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Impacts of Chronic Circadian Disruption on Neural Structure and Cognition:

The Importance of Considering Sex and Age

by

Emilio Emilio Soto Soto

A dissertation submitted in partial satisfaction

of the requirements for the degree of

Doctor of Philosophy

in

Neuroscience

and the Designated Emphasis

in

Women, Gender, and Sexuality

in the

Graduate Division

of the

University of California, Berkeley

Committee in Charge:

Professor Lance J. Kriegsfeld, Chair

Professor Minoo Moallem

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Summer 2021

Abstract

Impacts of Chronic Circadian Disruption on Neural Structure and Cognition:
The Importance of Considering Sex and Age

By

Emilio Emilio Soto Soto

Doctor of Philosophy in Neuroscience

Designated Emphasis in Women, Gender, and Sexuality

University of California, Berkeley

Professor Lance Kriegsfeld, Chair

Circadian (from the Latin, *circa*-about; *diem*-day) rhythms temporally coordinate neural and hormonal systems on a daily schedule to maintain optimal health and functioning. In adults, disruptions to circadian functioning through poor sleep hygiene, night or rotating shift work, transmeridian travel and exposure to artificial light at night are associated with a number of disease states, including obesity and metabolic disease, diabetes, heart disease, cancer, deficits in cognitive functioning and reproductive dysfunction, among other maladies. However, whether circadian disruption negatively impacts typical development, leading to permanent deficits in brain physiology and behavior, has been minimally explored outside of early life disruption. In addition, most studies to date focus on male animal models whereas the present studies explore whether or not chronic circadian disruption (CCD) differentially impacts both sexes. The present studies identify adolescence as a period in which CCD has differential impact on brain and behavior depending on the sex of the organism. These findings further underscore the need for inclusion of both sexes in studies of CCD and to consider of the age at which disruption occurs.

In this thesis I also apply the concepts of *just science* and *good science* to teaching undergraduate biology students about biological sex. Broadly, *just science* establishes that science is created in an objective vacuum outside of most social influences, and that implications of science arise from the interactions with society after the fact. An important caveat of why the concept of *good science* is a promising prospect to explore in students from the biological sciences is that it is carefully crafted to not be *anti-science*. Some students reported how they had initially thought biological sex was unchangeable or always addressed in biomedical research but, given the new information from scientists and feminists scholars, they had changed their view. Others remained adamant that biological sex cannot be changed regardless of a person's identity. Thus, my findings suggest further exploration on bringing *good science* around biological sex to earlier stages of students' college education, as opposed to be hidden in specialized and advanced courses. In summary this thesis explores the impact of addressing diversity and complexity both in biology research and instruction to not over simplify or look over the destructive biases that have been identified.

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Chapter 1: General Introduction

Circadian (from the Latin, *circa*-about; *diem*-day) rhythms temporally coordinate neural and hormonal systems on a daily schedule to maintain optimal health and functioning. Although circadian rhythms are endogenously generated, these cycles are synchronized to environmental time by the daily light:dark cycle and negatively impacted by modern artificial light exposure levels (Fonken and Nelson, 2014; Bedrosian and Nelson, 2013). In adults, disruptions to circadian functioning through poor sleep hygiene, night or rotating shift work, transmeridian travel and exposure to artificial light at night are associated with a number of disease states, including obesity and metabolic disease, diabetes, heart disease, cancer, deficits in cognitive functioning and reproductive dysfunction, among other maladies (Soulle et al., 2018; Ball et al., 2016; Bedrosian et al., 2015). In recent years, there has been a growing concern over the impact of circadian disruption on the progression of neurodegenerative diseases and considerations of circadian timing have pronounced implications for maximizing treatment efficacy of those afflicted (Gotlieb et al., 2018; Abbott et al., 2018; Karatsoreos, 2011). However, whether circadian disruption negatively impacts typical development, leading to permanent deficits in brain physiology and behavior, has been minimally explored outside of early life disruption. In addition, most studies to date focus on male animal models whereas the present studies explore whether or not circadian disruption differentially impacts both sexes. This chapter begins with an overview of the impact of circadian disruption in mammals, focusing on learning and memory, the main output being measured in this thesis. Likewise, how sex and age influence the impact of circadian disruption is considered. The conclusion of the chapter outlines questions addressed within the research chapters of the dissertation.

1.1 Impact of Circadian Disruption

1.1.1   Circadian Rhythms of Master and Subordinate Oscillators

Circadian rhythms are generated by highly-conserved biological time-keeping mechanisms, colloquially called "clocks", that coordinate neural and hormonal systems both internally and with the environment to maximize fitness (Zeng et al., 2021; DeCoursey and Krulas., 1998; Ouyang et al., 1998). Circadian rhythms are endogenously generated through transcription-translation feedback cycles of clock genes and their protein products (Takahashi, 2015). Briefly, the core feedback loop begins with the clock protein, CLOCK, binding to BMAL1 to drive the transcription of the Period (*Per1/Per2*) and Cryptochrome (*Cry1/Cry2*) genes starting early in the morning. At night, the PER and CRY proteins feed back to repress CLOCK:BMAL1-mediated transcription until the next morning. Circadian timekeeping is present in all cells of the body to time relevant behavioral and physiological processes (Bujis et al., 2013). These cycles are synchronized to environmental time by direct retinal projections to the master clock in the suprachiasmatic nucleus (SCN) of the hypothalamus in mammals (Kim et al., 2019; Moore and Eichler, 1972; Stephan and Zucker, 1972). The key players for this relay of environmental light to the SCN are the melanopsin expressing intrinsically photosensitive retinal ganglion cells (Rupp et al., 2019;Berson, 2003; Abrahamson and Moore, 2001; Provencio et al., 2000). The SCN integrates information from the daily light:dark cycle and its output relays this information to subordinate oscillators throughout different brain areas and peripheral organs circadian control of hormones and multisynaptic communication via the autonomic nervous sytem (Bujis et al., 2017;Bujis et al., 2014;Kay et al., 2010). Thus, each subordinate oscillator integrates this time stamped information provided by the

master clock in the SCN with site-specific timing to coordinate physiological processes (Brown et al., 2019).

The SCN is superior in maintaining molecular timekeeping at a tissue level when compared to peripheral oscillators due to its unique ability to maintain coherence among its ~20,000 cells (Yoo et al., 2004; Tei et al., 2000; Kay et al., 1997; Menaker and Tosini, 1996). Cell culture experiments recording oscillations of transcription-translation cycles of the SCN can be maintained in culture, while peripheral oscillators dampen their oscillations with varying time courses depending on the organ (Davidson et al., 2009). In a study of rat neuronal cultures, different brain regions exhibit similar properties, with extra-SCN brain loci maintaining molecular rhythms for varying times (Chun et al., 2015; Michikazu et al., 2002). Whether or not these rhythms dampen due to loss of cellular clock function or loss of cellular coherence was examined by looking at rhythms of cultured fibroblasts at the individual cell level (Welsh et al., 2004). This study established that, when tissue-level rhythmicity is dampened, single cell oscillations are maintained, indicating that tissue-level rhythmicity is lost due to loss of cellular coupling. The fragility of cellular coupling outside of the SCN could explain the widespread pathology from circadian disruption depending on the type and duration of disruption.

1.1.2   Ramifications of Circadian Disruption

In humans, chronic disruptions to circadian rhythms during adulthood through poor sleep hygiene, night or rotating shift work, transmeridian travel and exposure to artificial light at night are associated with a number of disease states, including obesity and metabolic disease, diabetes, heart disease, cancer, schizophrenia, deficits in cognitive functioning and reproductive dysfunction, among other maladies (Reviewed in Karatsoreos., 2014; Evans et al., 2013). Chronic circadian disruption (CCD) in adult rodent models leads to impairments in learning/memory and behavior accompanied by structural changes in brain areas associated with affected behaviors, with a focus on the hippocampus and hippocampus-dependent behavior (reviewed in Smarr et al., 2014). The hippocampus has been proposed as the main coordinator of circadian influenced memory functions (Lehr et al., 2021; Obrietan et al., 2018). Targeted suppression of clock genes in the hippocampus influenced learning and memory (Wardlaw et al., 2014). For example when utilizing a genetic knock out of Bmal1 clock gene in excitatory forebrain circuits affect acquisition of the Barnes maze or novel object location recognition (Snider et al., 2016).

In adult mice, CCD impacts components of the hippocampal-prefrontal circuitry that have been implicated in rodent and human models of schizophrenia and other cognitive disorders (reviewed in Sigurdsson and Duvarci, 2015). The effects of CCD on the medial prefrontal cortex (mPFC) leads to decreased cognitive flexibility in a Morris water maze task, though it does not influence initial spatial learning in adult male mice (Karatsoreos et al., 2011). Likewise, circadian disruption is associated with structural changes in total dendritic spine numbers and dendritic apical length in layer II/III of the mPFC, suggesting marked reorganization of neural circuitry following circadian disruption. CCD also impacts hippocampal morphology and hippocampal-dependent spatial learning (Gibson et al., 2011). Specifically, female hamsters exposed to CCD exhibit impaired spatial learning in a conditioned place preference task (CPP) accompanied by a cortisol-independent reduction in neurogenesis and cell proliferation in the hippocampus. These behavioral deficits persist for at least 30 days following cessation of CCD, supporting the idea that behavioral effects of CCD are long-lasting. Taken together, these findings support the notion that hippocampal and pre-frontal cortex structure, and behaviors dependent upon these loci, are

sensitive to CCD during adulthood and that the consequences of CCD persist well after recovery from the disruption.

1.2 Factors influencing the Impact of Circadian Disruption

1.2.1   State of Development and Sex

Although the impact of CCD during adolescence has not been explored, several studies have shown that circadian disruption *in utero* negatively impacts mouse development. For example, exposing pregnant mice to constant dim light (LL) results in reduced growth rates among their offspring, increased anxiety-like behavior in the mother, and increased fear responsiveness in the offspring as adults (Borniger et al., 2014). Similarly, increased anxiety-like behavior, but not spatial memory deficits, are observed in adult offspring exposed to LL perinatally (i.e., late prenatal and early postnatal) (Roman and Karlsson, 2013). In contrast, when constant light disruption began earlier in pregnancy, extending for 18 days postnatally, spatial memory deficits were observed in rats; anxiety-like behavior was not examined (Vilches et al., 2014). Finally, findings from our laboratory indicate that CCD during the last two weeks of pregnancy and/or the first three postnatal weeks of life increases anxiety-like behavior in an open field and elevated plus maze in offspring and decreases sociability behavior regardless of sex once they reach adulthood (Smarr et al., 2017). These disparate findings likely result from the use of different behavioral assays to observe spatial behavior and/or the different durations and developmental periods of circadian disruption (reviewed in Meyer et al., 2006). Nonetheless, these studies provide the impetus for systematically exploring the impact of CCD on the brain during sensitive stages of development and assessing the potential long-term consequences on brain and behavior in adulthood.

Adolescence marks a time when events at distinct critical developmental periods drive the transition from the juvenile to adult stage of an organism. The most notable change is sexual maturation, a process driven by sex-steroid hormones that promote the notable changes in both the brain and behavior of an individual (Schneider et al., 2013; Schulz et al., 2009; Sisk and Zher, 2005; Romeo et al., 2002). The marked development of the brain that occurs during puberty and adolescence is particularly sensitive to perturbations that can permanently and negatively affect brain development and behavior (reviewed in Spear, 2015). This period of development is also a time during which an organism more easily extinguishes previously-learned behaviors and learns new strategies required to adapt to novel conditions, an aspect of cognitive flexibility. Cognitive flexibility is dependent on the mPFC, which matures significantly by the end of adolescence, close to early adulthood, and is greater in juvenile and adolescent rodents than in adults (reviewed in Delevish and Wilbrecht, 2021; Johnson et al., 2011; McAlonan K., Brown, 2003). On the other hand, the limbic system is believed to develop principally during early and mid-adolescence. This belief in part is based on findings that established the effects of sex hormones during puberty on increased hippocampal neural plasticity and neurogenesis (reviewed in Kennard and Woodruff-Pak, 2011; Bondolfi et al., 2004). Spatial memory is dependent on the hippocampus generally decreases with age as neurogenesis declines (reviewed in Sohi et al., 2016; Praag et al., 2005; Kempermann et al., 1998; Kempermann et al., 1997). Taken together, these studies point to a critical developmental window for the hippocampal-prefrontal cortex network during adolescence and a critical window of influence of gonadal-steroidal hormones, suggesting vulnerability to CCD at this time point.

Regardless of the modulation of gonadal-hormones on the hippocampal prefrontal cortex network, sex differences on the impact of circadian disruption were not addressed in early studies of CCD (Karatsoreos et al., 2011; Gibson et al., 2010). Instead, the research conducted focused in male animals exclusively, with the exception of one study from our lab that focused on female animals (Gibson et al., 2010). Recent publications that have looked at the impact of circadian disruption on both sexes has yielded conflicting results. Male adults exhibit more anxiety like behavior than female adults in the passive avoidance test after extended exposure to dim light during adolescence (Borniger et al., 2014). In contrast, recent studies by our group show that the effects of chronic circadian disruption *in utero* affected anxiety and social behavior in offspring of both sexes (Smarr et al., 2017). Hence, data so far indicate that circadian disruption could have differential impact depending on the sex of the individual and the developmental period that the disruption targets.

1.3 Thesis Questions

Adolescence is a time of rapid neural development and maturation, including synaptic remodeling and pruning, during which the CNS is sensitive to external and internal perturbations. Thus, my proposed studies explore whether CCD targeted to critical periods of pubertal and adolescent development have more pronounced and long-lasting effects on the hippocampal-prefrontal network structure and dependent behaviors than the same circadian disruption in adults. The working hypothesis of the experiments was that CCD during adolescence will have more severe impact on behavior relative to adulthood disruption. To test this possibility, I targeted circadian disruption to two different life-history periods: adolescence, and adulthood. I then examined the effect of CCD on (1) hippocampal-prefrontal network-dependent behaviors, and (2) cellular changes in the separate components of the hippocampal-prefrontal network. My studies were designed to allow an examination of the magnitude and permanence of changes in behavior and brain structure following temporal perturbations restricted to adolescent or adult developmental time points.

Chapter 2: The Impact of Chronic Circadian Disruption on Spatial Learning, Cognitive Flexibility, and Anxiety Like Behavior

2.1 Introduction

Circadian rhythms are ancient patterns that have developed as an adaptation to the 24-hour solar cycle of light and darkness. Optimal synchronization of physiology and behavior to light conditions allows organisms to thrive, improving fitness and survival (Rubin et al., 2017 Saint Paul and Aschoff, 1978; Pittendrigh and Minis, 1972). In mammals, light input is transmitted to a master circadian pacemaker in the suprachiasmatic nucleus (SCN) in the hypothalamus via specialized ganglion cells expressing the photopigment melanopsin (Morin and Allen., 2006). The molecular scaffold that allows biological time keeping is present in every cell in the brain and body, with the SCN maintaining system-wide coordination of these independent oscillators (Takahashi et al., 2015; Buijs et al., 2013). This hierarchy of control allows for coordinated recovery of biological timekeeping, system wide, during times of circadian disruption (Buijs et al., 2013; Butler et al., 2010). However, repeated circadian disruption leads to a loss of this rhythmic coordination and is associated with an array of physical and mental health concerns (Walker et al., 2021; Evans and Davidson, 2013; Karatsoreos, 2012).

The advent of electricity and the widespread application of artificial light and blue-light-emitting portable devices, along with little exposure to sunlight during the day, has led to relatively chronic circadian disruption in our modern society (Fonken and Nelson, 2014; Evans and Davison 2013; Bedrosian and Nelson, 2013; Moore-Ede et al., 1983). The implications of the growing exposure to light after sunset are serious enough that the American Medical Association made a policy statement on the risks and illness resulting from light exposure at night (Stevens et al, 2013). In addition, changes in social responsibilities and work schedules across the week, termed social jet lag, further contribute to circadian disruption (Viera et al., 2021; Witmann et al., 2006). The circadian timing system can recover from singular occurrences of circadian disruption, such as a single shift of the light:dark cycle (Gibson et al., 2010; Davidson et al 2009), with negative impact (e.g., cognitive deficits) lasting the extent of recovery (typically requires one day for every hour of jet lag). However, the effects become severe and long lasting when individuals are exposed to chronic circadian disruption (CCD). In these cases, numerous illness are comorbid with circadian disruption, ranging from physiological (e.g., diabetes, heart disease) to mental (e.g., depression) health (Karatsoreos, 2019; Roenneberg et al., 2016, Moore-Ede et al., 1983).

Our laboratory pioneered studies exploring the effects of CCD on brain health and cognitive function. Specifically, in adult female hamsters, our group previously showed that CCD caused impairment of conditioned place preference memory well after the cessation of the disruption (Gibson et al., 2010). Likewise, CCD resulted in reduced neurogenesis in the hippocampus that was not dependent on changes to gonadal hormones or cortisol levels. In a study by another group, CCD led to decreased cognitive flexibility in a Morris water maze task and shortened the length of apical dendrites in the prefrontal cortex, though it did not influence initial spatial learning (Karatsoreos et al., 2011). Given the substantial impact of simulated jet lag on adult animals, our group asked whether or not early life development may be negatively impacted by CCD. In this set of experiments, exposure to CCD during pregnancy, or from birth until weaning, resulted in offspring with shallower growth curves, increased anxiety-like and perseverative behavior, and reductions in social interactions in adulthood (Smarr et al., 2017). Significantly, paternal CCD before mating has recently been shown to affect offspring development and have long-term metabolic implications, such as hyperglycemia and disrupted

corticosterone rhythms (Maximillian et at., 2021). These findings indicate that CCD during development, even at the level of the gamete, has long term, likely permanent, effects.

