leq 0.039$; Diestrus, $^{*}p \leq 0.015$). Comparing time freezing with the social target, metestrus mice displayed higher time compared to proestrus ($^{+}p \leq 0.002$), and diestrus ($^{!}p < 0.001$) animals.

CHAPTER FOUR

Discussion and Conclusion

Until recently, most scientific experiments conducted during human history, have not included females. This is primarily because females express cyclic reproductive and endocrine physiology, which can impact behavior and physiological response. This means that for almost all the preclinical research (experiments done on animals rather than humans) that has ever been done, approximately one-half of any population has been ignored with respect to health. This is a particular problem for maladies and disorders that are more prevalent in females than in males. Recently, new behavioral paradigms and animal models have been designed to include female subjects. To overcome this problem (the lack of female inclusion), it is necessary to conduct experiments that examine the contribution of reproductive cycling on prevalent clinical and veterinary disorders such as mood/affective disorders. During the experiments reported here, a new social interaction Go/No Go paradigm, called the Stress Alternatives Model was employed, in which mice were found to be in in all four stages of the estrous cycle following socially aggressive interactions. This suggests that the stressful conditions in the SAM did not repress cyclicity. The main goal of the study was to determine if there is a relationship between stages in the estrous cycle and behavior experienced after social stress using the SAM paradigm with fear-conditioning and the Social Interaction/ Preference Test (SIP). During the first four days of the SAM, three variables were tested: time spent in center, attention to escape route, and conflict freezing behavior. The data show that there were no significant results for time mice spend in the center, conflict

freezing behavior, or Escape Motivation (measured as attention to the escape route) over all estrous phases. As such, many stress-related behaviors did not appear to be influenced by reproductive cyclicity. However, for attention to escape route, diestrus mice exhibited more attention to the escape route on the fourth day of the SAM when compared to their own behavior days 1, 2, and 3, which indicated that there may be some effect of reproductive cycling on stress-related behavior.

On the fifth day, the female mice were exposed to the SIP test and three variables were analyzed: social preference, social avoidance, and freezing behavior. The data show that proestrus mice are the only ones that showed increase time near social target compared to novel target, thus displaying social preference. Metestrus mice were the only ones that showed an increased time in corners with social target compared to novel target, demonstrating social avoidance. Lastly, mice in estrus, metestrus, and diestrus (but not proestrus) showed increased freezing when social target was present. Metestrus mice demonstrated higher freezing time than proestrus and diestrus mice.

Following the SIP test, we conducted the Fear Conditioning analysis in which contextual fears (freezing prior to tone) and cued fears (freezing during/after tone) were distinguished. All mice exhibited increased cued fears. However, proestrus mice displayed reduced contextual freezing response compared to estrous mice and reduced cued freezing response compared to metestrus mice. While no significant differences were observed between Estrus, Metestrus, and Diestrus, there were mild variations in cued fear expression with metestrus mice having the highest freezing time.

The primary findings come from the Fear Conditioning analysis, where proestrus mice displayed reduced contextual and cued fear responses compared to the other

animals, and the SIP test, where proestrus mice showed an increase in social preference and metestrus mice showed an increase in social avoidance and enhanced freezing behavior. For proestrus mice, an increase in social preference and reduced fear responses show reduced fear of a novel aggressor and reduced depressive behavior. For metestrus mice, an increase in social avoidance and increased freezing behavior demonstrates characteristics of depression.

In proestrus mice, E_2 levels rise during the day and LH and FSH increase at night, thus signaling ovulation in the next stage.[4] This rapid increase in E_2 have been shown to have an effect on proestrus animals where they demonstrate anti-anxiety and anti-depression behaviors.[19] Increased LH and FSH signal ovulation, which cause a decrease in E_2 levels and lead to a negative emotional state similar to depression. Interestingly, as ovulation occurs during estrus, the prior stage, proestrus, has a mostly positive effect on the mood of the animal. This could be beneficial as a reproductive measure. Proestrus is considered the pre-ovulatory phase, prior to potential mating. So, it could be advantageous for a female mouse to be more explorative and therefore have a reduced fear response which increases the potential mating probability.

This reduced fear response and explorative behavior also coincides with proestrus mice displaying increased social preference and less social avoidance. Mice in proestrus are considered to be more sexually receptive compared to later stages in the estrous cycle[20] and may behave in a such a manner to increase the probability of mating. During this stage, female mice may be less hesitant to approach, or be approached by, a novel male. Thus, they display an increased social preference.

During the metestrus stage of the estrous cycle, mice exhibited more depressive behavior by showing an increase in freezing behavior and social avoidance. This could be due to the hormonal changes in this stage; metestrus occurs after ovulation/estrus and E_2 levels are low. The low concentration of E_2 may cause or allow female mice to exhibit depressive behavior. Experimental elevation of E_2 levels has been interpreted to produce anti-depression and anti-anxiety responses in female mice, and the experimentally reduced E_2 concentrations appears to promote anxious and depressive behaviors.[19] Mice in this stage have either already mated or missed their chance to mate and are not sexually receptive, so they might avoid other mice to protect themselves and potential offspring from harm.

Our results suggest that it is important to consider reproductive cycling when researching affective/mood disorders such as depression in preclinical studies involving females. During the estrous cycle, the flux of hormones, such as E_2, from stage to stage seem to cause changes in behavior after social stress. Further research is needed to investigate the specific relationship between hormone concentrations and behavior since this experiment only focused on the relationship between estrous cycle stage and behavior. It is worth noting that hormone activity is inherently part of each stage, but specific hormone levels were not measured in this experiment. For those reasons, we cannot make a direct link from plasma hormonal concentrations to the observed behavior. The data from this experiment show that proestrus mice exhibit better coping ability with social stress as they exhibit diminished fear responses and increased social preference compared to mice in other stages of the estrous cycle. Metestrus mice displayed increased depressive behavior marked by their increase in social avoidance and increased

freezing behavior compared to mice in other stages. Mood/affective disorders can affect both males and females, so both sexes should be used in research. However, females are used in behavioral studies, reproductive cycles must be considered as a potential variable. Animals in an earlier/pre-ovulation stage of the estrous cycle like proestrus show anti-depressive behaviors and animals in a later/post-ovulation stage like metestrus display depressive behaviors. Using this information, it could be theorized that we could predict when depressive or anti-depressive episodes occur in females and perhaps by changing their behavior during these times, it could have an effect on the prevalence of a mood disorder.

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