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ORAL CONTRACEPTION AND COGNITION

By

Kathleen J. Gerencer

B.A. Earlham College 2017

A THESIS

Submitted in Partial Fulfillment of the

Requirements for the Degree of

Master of Arts

(In Cognitive Psychology)

The Graduate School

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August 2023

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ORAL CONTRACEPTION AND COGNITION

By Kathleen J. Gerencer

Thesis Advisor Dr. Thane Fremouw

An Abstract of the Thesis Presented
in Partial Fulfillment of the Requirements for the
Degree of Master of Arts
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Oral contraception is currently used by over 100 million women worldwide.

Women utilize contraception not only to prevent pregnancy but also to manage a wide range of health concerns, such as acne and polycystic ovarian syndrome. Although this medication has granted women bodily autonomy, helped them attain higher levels of education, and helped them enter the workforce in greater numbers, little is known about the consequences outside of the intended contraceptive effects, specifically the cognitive and behavioral consequences.

Moreover, because doctors can prescribe contraception after the first menstrual cycle and during puberty, it's possible that this critical window of development could be altered longitudinally as a synthetic form could change the naturally occurring hormonal levels that are in flux during this time. The present research sought to determine the effects of synthetic and naturally occurring estrogen and progesterone on behavior and cognition at a critical developmental period. To determine these effects, we used a mouse model of oral contraception and based the concentration on a commonly used contraceptive. After 31 days of administration with a combination levonorgestrel and ethinyl estradiol solution, we conducted two behavioral tests. First, we used the elevated plus maze to determine anxiety-like behaviors. We then explored fear

conditioning and extinction by utilizing a three-day contextual fear extinction protocol with an additional cued fear recall day. Based on the existing literature, we hypothesized (1) animals treated with a combination OC would demonstrate decreased anxiety and fear conditioning and extinction compared to control animals. (2) Naturally cycling mice in diestrus would experience increased anxiety and fear conditioning and extinction compared to mice in estrus. (3) Animals treated in the potentially critical window during puberty would demonstrate significantly different anxiety and fear conditioning and extinction behaviors.

DEDICATION

To my parents and my advisor Thane for offering me support and believing in my abilities.

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CHAPTER 1

A BRIEF HISTORY OF WOMEN IN CLINICAL RESEARCH

Introduction

Clinical research regarding the mental and physical health of women has often been reductive and sparse. With roots dating back to 1900 BC, ancient Egyptians and Greeks asserted that mental disorders in women were directly attributable to hysteria, a phenomenon in which the uterus spontaneously moved throughout the body, causing various ailments (Tasca et al., 2012). As time went on, although different theories developed, hysteria became the overarching diagnosis for a wide range of health concerns in women that doctors did not understand (Tasca et al., 2012). On the contrary, research regarding men was vast and in-depth. Medicine focused on diseases that affected men and did so from a male perspective. Researchers who were mostly men studied men, male animal models, and developed experiments using cells from males. The understanding of male dominated diseases and the physiological functions of the male body grew, and research continues to advance. Conversely, women were historically ignored, as scientists either did not recognize the importance of studying women or believed that their physiological mechanisms, such as the fluctuations in female sex hormones, would be too complex to include in experiments (Holdcroft, 2007). Ultimately, women were left out of crucial clinical research. When left unstudied, these gaps in knowledge have and will continue to lead to dangerous outcomes regarding the health and well-being of women.

A notable instance of the danger caused by the lack of research including women, is illustrated by the drug Thalidomide. In the 1950s, preclinical testing of the drug in rodent models led to the assumption that the sedative was nontoxic to humans, and further tests were not conducted (Rehman et al., 2011). As a result, pregnant women taking this medication to reduce their nausea had miscarriages, stillbirths, or gave birth to children with significant limb

deformities. In response to this incident, policymakers in 1977 broadly excluded women of "childbearing potential", meaning all premenopausal women, from the early phases of clinical research (Liu & Mager, 2016). Moreover, because clinical trials did not include women, researchers did not know of the effects, leading to gross harm and death for those taking the drug. As the gaps in the literature widened, there continued to be little data on how drugs affected women and women's health in general. The lack of knowledge was also associated with consequences such as harmful responses to medication (Liu & Mager, 2016; Zucker & Prendergast, 2020). For example, data revealed that women who were given the same dose of drugs as men often experienced higher rates of nausea, cognitive deficits, cardiac abnormalities, and even acute liver failure. Research continued to reflect society's view of wanting to protect women while simultaneously undervaluing and erasing their existence.

As time went on and protests erupted, clinicians and researchers began to shift towards acknowledging the importance of including women and females in studies. Beginning in the 1980s, the US Public Health Task Force on Women's Health Issues published a report stating that the lack of women in research was detrimental (Mirin, 2021). This report also urged scientists and clinicians to investigate illnesses that more commonly affected women. Following the recommendations of the National Institutes of Health (NIH) advisory committee, the NIH created a section in their *Guides for Grants and Contracts* that encouraged researchers to include women and urged them to provide rationale when they chose not to (Liu & Manger, 2016). While these suggestions were important, it was not until the early 1990s that actions and funding began to reflect the shift in values. In 1991, the NIH launched a large 15-year study consisting only of female participants, and the FDA repealed the policies created in the 1970s that banned previously mentioned women from studies (Merkatz et al., 1994). Eventually, the US Congress

turned policy into law with the addition of the 1993 NIH Revitalization Act, which mandated the inclusion of women and other minority groups in research. While science and medicine have made strides in understanding women's health and female bodies, there are still fundamental areas that need to be investigated.

Past Literature and Focus

To date, one of the largest clinical research studies addressing women's health was launched at the recommendation of the Public Health Task Force on Women's Health Issues and the first female director of the NIH, Dr. Bernadine Healy. Beginning in 1991, the Women's Health Initiative (WHI) sought to address potential treatments and dietary changes that could alleviate illness and decrease mortality in women between the ages of 50 and 70. The study recruited over 160,000 women, including over 26,000 for hormone research.

In the hormone trials of the WHI, clinicians administered HRT in the form of estrogen alone or in combination with progesterone to reduce the risk of illnesses such as cardiovascular disease and cancer (Parker, 2013). In a shocking turn of events, results revealed the opposite of what researchers had expected, and the combination progesterone form instead led to an increased risk of stroke, heart disease, blood clots, and breast cancer (Paciuc, 2020). These results led the hormone section of the WHI to a premature stop, and many women also stopped accepting hormone therapy as a treatment post menopause. In response to this study, postmenopausal women potentially reduced their chance of having a stroke or developing a cardiovascular disease and were able to do so after learning of the effects correlated with hormone treatment. Furthermore, the greater health risks associated with combination hormone therapy led scientists to examine the safety and effects of estrogen more extensively.

Although this study was considered a landmark in women's health research, participants consisted of older women who had completed menopause and had a very different set of health concerns compared to younger women (Manson et al., 2013). On the one hand, this study paved the way and highlighted the importance of clinical research addressing women's issues. On the other hand, a critical group was left out, i.e., women of childbearing potential, and again, a gap remained in our understanding of the processes and illnesses related to women. While the WHI and subsequent studies proved important for treating older women, they were not representative of women as a whole. For example, in younger women, endogenous hormones (hormones originating from the body) are present at higher levels compared to their levels during menopause.

Though hormones fluctuate throughout a woman's lifetime, there are events that lead to major changes, such as puberty and pregnancy. During these times, it has also become more common for women to seek to regulate their hormones or take action to prevent pregnancy via contraceptive methods like hormone administration. In the hormone trials in WHI, those who had an intact uterus, theoretically still produced higher levels of estrogen compared to hysterectomized individuals and had higher health risks when treated with estrogen and progesterone. As younger women produce even higher levels of estrogen, it's possible that this risk may also increase. On the contrary, more recent data has revealed that hormones may be suitable for short-term treatment in younger menopausal women, but again, there has not been a large-scale longitudinal study addressing young premenopausal women's health and factors such as hormones, so the effects are largely unknown.

While postmenopausal research is important, it excludes a large group potentially affected by changes in hormonal levels, especially if they are prescribed a synthetic medication

to alter these levels, as is the case for oral contraception. As research has only begun to touch on the effects of hormone therapy in younger women, it's possible that one could go farther in hypothesizing that factors such as hormone administration in adolescence could even affect the brain and potentially lead to permanent changes in cognition and behavior. Moving forward, it is essential that clinical and scientific research include groups that have previously been intentionally left out or ignored in the past to better understand and improve health outcomes for women in the future.

"The Pill" and Other Forms of Hormonal Contraception

While older women were being administered hormones to regulate postmenopausal health concerns, younger women also sought help to find a way to mitigate issues related to menstruation and pregnancy. Beginning in the late 1950s, researchers created and tested medication that regulated women's menstrual cycles, by altering endogenous hormone levels and found that it had contraceptive side effects. In the 1960s, the pill was approved as a contraceptive method. The first formula contained 10,000 μ g of progestin (a synthetic form of progesterone) and 150 μ g of estrogen. Due to the initial high dose, many women experienced adverse health effects such as nausea, dizziness, vomiting, and even blood clots (Liao & Dollin, 2012). Today, hormone levels fall into the range of 50–150 μ g of progestin and 20–50 μ g of estrogen.

Mechanistically, oral contraception has two intended effects: it prevents ovulation and thickens cervical mucus (Rivera et al., 1999). Combination pills (estrogen and progestin) function as a negative feedback loop. Higher levels of estrogen and progesterone stop the release of follicle-stimulating hormone (FSH) and luteinizing hormone (LH) from the pituitary. This action then stops estrogen release from the ovaries and effectively prevents ovulation and

implantation of the ovum. Additionally, progestin maintains a thick cervical mucus, which creates a harsher environment for potential embryo implantation.

Most of the research conducted to address the safety and effectiveness of oral contraception was similar to previous clinical studies in that a large subset of women were left out (Petitti & Sidney, 2005). Though studies such as the Walnut Creek Contraceptive Drug Study in the early 1970s did include younger women, researchers instead focused on either pregnant women or those who had recently given birth. Furthermore, the data that was collected addressed factors such as birth defects and cardiac-related measures. While the findings were not indicative of increased health risks, critics of the study have suggested that the study is flawed because it did not include enough women actually taking the medication (Okie, 1981). Again, like previous research, this study, though large and longitudinal, was incomplete and did not address additional health factors or a representative population.

Today, there are over forty different formulations of hormonal birth control in pill form approved in the US (Allen et al., 2016). The composition of each medication can vary depending on the type of synthetic estrogen and progesterone used. For example, some pills are made up of ethinyl estradiol and levonorgestrel, while others contain estradiol valerate and dienogest. Additionally, formulations vary in concentration, ranging from a "low dose" of 35µg or less of hormones to higher doses of 50ug or more.

OC is either delivered in a monophasic or multiphasic manner, in which the same amount of drug is delivered or a varying amount. To decrease the side effects associated with the monophasic form, biphasic and quadriphasic pills were created (Van Vliet et al., 2006). However, the differences in side effects and overall effectiveness between the forms are largely

unknown, and many women continue to use a variety of options without being well-informed due to the gaps in the research.

As oral contraception became more widely known and used by women across the US and the world, other forms of hormonal contraceptives were also being developed. Like oral contraceptives, Intrauterine Devices (IUDs) also serve to prevent pregnancy. However, unlike a daily pill, this is a T-shaped device placed internally past the opening of the cervix and into the uterus. The hormone functioning to prevent pregnancy in the IUD is progesterone (progestin), which, again, thickens cervical mucus to prevent implantation. Unlike the different formulations of OCs, IUDs do not contain estrogen. Also, unlike the standard 28-pill regimen containing both active (estrogen /progestin) and an inactive (placebo), IUDs offer continuous contraception for up to 5 years, releasing an average of 20µg of progestin per day.

Another common alternative form of hormonal contraception includes the injection of synthetic progesterone. Known as Depo-Provera, this contraceptive is injected intramuscularly. After a 1mL 150mg/mL injection of medroxyprogesterone acetate, levels continue to rise for three weeks to reach serum concentrations of 1-7ng/mL. The contraceptive effects of this type of administration are said to last approximately three months (Pfizer, n.d.).

A third common form of contraceptive involves a small rod implanted by a physician under the skin of the upper arm. Known in the US as Nexplanon, this method functions to prevent ovulation and pregnancy by releasing 68 mg of progestin over 5 years, with an average release of about 40µg of progestin per day.

Since their creation in the 1960s, "the pill" and other forms of hormonal contraception have given women the agency to choose when and if they want to conceive. In addition to relieving medical symptoms caused by polycystic ovarian syndrome and endometriosis,

hormonal birth control has been correlated with a record number of women entering the workforce, women obtaining higher levels of education, and larger numbers of women in male-dominated careers such as medicine and law (Goldin, 2002). Although contraception is a useful tool to treat illnesses and prevent pregnancy, there is not substantial evidence to support its safety or indicate potential side effects. Currently, research in the field indicates that synthetic hormones within OC could cross the blood-brain barrier, act directly on endogenous estrogen and progesterone receptors, and alter neural activation and organization in vivo (Taylor, 2021). Clinical research conducted to address women's health has historically been sparse and unrepresentative. Contraceptive research has not been an exception to this narrative. While research does exist and the field is expanding, there is a potential for long-lasting effects associated with contraceptive use, and this area needs to be studied to directly address the safety and comprehensive effects of this medication.