CCD during pregnancy likely impacts fetal development through disruptions to maternal hormonal timing. Maternal hormonal cycles program the circadian rhythms of the fetus during gestation (Bates and Herzog, 2020; Varcoe et al., 2018). Specifically, hormonal and nutritional cycles of maternal food intake can synchronize peripheral oscillators in the fetus and postnatally (Canaple et al., 2018). Consistent with our findings, postnatal care by a non-disrupted foster mother can improve the health of offspring that has been genetically and experimentally manipulated to have weakened cellular clock (Olejníková et al., 2018). Another study of milder CCD show that exposing pregnant mice to constant dim light (LL) results in reduced growth rates among her offspring, increased anxiety-like behavior in the mother, and increased fear responsiveness in the offspring as adults (Borniger et al., 2014). Similarly, increased anxiety-like behavior, but not spatial memory deficits, was observed in adult offspring exposed to LL perinatally (i.e., late prenatal and early postnatal) (Roman and Karlsson., 2013). In contrast, when constant light disruption began earlier in pregnancy, extending for 18 days postnatally, spatial memory deficits were observed in rats; anxiety-like behavior was not examined in this study (Vilches et al., 2014). Thus, it appears that CCD can have a long term in impact in early life by disrupting normal development, likely through alterations in maternal hormonal cycles and/or maternal care behavior.

Adolescence marks a time when events at distinct critical developmental periods drive the transition from the juvenile to adult stage of an organism. The most notable change is sexual maturation after puberty, a process driven by sex-steroid hormones that promotes the notable changes in both internal and external traits of an individual, such maturation of sex organs and hormonal cycles (Schulz and Sisk, 2016; Schulz et al., 2009). The marked development of the brain that occurs during adolescence is particularly sensitive to perturbations that can permanently and negatively affect brain development and behavior (Fuhrmann et al., 2015; Spear, 2000). Nearing the end of adolescence the brain limits the exalted brain plasticity of this period with enhanced inhibition through outputs to most brain regions, modifying the balance of inhibition and excitation (Takesian et al., 2013). Adolescence is also a time during which an organism more easily extinguishes previously-learned behaviors and learns new strategies required to adapt to novel conditions, an aspect of cognitive flexibility. Cognitive flexibility is dependent on the medial prefrontal cortex (mPFC), a brain region that matures significantly by the end of adolescence, and is greater in juvenile and adolescent rodents than in adults (reviewed in Delevish and Wilbrecht, 2021; Johnson et al., 2011). The limbic system is believed to develop principally during early and mid-adolescence, in part, based on findings establishing effects of sex hormones at puberty on hippocampal neural plasticity and neurogenesis (Mahmound et al., 2016; Kennard and Woodruff-Pak, 2011). Likewise, inhibitory neurotransmission from the PFC increases onto cingulate pyramidal neurons during peri-pubertal development in female mice andthis change is blocked by pre-pubertal ovariectomy (Piekarski et al., 2017). Spatial behavior dependent on the hippocampus generally decreases with age (Foster et al., 2012; Lamberty and Gower, 1993) as neurogenesis declines (reviewed in Shoji et al., 2016). Taken together, these studies point to adolescence as a critical developmental window for the hippocampal-prefrontal network, suggesting vulnerability to environmental insults, including CCD, at this time point.

Given that adolescence is a period of rapid neural development and thus is a period of life during which the CNS is sensitive to perturbations, this study explored whether CCD targeted to critical periods of pubertal and adolescent development has more pronounced and long-lasting

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effects on the hippocampal-prefrontal network structure and dependent behaviors than the same circadian disruption in adults. In order to test this question, circadian disruption was applied at two different periods: adolescence post-natal day 25 to 56 (p25-p56), and late adulthood (p90-p121). The effect of CCD on hippocampal-prefrontal network-dependent behaviors was explored using the Morris water maze task. Anxiety-like was behavior assessed by open field, and social behavior was assessed by a three-chamber sociability task. These studies were designed to examine the magnitude and permanence of changes in behavior following temporal perturbations restricted to key developmental time points. The working hypothesis of this experiment was that CCD during adolescence will have more severe impact on behavior relative to adulthood disruption.

2.2 Materials and Methods
2.2.1 Animals
All procedures were approved by the UC Berkeley Institutional Animal Care and Use Committee (IACUC) and are in accordance with NIH policies on the care and use of animals. Mice were housed under an LD 12:12 photocycle with *ad libitum* access to water and laboratory chow. Light onset and offset for breeding pairs and control experimental conditions occurred at 0800 h and 2000 h, respectively. Light intensity during the photo- and scotophases were ~400 lux white light and <1 lux red light, respectively. Humidity and temperature were held constant at 40% and 21 °C, respectively. Female wild-type (WT) C57BL/6 mice were mated between 2–3 months of age, with males 2–4 months of age. Weaning of male and female offspring was performed at post-natal day 21 (p21), followed by group housing of same sex littermates. At p21 mice were ear-punched for identification and separated by sex within litters to a maximum of 5 per cage. In litters with 6 same-sex siblings, animals were separated into groups of 3 and 3 to avoid isolation; offspring with litters of just 1 same-sex sibling were singly housed.

2.2.2 Circadian Disruption Protocol
Male and female adolescent (p25 to p55) or adult (p90 to p120) offspring were exposed to chronic circadian disruption (**CCD**; 6 hour phase advances of the 12:12 light:dark cycle every 4 days) or control lighting conditions (12:12 LD). This treatment resulted is 8 experimental groups adolescent or adult , female or male, and under control or CCD light conditions (Table 1). The timeline of experimental procedures is presented in (Fig. 1). All animals were injected with the thymidine analog, bromodeoxyuridine (BrdU), to label the dividing cell population. BrdU (50 mg/kg body weight; Sigma) was injected intraperitoneally (i.p.) 1 before after lights off, one day after every second phase advance (i.e., every 9 days) for the jet lag condition or at the same time and day for control mice. Multiple injections of BrdU were used to estimate the total population of newly- generated cells throughout the 30-day temporal disruption as well as to maximize the number of neurons surviving until maturation into the network.

2.2.3 Assessment of Spatial Learning and Cognitive Flexibility:
A modified 6-day Morris water maze (MWM) task was used to probe changes in spatial learning and cognitive flexibility in disrupted mice as used by (Karatsoreos et al., 2011). Testing occurred in a metal pool filled with water made opaque by the addition of tempera white paint and maintained lukewarm (~36ºC) between runs. The maze had four geometrical shapes that identified the four release points (north, south, east, west) and served as intra-maze spatial cues for the mice. The same experimenter ran all behavioral testing at the same relative time (1 h after lights off) in all groups. Each day all groups of animals were tested in a randomized sequence to avoid ordering

effects. The experimenter stood in the same part of the room to avoid the introduction of new cues. Each day of behavior consisted of four trials that ended after the mice found the platform or when 90 seconds had elapsed. There was a resting intertrial interval of one min.

Spatial learning was tested during days 1 to 3. On day 1 the mice were habituated and trained to find a 'flagged' platform visible above the water level. The platform for each mouse remained in the same location across days 1 to 3. If the mice were unable to find the platform after 90 seconds the experimenter would relocate the mice to the top of the platform, allowing them to learn the location. On days 2 and 3, the platform was submerged 0.5 inches to be hidden from sight and the flag removed, requiring the mice to use spatial memory to remember the platforms location. To measure cognitive flexibility, the platform was kept submerged but the location was switched to the opposing quadrant during days 4 to 5. This procedure required mice to learn the new location and inhibit visiting the old location of the platform. On day 6, the platform was removed and mice underwent four full 90 second trials to measure perseverance of visits to either of the previous locations of the platform. Ethovision software (Noldus, Inc.) was used to acquire the latency to reach the platform, distance to the platform, swim speed, time spent and entries to the old and new quadrants for each trial.

2.2.4 Assessment of Anxiety Like Behavior: **Open Field**

Mice were placed on the center of a 12" x 12" acrylic box and allowed to explore for 5 minutes. Ethovision software was used to acquire the time spent and entries to the border or the center of the arena. After each mouse completed the test, all fecal pellets were removed, the arena sprayed with ethanol, wiped with paper towels, and allowed to dry before the next mouse was tested. Mice were scored for time in center vs boarders of the arena and number of entries to each. For stereotyped anxiety-like movements, the sum of transitions from center to border regions was calculated.

2.2.5 Assessment of Sociability Behavior: Three Chamber Sociability Task

Two 3″ diameter cylindrical cages were set with centers 12″ apart in a 10″ × 18″ acrylic three chamber arena with ¼″ of bedding. The two cages were centered relative to the walls of the chamber so that a mouse could move completely around each cage. Cages were constructed of clear plastic, with vertical ¼″ × 2″ slits every ½″ of the circumference so that mice could only touch noses through the slits. Each mouse underwent 3, 5-minute trials. On the first trial, both cages were empty to assess baseline exploration and familiarize the mouse with the arena and cages. On the second trial, one of the cages housed an age and sex-matched "stranger 1", and the other cage was empty. On the third trial, one of the cages housed the same age and sex-matched "stranger 1", the other had a new same age and sex-matched "stranger 2". The new stranger on the third trial allowed the differentiation of antisocial behavior from exploratory behavior. Focal mice were placed into the center chamber for each 5 min trial. Ethovision software was used to acquire the total time the mouse spent in each chamber of the arena and time spent sniffing (i.e., nose contact) each of the two cages for each trial.

2.2.6 Statistical Analysis

All statistical analyses were performed using SPSS 28.0 (IBM Corp., Armonk, N.Y., USA). Body weight, cell count and FOS colocalization data were analyzed with three way analyses of variance (ANOVA). Linear regression was used to examine correlations between behavioral data. All

results were considered statistically significant if p < 0.05. Fisher's LSD post-hoc analyses were used to determine the significance between groups of all ANOVAs.

2.3 Results

2.3.1 CCD impacts spatial navigation in female animals in the Morris water maze

To assess the impact of CCD during adolescence or adulthood on spatial learning, the latency to platform during the first 3 days of the Morris water maze was analyzed (**Figure 2 and Figure 3**). All animals learn the platform location on day 1 with a visible platform. During day 2 and 3, animals rely on spatial memory to reach the same platform location, a platform now hidden under the opaque water. Examination of the latency to the hidden platform on day 2 did not show a main effect of age ($F(1,78)=0.05$, $p>0.05$) or lighting treatment ($F(1,78)=0.02$, $p>0.05$). However, an effect of sex was observed ($F(1,78)= 11.81$, $p<0.05$) with females taking longer to reach the platform than males (**Figure 2D**; $p<0.05$ in both cases). The same pattern was observed on day 3 with no effect observed for age ($F(1,78)=0.25$, $p>0.05$) or lighting treatment ($F(1,78)=0.60$, $p>0.05$), but females took longer than males to reach the hidden platform ($F(1,78)= 7.91$, $p<0.05$). Although there was not a main effect of CCD, an interaction between sex and treatment ($F(1,78)= 5.70$, $p< 0.02$) emerged, with females taking longer than controls (**Figure 4C**) to reach the platform on day 3 of testing if disrupted during adulthood ($p<0.04$).

2.3.2 CCD in males during adolescence diminishes cognitive flexibility in the Morris water maze

To assess the impact of CCD during adolescence or adulthood on cognitive flexibility, the latency to platform during the last two days of the Morris water maze was analyzed. During days 4 and 5 of the Morris water maze the platform is changed to the opposite location and kept hidden. The rate at which animals learn the new location of the platform and their entries to the old platform quadrant can be analyzed to examine cognitive flexibility and perseverance. Entries to the quadrant in which the platform was previously found is interpreted as a perseverative behavior indicative of lower cognitive flexibility. When analyzing the latency to platform on day 4 of testing (**Figures 2-4**), the first day of the reversal of the platform location, there is no impact of sex ($F(1,78)=1.95$, $p>0.05$), lighting condition ($F(1,78)=0.27$, $p>0.05$), or age ($F(1,78)=1.98$, $p>0.05$) effect. This is to be expected given that all animals are required to learn the new platform location.

When considering the entries to the error quadrant (**Figure 5**), there was no main effect of sex ($F(1,78)= 0.00$, $p>0.05$), age ($F(1,78)= 0.23$, $p>0.05$) or lighting treatment ($F(1,78)= 0.02$, $p>0.05$). However, there was an interaction between sex and lighting condition ($F(1,78)= 4.21$, $p<0.04$). A post hoc analysis revealed that the main driver of this interaction was that females disrupted during adolescence make fewer entries into the error quadrant (**Figure 5A**, $p< 0.05$). Examination of the ratio of time spent in the quadrant platform location over the time spent in the quadrant that previously held the platform did not reveal a main effect of sex ($F(1,78)= 0.02$, $p>0.05$), age ($F(1,78)= 0.02$, $p>0.05$) or lighting treatment($F(1,78)= 3.51$, $p>0.05$). As with entries to the error quadrant, there was an interaction between sex and lighting condition ($F(1,78)= 5.23$, $p<0.03$). This interaction is driven by males that were disrupted during adolescence spending more time in the quadrant that holds the new platform location relative to the quadrant in which the platform was previously located (**Figure 5B**, $p < 0.005$).

Neither of these effects on cognitive flexibility observed on day 4 appear to be driven by differences in velocity or distance traveled (**Figure 6C and D**). Females disrupted during

adolescence do not differ from controls in the total distance moved (p >0.05), although they show a slower average velocity when compared to controls (p< 0.04). In contrast, males that were disrupted during adolescence travel a shorter distance than controls (p< 0.005) and their velocity was not different from controls (p >0.05). Given that both male adolescent experimental groups spend the same amount of time in the arena, as shown by their similar latencies to platform (**Figure 4A and B**), this finding indicates that they are spending more time in the quadrant with the new platform location.

The differences observed in mice disrupted during adolescence on day 4 are not observed by day 5 of testing (p >0.05 for all instances). In general, disrupted animals exhibited a smaller difference when subtracting average latency to platform from day 4 from average latency to platform on day 5 ($F_{(1,78)}$= 4.98, p< 0.03) (**Figure 2B**). Thus, disrupted animals do not improve their latency to platform as much as control groups, with no main effects of sex ($F_{(1,78)}$= 3.37, p> 0.05) or age ($F_{(1,78)}$= 3.12, p> 0.05) being identified. However, males disrupted during adolescence exhibit more entries to the error quadrant on day 5 (**Figure 5B**, p< 0.02) even though they did not have a different velocity (**Figure 6D**, p> 0.05) than controls, and traveled a shorter distance (**Figure 6B**, p< 0.005). These findings reveal a persistent change in cognitive flexibility, with females disrupted during adolescence recovering by day 5 (**Figure 5B**). This finding contributes to the effect of age ($F_{(1,78)}$= 7.16, p< 0.01) and the interaction of sex and lighting treatment ($F_{(1,78)}$= 5.22, p, 0.03) for the entries to the error quadrant on day 5.

When considering the ratio of time in the quadrant with the new platform location over the time spent in the quadrant with the old platform location on day 5, a further effect on cognitive flexibility was uncovered. An main effect of age ($F_{(1,78)}$= 5.06, p< 0.03) and lighting treatment ($F_{(1,78)}$= 11.92, p< 0.001) was identified, with no effect of sex observed ($F_{(1,78)}$= 0.03, p> 0.05). Specifically, all female animals disrupted during adulthood exhibited a higher ratio of time spent in the quadrant with the new platform location (**Figure 5D**, p< 0.01). This finding may reflect an unexpected, heightened flexibility in disrupted females. Even though females disrupted during adulthood have a higher average velocity than controls (**Figure 6D**, p< 0.01), they do not show a difference in the total distance they transverse during day 5 (**Figure 6B**, p > 0.05). Thus, these differences are not due to the disrupted adult females exploring more of the maze because of their enhanced speed.

2.3.3 CCD increases perseverative behavior in all animals and impacts mean velocity in females during Morris water maze probe trial

To assess the impact of CCD during adolescence or adulthood on perseverance behavior in the absence of a platform, a probe day was employed on day 6 of the Morris water maze task. Examination of the ratio of time spent in the old platform quadrant (**Figure 7**) did not reveal a main effect of sex ($F_{(1,78)}$=0.88, p>0.05), or age ($F_{(1,78)}$=0.28, p>0.05). However, a significant effect ($F_{(1,78)}$=5.84, p<0.01) of lighting treatment was seen. An interaction between sex and lighting treatment ($F_{(1,78)}$=9.22, p<0.037) was also observed. All male animals disrupted during adulthood spent more time in the quadrant that the platform was located in during days 4 and 5 compared to the quadrant with the former location of the platform on days 1-3 (**Figure 7A and B**, p< 0.05). In contrast, all control mice spent a similar amount of time in both quadrants that previously had a platform. Thus, CCD increases the perseverative behavior in males disrupted during adulthood and not those disrupted during adolescence (p<0.01). Males disrupted during adolescence and females in any experimental group start searching quadrants for a new platform in its absence. No difference was observed in quadrant entries for any group, there is no observed

effect of sex (F(1,78)=0.64, p>0.05), age(F(1,78)=0.97, p>0.05), or lighting treatment (F(1,78)=2.78, p>0.05).

Examining the mean velocity of all groups did not reveal a main effect of sex (F(1,78)=0.09, p>0.05), or lighting treatment (F(1,78)=1.37, p>0.05). However, a main effect of age was identified (F(1,78)=1.37, p<0.007). Interaction analysis revealed a significant interaction of sex and age (F(1,78)=1.37, p<0.042), and an interaction between sex, age, and lighting treatment (F(1,78)=9.22, p<0.003). These interactions are explained by the differential impact of CCD during adolescence versus adulthood in females (**Figure 7D**). Females impacted during adolescence showed a lower mean velocity when compared to controls, while females impacted during adulthood showed an increased mean velocity (p<0.002). In addition, females disrupted during adolescence were slower than males disrupted during adolescence (p<0.038). These results, taken together, support that the observed changes in perseverative behavior in males is not due to the increased mean velocity, and that females do not show an increase perseverative behavior on the probe day even when they have different mean velocities and show a preference for the quadrant with the new platform location in day 5.

2.3.4 CCD impacts anxiety-like behavior differently depending on age and sex

To assess the impact of CCD during adolescence or adulthood on later-life anxiety-like behavior, an open field task was employed. Examination of the percent of time spent in the center of the arena relative to the border did not reveal a main effect of sex (F(1,78)=2.30, p>0.05), age (F(1,78)=3.02, p>0.05), or lighting treatment (F(1,78)=2.68, p>0.05). However, a significant interaction was observed for sex and age (F(1,78)=12.27, p<0.001) and for sex, age and group (F(1,78)=11.96, p<0.001). The sex by age and the sex by age by group interaction is driven by the fact that males spent more time in the center arena when exposed to CCD as adolescents, but not when exposed as adults (**Figure 8A**), with females of both ages were unaffected by experimental treatment. Pairwise comparisons revealed that males exposed to CCD as adolescents spent more time in the center arena than all other groups (p<0.01 in all cases). No differences were observed in terms of average velocity for any group (**Figure 8B**).

2.3.5 CCD eliminates female preference for a familiar and a novel conspecific, while disrupted male adults strengthen their preference for the novel mouse

The impact of CCD on sociability behavior was examined using a three-chamber sociability arena. In part one where both the left and right chamber contained empty cages, all mice did not show a preference between the chambers of the arena. In the second part of the test, a novel mouse was introduced into one of the cages in either chamber while the remaining cage was left empty. All experimental groups showed a preference to the room with the stranger mouse (p>0.05 in all cases). In the final part of this task, a novel mouse was placed into the empty cage in the arena to discern the preference between the previous stranger, now familiar with the experimental mouse, in contrast to the new stranger. Analysis of the cumulative duration of time spent in the chambers with the caged mice did not reveal a main effect of sex (F(1,78)=1.92, p>0.05), age (F(1,78)=1.38, p>0.05), or lighting treatment (F(1,78)=2.88, p>0.05). However, a significant interaction between sex and treatment was observed (F(1,78)=4.53, p<0.001). Given that age did not show any significant interaction, groups were collapses over age (**Figure 9A**). While control females exhibited a greater preference for the stranger over the familiar mouse, females that underwent CCD exhibit no preference (p<0.05). Males shows a slight preference for the stranger under both conditions (p<0.05).

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A similar pattern is observed when subtracting the latency to first nose poke interaction with the familiar mouse from the latency to first nose poke interaction with the stranger mouse (**Figure 9B**). Analysis of this difference in latency to the first interaction with the novel caged mice not reveal a main effect of sex ($F(1,78)=1.92$, $p>0.05$), age ($F(1,78)=1.38$, $p>0.05$), or lighting treatment ($F(1,78)=2.88$, $p>0.05$). However, a significant interaction between sex and treatment was observed ($F(1,78)=12.66$, $p<0.001$). As observed with the difference in cumulative duration, the latency to first nose poke for disrupted females is similar for both the familiar mouse and stranger mouse regardless of their age. In contrast, female controls interact faster with the stranger mouse and take longer to interact with the familiar mouse. In addition an interaction between age and treatment was observed ($F(1,78)=6.32$, $p<0.015$), with males disrupted during adulthood showing a preference for interacting with the stranger, a finding in opposition to females and male adolescents.

2.4 Discussion

The present study examined changes in spatial memory, cognitive flexibility, anxiety-like and sociability behavior of male and female mice that underwent CCD during adolescence or adulthood. Our results uncovered an impact of CCD that depends on sex and age, with females taking longer to find the platform on day 3 of water maze testing when disrupted as adults. CCD does not appear to impact spatial learning when applied to males or adolescent females. As seen in previous studies (Vorgees et al., 2008; Hamilton et al., 2007), males exhibited a steeper learning curve, reaching the platform faster on days 2 and 3 of the spatial learning test. In contrast to spatial memory, CCD during adolescence but not adulthood, negatively impacted cognitive flexibility with males experiencing CCD during adolescence making more entries into the quadrant that previously contained the platform. Disrupted females, regardless of age, did not show any deficit in cognitive flexibility. CCD increased perseverative behavior in males and females regardless of the age of treatment, with mice experiencing CCD exhibiting perseverative behavior on the probe trial (i.e., spending more time in the quadrant that previously contained the platform on day 5). In addition, only males disrupted during adolescence showed increased impulsive behavior in the open field test, spending more time in the center of the arena than controls. Finally, CCD alters social behavior when delivered to female adolescents and adult males and females. Together, these findings reveal that the effects of CCD are sex and age dependent.

Previous findings from our group showed that circadian disrupted, adult female hamsters exhibited spatial learning and memory deficits (Gibson et al., 2010). Analogous findings are observed in adult females experiencing CCD in the present study, with an increased latency to find the platform on day 3. In contrast, CCD during adolescence did not impact spatial memory in female and spatial memory was unaffected by CCD when applied to males of either age. Our results corroborate that no spatial memory deficiencies were identified in males in a study utilizing the same modified water maze to study a different CCD protocol (Karatsoreos et al., 2011). In the present study, male mice experiencing circadian disruption during adulthood exhibited deficits in cognitive flexibility in agreement with previous findings in adult mice (Karatsoreos et al., 2011). In contrast, the recovery time after CCD may have been sufficient for females and adolescents to recover from the negative effects of circadian disruption on spatial memory (Davidson et al., 2009). Males disrupted during adulthood exhibited increased perseverative behavior on the probe day whereas male and female adolescent mice and adult females are less vulnerable to the impact of circadian disruption. Previous findings suggest that the brain is more sensitive to recreational drugs and social isolation during the adolescent period. However, studies examining the impact of

circadian disruption at this time have not been conducted (Kennarad and Woodruff, 2011), and increased plasticity during this stage of development may allow recovery from CCD (Fuhrmann et al., 2015). It is also possible that the recovery time is shorter in adolescents and adult females, permitting recovery from CCD by the time of testing (Roman and Karlson, 2013). The protection afforded during adolescence against CCD provides an opportunity to parse out the differential effects of circadian disruption from those of other stressors. Social isolation (Rivera-Irrizarry et al., 2020) has long lasting impact on prefrontal and limbic structures of males (McCool and Chappell, 2009; Whitaker et al., 2013; Butler et al., 2016) but not females (Butler et al., 2014) is implicated in cognitive flexibility and learning and memory. This suggests that CCD acts on the brain through pathways other than stress axis activation in adolescents and adult females. Previous work by our group showed that the impact of CCD was not dependent on glucocorticoids (Gibson et al., 2010). Future studies that include both female and male animals that examine animals directly following CCD in adults or adolescents are needed to examine recovery time and plasticity to determine why this disruption impacts individuals differentially based on age and sex.

The present findings suggest that females disrupted during adulthood may have a slight advantage in learning the location of the new platform when its location is switched. One previous study using the MWM to assess behavior after disruption only used male mice and no probe day (Karatsoreos et al., 2011). Future studies exploring the short- and long-term effects of CCD on cognitive flexibility using other tasks are needed to confirm this potential increase in flexibility behavior in adult females. Because studies to date applying CCD have only used male or female animals (Karatsoreos et al., 2011; Gibson et al., 2010) or did not observe sex differences (Smarr et al., 2017), further work is needed to examine sex differences in the impact of CCD. Likewise, MWM and radial arm maze have been shown to be susceptible by procedural variations (Hooge and Deyn., 2001; Brandeis et al, 1989), underscoring the need for standardization when exploring the impact of CCD on spatial learning and cognitive flexibility.

As expected, females exhibit poorer spatial memory than males, given that females were slower to reach the platform during these first three days. This finding reflects the male spatial advantage that has been reported in the literature (Vorgees et al., 2008;Markowska, 1999). Highlighting the importance of including the modified MWM used in the present study, other variations of the MWM have shown no sex differences (Fritz et al., 2017).

The present findings revealed that males disrupted during early adolescence show increased impulsivity in the open field test. Early life circadian disruption increased anxiety-like behavior in all mice 40 days after disruption, with no sex difference being found (Smarr et al., 2017). Studies utilizing dim light exposure in mice after p60 also show no impact in open field anxiety-like behavior (Borniger., eat al 2014) to increased time spent in center by males exposed to dim light at night during early life or adolescence (Cissé et al., 2017). Even though, dim light is a minor disruption compared to the present CCD protocol, these studies point to increased anxiety-like behavior or increased impulsivity when mice are disrupted during early life or adolescence. In line with our results, one study reported disrupted males to spend more time in the center of the open field (Cissé et al., 2017). These findings support the idea that male adolescent mice are more susceptible to the impact of CCD on impulsive behavior.

The findings regarding sociability behavior complement findings on the effects of early life CCD leading to social avoidance regardless of sex 40 days post CCD, a trait often considered a feature of autism spectrum disorder (ASD) (Smarr et al., 2017). This finding reinforces the notion that the impact of CCD depends on the age at which disruption occurs. Although losing the preference for social novelty is not as extreme as social avoidance, it has been reported in some

mouse models of ASD (Carter et al., 2011). In addition, loss of social novelty has also observed in mutant mouse strains that have impairments in synaptic vesicle docking and release but no other cognitive impairments (Drew et al., 2007) in a mouse model of schizophrenia (O'Tuahaigh et al., 2007) and in a mouse model for diabetes (Ueda et al, 2021). These conditions are part of the behavioral repertoire that have been linked with circadian disruption (Karatsoreos, 2014) or the diabetic mice phenotype of disrupted male mice (Karatsoreos eat al., 2011).

Taken together, the present studies identify adolescence as a period in which CCD has a different impact depending on the sex of the organism when compared to early life or adult disruption. Part of this differential impact could be due neuroprotection or plasticity (examined in Chapter 3) of spatial and perseverative behavior during adolescence, with a tradeoff of males being more vulnerable to increased impulsive behavior and females more vulnerable to changes in social interaction. The present findings underscore the need for inclusion of both sexes in future studies of CCD and considerations of the age at which disruption occurs. In addition, these data also highlight the need for researchers to report the age at which disruptions are performed and the specifics of the behavioral tests to ensure standardization across experiments.

2.5 Figures

Figure 1. Experimental timeline for all experimental animals starting from birth at postnatal day 0 (P0) until behavioral assessment and brain collection. The period of CCD was 30 days, followed by 14 days of recovery, and 9 days of behavioral assessment. For adolescent CCD, animals were either disrupted from P25-P56 for both females (n=14) and males (n=9), or maintained in static lighting conditions for both females (n=9) and males (n=14). For adult CCD, animals were either disrupted from P90-P121 for both females (n=9) and males (n=12), or maintained in static lighting conditions for adult controls for both females (n=9) and males (n=12).