CHAPTER 2

HUMAN LITERATURE

Over 100 million women worldwide currently use oral contraception (OC), and most women will use some type of hormonal contraception throughout their lifetime (United Nations, 2020). While synthetic hormones have been studied for contraceptive purposes and postmenopausal hormone replacement, little is known about their impact outside of the intended effects, and little is known about moderating factors such as the duration and timing of initial administration. Moreover, their effects on premenopausal women have not been examined in depth.

Given the extensive use of OC, it is imperative to understand the effects OC might have, including on neuroanatomy, behavior, emotions, and cognition. Similar to research on postmenopausal women and estrogen supplementation, the current literature on the cognitive and behavioral effects of exogenous estrogen and progesterone administration is contradictory and lacking. Currently, there are several opposing narratives within the realm of hormonal research regarding behavior and cognition, neuroanatomical differences, and some limited research relating to the developmental effects of hormone administration.

Development and Neuroanatomy

Endogenous estrogen and progesterone play a role in various critical processes. Endogenous estrogen can be classified into three forms: estradiol, estriol, and estrone (Ciu et al., 2013). These hormones are present at different points in a woman's life. For example, estradiol is the predominant form of estrogen found during puberty and into the childbearing years, whereas estrone is the main form of estrogen produced after menopause (Livingston, 2020). The additional form, estriol, is mainly produced during pregnancy. Although there are also different

forms of progesterone, the main endogenous substance utilized and synthesized by the reproductive system is 5-dihydroprogesterone (Livingston, 2020). While these hormones control reproductive events affecting the ovaries and uterus like menstruation, pregnancy, and menopause, there is also evidence of direct action and effects on the brain.

Beginning at puberty (which can vary in time of onset and duration but on average begins between 8 and 13 years old), levels of estrogen and progesterone increase. During this period, synaptic pruning takes place, and there is said to be an increase in myelination, helping to form emotional and cognitive processing areas. Lopez Moratalla et al. (2011) Additionally, this time marks the beginning of the menstrual cycle and monthly hormonal fluctuations that have potential activational and organizational effects and will continue in naturally cycling women until menopause. This is also the time when women can begin to use OC. While many women utilize this medication for contraception and to mitigate other health concerns, hormonal fluctuations are somewhat prevented as progesterone and estrogen are reduced via negative feedback (Rehbein, 2021). In turn, when the brain is supposedly shaped by large spikes in these hormones, the interference of this process may be associated with functional and activational changes that could have long-term effects.

After puberty, the next significant hormonal changes some women experience happen throughout and directly after pregnancy. During pregnancy, women experience a dramatic spike in progesterone and estrogen, which is comparably higher than the increase experienced during puberty. Following birth and during the post-partum period, progesterone and estrogen then sharply decline and eventually return to pre-pregnancy levels within 5 days (Rehbein, 2021). One of the final significant periods of hormonal change that women experience in menopause, which occurs on average between the ages of 44 and 55 years (NIH, n.d.), Research cites that the

main structural changes during this time involve higher-level cognitive processing areas and emotional processing areas (Mosconi et al., 2021). However, there is evidence that after menopause and the initial period of perimenopause, the brain can compensate for these changes and recover from any cognitive loss (Mosconi et al., 2021). Additionally, to counteract the undesirable changes or any health issues caused by this decline, many women are prescribed exogenous estrogen and sometimes progesterone supplements, also known as hormone replacement therapy (HRT).

The brain is a complex organ, and there are still gaps in the research on neuroanatomical changes, development, and OC use. As women undergo multiple hormonal changes throughout their lives, evidence suggests that alterations of structural and functional mechanisms within the brain are due to said hormones. Recognizing that there is room for future research, the most reported areas that involve changes are the prefrontal cortex, the hippocampus, and other components of the limbic system and emotional processing areas (Lisofsky et al., 2016; Zeidan et al., 2011; Albert et al., 2017; Hwang et al., 2015). Although these regions are mostly agreed upon, there are some controversies in the literature regarding volumetric changes and activational differences. Moreover, synthetic hormone administration, like HRT but more likely OC, could significantly interrupt and alter these processes as it interferes with endogenous hormone levels.

Estrogen and Progesterone

Researchers claim that endogenous estrogen is responsible for a range of effects such as poorer fear extinction recall, increased impulsivity, changes in hippocampal gray matter, and affect changes (Diekhof, 2015; Graham et al., 2013; Hampson & Morley, 2013; Albert et al., 2017; Dumas et al., 2010). Some research has suggested that these effects are context-dependent,

i.e., during fear conditioning or other anxiety-inducing situations (Hwang et al., 2015; Nasseri et al., 2020; De Bondt et al., 2013; Taylor et al., 2021). Alternative research asserts that there is little to no effect on cognitive or emotional processes due to estrogen (Renczés et al., 2020). Other studies that have focused on naturally cycling women at different points in their menstrual cycle (during times of either low or high estrogen levels) indicated changes in fear conditioning with lower levels of estrogen leading to impaired fear inhibition (Glover et al., 2013). In addition to estrogen-based changes, research also claims that progesterone affects mood and cognition in both similar and/or different ways to estrogen (Arélin et al., 2015; Herrera et al., 2019). Some research has asserted that progesterone facilitates functional connectivity between the hippocampus and dorsolateral prefrontal cortex, as determined by looking at the fluctuations in this hormone in naturally cycling women (Arélin et al., 2015). Moreover, this study hypothesizes that because these areas are related to learning and memory, it is possible or likely that progesterone significantly affects cognitive processes throughout the menstrual cycle.

While there are assertions that natural fluctuations lead to cognitive, behavioral, and neuroanatomical changes, the bulk of the literature on progesterone and estrogen maintains that the synthetic forms of these hormones can lead to more significant changes (Galvin & Ninan, 2014; Glover et al., 2013; Lundin et al., 2017). However, like the findings in naturally cycling women, data from studies conducted with synthetic progesterone and estrogen also provide evidence for the different functions of the two hormones. For example, De Bondt (2016) found that there were regional differences in gray matter that were negatively correlated with estradiol levels but not progesterone levels in women using OC. Another study determining the mood effects of combination OC found that progesterone, not estrogen, was associated with the stress response elicited by a cold pressor test (Herrera et al., 2019). Within this literature, experimental

results indicate that OC use is also associated with effects such as neural and activational changes, volumetric and neuroanatomical differences, cognitive changes in learning and memory tasks, and even changes in anxiety-like behaviors, (Gingnell et al., 2013; Lisofsky et al., 2016; Lundin et al., 2017; Radke & Derntl, 2016). Alternatively, other studies claim to have found no differences in any of these areas caused by either estrogen or progesterone (Scheuringer et al., 2020; Beltz et al., 2019). Due to the inconsistencies in the literature and the wide range of effects, it's important for studies to continue to address both endogenous and exogenous hormones and the impact that they have on cognition and behavior.

Anxiety and Depression

Contemporary data suggests that women are twice as likely as men to be diagnosed with anxiety or depression (Mayo Clinic, 2019). This higher prevalence could be due to a multitude of factors, such as unequal societal power structures or an increased workload with a profession and caring for children. However, some researchers point toward oral contraceptives as a potential cause of these disorders (Mayo, 2019; Lunden et al., 2017).

Although results vary across studies, one narrative surrounding depression and anxiety is that women with a past medical history of mood-related symptoms often experience either a reoccurrence or worsening symptoms when taking OC. Compared to non-users, OC users had less variability in mood overall across the menstrual cycle and menstruation (Oinonen & Mazmanian, 2002). However, when participants did experience negative mood effects and affect changes, these changes were associated with pre-existing depression, psychiatric disorders, and premenstrual mood disorders (Oinonen & Mazmanian, 2002). Similar results were presented in a case study when a woman with a history of depression and anxiety experienced feelings of fear and unreality after taking OC (Ushiroyama, 1992). Moreover, data from Borgström (2008)

suggests that women with Premenstrual Dysphoric Disorder who suffer from anxiety, depression, and other mood disorders often experience worsened symptoms when taking OC. Additional evidence for the connection between OC and mood is illustrated in a study by Hamstra et al. (2017), in which women who were free of prior mood disorders and associated symptoms experienced fewer negative emotions and mood shifts when taking OC (Hamstra et al., 2017; Skoglund, 2016). However, these studies also found that women using a combined OC pill had a higher incidence rate of depression compared with women using other forms of contraceptives. Again, the data illustrates a connection between mood disorders and OC use. While the connection between previous mood disorders, worsening symptoms, and OC usage is apparent in several studies, little is known about the mechanism and direction of causality between the factors.

Other studies on OC and anxiety maintain that hormone administration leads to mood effects without mentioning the history of mood disorders. In a study where women were administered either OC or a placebo, Lunden et al. (2017) suggested that OC use led to increases in anxiety, mood swings, and irritability. Moreover, further analysis revealed that these differences were mostly in women who had a history of adverse mood effects associated with OC use. Results also demonstrated that there were differences between cycle phases in OC women. OC women in the intermenstrual phase had significant mood side effects compared to women in the premenstrual phase. Also, women in the premenstrual phase experienced a slight improvement in their scores of depression. While these results were associated with smaller effect sizes, women experience different endogenous hormone levels throughout their cycles. Because of this, the changes in mood associated with hormonal changes throughout the month in naturally cycling women could further reinforce the validity hypothesis that the administration of

synthetic hormones leads to mood effects. To determine the directionality of these outcomes, it is important to continue to explore this area in a behavioral and cognitive context to detect the time-sensitive effects concerning OC usage at different points in the menstrual cycle but also over a longer period, such as during puberty and adolescence. In another study concerning fear conditioning context, injecting participants with saline to assess fear learning did not lead to a decrease in fear learning compared to naturally cycling (NC) women and men (Merz et al., 2012).

Alternative data suggests that hormone administration is associated with decreased anxiety and a blunted stress response (Lisofsky et al., 2016; Simone et al., 2015). A neuroimaging study found that OC women had a blunted stress response compared to NC women, as indicated by their performance on the Trier Social Stress Test and functional magnetic resonance imaging (Sharma et al., 2020). Ambruster et al. (2017) found similar results when measuring skin conductance and startle responses to stimuli. Compared to NC women, those taking oral contraceptives had dampened responses, indicating potential differences in the mechanisms related to stress. Analyses also revealed that other physiological measures pointed towards a more positive affect in OC users compared to NC women. In a neuroimaging study, Lisofsky et al. (2016) also found that contraceptive use was associated with a more positive affect compared to NC women. Contrary to these findings, a separate study asserted that women taking OC did not experience a decrease in stress hormones compared to NC women in a social setting (Pedersen et al., 2023). Researchers suggested that the decrease in endogenous progesterone levels when taking OC was related to differences in stress hormone levels recorded between NC and OC women. Furthermore, the NC group experienced increased stress hormone levels during times of metestrus and diestrus compared to proestrus and estrus. (Pedersen et al.,

2023). The contradictory nature of this literature compared to the previous studies reporting increased stress suggests that OC has a wider and more complex range of effects on mood-related symptoms, and there is room for further studies to investigate these areas.

Within the human literature, although some studies indicate significant findings, it's difficult to parse apart the differential effects of the two hormones. Due to economic factors, most of these studies related to progesterone and estrogen have been conducted in rodent models. Ultimately, animal research allows scientists to directly manipulate these components and control for any outside factors that may be influencing results. Although rodent models are not exact representations of human women, most of the animal literature also follows the narrative of contradictions and mixed findings regarding the effects of OC usage and behavior, cognition, and neuroanatomy.

CHAPTER 3

ANIMAL LITERATURE

Introduction

The clinical and scientific research communities have historically used animal models as an economically viable and easier-to-control study population compared to using human participants. More specifically, mice are a practical choice for hormonal research due to their ability to reproduce quickly and the shorter length of females' estrous cycles. In humans, women's menstrual cycles usually fall into the range of 28 days, whereas mice have an estrous cycle of 4-5 days. This shorter window allows scientists to obtain a general picture of how sex hormones might be affecting brain structures and behavior when hormones increase or decrease and do so in an efficient time frame (The Jackson Laboratory & Yeadon, J., 2014).

Studies indicate that the main effects of the synthetic administration of progesterone and estrogen include behavioral changes involving anxiety and depressive-like behaviors, as well as neuroanatomical changes where certain brain structures differ in volume (Nasseri et al., 2020; Lisofsky et al., 2016; Hwang et al., 2015). Although there is some agreement in the literature that these areas encompass the main effects of hormone administration, research has continuously found contradictory results, and our lack of knowledge on this subject leaves room for exploration.

Development and Neuroanatomy

In normal development, the activation and organization of the brain are shaped in a sex hormone-dependent manner (Schulz et al., 2009; Wisniewski, 1998; Premachandran et al., 2020). The period of approximately one week before and one week after birth is commonly thought of as the "organizational period," in which gonadal hormones such as estrogen and

progesterone, among other signaling molecules, act on the brain to alter tissue organization and ultimately function (Premachandran et al., 2020). Later in life, i.e., during adolescence, there is a second surge of gonadal hormones, which is characterized as the "activational period". This phase functions to promote sex-typical physiology and behavior into adulthood.