Period of CCD or Control	Period of CD or control (30 d)	Behavioral Assessment (9d)
Adolescence	P25-P56	P70-P80 (14d after CCD)
Adulthood	P90-P121	P135-145 (14d after CCD)
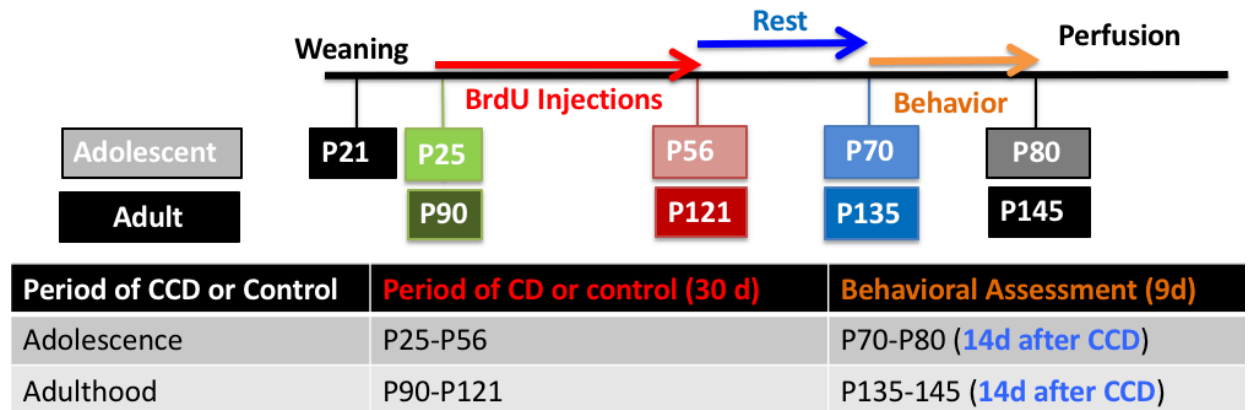
Figure 2. Mean (± SEM) average latency to platform for days 1 through 5 of the Morris water maze in male and female mice exposed to CCD or static lighting conditions during adolescence or adulthood. Data were analyzed by light condition (A, B) Age (C) and Sex (D) * = significantly greater when comparing adolescent disruption (P75) to adult disruption (P145) (C) or females to males (D), or significantly less when comparing all experimental to control lighting conditions (B), $p<0.05$ in all cases.
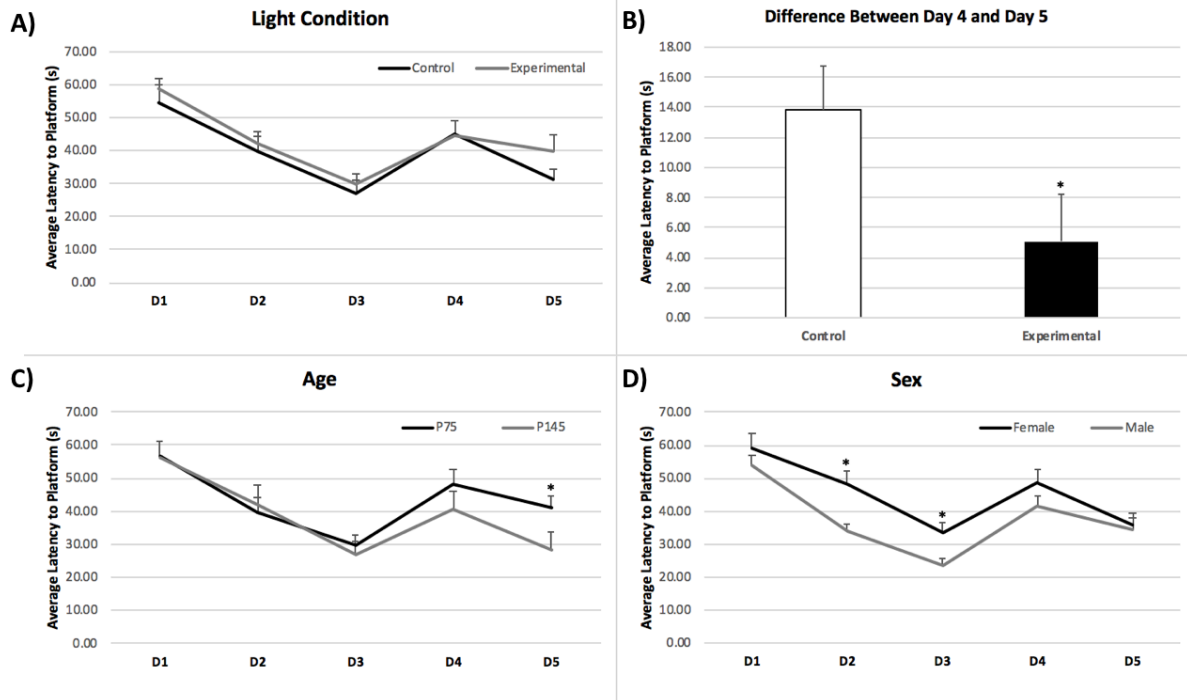
Figure 3. Mean (± SEM) average latency to platform for days 1 through 5 of the Morris water maze in collapsed over male and female mice exposed to CCD or static lighting conditions during adolescence or adulthood. Data were analyzed by age of disruption for adolescents (A) and adults (B) or by sex and treatment for females (C) and males (D).
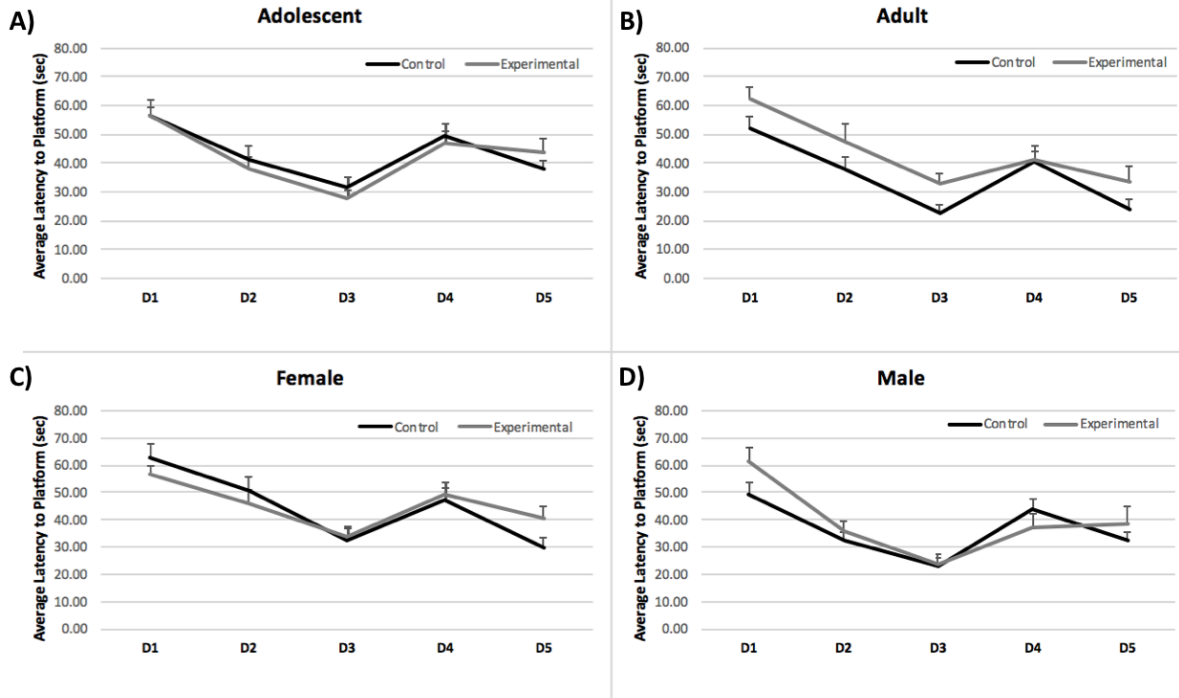
Figure 4. Mean (± SEM) average latency to platform for days 1 through 5 of the Morris water maze for all experimental groups. Data were analyzed by age of disruption for adolescents (A, B) and adults (C, D) * = significantly greater than disrupted animals in the experimental condition, $p<0.05$.
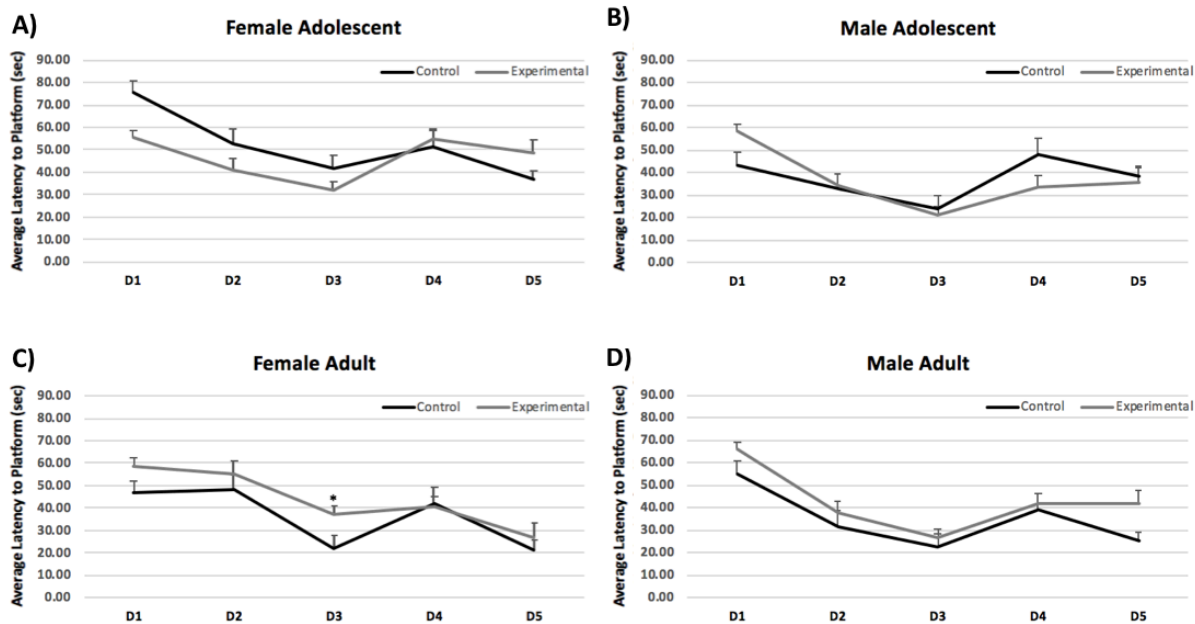
Figure 5. Mean (± SEM) cognitive flexibility measurements for platform reversal days 4 and 5 of the Morris water maze. Average entries to error quadrant (A, B) and ratio of time spent in quadrant with the new platform location over the time in quadrant where the old platform location was located (C, D). * = significantly greater than controls, $p<0.05$.
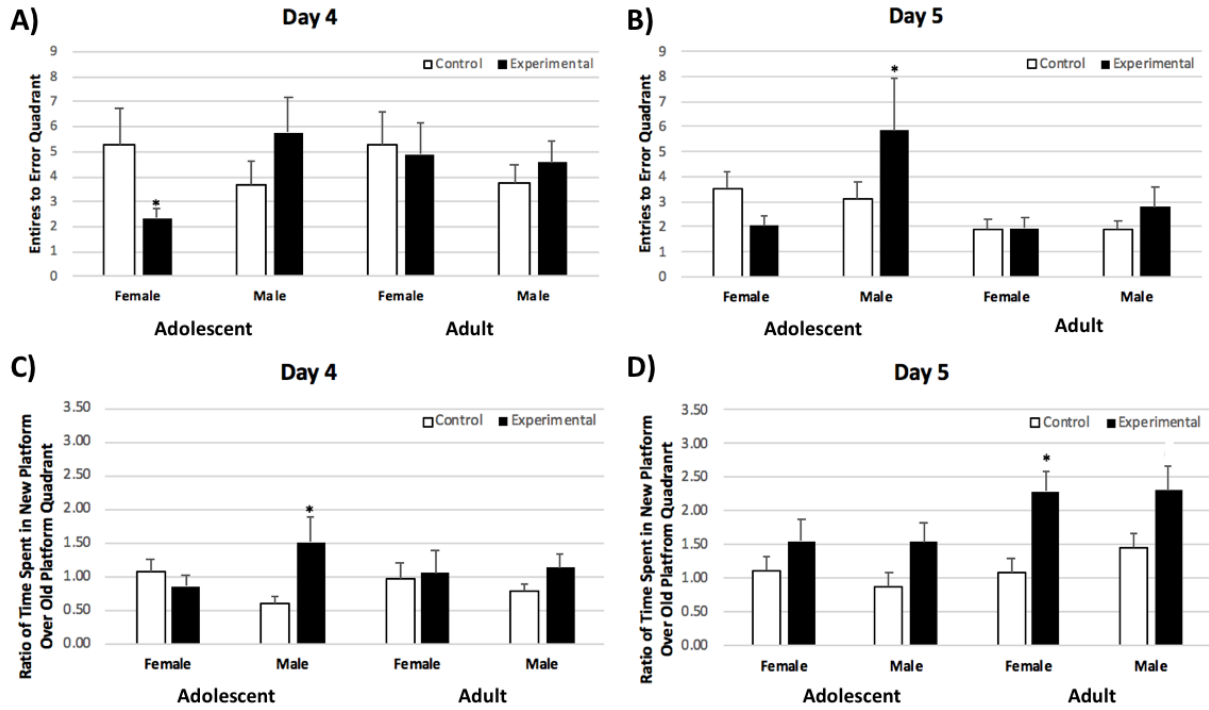
Figure 6. Mean (± SEM) measurements for average distance and velocity on the platform reversal in day 4 and 5 of the Morris water maze. Average distance (A, B) and average velocity (C,D) are depicted. * = significantly greater than the control group, ** = significantly less than the control group p<0.05.
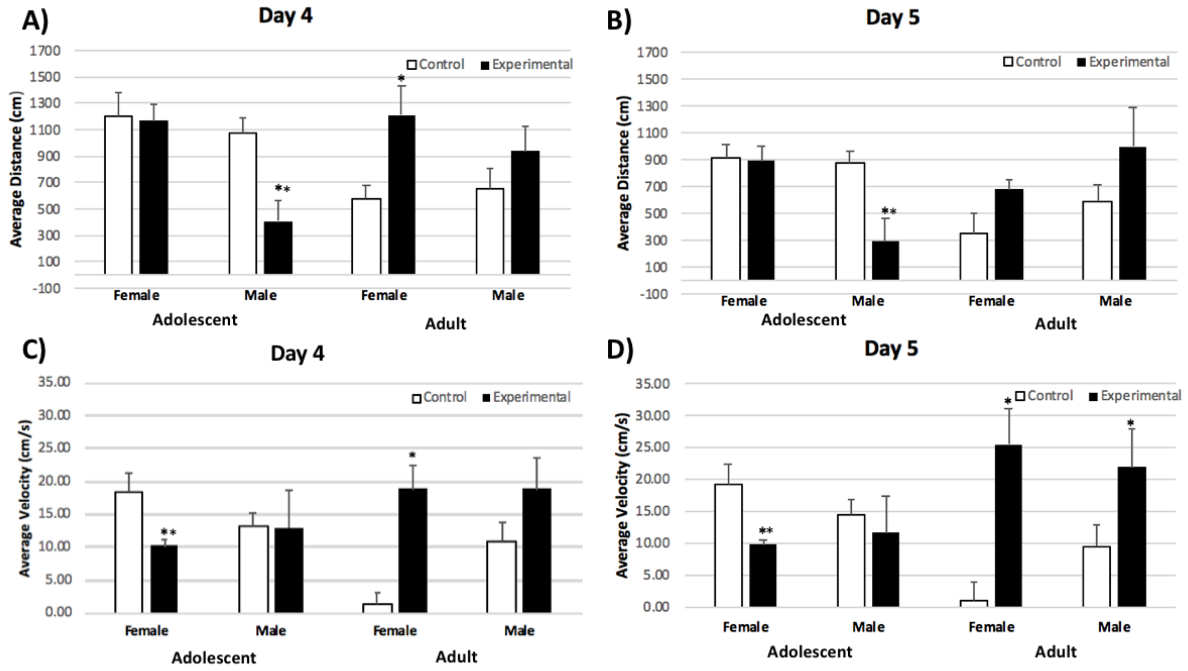
Figure 7. Mean (± SEM) of measurements for the probe day (day 6) of the Morris water maze. Average ratio of time spent in the new platform quadrant over the time spent in the old platform quadrant (A). Average ratio of new platform location crossings over old platform location crossings (B), average distance (C), and average velocity (D). * = significantly greater than the control group, ** = significantly less than the control group $p < 0.05$
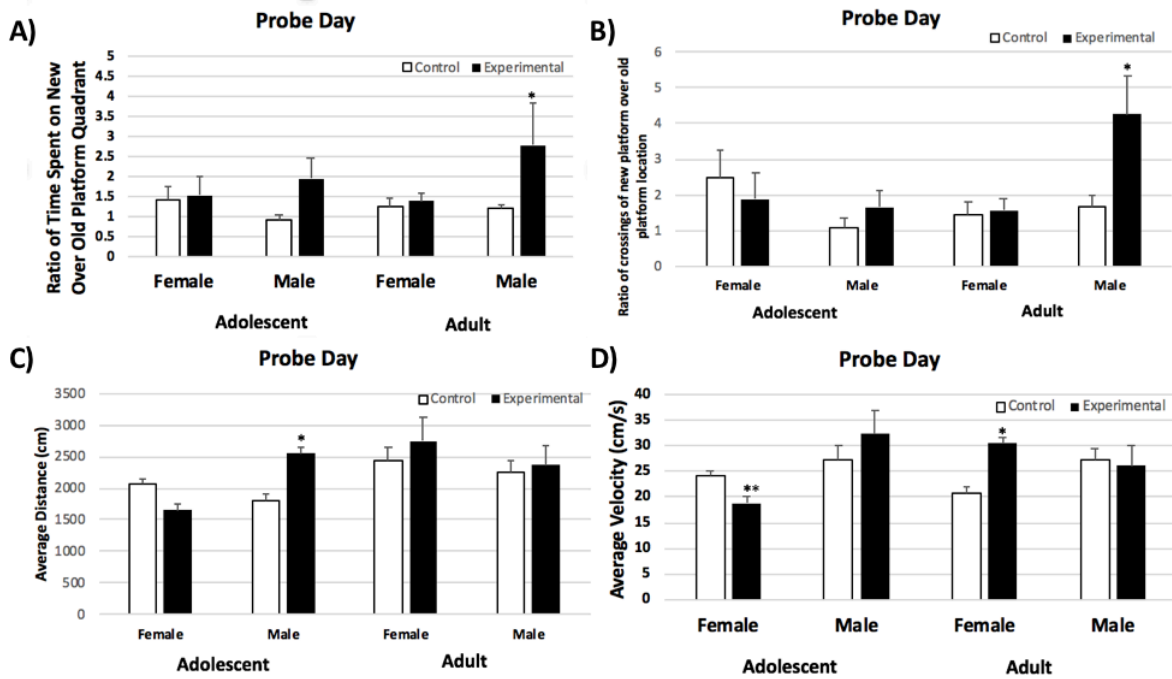
Figure 8. Mean (± SEM) open field measurements, including percent time spent in the center arena relative to the borders of the arena (A) and average velocity in center arena (B). * = significantly greater than all other groups, p<0.05.
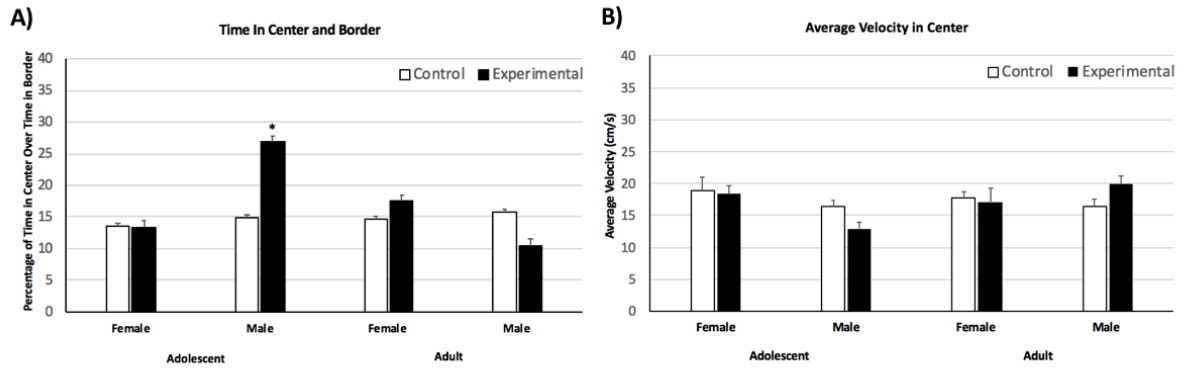
Figure 9. Three chamber sociability task: Mean (± SEM) difference between the cumulative time spent in the chamber with the stranger and the time spent in the chamber with the familiar mouse (A) and difference between the latency to the first interaction with the stranger and the latency to the first interaction with the familiar mouse. * = significantly greater than the control group, **= significantly less than the control group p<0.05
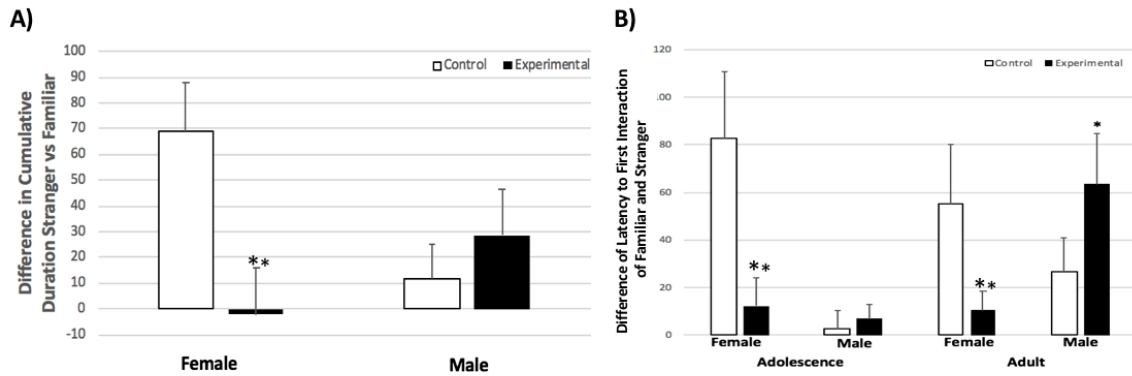
Chapter 3: The Impact of Chronic Circadian Disruption on Neurogenesis, Parvalbumin Interneurons, and Perineural Nets in the Hippocampus and Prefrontal Cortex

3.1 Introduction

Organisms living longer than 24h utilize an endogenous circadian timing system to coordinate their physiology and behavior with changing environmental conditions and requirements throughout the day. By anticipating environmental conditions, animals can prepare accordingly rather than directly responding to predictable changes throughout the day (Antle and Silver, 2009). Repeated daily, temporal adaptation strengthens associations with phases throughout the day and allows optimal utilization of resources only available during restricted time windows. Thus, it comes as no surprise that circadian rhythms influence every aspect from physiology and behavior (reviewed in Karatsoreos, 2019). The advent of electricity and widespread use of artificial light and portable light-emitting devices, along with limited levels of light exposure during the day, has led to a relative state of chronic circadian disruption in our society (Evans and Davison, 2013; Moore-Ede et al., 1983). Although the circadian timing system can recover from singular occurrences of circadian disruption, chronic circadian disruption leads to long term behavioral and physiological deficits even after the circadian system has recovered (reviewed in Karatsoreos, 2011).

Behaviors that are impacted by CCD, including spatial memory (Jin and Stephen, 2015; Floresco et al., 1997), social memory (Philips et al, 2019) and cognitive flexibility (Mála et al., 2015) are mediated by projections between the hippocampus and pre-frontal cortex (PFC) (Chen et al., 2021). Although cognitive flexibility and reversal learning are thought to be principally dependent on the PFC, the hippocampus is likely involved as hippocampus neurogenesis has been implicated in reversal learning and perseverance behavior in some studies (Burghardt et al., 2012; Garthe et al., 2009). CCD decreases neurogenesis (Horsey et al., 2020; Gibson et al., 2010) and NMDA receptors in the hippocampus (Vilches et al, 2014) and is accompanied by impairments in spatial memory. Likewise, CCD results in a reduction in number of apical dendrites in the PFC (Karatsoreos et al., 2011) associated with changes in perseverative behavior. In addition, exposure to CCD during pregnancy, or from birth until weaning, results in offspring with shallower growth curves, and anxiety-like behavior and decreased sociability in adulthood (Smarr et al., 2017), both of which are regulated by interactions of the prefrontal hippocampal network (Philips et al., 2019). Similarly, other studies show that exposing pregnant mice to constant dim light (LL) results in reduced growth rates among her offspring, increased anxiety-like behavior in the mother, and increased fear responsiveness in the offspring as adults (Borniger et al., 2014). Moreover, increased anxiety-like behavior, but not spatial memory deficits, are observed in adult offspring exposed to LL perinatally (i.e., late prenatal and early postnatal life) (Roman and Karlsson, 2013). Together, highlight the need to further explore whether the hippocampal prefrontal network is particularly vulnerable to the impact of CCD during sensitive periods of development.

The hippocampus is believed to be developing principally during early and mid-adolescence, in part, based on findings establishing effects of sex hormone at puberty that correlate with increased hippocampal neural plasticity and neurogenesis (reviewed in Kennard and Woodruff-Pak., 2011); spatial behavior dependent on the hippocampus generally decreases with age as neurogenesis declines (reviewed in Sohi et al., 2016). The most notable structural maturation of the hippocampus in mice is a sharp decrease in neurogenesis and an increase in apoptotic cells during adolescence during p30 to p60 (Abdallah et al., 2010). Newborn neurons take about 30 days to integrate into the hippocampal circuitry and be considered mature (Denoth-Lippuer and Jesseberger, 2021). Exploring the long-term effects of decreased neurogenesis caused

by CCD, and the developmental decrease in neurogenesis during adolescence, are needed to gain a complete understanding of the impact of circadian disruption. Thus, further studies exploring the impact of CCD during development and adulthood are needed (Smarr et al., 2017; Vilches et al., 2014; Borniger et al., 2014; Roman and Karlsson, 2013; Horsey et al, 2019; Kott et al., 2012; Karatsoreos et al., 2011; Gibson et al., 2010).

Perseverative behavior and sociability rely on the medial PFC (mPFC), a brain region that matures significantly throughout adolescence (reviewed in Delevish and Wilbrecht, 2021; Johnson et al., 2011). Throughout the period of adolescence, the mPFC stabilizes synapses as it undergoes a loss of neurons known as neuronal pruning (Markham et al., 2007). The majority of neuronal pruning occurs during p35 to p45 in rodents and has been suggested to be regulated by hormonal changes during adolescence, potentially through different mechanisms in females and males (Willing and Juraska, 2013). Functionally, inhibitory and excitatory balance matures at this time in the mPFC through refinement of GABAergic neuronal function (Caballero et al., 2016). More specifically, parvalbumin positive (PV) GABAergic interneurons mature at this time and regulate inhibition of mPFC pyramidal neurons (Delevish et al., 2015; Packer and Yuste, 2011). The activity of PV interneurons is remodeled during adolescence in the mPFC (Wang and Gao., 2010) and regulates the termination of sensitive periods of synaptic plasticity (Hench 2005). PV interneurons regulate cognitive flexibility (Sparta et al., 2014), fear learning (Courtin et al., 2014), and social behaviors (Bicks et al., 2020), all of which develop to their adult state throughout adolescence. PV interneuron excitability is modulated by the protein scaffold known as perineural nets (PNN) that assemble through development (Spikjer et al., 2017). The appearance of PNN increases from pre-adolescence to adulthood (Baker et al., 2017). PNNs stabilize synaptic plasticity in sensory cortices and influence behavior (Favuzzi et al., 2017). However, it is not clear how PV interneurons shape the behavioral changes observed during adolescence (Piekarski et al., 2017).

In addition to the well-established developmental changes in mPFC network, the hippocampus has been proposed as the main coordinator of circadian-influenced learning and memory (Lehr et al., 2021; Obrietan et al., 2018). Hippocampal circadian rhythms were first considered to be involved in memory processing when the genes that maintain circadian rhythms, referred to as clock genes, were shown to be expressed in a circadian pattern within this brain region (Wang et al., 2009). Later studies identified clock genes in most brain areas including the PFC (Chun et al., 2015). Targeted suppression of clock genes in the hippocampus negatively impacts learning and memory (Snider et al., 2016; Wardlaw et al., 2014) and inhibits neurogenesis (Malik et al., 2015). In addition, CCD negatively affects hippocampal differentiation and hippocampal-dependent memory (Craig and McDonald, 2008; Evan et al., 2001). Thus, the present study focused on identifying the long-term consequences of CCD on the hippocampal-prefrontal network.

Given that adolescence is a time of rapid neural development and a period of life in which the CNS is sensitive to perturbations, this study explored whether CCD targeted to critical periods of pubertal and adolescent development have more pronounced and long-lasting effects on the hippocampal-prefrontal network structure than the same circadian disruption in adults. In order to test this hypothesis, CCD was applied at two different periods: adolescence (p25-p56), and late adulthood (p90-p121). All animals were injected with the thymidine analog, bromodeoxyuridine (BrdU), to label the dividing cell populations during their disruption or control rest period. These studies were designed to examine the magnitude and permanence of changes in the brain following temporal perturbations restricted to a key developmental time point. The working hypothesis of

this experiment was that CCD during adolescence will have more severe impact on the number of mature BrdU labeled neurons in the hippocampus and the number of PV interneurons and PNN in the PFC, relative to adulthood disruption.

3.2 Materials and Methods

3.2.1 Animals

All procedures were approved by the UC Berkeley Institutional Animal Care and Use Committee (IACUCs) and were in accordance with NIH policies on the care and use of animals. Mice were housed under an LD 12:12 photocycle with *ad libitum* access to water and laboratory chow. Light onset and offset for breeding pairs and control experimental conditions occurred at 0800 h and 2000 h, respectively. Light intensity during the photo- and scotophases were ~400 lux white light and <1 lux red light, respectively. Humidity and temperature were held constant at 40% and 21°C, respectively. Female wild-type (WT) C57BL/6 mice were mated between 2–3 months of age, with males 2–4 months of age. Weaning of male and female offspring was performed at post-natal day 21 (p21), followed by group housing of same sex littermates. At p21 mice were ear-punched for identification and separated by sex within litters to a maximum of 5 per cage. In litters with 6 same-sex siblings, animals were separated into groups of 3 and 3 to avoid social isolation; offspring with litters of just 1 same-sex sibling were singly housed.

3.2.2 Circadian Disruption Protocol

Male and female adolescent (p25 to p55) or adult (p90 to p120) offspring were exposed to chronic circadian disruption (**CCD**; 6 hour phase advances of the 12:12 light:dark cycle every 4 days) or control lighting conditions (12:12 LD). This treatment resulted is 8 experimental groups adolescent (**adol**) or adult (**adu**), female or male, and under control (**cont**) or CCD light conditions (**Table 1**). The experiment lasted 55 days with mice being perfused and brains extracted on the last day (**Figure 1**). Offspring were weighed every 2–3 days at the start of their exposure to control conditions or CCD. All animals were injected with the thymidine analog, bromodeoxyuridine (BrdU), to label dividing cells. BrdU (50 mg/kg body weight; Sigma) was injected intraperitoneally (i.p.) 1 before after lights off, one day after every second phase advance (i.e., every 9 days) for the jet lag condition or at the same time and day for control mice. Multiple injections of BrdU were used to estimate the total population of newly-generated cells throughout the 30-day temporal disruption as well as to maximize the number of neurons surviving until maturation into the network.

3.2.3 Brain Perfusion and Preservation

Mice were deeply anesthetized with sodium pentobarbital (200 mg/kg, Med-Pharmex, Pomona, CA) and perfused transcardially with approximately 30 ml of 0.9% saline, followed by approximately 100 ml of 4% paraformaldehyde in 0.1 M PBS (pH 7.3). Brains were postfixed for 4 h in 4% paraformaldehyde followed by cryoprotection in 30% sucrose in 0.1 M PBS at 4°C until sectioned. Brains were sectioned in the coronal plane at 40 μm on a Leica 3050S cryostat and stored at -20°C in an ethylene glycol and sucrose-based antifreeze until immunohistochemistry was performed.

3.2.4 BrdU/NeuN Double Label Immunofluorescence

To visualize the colocalization of BrdU with the mature neuron marker, neuronal nuclear protein (NeuN), double-label immunofluorescence was performed on separate sets of every fourth 40 μm brain slice. Free floating sections were washed in PBS, incubated for 30 min in 0.5% hydrogen peroxide, washed in PBS again, and then blocked for 1 h in 3% normal goat serum suspended in 0.1% Triton X100 (PBT). Sections were then incubated for 48 h at 4°C in a rat polyclonal anti-BrdU antibody (1:500;ABCAM) with 1% normal goat serum in PBT. After incubation in the primary antibody, sections were washed in PBT, incubated overnight in Cy-2 Donkey Anti-Rat (1:200, Jackson Laboratories). Next, sections were washed with PBT and incubated for 48 h at 4°C with a mouse anti-NeuN primary antibody (1:1,000 MilliporeSigma) and 2% normal donkey serum in PBT. Sections were then washed with PBT and labeled with the fluorophore Cy-3 donkey-anti-mouse (1:200, Jackson Laboratories). Finally, sections then washed with PBS and mounted on gelatin-coated slides, dehydrated and cleared with xylene, and cover slipped.

3.2.5 PV/PNN Double Label Immunofluorescence

To visualize the colocalization of PV interneurons with PNN, double-label immunofluorescence was performed on separate sets of every fourth 40 μm brain slice. Free floating sections were washed in PBS, incubated for 10 min in 0.5% hydrogen peroxide, washed in PBS again, and then blocked for 1 h in 3% normal goat serum suspended in 0.1% Triton X100 (PBT). Sections were then incubated for 48 h at 4°C in a rabbit polyclonal anti-PV antibody (1:4,000; Thermo Fisher Scientific) with 1% normal goat serum in PBT. After incubation in the primary antibody, sections were washed in PBT, incubated overnight in Cy-3 Donkey Anti-Rabbit (5:1,000, Jackson Laboratories). Next, sections were washed with PBT and incubated for overnight at 4°C with wisteria floribunda agglutinin (WFA) (1:1,000; Vector Technologies) and 2% normal donkey serum in PBT. Finally, sections then washed with PBS and mounted on gelatin-coated slides, dehydrated and cleared with xylene, and cover slipped.

3.2.6 Microscopy and Quantification

To examine the percentage of NeuN cells expressing BrdU or PV cells surrounded by PNN, sections were examined at the conventional light microcopy level using the standard wavelengths for Cy-2 (508 nm) and Cy-3 (568 nm) with a Zeiss Axioscan slide scanner (Thornwood, NY). Every eighth section starting at the dentate gyrus was examined for colocalization of BrdU and NeuN. Every fourth section starting at the fornix was examined for colocalization of PV cells surrounded by PNNs. Each label was captured as a single image at 200x magnification without adjusting the plane of focus between captures and then 12 superimposed digitally. Brain areas were examined by two observers blind to the experimental conditions using Zen Blue software (ZEISS Group, Oberkochen, Germany) to view the Cy-2 and Cy-3 channels independently or together. In the hippocampus, a cell was considered to be double labeled if Neun and BrdU were expressed in the nucleus without extending beyond its borders. In the PFC, a cell was considered to be double labeled if PV was expressed in the cytoplasm and WFA was expressed around the cell. Cells without a clearly identifiable nucleus were not included in analysis. To confirm that conventional light microscopy did not result in false positives, a subset of NeuN and PV cells were also examined using a Zeiss Axiovert 100TV confocal microscope with a Zeiss LSM 510 laser-scanning confocal attachment. The sections were excited with an argonkrypton laser using the standard excitation wavelengths for Cy-2 and Cy-3. Stacked images were collected as 0.8μm

multitract optical sections. Using the ZEN Black software (Zeiss), the channels were digitally superimposed and cells were categorized as single or double labeled using the definition described above. One section each from 2-3 animals per group was confirmed in this manner.

3.2.7 Statistical Analysis
All statistical analyses were performed using SPSS 28.0 (IBM Corp., Armonk, N.Y., USA). Body weight, cell counts and colocalization data were analyzed with three way analyses of variance (ANOVA). Linear regression was used to examine correlations between behavioral data. All results were considered statistically significant if $p < 0.05$. Fisher's LSD post-hoc analysis were used to determine the significance between groups.

3.3 Results

3.3.1 CCD decreases the amount of PV interneurons and BrdU-labeled cells in the dentate gyrus in male mice disrupted during adolescence
The impact of CCD during adolescence or adulthood on the number of BrdU and PV cells and PV cells co-labeled with PNN in the dentate gyrus (DG) and PV and PV cells co-labeled with PNN in the hilus was examined to determine the impact of adolescent and adult CCD on the hippocampus. There is no main effect of sex ($F(1,78)=0.15$, $p>0.05$), lighting condition ($F(1,78)=0.04$, $p>0.05$), or age ($F(1,78)=1.98$, $p>0.05$) on the average number of PV interneurons in the hilus (**Figure 2A**). However, there was a significant effect of age ($F(1,78)=8.13$, $p< 0.01$) with p140 animals having more PV interneurons co-labeled with PNN in the hilus than p80 (**Figure 2B,C**) mice ($p<0.05$). There was no main effect of sex ($F(1,78)=1.75$, $p>0.05$), lighting condition ($F(1,78)=1.13$, $p>0.05$), or age ($F(1,78)=0.12$, $p>0.05$) on the number of PV interneurons in the DG (**Figure 3A**). However, there was an interaction of sex, age, and group ($F(1,78)=5.94$, $p< 0.02$). Post hoc analysis revealed that the main driver of this interaction were males that were disrupted during adolescence had fewer PV interneurons than controls ($p< 0.02$). In contrast, there was no impact of sex ($F(1,78)=0711$, $p>0.05$) or lighting treatment ($F(1,78)=0.09$, $p>0.05$) on the percentage of PV interneurons co-labeled with PNN in the DG (**Figure 3B**). The percentage of co-labeled PV interneurons differed by age, with PV interneurons co-labeled with PNN in the DG ($F(1,78)=4.59$, $p< 0.04$) increased in p140 animals than p80 mice (**Figure 3C**). Lastly, there was no main effect of sex ($F(1,78)=3.90$, $p>0.05$) or lighting treatment ($F(1,78)=1.66$, $p>0.05$) on the number of BrdU cells all co-labeled with mature neuronal marker NeuN in the DG. However, there was a main effect of age on BrdU neuron numbers in the DG ($F(1,78)=4.97$, $p< 0.03$) and an interaction with lighting treatment ($F(1,78)=7.10$, $p< 0.01$). This interaction was attributed to the lower number of BrdU-labeled neurons in males disrupted during adolescence compared to controls (**Figure 3D**).

3.3.2 CCD decreases the amount of PV interneurons co-labeled with PNN in the infra limbic, pre-limbic, and dorsal penduncular region of the PFC in male mice disrupted during adolescence with increased co-labeling in the infralimbic cortex of animals disrupted during adulthood.
To assess the impact of CCD during adolescence or adulthood on the PFC, the number of PV+ cells and PV+ cells co-labeled with PNN were examined in the infra limbic (IL), pre-limbic (PrL), and dorsal penduncular (DP) regions of the PFC. In the IL (**Figure 4A**) there is no main

effect of sex (F(1,78)=1.78, p>0.05), but there was a main effect of lighting condition (F(1,78)=9.29, p<0.005) and age (F(1,78)=6.37, p<0.01) . These main effects show that males disrupted during adulthood have fewer PV interneurons than controls (p<0.001). When extending analysis to PV interneurons co-labeled with PNN (**Figure 4D**) in the IF there was also an impact of age (F(1,78)=4.94, p<0.03), but not lighting condition (F(1,78)=3.55, p>0.05) or sex (F(1,78)=0.92, p>0.05). An interaction between lighting condition and age was also observed (F(1,78)=12.90, p<0.001) and explained by disrupted adults having a higher numbers of PV interneurons co-labeled with PNN (p< 0.04 in both instances).

Regarding the number of PV interneurons in the PrL (**Figure 4B**), there was no main effect of sex (F(1,78)=2.88, p>0.05), lighting condition(F(1,78)=3.10, p>0.05), or age (F(1,78)=0.20, p>0.05). The same was true when analyzing PV interneurons co-labeled with PNN in the PrL, with no main effect of sex (F(1,78)=0.75, p>0.05), lighting condition(F(1,78)=3.75, p>0.05), or age (F(1,78)=2.20, p>0.05). However, there was an interaction between lighting condition and sex (F(1,78)=4.58, p<0.04). Post-hoc analysis pointed at males disrupted during adolescence having a lower number of PV interneurons co-labeled with PNN (**Figure 4 E**)(p< 0.01). Following a similar patter to PrL, the number of PV interneurons in the DB (**Figure 4C**) was not impacted by sex (F(1,78)=2.88, p>0.05), lighting condition (F(1,78)=3.10, p>0.05), or age(F(1,78)=0.20, p>0.05). PV interneurons co-labeled with PNN are impacted by age (F(1,78)=5.13, p<0.03) , but not of sex (F(1,78)=0.25, p>0.05) or lighting condition (F(1,78)=1.53, p>0.05) or sex (F(1,78)=0.25, p>0.05). An interaction between lighting condition and age (F(1,78)=6.65, p<0.01), once again, appears to be driven by males that were disrupted during adolescence (**Figure 4 F**) exhibiting a smaller amount of PV interneurons co-labeled with PNN (p<0.0001).

3.4 Discussion

The present study examined changes in the PV+ cells in the PFC and hippocampus, PV+ cells co-labeled with PNN, and BrdU-labeled neurons in the PFC, and hippocampus of male and female mice that underwent CCD during adolescence or adulthood. Our results uncovered an impact of CCD that depends on sex and age, with females disrupted during adolescence not exhibiting any cellular changes in any of the examined brain regions. In contrast, males disrupted during adolescence show a decrease in PV+ interneurons and neurogenesis in the dentate gyrus of the hippocampus, and a smaller percentage of PV+ interneurons surrounded by PNN in all PFC regions. In addition, animals disrupted during adulthood, regardless of sex, exhibited an increase in PV+ interneurons surrounded by PNN in the IL region of the PFC. Together, these findings reveal that the effects of CCD on the PFC and hippocampus are sex and age dependent.