As estrogen and progesterone play a role in differentiation and activation, it's important to determine the mechanism and location of action for these two hormones. Animal experiments have proven useful in determining that estrogens are not only found and synthesized within the brain but are also able to act extra-nuclearly at synapses to alter transmission and potentially synaptic plasticity (Woolley, 2007; Krezel et al., 2001; Sheppard et al., 2019). Studies utilizing rodent models also suggest differences due to estrogen and progesterone regarding neuronal plasticity. For example, for the past 30 years, the research community studying the effects of estrogen has generally agreed that endogenous estrogen and progesterone alter the physiology of neurons, specifically at the synapse (Woolley, 2007; Taylor et al., 2021). Research in rodent models has illustrated that the density of dendritic spines varies by approximately 30% in the hippocampus over the course of the estrus cycle (Taylor et al., 2021). Also, when progesterone and estrogen are suppressed, such as after an ovariectomy, there is a significant loss in dendritic spine density, as demonstrated in rat studies (Taylor et al., 2021). Translationally, it is possible that changes in the levels of endogenous estrogen, for example, during different periods in the estrous cycle, are one explanation for the differences in functional connectivity in humans in areas such as the hippocampus and the amygdala (Krezel et al., 2001; Sheppard et al., 2019). The basis of these generally agreed-upon changes due to endogenous hormones suggests the potential for changes with the use of exogenous synthetic hormones.

Further supporting the idea that estrogen is responsible for changes in synaptic plasticity and transmission, studies have found that in mouse models, synaptic transmission via glutamatergic neurons and non-NMDA receptors increased during times of lower estrogen, such as diestrus (Galvin & Ninan, 2014). This study also revealed that when researchers activated estrogen receptor beta (ER), the subsequent decrease in transmission during times of higher endogenous estrogen (proestrus) was rescued (Galvin & Ninan, 2014). However, this data contrasts with other results, demonstrating an increase in synaptic transmission and plasticity coinciding with increases in estrogen (Sheppard et al., 2019). The authors indicate a possible explanation in that they were looking specifically at endogenous estrogen in rodent models, whereas, in other experiments, exogenous estrogen has had the opposite effect. These results illustrate a possible difference between animals that naturally cycle and those that are administered hormones. Furthermore, the differences in these findings highlight the importance of determining the effects associated with exogenous estrogen administration vs. endogenous. Because women are treated with the synthetic exogenous form of estrogen in OC, it is possible that synaptic transmission could be affected due to a negative feedback loop. However, little is known about these specific effects, this mechanism, and other affected areas such as behavior and additional factors influencing outcomes such as time and duration of administration.

Regarding volumetric differences, the areas of the brain most affected by progesterone and estrogen over a female's lifespan are thought to be the limbic system, hippocampus, and prefrontal cortex (Galvin & Ninan, 2014; Pappas et al., 1979; Premachandran et al., 2020; Schulz et al., 2009; Wisniewski, 1998). Some research has indicated that there are more subtle differences across the naturally occurring estrus cycle (Sheppard et al., 2019). For example, findings indicated lower hippocampal volume during the menstrual phase compared to the

preovulatory phase. More specifically, volumetric differences in both the white and gray matter were found within these structures (Galvin & Ninan, 2014; Pappas et al., 1979; Schulz et al., 2009; Sheppard et al., 2019; Taylor et al., 2021; Wisniewski, 1998; Data from rodent models has also demonstrated that volumetric changes can occur in the hippocampus within a 24-hour period, indicating a time-specific component related to hormones and changes in the brain (Taylor et al., 2021).

On a larger scale, researchers have found that exogenous estrogen and progesterone affect total cortex volume in different ways (Pappas et al., 1979). Results from an experiment utilizing exogenous hormone administration found that treatment with estrogen led to a comparatively thinner cortex compared to naturally cycling rats, while progesterone administration was associated with a slightly thicker cortex. Similarly, Pappas et al. (1979) found that rats ovariectomized following postnatal day 1 had significantly thicker cortices compared to controls. Furthermore, when mice were treated with estrogen, their cortices were also comparatively thinner than controls. While there is some evidence for changes in neuroanatomy, there is no consensus on the general effects of estrogen and progesterone, which remain unclear and require further research to clarify the validity and causality of these differences.

Timing

A benefit of using rodent models for hormonal research is the ability to study longitudinal effects over a shorter period of time. Within the human and rodent literature, research provides evidence of a critical period in development that is influenced by sex hormones (Juraska & Willing, 2017; Piekarski et al., 2017; Schulz et al., 2009). Furthermore, exposure to estrogen and progesterone specifically at this critical time period is what drives these significant behavioral and anatomical changes (Piekarski et al., 2017; Woolley et al., 2007; Zoladz et al., 2019). For

example, there is evidence for behavioral effects related to the onset of puberty involving estrogen and progesterone increases. For example, a study determining the relationship between psychosocial stress and ovarian hormones found that ovariectomized mice had a significantly greater startle response and heightened anxiety on the elevated plus maze compared to their intact counterparts (Zoladz et al., 2019). This study also included a measure of chronic stress, in which they were handled daily and exposed to social instability. Results indicated that chronic stress increased anxiety in all groups, irrespective of estrus stage. It is possible that stress plays a moderating role in tests of anxiety-like behavior that leads to muted differences between groups with different hormone concentrations.

In another study, data illustrated that hormone treatment at pubertal transition was associated with location-specific maturation, such as the frontal cortex (Piekarski et al., 2017). These changes then led to a change in performance on a reversal learning task. Moreover, in a second experiment, researchers demonstrated that these changes were time- and hormone-specific. In the second experiment, their findings illustrated that prepubertal but not postpubertal gonadectomy in mice blocked the signaling that typically occurs during prepubertal development to decrease inhibitory neurotransmission in the frontal cortex, further supporting the dynamic interplay between hormones and critical time periods or windows of development. Additionally, one study found that mice shipped from their distributor between four and six weeks of age did not respond to or develop typical sexual behaviors following estradiol and progesterone injections compared to their control counterparts (Blaustein & Ismail, 2013). Although it was proposed that the cause of this abnormality was likely stress, they cited that this response was specific to a certain timing factor and led to changes in behavior in response to hormones. It is possible that alternative changes, such as synthetic hormone administration at this critical period,

could also lead to behavioral changes. Although the specific time point(s) remains relatively unclear, some studies have found that during the onset of puberty, when progesterone and estrogen levels spike, there is a significant neuronal loss in the medial prefrontal cortex in female mice (Willing & Juraska, 2015). Translationally speaking, it is crucial to determine if the timing of hormone administration significantly affects behavior and neurodevelopment, as many women are prescribed contraception soon after beginning menstruation or over the course of puberty.

Overall, there seems to be strong evidence that endogenous progesterone and estrogen play an important role in development and that there may be specific timepoints at which their role is particularly impactful. Thus, it's possible that synthetic hormone administration could significantly alter the behavior and neuroanatomy of females entering or at the time of adolescence; however, no large-scale human studies have looked at the effects of OC at pubertal onset. Further research is necessary to determine the nuances of these effects as well as any potential long-term consequences.

Emotional Cognition and Behavior

As progesterone and estrogen drive differentiation and activation and may do so at a specific time, it's possible that a disturbance in these levels, for example when patients and animal models are administered oral contraception, could also alter emotional processing, leading to mood disorders and a change in behavior. One of the predominant narratives within the rodent hormonal contraceptive literature asserts that progesterone and estrogen administration affect behaviors related to anxiety, depression, and learning specific to fear-related behaviors (Koebele et al., 2021; Simone et al., 2015; Paris et al., 2014; Flores et al., 2019; Krezel et al., 2001). Several studies looking at the effects of these hormones on anxiety indicate that there is an opposing effect, suggesting that progesterone reduces anxiety while estrogen

promotes anxiety-like behaviors (Paris et al., 2014; Flores et al., 2019). When administered alone, progesterone significantly reduced anxiety-like behaviors on an elevated maze and marble-burying task. However, when co-administered with estrogen, this effect dissipated (Flores et al., 2019). Similarly, when synthetic progesterone was administered to an experimental mouse model with increased anxiety, this injection ameliorated these behavioral effects. On the contrary, evidence from naturally cycling mice instead suggests that an increase in anxiety is associated with higher progesterone, such as during diestrus, whereas lower anxiety is associated with proestrus, when progesterone declines. Evidence for this was illustrated when naturally cycling mice had significantly higher anxiety during elevated plus maze testing (Paris et al., 2014).

Some studies suggest that the anxiolytic effect associated with estrogen and progesterone is due to a specific estrogen receptor (Krezel et al., 2001). Results revealed that knockout mice for estrogen receptor beta (ER) but not estrogen receptor alpha (ER) experienced increased anxiety-like behaviors. These findings could demonstrate a differing effect between receptors, and, depending on the specificity of the synthetic hormone, the administration of exogenous hormones could lead to increased anxiety. Additionally, other data has revealed that progesterone is related to and responsible for increased anxiety. For example, Porcu et al. (2017) used levonorgestrel, a third-generation progesterone and testosterone derivative, either in combination with ethinyl estradiol or alone to determine behavioral effects. Unlike previous studies, results indicated that the administration of levonorgestrel but not estradiol led to increased anxiety-related behaviors. It's possible that the type of progesterone led to these effects, as other papers used alternative progesterone forms such as P4, which is characterized as endogenous and may have interacted differently with internal systems and led to contrasting results (Paris et al., 2014).

Further contradicting the above findings, one study found that the combination of estrogen and progesterone rather than one or the other reduces anxiety-like behaviors (Koebele, 2021).

However, this study used ovariectomized mice as a model for transitional menopause, and the decrease in interactions between endogenous and exogenous hormones could have altered the results. The narrative surrounding the effects of synthetic estrogen and progesterone on various behaviors, including anxiety-related behaviors, is complicated. Additionally, the differences in the age of treatment initiation and treatment duration between studies could have further complicated the results. For example, several of the studies did not report the age of treatment initiation at all; others treated mice between 10 and 11 weeks. (Flores et al., 2019; Krezel et al., 2001; Paris et al., 2014; Porcu et al., 2017). The gaps in the overall understanding of these effects leave room for exploration in terms of variables such as duration, type of synthetic hormone used, and the possible contrasting effects of either hormone.

Behavioral Tests

To determine possible differences in behaviors like anxiety associated with hormone administration, several tests are commonly used and are thought to act as translational models that reflect the possible dysfunction in processing in disorders such as post-traumatic stress disorder or other anxiety and mood disorders. A common construct thought to be associated with anxiety disorders is the inability to decipher between safe and non-safe cues in relation to a threat (Graham et al., 2011). Additionally, researchers and clinicians also believe that there are defects in the ability to extinguish and inhibit fear or anxiety-like responses in the presence of a threat. As the natural fear response in rodents is freezing, when mice exhibit this behavior in a test setting such as the elevated plus maze or a light/dark box test, researchers can apply and

hypothesize that increases and decreases in freezing correlate to increases and decreases in anxiety (Curzon et al., 2009).

Although not designed to directly measure anxiety, two behavioral tests related to anxiety as well as learning and memory capabilities are fear conditioning and fear extinction (Beckers et al., 2013; Graham et al., 2011). Furthermore, the behaviors associated with fear conditioning, such as anxiety-like behaviors, fear inhibition, and fear extinction, are said to be modulated by progesterone and estrogen, both exogenous and endogenous (Maeng et al., 2017; Zeidan et al., 2011; Shumake et al., 2014). Research has found that estrogen enhances fear extinction, citing rats administered estrogen (E2) who experienced decreased fear responses after repeated exposure to a fear cue without reinforcement compared to other groups (Maeng, 2017). Further evidence for these findings is indicated in another study involving naturally cycling rats in cycle phases with higher estrogen; these rats had faster fear extinction capabilities compared to rats in stages with lower estrogen (Tang & Graham, 2020).

These findings further complicate the narrative surrounding hormones and anxiety-like behaviors, as previous research has suggested that estrogen increases extinction behavior, whereas this data asserts that estrogen is responsible for the reduction of fear-associated behaviors, which could also be interpreted as anxiety-like behaviors in rodents. This lack of clarity regarding the different mechanisms and functions of estrogen and progesterone further solidifies the need for additional research in this area.

Major gaps in the research include addressing changes concerning the administration at critical developmental time points, determining when those critical time periods are, the behavioral and cognitive effects, and neuroanatomical changes. The literature surrounding the effects of estrogen and progesterone is similar to human research in that results are contradictory,

with mixed findings throughout. These unanswered questions need to be addressed to establish a better picture of the risks and potential short- and long-term effects of endogenous hormones, but more importantly, in the context of this research, the effects of exogenous hormones, specifically hormonal contraception and synthetically administered progesterone and estrogen.

CHAPTER 4

THE PRESENT STUDY

Introduction

Throughout their lifetime, women will choose to use hormonal contraception for a variety of reasons, from managing health concerns such as polycystic ovarian syndrome to preventing pregnancy (Powell, 2017). Although doctors can prescribe this medication following the beginning of their first menstrual period, women begin taking hormonal contraception at different times in their lives for various durations. Today, one of the most commonly used forms of contraception among women worldwide is oral contraception (Borgström et al., 2008; de Wit et al., 2020). While there are numerous formulations, many contain either synthetic progesterone or a combination of synthetic progesterone and estrogen. Endogenously, progesterone and estrogen work together to regulate female reproductive organs and play a role in various other areas, such as the brain and behavioral processes (DeMayo et al., 2002). Exogenously, little is known of the repercussions outside of the intended contraceptive effects. Furthermore, even less is known about potential moderating factors such as the age of first administration and duration of administration and whether they could have significant behavioral effects.

As mentioned before, scientists believe that natural estrogen and progesterone in the female body facilitate processes related to fear, anxiety, and stress and could additionally be related to learning and memory (Arélin et al., 2015; Herrera et al., 2019; Lisofsky et al., 2016; Lundin et al., 2017; Radke & Derntl, 2016; Zoladz et al., 2019). In the mouse model, research findings are similar, as mice who were ovariectomized behaved significantly differently on anxiety-related tasks compared to their intact counterparts (Zoladz et al., 2019).