In the hippocampus, PV+ interneurons surrounded by PNNs increase around p35 in mice, stabilizing at the end of adolescence, with disruptions to PNN maturation seen in some mouse models of psychiatric disease (Dominguéz et al.,2019; Piskorowski et al.,2016). PV interneurons in the DG of the hippocampus promote neurogenesis and survival of new neurons in the same region (Song et al., 2012), while the suppression of PV decreases neurogenesis and survival (Song et al., 2013). The present results corroborate the increase of PNN in adult mice, while revealing an impact of CCD on the numbers of both PV+ and new neurons in the hippocampus only in male mice disrupted during adolescence, without impacting the percentage of PV+ cells expressing PNN in any experimental group. The cellular composition and functioning of the DG is important in regulating anxiety-like behavior (Anacker and Hen, 2017:McEwen et al., 2016); mice that have greater DG inhibition show less anxiety-like behavior (Botterill et al., 2020). Taken together, these findings suggest that the decreased number of PV and neurons of the DG is a possible mechanism

by which males disrupted during adolescence exhibit enhanced anxiety like behavior in Chapter 2. In addition, increased anxiety-like behavior observed in many psychiatric diseases is associated with fewer PV interneuron numbers in the hippocampus (Piskorowski et al.,2016). These findings provide a potential link between CCD and the development of psychiatric illness that has been reported in the literature (Karatsoreos, 2001).

Previous findings from our group showed that circadian disrupted, adult female hamsters exhibited spatial learning and memory deficits, accompanied by decreased neurogenesis in the DG (Gibson et al, 2010), and other studies have since expanded on these findings (Kott et al., 2012; Fujioka et al., 2011). Given that several studies have suggested that excitation from the DG is important for spatial functions (GoodSmith et al., 2019 ; Senzai and Buzsaki, 2017; Soltesz et al., 1993), it was surprising that a change in neurogenesis was not seen in disrupted female animals, specifically female adults disrupted during adulthood that showed spatial memory deficits in the Morris water maze task in Chapter 2. However, CCD may cause impairments in spatial memory by regulating the functionality hippocampal cell-to-cell communication (McMartin et al., 2020) and/or NMDA receptor level (Vilches et al., 2014). Such a mechanism would not be surprising given the range of genes influenced by circadian control (Krishnan and Lyons, 2015;Mulder et al., 2013) and the potential long term neuronal consequences of disrupting these genes in periods of development (Schulz and Sisk, 2016). Relative to adult female hamsters investigated previously, adolescent female mice may be resilient to the cellular and behavioral impact of CCD, with females disrupted during adulthood showing altered spatial navigation without accompanying cellular changes in the DG. Future studies that characterize functional differences in the DG and hippocampus of disrupted and control animals would help to uncover such a specific mechanism of control.

As mentioned previously, CCD in adult male mice shortens the length apical dendrites in the PFC, and impacts reversal learning in mice (Karatsoreos et al., 2011) in addition to changing the rhythm of learning and memory genes of the PFC to similar levels of aged control adult mice (Naomi et al., 2019). Our findings expand upon these results by showing that CCD reduces the percentage of PV cells surrounded by PNN in both the IL, PrL, and DP areas in male mice disrupted during adolescence. In addition, this cellular change mirrors the functional change in reversal learning errors made by males disrupted during adolescence in Chapter 2. Males disrupted during adulthood were the only group that showed a decrease in PV+ cells in the IL of the PFC associated with impairments in reversal learning only on the probe day of Morris water maze testing, while males disrupted during adolescence showed deficits during the last days of the water maze but not on the probe day (see chapter 2). In contrast, females disrupted during adulthood showed an increase in PV+ cells co-labeled with PNN in the PFC with behavioral deficits observed in spatial memory and social behavior, underscoring the relevance of age and sex when studying the impact of CCD (Drzewiecki et al., 2020).

Adolescence coincides with the onset of puberty. Both neuronal and synaptic pruning during adolescence are mediated by hormonal changes during puberty (Markham et al., 2007; Vaneden and Uylings, 1985). In addition, pre-pubertal females exhibit increased neuron numbers in the PFC compared to post-pubertal controls (Willing and Juraska, 2015) and prepubertal gonadectomy in females, but not males, prevents this neuronal loss (Koss et al., 2015). Thus, it is possible that the protection of adolescent females to the impact of CCD is due to protective effects of gonadal hormones such as estradiol (Russel et al., 2019; Engler-Chiurazzi et al., 2016). Earlier studies from our group showed that CCD did not affect the onset of puberty in mice disrupted during early life, regardless of sex (Smarr et al., 2017). However, future studies looking at the

short term and post recovery impact of a narrower CCD disruption in pre- and post-pubertal animals could help unveil the role of puberty on CCD and aid our understanding of how CCD impacts adolescence. The present study identifies that males disrupted during adolescence have a decrease in mature neurons in the hippocampus, along with PV and PNN in both the hippocampus and prefrontal cortex 25 days after the last day of CCD. Thus, these future shorter term studies could identify when we start to see these changes in neurogenesis, PV, and PNN in the hippocampal prefrontal network in males disrupted during adolescence. In addition, we would be able to assess the progression of the observed cellular changes on behavior in earlier timepoints post CCD or with more targeted or shorter disruptions.

Changes in melatonin and the stress axis both impact neurogenesis (McEwen et al., 2016), change during adolescence (Yang et al., 2021; Onaloapo and Onaloapo, 2017; Romeo, 2013), and influence or are influenced by circadian control (Karatsoreos 2019; Gillette and McArthur et al., 1996). However, the studies conducted by our group have been done using mouse strains with blunted melatonin rhythms, with C57BL/6 mice (Roseboom et al., 1998) used in the present study; and BALB/c mice (Kennaway et al., 2002; Vivien-Roels et al., 1998) used in our early life CCD study (Smarr et al., 2017). While using melatonin deficient mice allows parsing out the impact of disruptions to melatonin as a mechanism responsible for any deficits observed, the impact of CCD on melatonin and downstream outcomes cannot be determined (Bonmati-Carrion et al., 2014). In addition, changes in the activity of the hypothalamo-pituitary adrenal (HPA) axis contribute to the effects of CCD and were not controlled herein (Kinlein and Karatsoreos, 2020). However, previous work by our group established that, at least in hamsters, HPA axis activity was inconsequential (Gibson, et al., 2010) even though higher cortisol levels reduce neurogenesis (Cerqueira et al., 2005; Wellman et al., 2001). Future studies, expanding on the impact of CCD in animals with normal melatonin production and studies in which the stress response is also manipulated will help elucidate the contribution of these physiological systems to the impact of CCD.

Taken together, the present studies identify adolescence as a period in which CCD has a different impact depending on the sex of the organism when compared to early life or adult disruption. Part of this differential impact could be due neuroprotection or plasticity of the cellular structures of the PFC and hippocampus during adolescence, with males being more vulnerable than females potentially because of the protective effects of estrogen. The present findings underscore the need for inclusion of both sexes in future studies of CCD and considerations of the age at which disruption occurs. In addition, these data also highlight the need for researchers to report the age at which disruptions are performed and the specifics of the behavioral tests to ensure standardization across experiments.

3.5 Figures
Figure 1. Experimental timeline for all experimental animals starting from birth at postnatal day 0 (P0) until behavioral assessment and brain collection. The period of CCD was 30 days, followed by 14 days of recovery, and 9 days of behavioral assessment. For adolescent CCD, animals were either disrupted from P25-P56 for both females (n=14) and males (n=9), or maintained in static lighting conditions for both females (n=9) and males (n=14). For adult CCD, animals were either disrupted from P90-P121 for both females (n=9) and males (n=12), or maintained in static lighting conditions for adult controls for both females (n=9) and males (n=12).

Period of CCD or Control	Period of CD or control (30 d)	Behavioral Assessment (9d)
Adolescence	P25-P56	P70-P80 (14d after CCD)
Adulthood	P90-P121	P135-145 (14d after CCD)
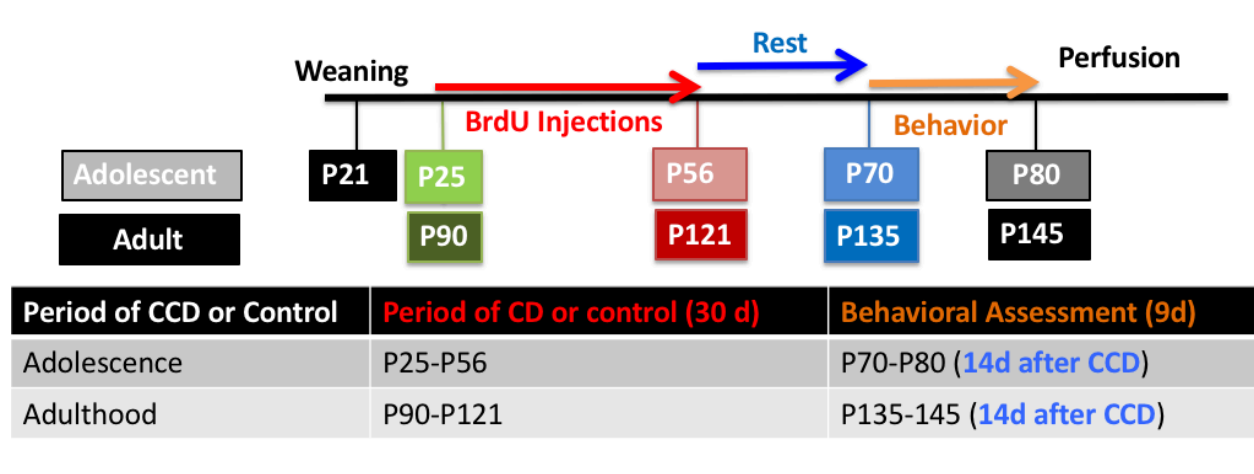
Figure 2. Mean (± SEM) number of PV+ interneurons (A), percentage of PV+ interneurons co-labeled with PNN (B), and PV+ cell numbers for all animals collapsed over sex and treatment (C). * = significantly greater compared to P80, $p < 0.05$
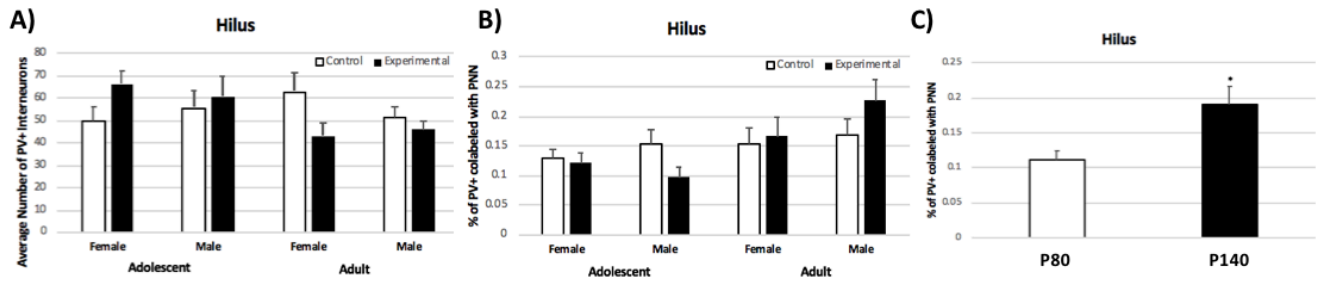
Figure 3. Mean (± SEM) number of PV+ interneurons (A), percentage of PV+ interneurons co-labeled with PNN (B, C), and BrdU/NeuN positive neurons (D). * = significantly greater when compared to P80, $p < 0.05$, ** = significantly smaller than control group, $p < 0.05$
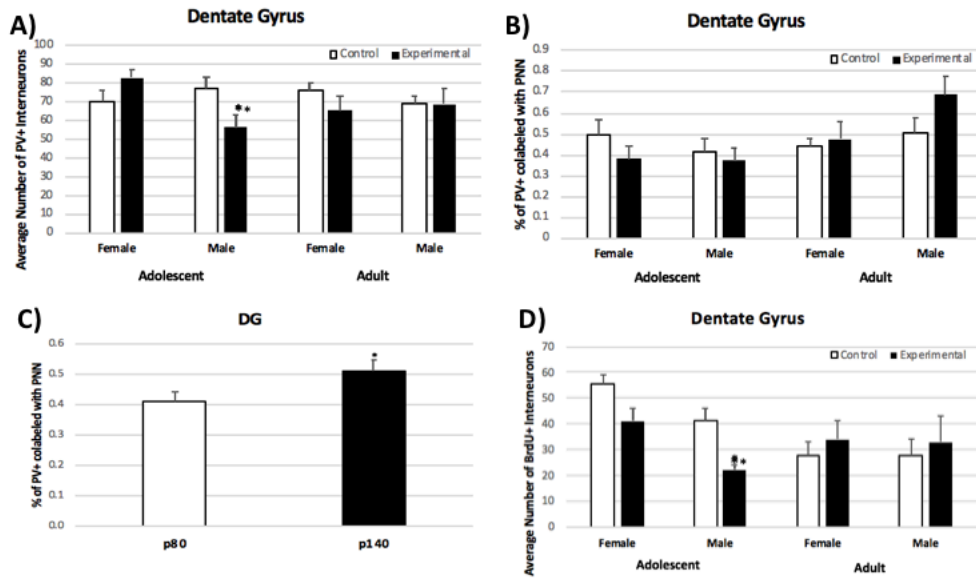
Figure 4. Mean (± SEM) PV+ interneurons, PV+ cells co-labeled with PNN, and BrdU positive cells in the infralimbic (IL), pre-limbic (PrL) and Dorsal Peduncular region (DP) of the PFC. Average number of PV+ interneurons (A, B,C), percentage of PV+ interneurons co-labeled with PNN (D, F, G), * = significantly greater than controls, $p < 0.05$; **= significantly smaller than controls, $p < 0.05$
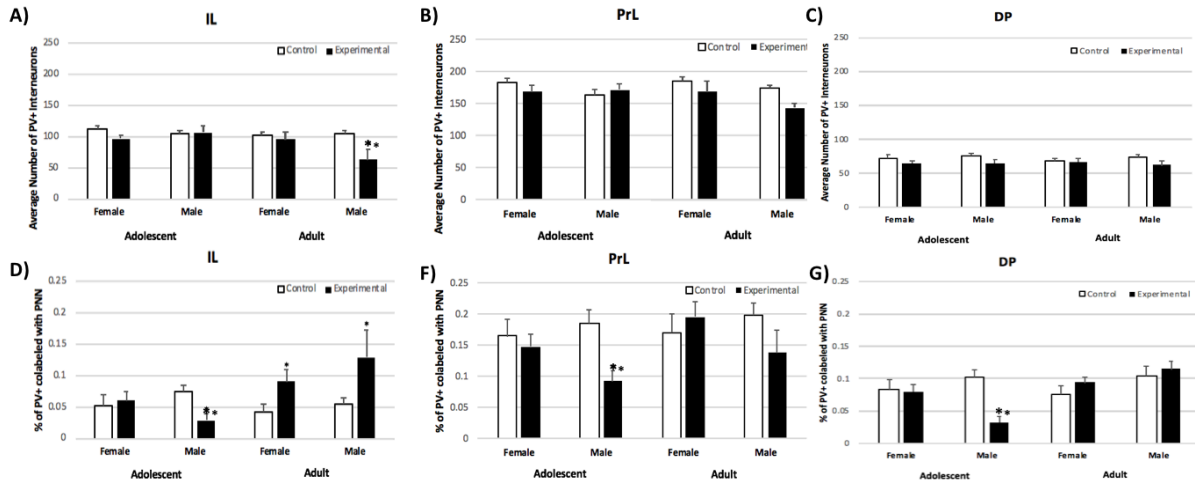
Chapter 4: Beyond "Just Science": Interdisciplinary Teaching of Sex to *Biology Students*

"Sex has always been as socially constructed as gender." Judith Butler

"The belief that non-human female mammals are intrinsically more variable than males and too troublesome for routine inclusion in research protocols is without foundation." Annaliese K. Beery and Irving Zucker

4.1 Introduction

One common feedback I get from students I have taught in the natural sciences for the past 5 years is that they were unaware biological sex was not the same as gender. This misunderstanding is due to their previous coursework in Biology or/and what they were taught in their home. In this chapter I apply the concepts of *just science* and *good science* to teaching undergraduate biology students about biological sex. Broadly, *just science* establishes that science is created in an objective vacuum outside of most social influences, and that implications of science arise from the interactions with society after the fact. I extend *just science* to describe biological sex as either (1) a fact of nature to be studied exclusively by scientists, or (2) a social construct sustained by a biased framework used by scientists. However, *good science*[1] as employed by Charis Thomson calls for sciences that are more intertwined with ethics, rather than sciences that just "have ethics". The concept of *good science* is useful to explore interdisciplinary teaching about biological sex because of the intertwining of **ethical, social, and legal** issues that co-exist with any description of biological sex. I extend *good science* to refer to the intertwined knowledge between Biology and feminist critiques of science studies. Biological sex through the lens of *good science* acknowledges social and ethical dimensions in science instruction and research. Therefore, I propose that the academic instruction of *good science* should be foundational to the introductory or general courses of the biological sciences.

The lens of "just science" applied to the realm of biological sex maintains disciplinary boundaries and places scientific research as superior to interdisciplinary scholarship. For example, let us consider the quotes by Butler juxtaposed with those by Beery and Zucker at the opening of this chapter. The first interpretation, from Butler's statement that "sex has always been socially constructed as gender" one might infer that the lack of "too troublesome" female animal models in scientific research, pointed out by Beery and Zucker, is not due to these alleged variations in biological sex but rather due to sexism in science culture and research. Feminist scholarship such as that of Donna Haraway and Fausto Sterling[2] makes the case for biological sex as a social construct based on biological research findings and a feminist critique of science studies. Given growing discussion around intersex or/and those with disorders of sexual development, it has become clear that biological sex does not align in a male and female binary, building a case for Butler's claim. In a second interpretation, one might give more weight to the statement of Zucker and Beery as scientists and dismiss Butler's statement coming from a "non-scientist". After all, there is an extensive body of scientific research regarding sexual dimorphisms, referring to traits that differ in males and females such as gamete size. Intersex cases make our scientific understanding of sexual dimorphism more complex; however, intersex individuals account for about less than one percent of the population. Therefore, intersex does not sufficiently invalidate sex dimorphisms that dominate biological science research[3] to this day. Thus, I concur with Fausto Sterling and other authors that our current knowledge on biological sex validates both the social

construction of biological sex and the importance of biological sex as a biomedical research area[4]. This balance of perspectives is not possible without applying the concept of *good science*.

If we apply the lens of *good science* to the interaction between the aforementioned quotes, we could explore how they might build on each other. For example, the context of performance theory in Butler's work describes that a repetition of acts, unconscious or conscious, is what holds together certain concepts like *biological sex*. This sequence of ever-changing acts could challenge the seemingly unchanging truth of biological sex, which is defined at times as exclusively determined by chromosomal sex or some biological measurement. Therefore, under the lens of performance theory, biological research on sex is an iterative enactment of scientists' social understanding of biological sex and not solely an empirical truth outside of social influence. The impact of social biases of biological sex has arguably led researchers to not use female animals in their studies for the past decades, with the justification that females are more variable because of their estrous cycle, despite female cycling being a disproved source of variation for most biological measures[5]. In summary, I aim to challenge two practices across disciplines sorted by *just science* by using an interdisciplinary analysis guided by *good science*. The first practice I challenge is based in feminist studies: the absence of engagement with social constructionist or feminist science studies when teaching science majors about biological sex. The second practice I challenge is based in the current research practices in the biological sciences, and this is the lack of inclusion of female animals in scientific research.

The first practice can be observed in the contents of General Biology and Genetics courses of undergraduate science majors. When looking over the most common textbooks used for these types of courses there is a complete absence of any interdisciplinary perspective or introduction to the complexities of topics such as biological sex[6]. Instead, we have an elaboration of the different types of sexual reproduction and the genetics of sex. Thus, implying and enforcing the notion of a science devoid of social influence that has been heavily critiqued by feminist scholars. In my work I introduce students to these perspectives so that they can contextualize the knowledge they have gained from science and their upbringing regarding biological sex. This interaction between upbringing and biological science education can be observed, for example, in the case of the compulsory surgeries intersex bodies were subjected to by doctors that seeked to affirm the male and female binary held by their biases and not biology.

The second practice is the exclusion of female animal models has serious implications regarding the rigor behind the study of biological sex. This culminates in scientific bodies of knowledge, such as pharmacology, that do not account for any traits under the category of *biological female*. This has created circumstances such as the exponentially higher risk of women to suffer side effects from certain medications when compared to males, given that the foundational science done in animal models is not accounting for female physiology. However, the research that has systematically excluded female animals was allegedly being objective about sex, given their assumption that results found in males could be applied to females without having to untangle the variation of the estrogen cycle [7]. The repetition of this ideal were the reiterations of acts that created a female animal model that was too "variable" to be accounted for in research. This body of knowledge about female biological sex that was sustained by social bias, along with no research to back it up or research that made blanket statements about female biology and the "erratic" effects of estrogen.[8] Therefore, the researchers are excluding a category of female biological sex in their research based on their biased understanding. This occurrence does not dismiss the biological process co-existing with bias, such as the biological sex differences that have led to females to have an increased risk of suffering side effects from medication or the effects of the estrous cycle

on other biological variables. However, we make sense of these biological processes with our social understanding intertwined with the research practices to describe biological sex and in its interactions with other biological factors. Thus, these biases have literally created a physical reality in which biological female individuals are more prone to suffering from pharmacological treatments[9]. This reality would not necessarily exist under a science that was more aware of *good science* and "just science" in general or more specific to biological sex.

In order to explore *good science* in the Biological Sciences classroom I provide three examples illustrating my argument about good science in the biological sciences classrooms based on my experiences in academia. First, I will use my personal experience in academia to underline some problems and contradictions of just science by exploring the tensions I have experienced in my interdisciplinary journey as a *maricón* through my academic career. Depending on the discipline and academic space, these tensions of me "being seen as an outsider or insider" in higher education by professors and students in Biology, highlight different potential roadblocks to *good science*. Second, I will explore how the nature of science makes instruction regarding the *good science* about biological sex a possible component of the general biology instruction with an interdisciplinary framework. Third, I will provide evidence of the biological sciences classroom where I introduce a lecture based on *good science* and culturally responsive teaching, demonstrating how the classroom offers a textual site for linguistic analysis and reflection[7]. My classroom analysis will focus on contrasting students pre-lecture and post-lecture descriptions of biological sex. The research question behind this analysis is "How biology students describe biological sex and in which ways their understanding is informed by biological sex as socially constructed?". I then elaborate on how student responses align with the framework of *good science* or/and *just science* that I previously described. Lastly, I discuss the potential benefits of further studying the impact of including *good science* in introductory or General Biology courses, based on the responses by the student's post-lecture.

The rest of this chapter will focus on three examples illustrating my argument about good science in the biological science classrooms. First, I will use my personal experience as an openly queer person trying to understand the relevance of biological sex to my gender and sexuality to underline some of the problems and contradictions of *just science*. Second, I will explain how I apply the nature of science to teach *good science* and culturally responsive teaching about biological sex. Third, I will provide evidence of the impact the concept of *good science* about biological sex has on students by analyzing their knowledge prior and after to my lecture. The research question behind this analysis is "How biology students describe biological sex and in which ways their understanding is informed by biological sex as socially constructed or a fact outside of social influence?" Lastly, I discuss the need to further explore and research the potential benefits of including *good science* in introductory or General Biology courses given the post-lecture responses of the students and my experience teaching at UC Berkeley for the past 5 years.

4.2 Experience as Evidence: "The Loud Maricón Scientist from Another Country That Does Not Do Science."

"I implore to all of you, future doctors and scientists of color, do not keep the arts and humanities outside of your scholarship." Maya Angelou, Keynote Speaker ABRCMS 2010

"I would completely remove your Woman, Gender, and Sexuality Studies from your resume as you apply to graduate school, it will take away from all the scientific research you have done

in the eyes of some admission committees. It is better to play it safe. Various summer and academic undergraduate advisors

***"When are you going to talk about actual science in laboratory meetings instead of this teaching stuff*?"** Various coworkers

My experience as one of the few queer minority students in my department is evidence of the opposition to *good science*, and how at times queer voices are undervalued in science. Thus, I have selected a few key moments through my education that have highlighted obstacles to *good science* and feminist critiques of science to be incorporated into the biological sciences discussions in higher education. My interdisciplinary work throughout graduate school has been met with considerable opposition, and my resistance to this opposition demonstrates the institutional barriers to *good science* from those that are "classically trained" to do Biology research. Along the way, I have faced faculty members questioning why I am not doing "real science" or, in the terms of my chapter, *just science*. I have been questioned regarding the need of incorporating my *good science* to scientific meetings or biology instruction. In some of my laboratory meeting presentations I have even been accused of being anti-science, because I try to incorporate the critiques of Historians of Science, Science Philosophers, and/or Feminist Scholars whenever I talk about my scientific research. I have been told by professors and colleagues in my department that the only reason I bring my background into a conversation is because I am not able to produce *just science*. This view of just science is lines with the common landscape portrayed in biomedical research that is dismissive of feminist or science historians' critiques of science[10]. This adversity has been incorporated to my own course-annotated syllabus for an interdisciplinary course focused on *good science* regarding biological sex for the Biological Science's curriculum. It is from this syllabus that I extracted the lecture that I will implement and analyze *good science* in the biology classroom for this chapter.

Primarily, I use these conversations around *just science* to shed light on the conflicts from which my scholarship emerges. These conflicts reflect various debates about *good science* regarding biological sex in the Biological Sciences classroom, such as the broader context of the history of science being irrelevant or harmful to train future scientists[11]. Secondarily, I want my scholarship to be transparent about its foundation and its limitations. I am primarily a neuroscientist who has maintained a training in feminist science studies throughout my undergraduate and graduate education with the goal of highlighting the need for more dedicated efforts to teach *good science* in the biological science classroom for the past five years. However, in order to promote social change, some of the unwritten rules of biology scholarship in academia have to be shed. One example of these unwritten rules is the assumption that a biology thesis has to be limited to the research performed in the laboratory.[12] This section of my chapter attempts to bring the raw life experience grounding this chapter and my scholarship, not only from the preface to this thesis or from the assumption that my experience is different just because I state that I am a queer Puerto Rican neuroscientist. In this section I am employing my privilege as a graduate student to revisit and reshape my hardships around my sex and gender into an academic intervention. This chapter is crafted from the imperative to not let the challenge of interdisciplinary studies be used as an excuse to not develop interdisciplinary scholarship within the Biological Sciences and the minorities that compose it.

I refer to myself as a *maricón[13]* because this is a common curse word in Puerto Rico to designate someone that is being feminine or flamboyant, equivalent to *fag* in the English language.

It is also used to designate someone that is being unreasonable or "evil". Growing up, I dreaded to be called *maricón*, as I identified as being masculine and rejected any hint of being gay. Additionally, this word was constantly used in comedy sketches or in often violent discussions between my parents or friends. Thus, I reclaim and identify as *maricón* to bring into focus the opposition to the intersectionality of my sexuality, ethnicity, and interdisciplinary scholarship.

Connecting my life experience with my academic development goes in line with my teaching philosophy of "personal is political", as I have learned from feminist scholars. I highlight how the power behind our narratives connect with bigger-picture social and political discourses, as it is used by feminist activists. However, my past attempts to adopt the "personal is political" in my discussion sections and instruction in Biology have been unfruitful. Many of my past students have communicated that for them *the political* opposes *being professional* in science. Therefore, I prefer to use "personal is professional" in my teaching to biology majors, to help students understand the notion that "personal is political" without them shutting down if they hold the notion that politics and science are in opposition to one another, thus connecting with students' goal of becoming future professionals. In addition, "personal is professional" allows me to highlight the extent that being a *maricón* has shaped my academic path.

Through high school and college, I was exposed to academics, biomedical research projects and summer research internships. At first I thought that understanding gender and sex at the biological level would grant me *good science* as a leverage to discard all the hate towards anyone being a *maricón*. Family members and society often refer to the observable nature of male / female genitalia for reproduction to justify their heteronormativity and homophobia. In my second year at college, I was unsatisfied with the course material and the prestigious research opportunity programs for minorities[14] that focused exclusively on *just science*. I started to take courses on the intersection of science, sexuality, and feminism in order to address this shortage of *good science* in the biological sciences. I was further motivated by a quote by Maya Angelou that I came across when I was a sophomore attending the Annual Biomedical Conference for Minority Students' 10th year anniversary. This propelled me to gather tools from different disciplines in order to understand the interactions between science and society regarding gender and sex. This interdisciplinary knowledge allowed me to contextualize the pressure I endured as I grew up in a heteronormative society and how my education in *just science* impacted any other *maricones* looking for refuge from society's judgement in the biological sciences. Collecting as many interdisciplinary lenses agglomerated under the Woman, Gender, and Sexuality Studies to create *good science* and questioning *just science* became my main interest in the last years of my undergraduate training as a neuroscience researcher.

Two examples of experiences in my graduate school career portray the roots of *good science* and *just science* in the academic circles that research biological sex. The first one was the Seventh International Symposium on Vertebrate Sex Determination at Kona, Hawaii in 2015. The conference was attended by the top researchers that focus on sexual differentiation or disorders of sexual development. The professional and serious atmosphere of the conference pervaded every presentation and discussion, except in the talks that introduced perspectives from activists and patients regarding the disorders of sexual development. To me, the most memorable aspect from this conference was how various senior researchers laughed at a presentation that incorporated the perspectives of individuals with disorders of sexual development. The presenter reminded the audience that some groups build their identities around their own disorders of sexual development, and that the work being done at any level can grow and develop from open communications with the community. Some senior researchers nearby added to their laughter a statement mocking the

idea of building an identity around a disorder of sexual development. Namely they described a person with hypospadias, a condition characterized by the urethra opening being in the shaft of the penis, trying to pee in a men's bathroom and accidently urinating on the person next to them. They stated that these are just disorders, not something to build an identity on, and continued to dismiss their activism as misinformed science. The laughter and the haste to discard people's identities that are beyond the binary of male and female was shocking to me. The separation of the social implications of science on society made by these researchers are a good example of *just science* regarding biological sex.

The second experience I want to consider was at the Interdisciplinary Conference - A Critical Moment: Sex/Gender Research at the Intersection of Culture, Brain, & Behavior in 2015. This conference featured key researchers from Molecular Biology, Psychology, Sociology, Anthropology and Film Studies, many of which were part of Women, Gender, and Sexuality Departments at their home institutions. The conference was divided into panels that had one person from each of these diverse areas of study. Each panel was followed by questions from the audience. In this instance, the crowd was always respectful and open to the intersectionality of work around Sex and Gender, and every aspect of this conference was founded in *good science*. However, another problem was clear. Nobody was actually asking questions to the panelists. The moderators only asked questions to those panelists within their own field, not crossing disciplinary boundaries. These tensions made me wonder what kind of training or intervention is needed in order to facilitate interdisciplinary conversations around *good science*, thus feeding my enthusiasm to keep working on the design for a curriculum tailored for the biological sciences that could create and promote tools that enable these conversations, all while validating unique student experiences as data that can be used to keep building higher education with diversity in mind.

4.3 Those who can't… hyper-specialize and employ interdisciplinary ignorance?

"Science is a way of knowing and there are other ways of knowing. Science indicates what can happen in natural systems, not what should happen." **Next Generation Science Standards, Nature of Science, High School Learning Objectives 2013**

"Much false debate could be avoided by careful consideration of whether or not alternative hypotheses are or are not mutually exclusive alternatives." **Scott A. MacDougall-Shackleton**

It is a common expression that "those who can't do research… teach". I argue that promoting *just science* is done by those who hyper-specialize in research and cannot see the relevance of other disciplines, including pedagogy actively tailored to student's interests. These two quotes target different audiences but address similar problems of hyper-specialization in Biology that are reflected in the General Biology textbooks such as Campbell's Biology. The first was intended for K-12 high school science teachers, and the second is from a specialized review for the field of behavioral neuroendocrinology, yet they are both addressing the same problem of *just science*. For the first quote, the nature of science is a learning objective about what science can do, what it cannot do, and how it interacts with society through K-12 education.[15] Thus, it encourages students to understand the limitations of science and seek knowledge from other disciplines as they interact in society. However, the nature of science has been defined in different ways and described as one of the most challenging topics to incorporate to science instruction as teachers report that they are not trained on how to teach it. Hence, it becomes imperative to further

develop a framework to understand and teach the nature of science. I propose that the nature of science is a foundational block to teach *good science* since it entails an awareness of the interconnectivity of science, other disciplines, ethics, and society.

The second quote names false debates amongst scientists as those debates would not happen if the scientists involved made an effort to identify whether their hypotheses are mutually exclusive of one another. For example, one scientist might argue that the genetics in sex chromosomes define biological sex, while another could go into a debate regarding how hormonal levels are actually determining biological sex. These two ideas are referring to different layers or reductions of how biological processes work and can be studied. For example, hormonal studies and genetic studies do not look at the same aspect of biological sex, so a debate that possess them as mutually exclusive would be deemed a false debate. Thus, the question arises of how students in STEM respond to the concept and application of *good science* that would disambiguate such debates and promote interdisciplinary learning.

These debates are relevant to *good science* because they highlight the current challenges of integrating knowledge between closely related disciplines, such as genetics and endocrinology in Biology. Therefore, the challenge of integrating more distant disciplines and ways of knowing in *good science* is even more evident given different epistemologies. Regardless of this difficulty, interdisciplinary work has been done around biological sex integrating different disciplines in a successful way, such as the work by Fausto Sterling's work on intersex[15], Gillian Einstein's work on the primary literature of the interactions of sex and the brain[16], and Sari van Anders' work on the psychology of gender[17]. These three scholars have department appointments in both their Science and Woman, Gender, and Sexuality Studies departments in their home institutions and offer elective courses that explore their particular intersection of their disciplines. Courses of this interdisciplinary nature impacted my personal development as a scientist. I developed my own annotated syllabus based on *good science*. My work explores the impact of one lecture that could be incorporated into a General Biology classroom as a gateway to bring attention to *good science*, using the social construction of biological sex as an interdisciplinary case study. As mentioned before, I recognize that the *just science* regarding biological sex has been tied to various social problems around sex and gender. Furthermore, biases around biological sex have fostered the exclusion of female mammalian animal models in laboratory research, negatively impacting the quality of science and women's health, according to the studies and perspectives presented by Irving Zucker and others[18]. Thus, my objective is to teach future scientists that scientific research on biological sex goes hand in hand with interdisciplinary scholarship by incorporating *good science* in General Biology instruction.