Although there is contradictory evidence within the human and animal literature, several studies have suggested that when animals and humans are treated with OC, anxiety and stress responses are dampened (Ambruster et al., 2017; Lisofsky et al., 2016; Merz et al., 2012; Sharma et al., 2020 Simone et al., 2015). Other findings assert that women experience worsening symptoms related to fear, depression, and anxiety compared to women who are naturally cycling (Borgström, 2008). However, these effects were only seen when women had a previous history of mood disorder symptoms. The animal literature on synthetic hormones, though similarly contradictory, also indicated a dampened stress and anxiety effect when animals were treated with OC (Flores et al., 2019; Koebele et al., 2021; Paris et al., 2014; Simone et al., 2015).

Much like the synthetic OC literature, the effects of endogenous progesterone and estrogen offered contrasting evidence. Although, there were multiple studies illustrated higher anxiety and less fear inhibition during times when estrogen was low and progesterone was high (diestrus) compared to when estrogen was high and progesterone was low (estrus) (Gavin & Ninan, 2014; Paris et al., 2014; Tang & Graham, 2020).

Additional components that complicate this narrative include the time point and duration of administration. Data from animal models reveal a potentially critical period in which their responses and behaviors are altered in relation to hormone administration (Blaustein & Ismail, 2013). During puberty, humans and animals experience large shifts in their reproductive hormones that lead to changes in their brains and behavior (Blaustein & Ismail, 2013). If women begin taking hormonal contraception during sensitive periods like puberty, their behavior and even the organization and activation of their brains could be significantly affected, and it is possible that these changes will persist long-term.

In this study, we addressed the influence of a combination estrogen and progesterone oral contraceptive on a sensitive period for an extended duration. Using mouse models to minimize external factors, we administered the hormones in a glucose solution daily. We initiated administration at the beginning of puberty, a time period that is potentially susceptible to significant changes when hormones are altered (Lisofsky et al., 2016; Merz et al., 2012; Simone et al., 2015). To determine changes in anxiety-related behaviors, we utilized the elevated plus maze. To determine changes in learning and memory and fear-related behaviors, we used a fear conditioning protocol along with two tests of fear extinction (contextual and cued). Although these tests are not directly translatable to humans, they represent the closest behaviors in this animal that could be interpreted as anxiety, fear, or learning and memory and help determine changes in cognition in general.

Based on the previous literature, we hypothesized (1) animals treated with a combination OC would demonstrate decreased anxiety and fear conditioning and extinction compared to control animals. (2) Naturally cycling mice in diestrus would experience increased anxiety and fear conditioning compared to mice in estrus. (3) Animals treated in the potentially critical window during puberty would demonstrate significantly different anxiety and fear conditioning and extinction behaviors.

Materials and Methods

Experimental Groups. Adolescent C57B/6J female mice (10–11 weeks of age) were distributed into 2 groups: a control group (control, n= 23) treated with 30% glucose in distilled water, and an oral contraceptive group (OC, n=15) treated with a solution of levonorgestrel (.1mg LG) and ethinyl estradiol (.02mg EE) at a dose of 5.2mg/kg in the 30% glucose distilled water solution. Dosing for the OC group was based on a commercial formula and roughly on the average weight

of a thirteen-year-old female, as this is a common age of pubertal initiation (Lacroix, 2023). Additionally, a recent study tested several different concentrations to determine which best suppressed hormonal cycling and prevented pregnancy in mice (Isono et al., 2018). Reports indicated that the two-fold concentration of 5.2mg/kg was sufficient to suppress estrus cycling without changing the morphology of the vaginal cell wall. Both groups were treated for 31 days before behavioral testing began, and treatment continued during behavioral testing. Daily vaginal lavages using distilled water were performed to determine the estrus cycle stage in control mice and whether the OC mice were cycling. Animals were kept on a 12:12 hour light/dark cycle in a temperature-controlled room. Food and water were available ad libitum. For a subset of analysis, mice in the control group, were broken up into subgroups (estrus and diestrus) to explore potential behavioral differences based on naturally cycling hormonal differences within the control group mice. We chose these two estrus stages because there are elevated levels of progesterone in diestrus and elevated levels of estrogen during estrus. The use of mice was approved by the University of Maine Animal Care and Use Committee.

Behavioral Tests. Beginning the fifth week of hormonal administration (day 31), several behavioral tests were conducted. All behavioral tests were conducted in dedicated procedure rooms meant to decrease external stimuli such as lights or noises that would interfere with or confound results. At the end of each behavioral test, the animals were returned to their home cage. Researchers who were blind to experimental conditions analyzed all behavioral data. ANY-maze technology was used to record the elevated plus maze (EPM).

Elevated Plus Maze (EPM). To assess anxiety-like behaviors, we utilized the elevated plus maze and recorded time spent in the open and closed arms. At the beginning of each testing session, the mouse was placed in the center of the structure, facing one of the closed arms. The

mouse was allowed to explore freely for 5 minutes and recorded with a 720-p webcam. During this time, time spent in open arms, time spent in closed arms, time spent immobile, and the total distance traveled were automatically coded by ANY-maze video analysis software.

EPM: Apparatus. The "+"-shaped testing structure (ANY-maze) was elevated 50cm from the floor and consisted of two closed arms and two opened arms with identical 80cm x 5cm x 35cm dimensions (ANY-maze) perpendicular to each other and an open center area (5cm x 5cm) between the four arms. As a result, the maze is broken down into three areas: open arms, closed arms, and the center area. The entire maze was gray, and the lighting in the room was set to 130 lux.

Figure 1

Elevated Plus Maze



Note. Image from ANY-maze

Contextual Fear Conditioning (CFC). The following fear conditioning and extinction protocol was similar to Femouw et al., 2012 with an additional day of contextual fear testing added to increase the duration of assessed contextual fear extinction. This procedure allowed for the measurement of contextual fear memory, contextual fear extinction, cued fear memory, and cued fear extinction. Similar to the elevated plus maze, this behavioral test does not exactly translate to the human experience of fear. However, this is a generally agreed upon test of conditioning and extinction.

CFC: Apparatus. Fear conditioning was conducted in a Med-Associates test chamber (St. Albans, VT; 32cm wide × 25cm deep × 25cm high). The front, top, and back walls of the test-chamber were plexiglass, and the two sidewalls were aluminum. One of the aluminum walls contained a circular light (2.5cm diameter and 18cm above the floor). A speaker was embedded in the other wall. The floor was composed of stainless-steel bars (2mm diameter, 8mm center to center). The test chamber was housed in a larger Med-Associates sound-attenuating cabinet equipped with a ventilation fan which produced ~65dB of background noise. Behavior was recorded at 30 frames per second via a Firewire CCD video camera (Med-Associates, VID-CAM-MONO-4A; 640 × 480) with a near-infrared filter (Med-Associates; VID_LENS_NIR-1). A near-infrared light (Med Associates, NIR-100; 940nm) embedded in the top of the sound-attenuating cabinet was used as a video light source during all conditions. Freezing was assessed with Med-Associates' Video Freeze software (SOF-843). In brief, the software generates a motion index by comparing each successive video frame on a pixel-by-pixel basis and compares those changes to the noise inherent in the video as assessed via a reference video sample taken just prior to the mouse being placed in the test chamber (Fremouw et al, 2012). Freezing was defined as a motion index of 18 or less for at least 1 s as suggested and validated by

Anagnostaras, et al. (2010). We note that previous testing in our lab indicated that motion indexes between 17 and 22 are all highly correlated with hand coding (Fremouw et al., 2012).

CFC: Procedure. One week following the EPM testing mice were run on a four-day fear conditioning procedure.

Day 1: Conditioning. Mice were individually transported from the colony room to the testing room in a standard clear housing cage and placed in the conditioning chamber. All lights in the rooms and hallway during the transportation to the fear chamber were on. Conditioning consisted of a 2-min baseline period followed by three tone-shock pairings: 30s tone (3 kHz, 85 dB) co-terminating with a 2s foot shock (0.75mA scrambled, constant current). Each tone-shock pairing was separated by 30s. Mice remained in the conditioning chamber for 2 minutes after the last tone-shock pairing (post-shock period). Thus, the conditioning session lasted a total of 6 minutes and 30s. The circular light embedded in one of the aluminum walls was lit during the entire conditioning session. The transportation cage and the conditioning chamber itself were wiped down with a 70% alcohol solution between each animal. A small amount of 70% alcohol was squirted in the drop pan of the chamber.

Days 2 and 3: Context testing. On days two and three, mice were tested for conditioned fear to the training context. The procedure was exactly the same as day one with the exception that no tone or foot shock occurred and the session duration was 5-min.

Day 4: Cued testing. On day 4, mice were tested for conditioned fear to the tone. To minimize the contextual cues associated with shock during the cued testing, the transportation procedure was changed, and the chamber was modified. Mice were individually transported from the colony room to the testing room in an opaque white plastic rectangular tub and the lights were off during transportation. A black plastic triangular tent (Med-Associates) was placed in the

chamber and the metal grid floor was covered with a rigid white plastic sheet (Med-Associates). The chamber was not illuminated by the circular light and the transportation cage and the conditioning chamber itself were wiped down with a 4% vinegar solution rather than 70% alcohol between each animal. A small amount of the vinegar solution was squirted in the drop pan of the chamber. Cued testing consisted of a 2-min baseline, followed by 3-mins of the tone used during conditioning (day one).

CHAPTER 5

RESULTS

Data Inspection

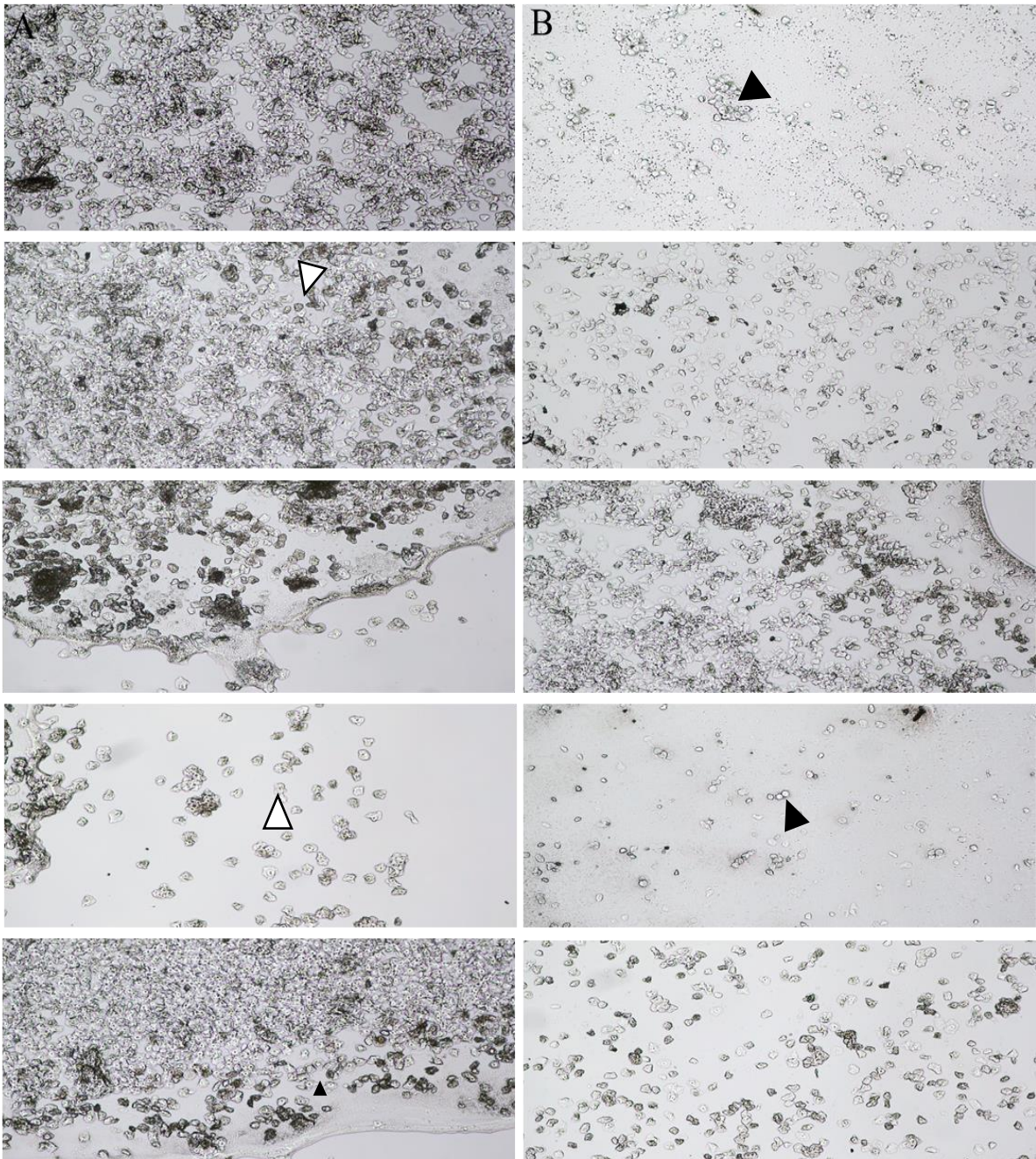
Prior to analysis, data was examined to determine possible errors and outliers that could alter results. Data points were excluded if they exceeded the set z-score of +/- 3.29. Z-scores were calculated for each data point and at this limitation, no data points were excluded.

Manipulation Checks

Vaginal smearing. Vaginal cells were collected daily to determine if treatment with oral contraception successfully halted the estrus cycle. The cells were collected using a vaginal lavage protocol, taken from McLean et al. (2012). The researcher inserted the tip of a 20uL micropipette into the vaginal opening of the mouse and gently depressed distilled water several times and then deposited the fluid onto a gelatin-treated slide. The estrus cycle was visually identified via microscope. Daily imaging revealed a pattern in which OC (oral contraception) animals remained in a persistent estrus stage following treatment, while control animals continued to cycle. However, some animals in the control group demonstrated multiple days of certain cycle stages (typically each stage would last about 12 to 48 hours) which could have been due to a variety of external factors such as potential stressors like external noise and temperature changes. Evidence of the persistent estrus in the OC animals can be viewed in *Figure 2*. Fifteen out of fifteen animals in the OC group were characterized as not cycling, and the majority of time, if not all time, was spent in persistent estrus. Analysis was continued as planned to explore differences in behavior on the elevated plus maze and fear extinction protocols.

Figure 2

Estrus Cycle Images



Note. OC (A) and control (B) animal cycle stage over the course of five days. White Arrows: indicative of anucleated cornified cells indicative of estrus, Black arrows: leukocytes indicative of diestrus.

Statistical Analysis

Elevated Plus Maze.

To assess anxiety-like behaviors between treatment groups, we utilized the elevated plus maze. Historically, this test has been thought to demonstrate anxiety as mice innately avoid brightly lit, open space (Albani et al., 2015). The structure consists of four arms, two open, and two closed, and an open center area between the arms. Historically, the elevated plus maze has been interpreted as demonstrating increased anxiety when animals spend more time in the closed arm or less time in the open arm. The interpretation and analysis of this behavioral test are complicated in relation to anxiety as more time in the closed arm does not necessarily mean less time in the open arm, as they are two separate locations and a center portion. Animals may be spending more time in the closed arm compared to their control counterparts but also spending time in the center portion instead of the open arm, meaning that the difference between animals with regard to the open arm would not be different. We chose to exclude the time spent in the open center portion as there were times when mice were facing the closed arm or the open arm but remained in the center. It was difficult to determine what the different positions within that portion meant, and with little literature to aid in that interpretation, we decided that it was better to exclude this data than wrongfully interpret this ambiguous location.

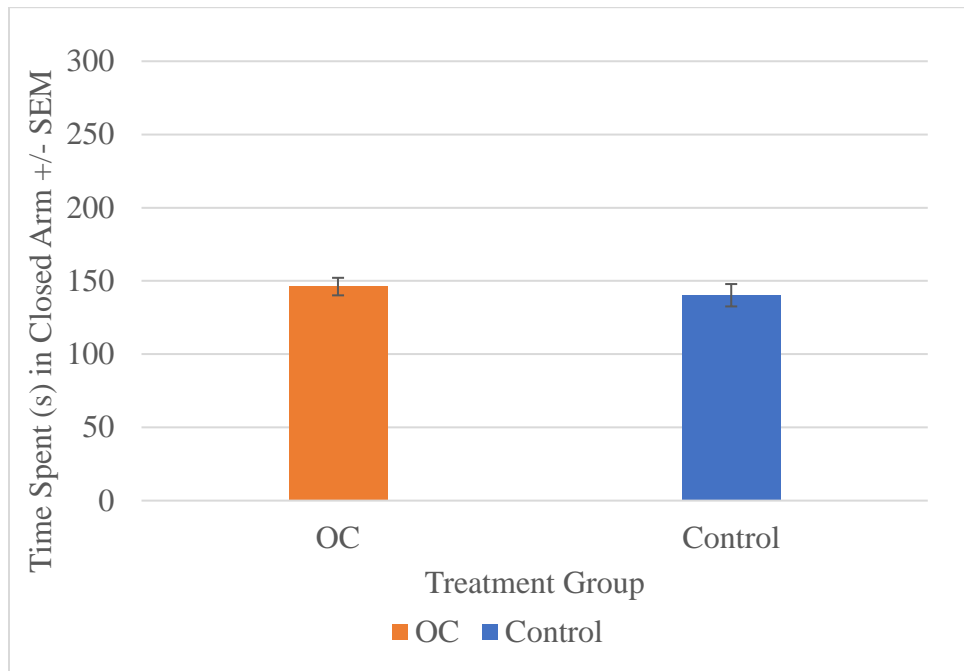
To analyze the time spent in the open arm and time spent in the closed arm, we conducted two t-tests to determine the differences between the OC and control groups. We also ran two t-tests to determine differences in the total distance traveled (m) and time immobile (s) as an additional measure of anxiety-like behavior, as mice often freeze when anxious. We did not include analysis of the time spent in the center area as we were looking to determine anxiety-like behaviors in which we coded as more time spent in the open or closed arm. During testing, one

animal from the OC group and four animals from the control group fell off the apparatus and were excluded from the analysis, $n_{\text{control}}=19$ and $n_{\text{OC}}=14$.

Analysis of the initial t-tests did not reveal significant differences between treatment groups in relation to the time they spent in the open arm [$t(31) = -.62, p = .268, d = .195$, small, homogeneity not assumed corrected], or time animals spent in the closed arm [$t(31) = .57, p = .285, d = .202$, small]. Analysis of the time spent immobile did not illustrate significant differences between the two groups [$t(31) = -1.01, p = .158, d = .359$, medium]. Similarly, there were no significant differences between groups with regard to total distance traveled [$t(31) = -.29, p = .384, d = .104$, small]. Results did not offer support for our first hypothesis that OC treated groups would demonstrate less anxiety compared to the control group as there were no significant differences between groups. While there were not differences between treatment groups, animals exhibited expected behavior as they spent more time in the closed arm compared to the open arm across the two groups, as seen by comparing the time spent in *Figure 3* and *Figure 4*.

Figure 3

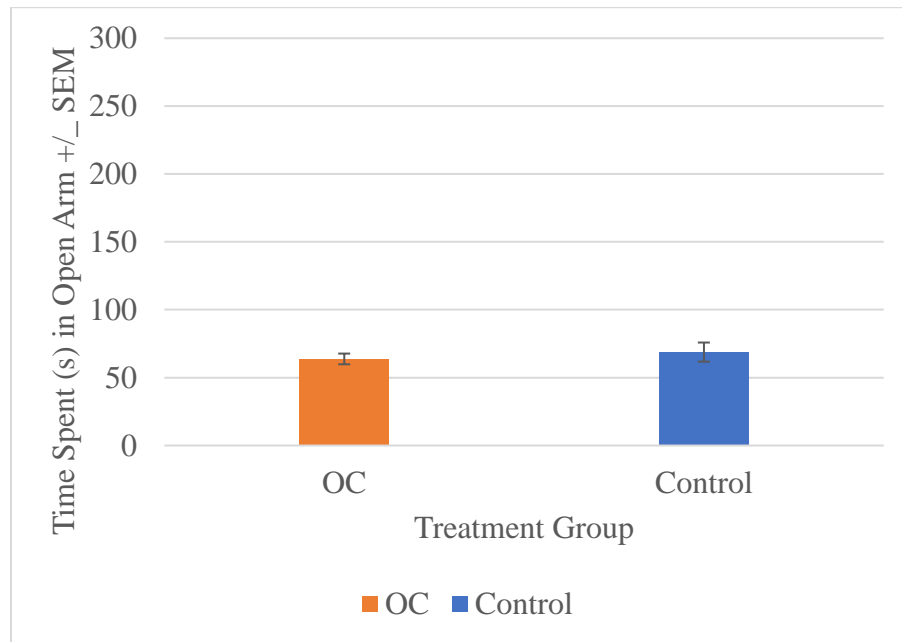
Mean Time Spent in Closed arm by Treatment Group Over 5 Minutes



Note. Error bars indicate +/- standard error mean.

Figure 4

Mean Time Spent in Open arm by Treatment Group Over 5 Minutes



Note. Error bars indicate +/- standard error mean.

Fear Conditioning.

To assess fear conditioning and extinction behaviors we utilized a three-day contextual fear conditioning protocol with an additional day of cued-recall testing. On the first day, the animal is expected to explore freely and exhibit minimal freezing before the conditioning period as the environment is dark and quiet and should not elicit freezing or anxiety-related behaviors (Fremouw et al., 2012). After the conditioning period, when the shock and tone are presented, animals are expected to show a large increase in freezing demonstrating a fear conditioning to the stimulus. During the last part of the second day and the third contextual day, animals are expected to freeze significantly less than on the first conditioning day as there is no presentation of the conditioned or unconditioned stimulus and the lack of fear behavior is further extinguished over time without this presentation. (Fremouw et al., 2012). On the cued recall day, animals are

expected to show very minimal freezing to begin with and then experience a large increase in freezing when the tone (cue) is played. This also depends on if cue was driving the fear conditioning or if it was the context. Contextual Recall: Day 1. As expected, there was virtually no freezing (%) during the first two minutes of the conditioning session [$t(28) = -1.37, d = .373$, small], indicating that the chamber itself did not induce fear. Importantly, percent time freezing during the last two minutes (after the three tone-shock pairings had occurred) suggests that both groups developed fear conditioning to about the same extent [OC ($M = 48.62, SD = 26.33$) and the control group ($M = 52.90, SD = 17.72$); $t(28) = -.529, p = .60, d = .964$ large]. Thus, the results from day one demonstrated successful fear conditioning and no significant differences between the groups.

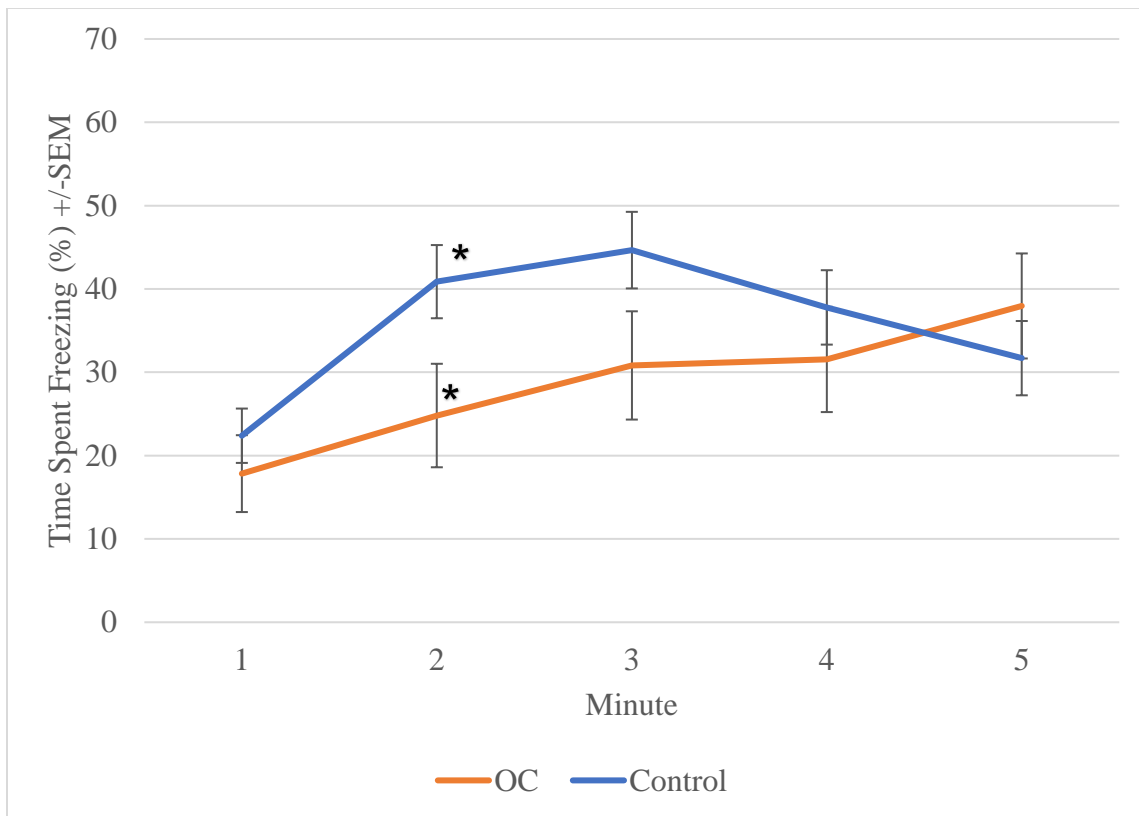
Contextual Fear Conditioning. Three two-way mixed methods ANOVAs [Treatment by Time] were conducted to compare freezing across time (minutes one through five) and treatment (OC and Control) for days 2, 3, and 4 to assess how fear conditioning changed within each day.

Contextual Recall: Day 2. On the second day, animals demonstrated some freezing in the initial two minutes leading up when the animals had been shocked on the previous day, however, it peaked for both groups at 3 minutes or later (at or after when they were shocked on day 1) as seen in Figure 5. .. A two-way mixed methods ANOVA revealed a significant main effect of time [$F(4, 112) = 7.66, p < .001, \eta_p^2 = .215$] but did not indicate a significant main effect of treatment [$F(1, 28) = 1.368, p = .252, \eta_p^2 = .047$]. Results also indicated a significant interaction between time and treatment [$F(4, 112) = 3.13, p < .05, \eta_p^2 = .101$]. Due to the significance and the disordinal nature (seen in *Figure 5*) of the interaction, the significant main effect of time could not be meaningfully interpreted. To determine the simple main effects of group, one-way ANOVAs were conducted at each time point. Results from the one way ANOVAs indicated that

there were significant differences at minute two [$F(1,28)= 4.45, p=.044, \eta_p^2=.137$] and a trend at minute three [$F(1,28)=3.02, p=.09, \eta_p^2=.097$], illustrating that at the second minute control mice ($M=40.87, SD=21.01$) demonstrated significantly more freezing compared to the OC mice ($M=24.81, SD=16.36$). Similarly, the trend also illustrated that control mice appeared to freeze more than their OC counterparts third minute. Regarding our first hypothesis, we did see a small hint of support. At minute two control mice froze significantly more than OC mice. This data potentially suggests that there were differences in fear conditioning between groups. However, we did not see significant differences between groups collapsed over five minutes.

Figure 5

Mean Freezing Time Across Five Minutes by Treatment Condition on Day Two



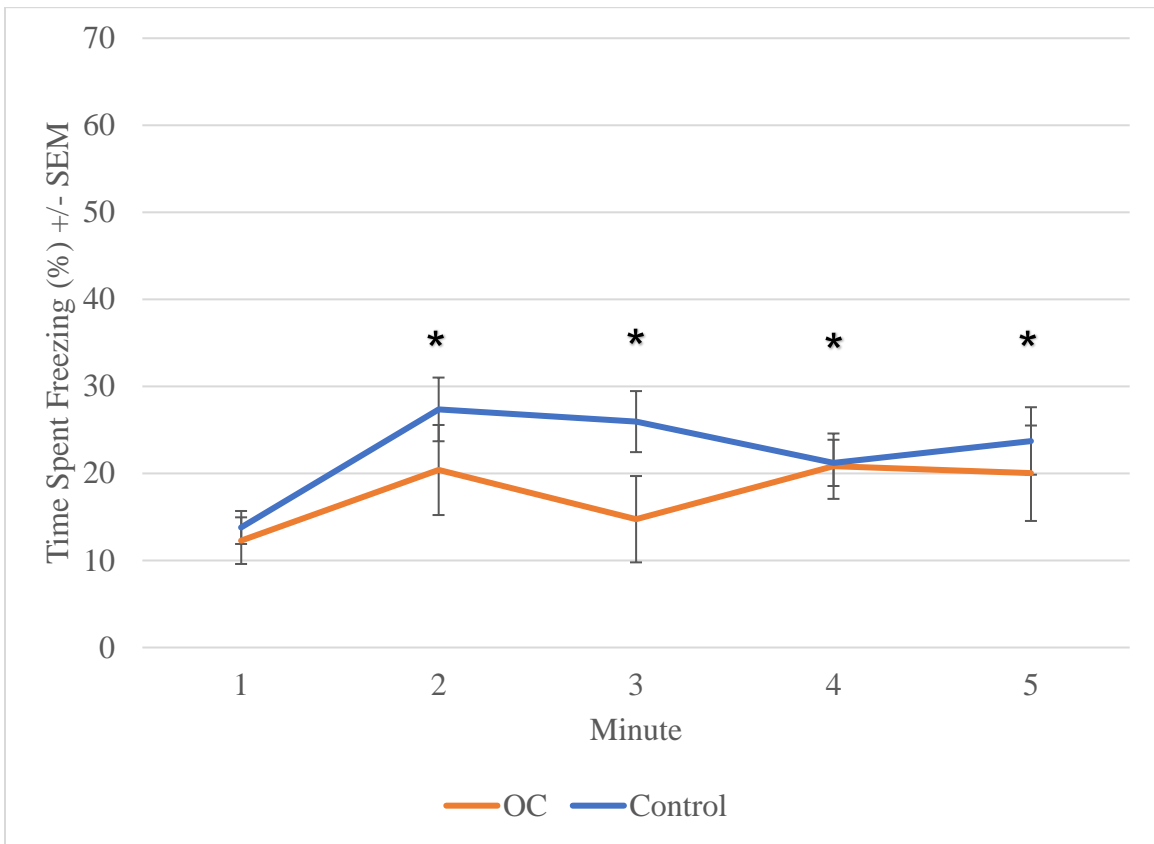
Note. Error bars indicate +/- standard error mean. * Indicates $p < .05$ between treatments

Contextual Recall: Day 3. Results illustrated a significant main effect of time on freezing [$F(3.14, 87.91) = 4.41$, $p < .05$, $\eta_p^2 = .136$ Greenhouse-Geisser corrected], but no significant interaction between time and treatment [$F(3.14, 87.91) = 1.20$, $p = .313$, $\eta_p^2 = .041$, Greenhouse-Geisser corrected] and no main effect between treatment group [$F(1,28) = 1.26$, $p = .271$, $\eta_p^2 = .043$]. Paired comparisons revealed significant differences in freezing between the first minute and minutes two, three, four, and five. No other differences in freezing between minutes were significant. This data did not offer support for our first hypothesis as the OC group did not demonstrate significantly less freezing compared to the control group.

While there were no significant differences between treatment groups over the four-day protocol, the overall behavioral pattern met expectations based on past precedent. After the first conditioning day, we expect to see an increase in freezing when the animal is placed into the same context on the second day (*Figure 5*) as they have formed a memory and are expecting the shock. After they do not receive the shock, fear extinction begins and freezing begins to decrease with significantly less freezing expected on the third day (*Figure 6*) as this continues to be reinforced without a shock pairing.. A two-way mixed model ANOVA [Treatment x Day] confirmed that freezing, collapsed over all five minutes, was lower on day three ($M = 21.03$, $SD = 11.17$) than on day two ($M = 32.15$, $SD = 15.26$). Results indicated a significant effect of day [$F(1,28) = 21.27$, $p < .001$, $\eta_p^2 = .432$] and did not indicate a significant interaction between day and treatment [$F(1,28) = .19$, $p = .66$, $\eta_p^2 = .007$].

Figure 6

Mean Freezing Across Five Minutes by Treatment Condition on Day 3



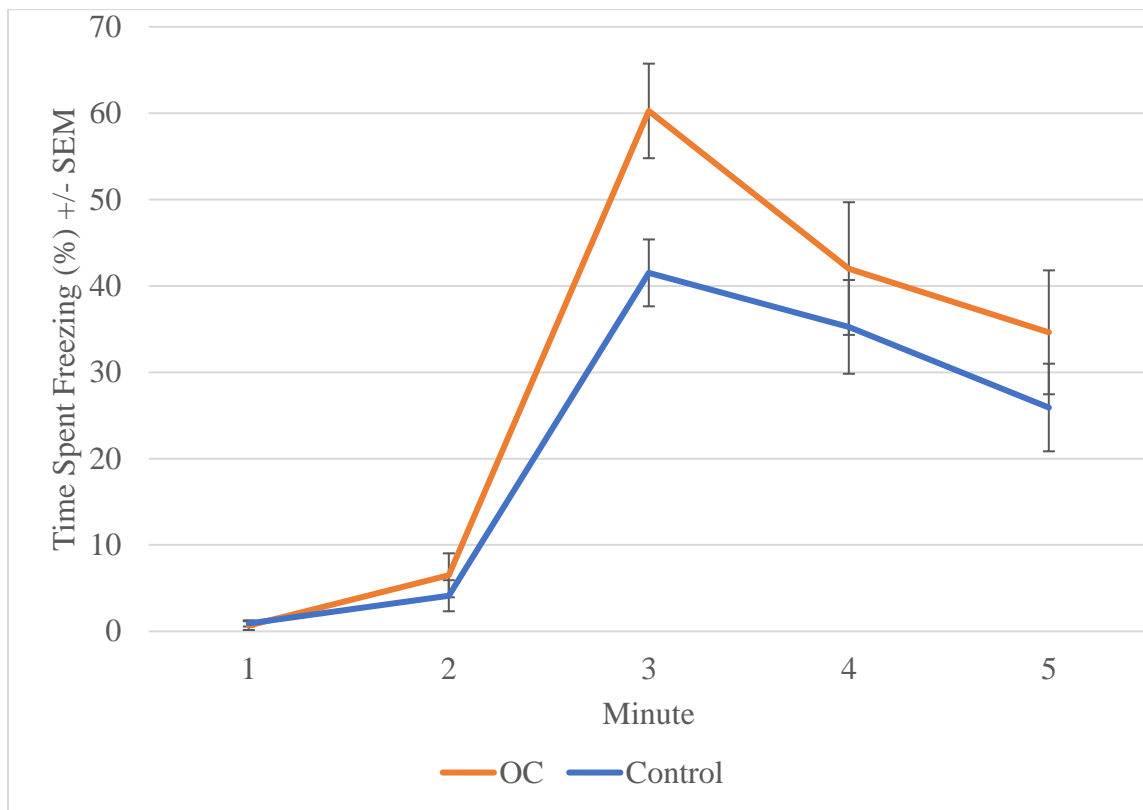
Note. Error bars indicate +/- standard error mean. * Indicates $p < .05$ compared to minute one

Cued Recall: Day 4. As with the other days, there was a significant main effect of time [$F(2.23, 62.24)=76.30, p < .001, \eta_p^2=.732$ Greenhouse-Geisser Corrected]. There was not a significant interaction between treatment and time [$F(2.23, 62.24)=2.20, p=.113, \eta_p^2=.073$, Greenhouse-Geisser Corrected] nor a significant main effect of treatment [$F(1,28)=2.13, p=.155, \eta_p^2=.071$]. Pairwise comparisons revealed differences between the first minute and minutes two, three, four, and five. There were also significant differences between the second minute and minutes three, four and five as well as significant differences between the third minute and minutes four and five. There was a trend for a significant difference between minutes four and five ($p=.079$). Again, while there were differences within the two groups, data did not support

our first hypothesis as the freezing levels within the OC group were not lower compared to the control group. Results from this analysis, again not significant between treatment groups, did conform to typical behavioral expectations. There was a large spike in freezing after the tone was played which illustrated a heightened fear response to the tone itself, followed by a decrease in freezing indicative of fear extinction to the tone in both groups.

Figure 7

Mean Freezing Across Five Minutes by Treatment Condition on Day 4



Note. Error bars indicate +/- standard error mean. *Indicates $p < .05$ compared to minutes one, two and three

Analysis of Controls by Estrus Stage,

In addition to examining differences between treatment groups, we also sought to determine if endogenous hormones in naturally cycling animals had a significant effect on

anxiety-like and fear-conditioned behaviors. Previous research in naturally cycling humans and mice has illustrated impaired fear inhibition and extinction and an increase in anxiety when estrogen levels are low, such as during diestrus (Flores et al., 2019). We chose to focus on animals in estrus and diestrus as levels of estrogen peak in the beginning of estrus and then progesterone levels peak at diestrus when estrogen levels are low.

Cycle stages were identified visually each day via microscope. There were some days in which the cycle was difficult to identify, as some vaginal smears may indicate a transitional period between stages (Herington et al., 2018). In the instances where the stage was either in a transitional period or identified as proestrus or metestrus, we chose to exclude those animals from analysis due to the low number of such animals. After excluding these animals, we had an n of 15 (9 estrus, 6 diestrus) over the entire five minutes.

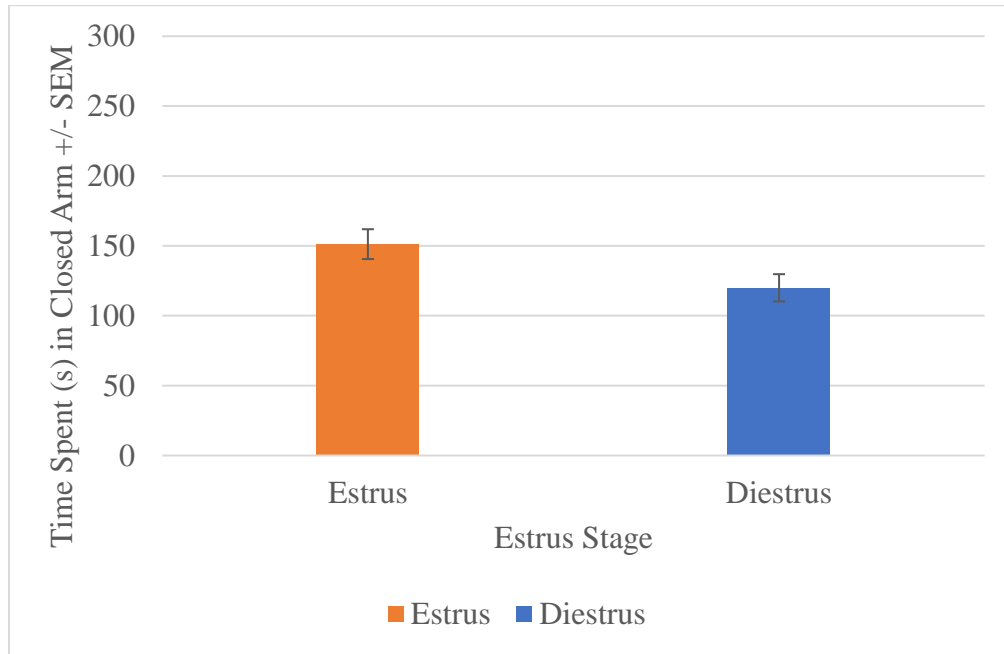
Elevated Plus Maze by Estrus Stage. To determine the differences in time between estrus cycle stages, we ran two t-tests for the open and closed arms over five minutes. In addition to time spent in each arm, we conducted a t-test for total distance traveled (m) as well as a t-test to determine the time immobile (s) over the five-minute trial.

Results for time spent in the closed arms illustrated a trend [$t(13)=2.03, p=.06, d=1.07$, large] suggesting that animals in estrus ($M=151.24, SD=32.01$) may spend more time in the closed arm compared to animals in diestrus ($M=120, SD=23.85$). There were no significant differences or trends between groups regarding time spent in the open arm [$t(13)=-.16, p=.87, d=.08$, small]. Analysis revealed that there were significant differences in the total distance traveled over five minutes [$t(10.41)=2.56, p=.027, d=1.137$, large, homogeneity not assumed corrected] indicating that mice in estrus ($M=13.75, SD=3.67$) traveled significantly further than mice in diestrus ($M=10.37, SD=1.22$). Analysis did not indicate differences in the time animals

spent immobile [$t(12.68) = -.89, p = .359, d = .473$, medium]. Results from the EPM potentially offer support for our second hypothesis as animals in estrus traveled significantly further compared to animals in diestrus. However, they did not spend significantly more time in the open arm or exhibit less time immobile compared to diestrus animals, so we are unable to confirm this hypothesis. Again, while there were only hints of support for our second hypothesis, we did see overall the expected behavior within both groups as animals spent more time in the closed arm compared to the open arm as illustrated by *Figure 8* and *Figure 9*.

Figure 8

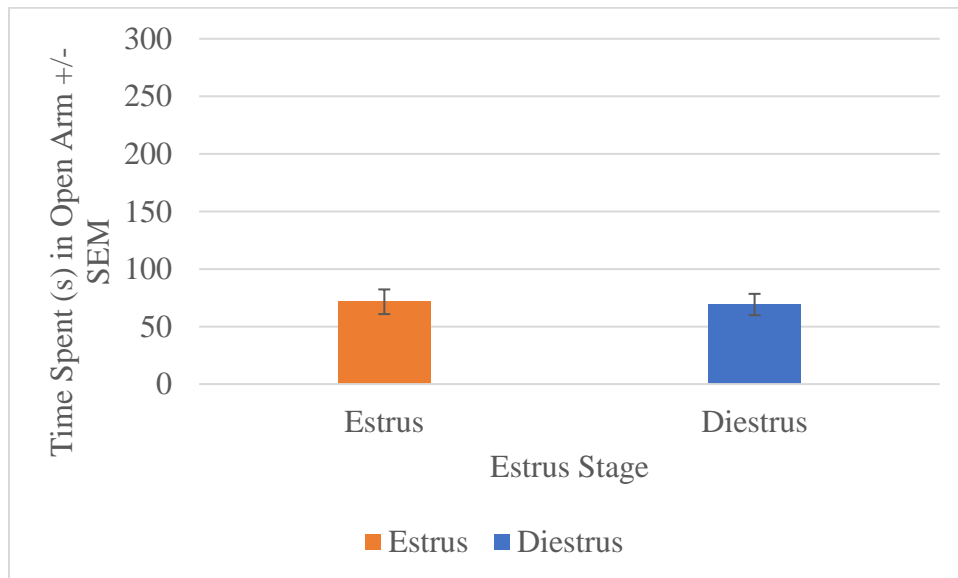
Mean Time spent in Closed Arm of EPM Split by Estrus Stage: All 5 Minutes



Note. Error bars indicate +/- standard error mean.

Figure 9

Mean Time spent in Open Arm of EPM Split by Estrus Stage: All 5 Minutes



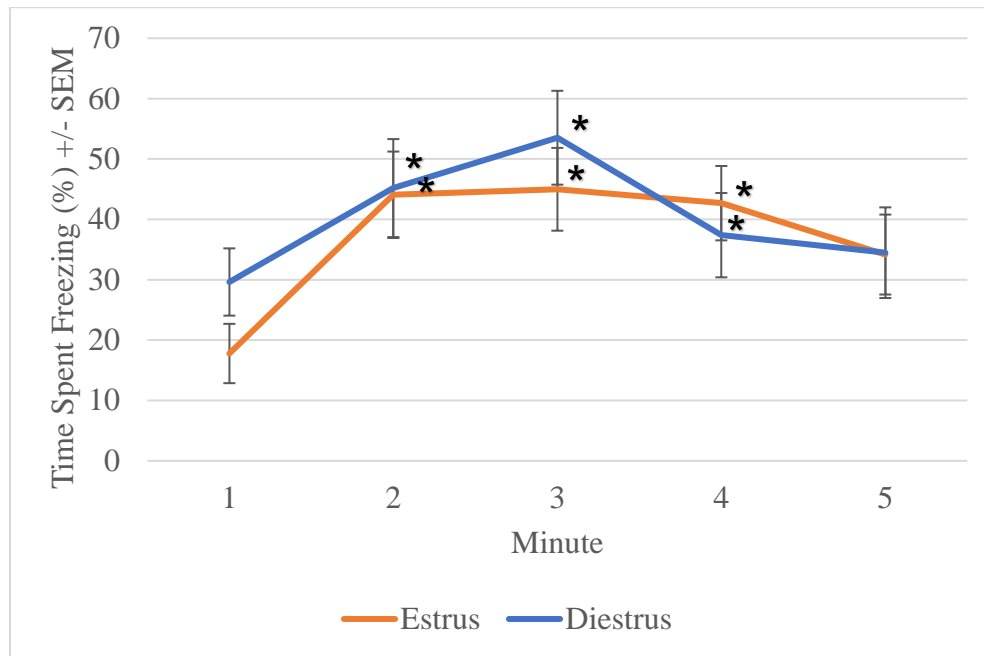
Note. Error bars indicate +/- standard error mean.

Fear Conditioning by Estrus Stage. To analyze fear conditioning by estrus stage, we repeated the same protocol of running a separate two-way mixed methods ANOVA [Estrus Stage (Diestrus and Estrus) by Time (Minutes 1-5)] to compare freezing across time (minutes one through five) on day two, three, and four.

Results illustrated a significant main effect of time on day two [$F(4,56)= 8.63, p<.001, \eta_p^2=.381$]. There was not a significant main effect of treatment [$F(1,14)=.19, p=.66, \eta_p^2=.013$]. Pairwise comparisons indicated that minute one was significantly different ($p<.05$) than minutes two, three and four. Results did not indicate a significant effect of estrus stage [$F(4,56)=1.03, p=.39, \eta_p^2=.069$]. Results from day two did not indicate support for our second hypothesis as animals in diestrus did not demonstrate significantly higher levels of freezing or anxiety-like behaviors compared to animals in estrus. Both groups of animals did exhibit the expected behavior, as there was an initial period of increased freezing when the shock as expected and then a subsequent decrease when it was not delivered (*Figure 10*).

Figure 10

Mean Freezing Time Across Five Minutes by Estrus Stage on Day Two

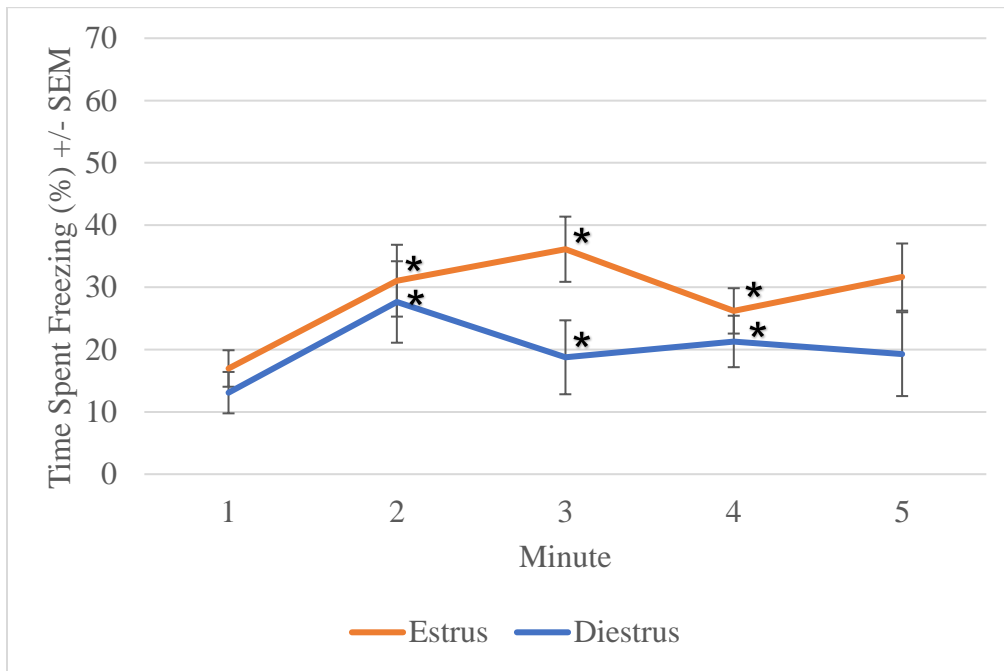


Note. Error bars indicate +/- standard error mean. *Indicates $p < .05$ compared to minute one

On day three, there was also a significant main effect of time [$F(2.41,33.74)=3.99$, $p=.022$, $\eta_p^2=.222$, Greenhouse-Geisser Corrected]. There was no significant interaction [$F(4,56)=1.25$, $p=.30$, $\eta_p^2=.082$]. Pairwise comparisons illustrated a significant difference between minute one and minutes two, three and four ($p < .05$). There was not a significant main effect of estrus stage on day three [$F(1,14)= 2.39$, $p=.144$, $\eta_p^2=.146$]. Again, we did not find support for our second hypothesis within these results. However, there was less freezing on day 3 for both groups compared to day two, as illustrated by *Figure 10 and 11* which demonstrates that both groups, as expected, began to extinguish their fear to the context when the shock-tone pair was not presented and there was not a threat present.

Figure 11

Mean Freezing Time Across Five Minutes by Estrus Stage on Day Three



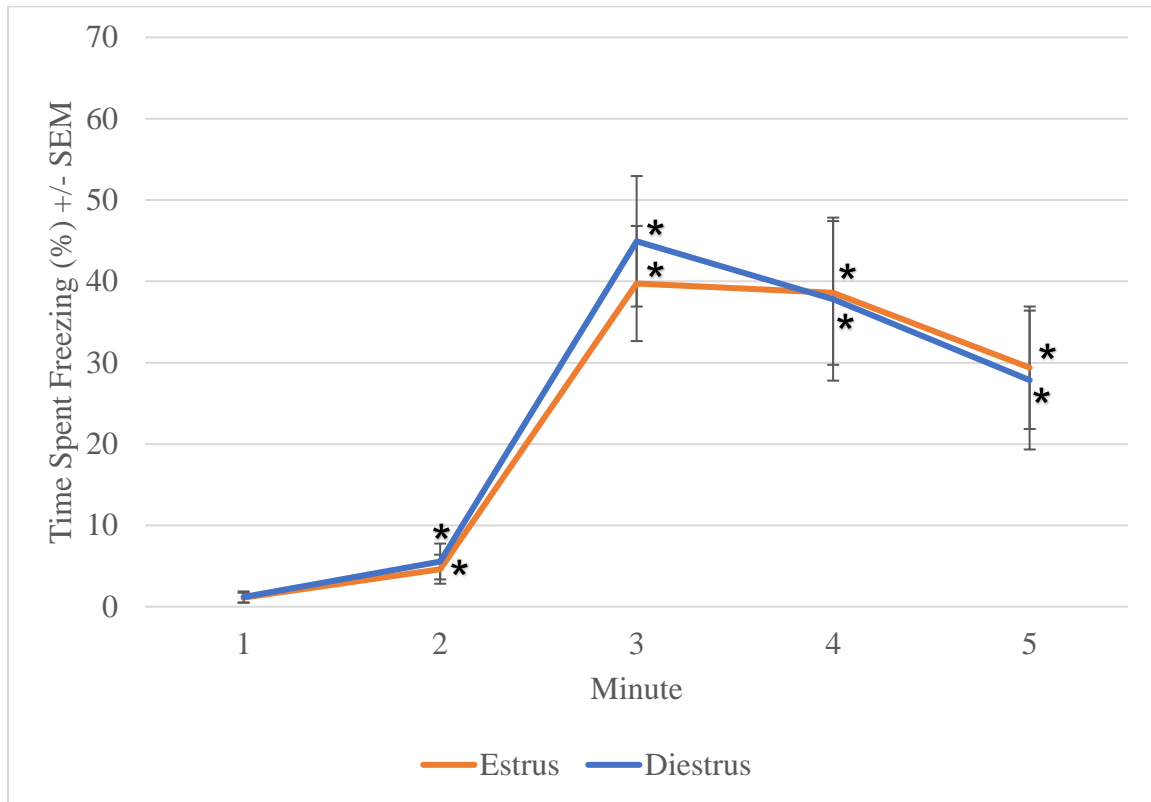
Note. Error bars indicate +/- standard error mean. *Indicates $p < .05$ compared to minute one and two

On the fourth day, there was a significant main effect of time [$F(2.09, 29.36)=30.152$, $p < .001$, $\eta_p^2=.683$, Greenhouse-Geisser Corrected]. There was not a significant interaction between minute and estrus stage [$F(2.09, 29.36)=.14$, $p=.87$, $\eta_p^2=.01$]. Pairwise comparisons indicated differences between minute one and minutes two through five. Additionally, there were also differences between minute two and minutes three, four, and five. While there was a significant main effect of time, analysis did not illustrate indicate a significant main effect of estrus stage [$F(1,14)=.013$, $p=.911$, $\eta_p^2=.001$]. Indicating that the cue was driving fear behavior (that cued fear conditioning occurred), animals experienced increased freezing after presentation of that tone. Our results revealed that animals in diestrus and estrus demonstrated significantly increased freezing at the third minute (*Figure 12*) compared to the first two minutes of testing

when the tone was not being played. As both groups demonstrated increased freezing, these results did not indicate support for our second hypothesis.

Figure 12

Mean Freezing Time Across Five Minutes by Estrus Stage on Day Four



Note. Error bars indicate +/- standard error mean. *Indicates $p < .05$ compared to minute one and two.

CHAPTER 6

DISCUSSION AND CONCLUSIONS

Present Study and Current Literature

This study sought to determine if administration of synthetic estrogen and progesterone at a critical time (just after puberty) would affect anxiety or fear like behavior in mice. we hypothesized (1) animals treated with a combination OC would demonstrate decreased anxiety and fear conditioning and extinction compared to control animals. (2) Naturally cycling mice in diestrus would experience increased anxiety and fear conditioning and extinction compared to mice in estrus. (3) Animals treated in the potentially critical window during puberty would demonstrate significantly different anxiety and fear conditioning and extinction behaviors.

Analysis of the time spent in the open arm and closed arm did not reveal significant differences between treatment groups. However, between naturally cycling animals in the control group, there was a trend that would suggest that animals in estrus spent more time in the closed arm compared to animals in diestrus. Analysis also revealed that mice in estrus traveled significantly further compared to their diestrus counterparts. On the contrary, the data did not illustrate significant differences in the time estrus and diestrus mice spent in the open arm.

Analysis of fear extinction data did not indicate significant differences between treatment groups on days three or four. In contrast, we did find significant differences between groups on minute two and a trend at the third minute on the second day. After comparing groups at each time point, we found that the control group froze significantly more than the OC treated mice. Results also suggested that control animals also froze more at the third minute; however, this was not significant. Increased freezing at these two minutes may suggest that control mice had significantly lower levels of fear extinction compared to OC mice. However, there were no

differences between groups at other time points. Furthermore, no significant differences in fear extinction were revealed within the control group between mice in estrus or diestrus.

Compared to the bulk of the literature on endogenous hormones, our results add to the contradictory nature of estrogen and progesterone. Previous studies have suggested that elevated levels of progesterone in diestrus decrease anxiety levels, whereas elevated levels of estrogen increase anxiety on tasks such as the elevated plus maze (Flores et al., 2019; Paris et al., 2014). Other researchers have asserted that estrogen does not have an effect on anxiety and fear-related behaviors (Renczés et al., 2020). If we were to interpret our findings strictly based on time spent in each arm as a measure of anxiety, the results may suggest that at times when estrogen is higher, anxiety is also higher. However, we also included measures of distance traveled and time immobile. If we are to understand that mice often freeze when they are anxious, then there are no differences between estrus and diestrus anxiety-like behaviors. On the other hand, mice in estrus also traveled significantly further compared to mice in diestrus. If we were to interpret this data based on freezing levels, it would suggest mice in estrus, when estrogen levels are high, are in fact less anxious compared to when progesterone levels are high. Overall, these results paint a complex picture, and to interpret this data as progesterone or estrogen definitively increasing or decreasing anxiety would not be meaningful. Instead, we suggest that estrogen and progesterone have a complex relationship that needs to be studied together as well as separately to assure that we can gain a better understanding of their distinct mechanisms.

It is possible that this data correctly represents the role that hormones play in behavior and cognition, although most findings from past research do not align with the findings in the present study. The more likely explanation is that there were issues in the initial experimental design and external moderating factors that led to these results.

Strengths

Data yielded minimally significant differences between groups in a test of fear extinction and did not demonstrate significant differences between groups during the elevated plus maze treatment. Furthermore, the data from the naturally cycling control mice was complex. However, there are still strengths to consider. Within the context of this field of research, this study has helped to close the knowledge gap regarding hormones and behavior. Although these results may not be representative of the larger body of literature, they do inform future research on how to better create a model of oral contraception and some key external moderating variables that may affect results. Ultimately, this is novel research, and the results produced in this study are meaningful and aid in the continuation of meaningful contraceptive and hormone research.

Limitations

Experimental Design Flaws.

First and foremost, it is plausible that our model of oral contraception did not significantly suppress the estrus cycle to the extent that behaviors would have been affected. In this experiment, our model was based on the concentration of a commonly used oral contraceptive and data from a recently published study that tested a combined oral contraceptive and the suppression of the estrus cycle at different concentrations in mice (Isono et al., 2018). This study claimed that the two-fold concentration was sufficient to suppress estrus cycling without changing vaginal cell wall morphology. However, during the experimental portion of this study, we discovered more recently published literature that suggested that translational dosing of OC is not necessarily the best method of treatment as steroid hormones are not always subjected to hepatic-first metabolism like other molecules and do not act locally (Lecasse et al., 2022). With this new information, it's possible that the dosing we chose was ineffective in

suppressing the cyclicity and significantly changing endogenous hormone concentrations, which would potentially explain the lack of differences between the two treatment groups. Additionally, in the present study, we administered a solution of levonorgestrel (.1mg LG) and ethinyl estradiol (.02mg EE) at a dose of 5.2mg/kg. Past studies that have found significant differences between treatment groups have utilized different concentrations ranging from .01ug/g estradiol and 2mg/g progesterone to an injection of 5ug estradiol and 250ug progesterone, even 4mg/kg progesterone and .09mg/kg estradiol. (Flores et al., 2019; Paris et al., 2014; Piekarski et al., 2017; Willing & Juraska, 2015). There is a possibility that these different concentrations were the driving factor behind the significant differences between the treated and control groups. In addition to the model of oral contraception, it is also possible that our manipulation check failed to correctly identify which animals were cycling and which were not. To determine the estrus stage, we utilized a vaginal smear protocol each morning between the hours of 7 and 10:00 a.m. While one of the benefits of mouse models is their short estrus cycle, this could also have been problematic. For example, the longest stage in the estrus cycle is diestrus, followed by estrus, and then proestrus metestrus. Because we were only smearing at a specific time, it's possible that the mice we identified as cycle suppressed were cycling, but those transitions occurred later in the day. Furthermore, it was difficult to identify the cycle stage on certain days as stages in mice are not as distinct compared to other animal models such as rats or hamsters (Herington et al., 2018). Due to the difficulty in identifying the estrus stage, there is a chance that on testing days the cycle was incorrectly identified, and when analysis was completed by estrus stage, the lack of significant results was due to researcher error.

It is also possible that lighting in the testing environment significantly altered behavior. In their natural habitat, rodents innately avoid brightly lit open spaces (Albani et al., 2015). In

our Elevated Plus testing room, the lighting was set to 130 lux, whereas others have used a range between 300 and 400 lux (Neuwirth et al., 2022). While we did want to significantly induce anxiety in the testing environment, we did not want to falsely create a setting in which animals would be overly anxious and misrepresent typical behavior. That being said, the lower level of light might not have been sufficient enough to induce anxiety to the appropriate threshold, and the lack of differences was not apparent because of this. However, lighting in testing environments has been grossly underreported and unstandardized, so it's possible that this did not significantly affect results (Neuwirth et al., 2022).

Physiological Stressors.

Although we attempted to limit external stimuli in the testing and housing environments, there were instances in which external noises and changes in temperature could have led to physiological responses that significantly affected behaviors during testing. Animals were housed in a 12:12 hour light/dark cycle in what we believed was a temperature-controlled room. There were several instances in which the heat spiked to abnormal levels due to mechanical issues outside of our control. While the core temperature of mice fluctuates when they are awake vs. asleep, extreme environmental temperature spikes could alter their core temperature and lead to physiological stress as mice do not have the ability to adjust to higher heat (Hankenson et al., 2018). In response to a higher external temperature, it's possible that behavior was significantly affected as the animals were attempting to regulate their physiological processes in addition to their psychological processes.

In the same vein, noises such as construction and loud voices could potentially alter behavioral outcomes. While we attempted to control noise by discussing our schedule with maintenance staff and testing behind a closed door, there were days prior to testing in which

construction was being performed on the floors above where animals were being housed. Additionally, although the animals were housed in a basement room, several staff members and students passed by the room and spoke at volume levels that were associated with behavioral effects. It's possible that this external noise led to increased anxiety in both the OC and control groups, and because of this, differences were not apparent between groups.

In addition to noise, it is possible that handling the mice every day either induced significant stress or significantly increased stress threshold over the four weeks of treatment. A previous study illustrated that mice exposed to chronic psychosocial stress daily significantly increased anxiety regardless of estrus stage (Zoladz et al., 2019). Similar to our study, researchers wanted to determine the role of gonadal hormones in anxiety-like behaviors. Results indicated that both gonadectomized and intact mice experienced heightened anxiety after chronic stress exposure. With regard to the present study, it is possible that handling the mice every day to administer oral contraception induced significant and chronic stress that led to the lack of differences between groups. On the contrary, it is possible that this constant handling had the opposite effect: the threshold for stress was increased, and tasks such as the elevated plus maze did not elicit a significant stress response.

Timing.

Part of our initial reasoning behind conducting the present study was to determine if there was a critical period, such as the beginning of puberty, that led to longer term and significant behavioral effects. Due to unforeseen circumstances, animal transportation issues, and a required acclimation period, we were unable to begin treatment until the animals were 10–11 weeks old. On average, mice enter puberty between 6 and 8 weeks. As we were unable to test at this time, we potentially missed a critical window in which treatment with synthetic hormones led to

significant and potentially long-term behavioral and cognitive effects. However, it is also possible that the pubertal window differs between mice, and the initiation at 10 weeks was in fact representative of that sensitive period, and hormone administration did not have an effect at this time.

In addition to the initiation of treatment at puberty, it is possible that transport during the pubertal window also significantly affected behavioral and cognitive results. In a previous study, researchers determined that mice shipped between four and six weeks did not develop typical responses to progesterone and estrogen injections compared to their control counterparts (Blaustein & Ismail, 2013). In our study, mice were shipped between seven and eight weeks old. While we did confirm that mice began cycling via vaginal smear, it is possible that shipment and introduction to a new housing environment during a critical developmental time led to changes related to hormone sensitivity that were not detected in vaginal smears, which then led to the lack of behaviors between groups.

A final timing-related factor that potentially led to the lack of behavioral results was the timing of treatment administration on testing days. In our study, we decided to conduct behavioral tests in the morning and then administer OC in the afternoon. It is possible that progesterone and estrogen had an immediate, shorter-term effect that was not illustrated as the treatment was administered approximately 17 hours later.

Recommendations for Future Research.

First, we recommend the continued formulation of a model of oral contraception at a dose that is both effective at suppressing the estrus cycle and does not alter anatomical structures such as the ovaries (Isono et al., 2018). In our model, although smears did indicate that the majority of treated animals were not cycling, it may be more beneficial to create a model of contraception in

which the animal is presenting as consistent diestrus or metestrus, the stages in which estrogen and progesterone, respectively, are lower. It is possible that this type of model would be more applicable to the human form and mechanism of contraception.

Second, we propose that there is a critical time in which hormone administration would alter the brain and behavior. As past research has indicated, puberty is a time of increased sex hormone concentration and neural organizational and activational development (Taylor, 2021). Past research has also suggested that synthetic hormone administration during puberty alters this development as well as cognition and behavior at this time (Gingnell et al., 2013; Lisofsky et al., 2016; Lundin et al., 2017; Radke & Derntl, 2016). We suggest further research to confirm this effect and to determine specifically how and where it is affecting cognition and behavior. Furthermore, as there are limited studies on the long-term effects of oral contraception in general, we feel that it would be important to study the longitudinal effects of the initiation of synthetic hormone administration beginning at puberty. Additionally, it is possible that there are also more immediate behavioral effects that should be studied in a shorter period of time compared to the larger gap between treatment and testing that we demonstrated. We also suggest that researchers use mice bred in house to control for the possible stress of transportation leading to differential behavioral effects.

Lastly, we implore future researchers to address other behavioral and cognitive areas that could potentially be altered by oral contraception. In this study, we addressed anxiety-like behaviors and fear learning and memory. Other cognitive and behavioral areas, such as those related to depression, are essential topics of future studies, as past research has indicated that hormone administration can lead to worsening mood disorder symptoms (Oinonen & Mazmanian, 2002).

Conclusions and Future Implications.

The present study sought to determine the differences in cognition and anxiety-like behaviors between animals treated with a combined oral contraceptive and control animals who were naturally cycling. We also inquired about potential differences in cognition and behavior between different stages of the estrus cycle in control animals. We used a model of oral contraception that consisted of a solution of levonorgestrel (.1mg LG) and ethinyl estradiol (.02mg EE) at a dose of 5.2mg/kg. Utilization of the Elevated Plus Maze offered an opportunity for us to translationally determine the effects of endogenous and exogenous hormones on anxiety. However, potential issues with the experimental design and a lack of significant differences suggest that the overall model of oral contraception used in this study could be improved for future use. Similarly, a lack of significant differences with regard to testing for fear extinction could have also been due to design and other moderating factors. While this study did not indicate significant differences between treatment groups or between estrus stages in naturally cycling animals, it did illustrate the importance of a representative model to strengthen hormone research in psychology. With knowledge of the limitations and the recommendations for continued inquiry into variables such as timing, and duration, we offer a starting point for a greater translational understanding of the scope of the effects that both synthetic and endogenous progesterone and estrogen have on female cognition and behavior.

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