The unifying framework proposed by MacDougall-Shackleton between the levels of analysis and biological levels of reductionism[19] in his review "The Levels of Analysis Revisited" works in unison with the feminist scholarship I have referred to in this chapter, but also with the training of scientific research. As argued by MacDougall-Shackleton, promoting scientists that understand different epistemologies based on their level of analysis and/or reductionism allows for false debates and hierarchies about different categories to be disambiguated or to not happen at all. In addition, it gives the proper framework to understand that as we move from different levels of analysis new questions can emerge that take into account each level and the types of questions that can be asked. One could apply MacDougall-Shackleton's framework to developing a lecture about biological sex by describing how sex is studied at the genetic level, at the neurological or psychological level, or at the behavioral level, all without assuming that one category is better at explaining biological sex than another. Once students understand these different levels of analysis

for biological sex, students can apply this framework when studying another subject by questioning how it is studied. Most importantly, students would discover that one level of analysis is not more important than another for determining biological sex. This framework aims to promote a level of academic rigor that fosters *good science*, the analytical skills useful for research, and knowledge about the nature of science.

In pedagogical research, the propensity of using biological sex to justify biased and binary categories of sex and gender is a phenomenon known as essentialism. Essentialism regarding biological sex can potentially be put into question with the levels of analysis and *good science*. Addressing essentialism is relevant as it has merged with neuroscience knowledge to create a neurogenetic essentialism[20]. This specialization of essentialism has been used to justify the lack of women in STEM because of their female brain being less structured than those of men for the subject matter by leaders in the field. Therefore, highlighting another known hazard of this type of essentialism when it is mixed with *just science* as I pointed out with female mammals inclusions in research and intersex surgery, which literally materializes the social biases held by those in power in the bodies and lives of those being oppressed. After such materialization and creation *just science* is used to justify the "objectivity" and unchangeable "essence" of the biases created, as explained by the concept of situated knowledge coined by Donna Harraway.[21]

In the lecture I designed I present students with a case study of intersex individuals being mutilated or/and having surgical intervention by doctors that needed to reproduce their imaginary categories of female and male, that they justify with generalized observations and social understanding[22]. Intersex conditions can be hazardous to the individual's health, but this is not the case with all of the intersex phenotypes[23]. Thus, providing an example of how different levels of sex are simplified or ignored, and how our social biases can manifest bodies as we wish them to look in order to call them natural and healthy. The other example I provide is the lack of female animal mammal inclusion in research with the excuse that their estrous cycle makes them more variable[24]. This belief had not been sustained by research findings for every field, and when research was done to investigate this bias it was found that if anything males are more variable in certain traits. Therefore, biological sex can influence certain traits, but it is not as simple as saying that females or males are more variable, as it depends on the trait being studied[25]. However, studies during the past decade have been conducted primarily in male rodents. The data from male-centric mammalian research has been extrapolated to pharmacology and medicine for both male and female human subjects, which has led to more instances of pharmacological side effects in females when compared to males. These biases about males and females have manifested human bodies and research corpora that have caused harm to parts of society, while claiming to be objective. These manifestations of bias would face more obstacles and be questioned if the scientific community made understanding *good science* and the levels of analysis required for scientific training.

In the next section, I explore how students conceptualize biological sex as a binary or on a spectrum, including cognitive categories such as gender and sexuality by analyzing students pre-lecture and post-lecture responses. I start the lecture by sharing how my own background growing up as a queer Puerto Rican shaped my interest in biological sex and feminist critiques of science. The lecture I designed as a curricular intervention reveals to students how intersex individuals demonstrate that biological sex is more varied than the male and female binary. I also contextualize empirical phenomena we categorize under biological sex and how this materiality can be useful to analyze under the binary of male and female when we consider the impact of not including female animal in biology research. The issue arises when we define these categories as unchangeable

essences that remain the same across different levels of analysis and not acknowledging when new categories can arise such as intersex biological sex, non-binary gender, amongst the many variabilities. In my lecture I highlight the social construction behind the conceptualization of biological sex, promote *good science*, and deconstruct essentialism. Afterwards, I assess students' understanding of biological sex and the questions that pop up through a linguistic analysis of students' pre- and post-reflections.

4.4 Introducing Feminist Science and Experience as Evidence in the Biology Classroom

"*At birth, you are assigned a gender based on XY chromosomes, however, through sexual reassignment surgery, individuals have the ability to alter their biological sex.*" Student pre-lecture opinion

"*We know that the sex of an organism is defined and identified by how one is organized for sexual reproduction. An organism's sex as a male or a female is known by the way reproductive acts are organized. Things like surgery and hormones do not have the ability to change one into the opposite sex, as they can only affect appearances. I would like to include a quote as I was doing my research on this question and it says "changing sexes is a metaphysical impossibility because it is a biological impossibility.*" Student pre-lecture opinion

Academic studies have recently called attention on understanding biology students' *intuitive biological thinking* and improving students' academic achievement by questioning their, at times fixed, perceptions of biology.[26] In the same line of thought, exposing STEM students to the multiple layers of biological sex and how they are dependent on social constructs through *good science* and the levels of analysis, can help them question fixed views around sex that are not backed up by rigorous science. If these views are left unquestioned, they could lead to discriminatory behavior in academia and their home communities, such as homophobia, transphobia or misogyny based on their unandressed *intuitive biological thinking*.

The goal of this section is to showcase and analyze the students' reactions to the statement "Biological sex can NOT be changed" by comparing and contrasting trends in their responses, including their feedback and takeaways from the lecture. I am taking into consideration both semantics (choice of words as objects regardless of context) and pragmatics (choice of words as subjects in regards to context) in the way students formulate and frame their responses.[27] This linguistic analysis is not deterministic, and my aim is not to prove or disprove any fact but to showcase possible assumptions made by students regarding sex and gender in the context of Biology. Instead of using a strict semantic methodology to find objective meaning in students' work, which would result in possible fallacies and misunderstandings, I use a more simple and functional method of annotating, abstracting and analyzing a corpus consisting of the students' written work. There is, of course, overlapping between the students' responses and the reasoning behind those responses. For this reason I am splitting the analysis process into two parts: (1) looking at trends in agreement or disagreement with the statement, and (2) looking at the trends in the reasoning behind their responses and reactions. This analysis aims to identify obstacles faced by biology students when incorporating critical interdisciplinary perspectives in relation to biological sex.

My main research question is the following: "To what extent do college biology students consider biological sex as being determined at birth and unchangeable?" My other research questions are: (1) Prior to the lecture, to what extent do college Biology students describe

biological sex as unchangeable? (2) What stands out to biological sciences students from a lecture on the social construction of biological sex? (3) What questions do students have after the lecture? The first research question is investigated by quantifying the linguistic analysis of students' pre-lecture responses to the assessment question: Do you agree or disagree with the statement "Biological sex can NOT be changed. The second research question is studied by analyzing students' post-lecture responses to the following question: List at least 2 takeaways from Emilio's lecture on Biological Reductionism. For the third research question I used the following post-lecture prompt: Describe what was missing or unclear in today's lecture. From these analyses, I aim to use biological sex as a case study to identify the benefits and challenges of incorporating *good science* to the Biological Sciences classroom. Thus this preliminary study is aimed at exploring the potential benefits of further researching these questions with future increased sample size and more robust analysis.

4.4.1 Students Pre-lecture Responses to the question: Do you agree or disagree with the statement "Biological sex can NOT be changed"?

Students received this question two weeks prior to my lecture in the course and submitted their responses one week prior in order for me to adjust the lecture to their concerns. In the 5 years of giving this lecture I had never seen more than half of the class, 46 out of 83 students, disagree with the statement. The main reason for disagreeing was the awareness of sex reassignment surgery or hormone replacement therapy. In total, 27 students referenced this reasoning in their responses. The statement that best highlighted the power of surgery read as follows:

"*At birth, you are assigned a gender based on XY chromosomes, however, through sexual reassignment surgery, individuals have the ability to alter their biological sex.*"

In addition, students that disagreed would often highlight the importance of the individuals' choice to identify as they want and to pursue happiness. In total, 12 students responded to their disagreement with the statement by mentioning this reasoning. An example of this high regard for the individuals' self-identification can be read in the following quote:

"*Personally, in my opinion, I feel that it is not my place to tell someone what their biological sex is or tell someone that they can't change their biological sex. I have only met a couple of people who have changed their biological sex (they openly speak about their experiences) and from them I have learned that it really is their decision to make and in the long-run if it makes a person happy and allows a person to live their true authentic self, then in my opinion, who I am I to tell them that they can't change their sex or that their biological sex cannot be changed.*"

The other most common rationale to disagree with the statement was knowledge of the existence of animals that can change their sex depending on environmental conditions. In total, 10 students mentioned being aware of cases where this occurs in nature, and used this statement as evidence for their disagreement with the statement. One student wrote:

"*I typically appeal to logic with my reasoning and it is a fact that biological sex can be changed. In several animal species, individuals can change their biological sex dependent on what*

their current environment/ population calls for. Even in humans, sexual reassignment surgery exists."

Another variation pertaining to the Animal Kingdom came from students that had recently heard from Dr. Tyrone Haze, and the hormone disrupting effects of Atrazine in changing the sex of certain frogs. One student highlighted that:

" *Two weeks ago, I would have said that Biological sex cannot be changed. However, after hearing Professor Haye's lecture, I realize that Biological sex can be changed. A big discussion point from the lecture was the influence that Atrazine had on frogs. This toxin would change the frog's sex to make them hermaphrodites. Similarly, Professor Haye predicted that if men fell into river streams with Atrazine their biological sex would also change. Changes in the physical bodies of these men would arise. He also spoke about how communities were drinking contaminated water with Atrazine and these individuals were prone to undergo changes in their biological sex, which might be generational.*"

Thus, students that disagreed with the statement had a degree of knowledge prior to the course, or acquired in the course that allowed them to highlight how Biological sex can be changed, by focusing on individual agency, medical advancements, and biological sex fluidity in nature. All while not addressing sex chromosomes or sex identified at birth as an irrevocable determinant of biological sex, which was the main argument of students that agreed that biological sex cannot be changed.

Exploring the responses of the 21 students that agree with the statement, and thus, that biological sex can't be changed referred to this as a known fact of science that is not up for debate. For example:

"*When studying the biology of animals and other living organisms, they are referred to as either male or female. This applies to various different organisms such as plants or livestock and is even applied in a textbook definition of humans, although some people may think differently about this*"

From this view that sex is an unchangeable part of living beings then students focused on different layers of sex for their argument. In total, 18 out of the 21 students who agreed with the statement referenced this reasoning. Regarding genetics, most students focused on sex chromosomes. For example:

" *I agree with this statement given the information I currently have which is that either an XX or XY chromosome determines biological sex*"

Two students recognized that even with the existence of sex surgery, biological sex cannot be changed because no treatment can ever change an individual's sex chromosomes, as exemplified in the following response:

"*We know that the sex of an organism is defined and identified by how one is organized for sexual reproduction. An organism's sex as a male or a female is known by the way reproductive*

acts are organized. Things like surgery and hormones do not have the ability to change one into the opposite sex, as they can only affect appearances. I would like to include a quote as I was doing my research on this question and it says "changing sexes is a metaphysical impossibility because it is a biological impossibility."

This highlights the reproductive success rationale of other students, stating that regardless of what is done by the individual, their genetics and reproductive capabilities will never be changed. This is represented in the following statement:

" Yes, a man can undergo surgery to have his penis and balls converted into a vagina but is he then able to produce offspring and women can have her breast and ovaries removed and changed into a penis so at this point although they may identify as the opposite sex they are unable to reproduce. "

In summary, the students that agreed with biological sex not being changeable relied on individuals not being able to change their genetics or gain reproductive functions they did not have before.

Lastly, in the minority of 7 student responses there were some students that even though they understood that genetics cannot be changed, they also knew about the existence of surgeries and treatments of sex reassignment. However, they were unsure of how those interacted and what their response to the question would be. For example:

"I would usually agree that biological sex cannot be changed, but I am aware there are drugs used that can help transition from one sex to another. However, I am not really aware if the transition is 100% to another sex. So you are able to change biological sex, but to a certain extent from what I am aware of."

Therefore, even with students that have certain exposure to sex reassignment technology, the image of genetics being unchangeable can still be confusing for them.

When applying these results to my first research question to my main research question "To what extent do college biology students consider biological sex as being determined at birth and unchangeable?" I can observe that prior to the lecture there were already 46 students that disagreed with the statement in contrast to 28 students that agreed with the prompt. Thus, more than half of the class supported biological sex to not be solely determined by genetics or sex identified by doctors at birth. The 28 students that agreed with the prompt and thus supported that biological sex could not be changed brought up argumentation regarding how sex is determined at birth by your sex chromosomes, and later in life by the success of reproduction. Lastly, the 7 students who were unsure tried to make sense of their knowledge regarding how genetics can't be changed with the awareness of transgender individuals and the technology that allows for sex reassignment procedures. Thus, from this first iteration, we can already observe a gap in how students value their knowledge regarding sex chromosomes at the time to establish biological sex as unchangeable.

Given that I had student responses one week prior to giving my lecture and having analyzed their core arguments I modified my lecture to focus on 3 key points. First, I talk about how my identity as a queer Puerto Rican in the biological sciences shaped my interest and career path to be

interdisciplinary. Second, I discuss how to balance biological reductionism with the notion of a false debate in biology from Scott A. MacDougall-Shackleton. Through reductionism, biology breaks down different levels of life that differ in their scale and functions. It was important for me to acknowledge how useful biological reductionism is to study life. At the same time I acknowledged the tendency in Biology to ascribe greater value to the smallest scale that can be studied, which in current biology is genetics. Thus bringing into the equation the balance with the notion of false debates, or debates that pin different levels of reductionism as more or less important than the other, when they all explain a different layer of biology. Third, I bring into attention how scientists and medical doctors have replicated their biases about sex. The first example being the medical enforcement of a biological sex binary maintained by mutilating and classifying as sick intersex bodies in the past. The second example was the current data on the exclusion of female animals in biological research because of the assumption that females are always more variable. After the lecture, students submitted their responses to the following questions. "List at least 2 takeaways from Emilio's lecture on Biological Reductionism. Describe what was missing or unclear in today's lecture" which were analyzed for the following sections.

4.4.2 Students Post-lecture Responses to the question: List at least 2 takeaways from Emilio's lecture on Biological Reductionism.

I decided to ask students about their main takeaways post-lecture instead of directly reinstating the first question about agreeing or disagreeing about biological sex being unchangeable in order to give them the liberty to highlight what was memorable for them. The intent is for students to explain what they understood and then to expand on what they wanted to learn more about with the second part of the question. This study is not attempting to test percentage changes of students' opinions after the lecture, but rather to bring into attention how students interact with this topic and to hear their questions about interdisciplinarity in order to encourage possible future studies. There were three main focuses on the takeaways that students had post-lecture: the impact of bias on science and how to be mindful about it (the main takeaway with 47 students in total), the complexity of biological sex (21 students in total), and the need of diversity in science (15 students in total).

Under the category of the impact of bias in science I had two main subcategories. The first subcategory being comments about the impact of biological reductionism on intersex individuals or animal science. The second subcategory was on how to be mindful of biases and how to create *good science*. More than half of the students showed interest and concern on the impact of biological reductionism on intersex individuals. Students also highlighted how disturbing it was for fully trained scientists and medical doctors to not delve deeper into their biases for the sake of their research or patients. For example one student wrote:

"*A second key takeaway from Emilio's lecture was this concept that doctors were conducting intersex surgery and that research has had a selectivity for researching with male rats than female rats, because females are inherently variable. The information he gave about doctors conducting intersex surgery was astonishing and disturbing to me because their bias and perhaps insensitivity led them to categorize certain individuals as in need of an "emergency" surgery to fit the norm-definitions of man and woman. In a similar way, this selectivity with male rats research is catered to understand men's bodies. This lack of research to understand women's bodies, implies medicine and science has not been and continues to not tailor to the needs of women. As a*

future doctor, it is my responsibility to address any biases that might exist that will hinder science and its impact on patients. Furthermore, it's my responsibility to not make assumptions but instead to explore the ambiguous and be okay with ambiguity rather than attempting to objectify, false objective claims."

Students often cited how helpful learning about the layers or levels of biological sex is, showing surprise at the notion of how biases are held by doctors and scientists alike. This is something that appears to be barely addressed in some of the students' education in biology. One student wrote:

"*First of all I feel a lot more clear on the fact that gender is not defined by genetics the way I thought. I hold the view that we can change and choose our gender, but I was unaware of the diversity in nature that highlights this fact. I was also really stuck by when Emilio mentioned that doctors are people, hence they themselves had fallen into a trap of socially constructed gender and went to the extent of mutilating new-borns. I find this atrocious, but what was most sticking to me was Emilio's ability to help me see that this idea of binary gender is so ingrained in many cultures even doctor's who study biology all their lives failed to see, and sometimes still fail to see, that the reality is much broader.*"

This kind of remark at times was accompanied by linking the importance of understanding biological reductionism to better understand biological sex and avoid false debates. One student wrote:

"*One thing I learned about Emilio's lecture was that we need to be careful about biological reductionism. I don't think I knew what this meant before but, his explanation made sense to me and I understood why we should be skeptical about it. For example, I also had the idea that biological sex could be defined by chromosomes present (XY or XX). But, then I see then I understand that this is also a genetic view and that changing biological sex can be changed through hormones.*"

In addition students also mentioned that it was helpful to have an example of how nonsensical it would be to use genetics to define the body. One student wrote:

"*Emilio's discussion of biological reductionism was insightful, as I learned that there is more to the sexual biology of organisms than what is taught in my biology classes. Furthermore, I learned that reducing biology to physical aspects neglects many complicated, and important aspects of biology. I thought Emilio made an interesting point when saying that we don't refer to the eye as being feet, even though the genetic makeup may be the same. Similarly, we cannot identify males and females based solely on socially accepted constructs (gonads, behaviors, etc.).*"

Thus these comments highlight the possible deficiency in students' biological sciences education regarding how the biology they are studying is produced, the significance of the different layers that can be studied, or the impact of science on society.

The second category I used to analyze student responses was the complexity of biological sex. Under this category, students wrote about the many factors that can influence biological sex and the confusion between sex/gender. For example one student wrote about the new complexity of biological way in the following way:

" Another takeaway that I got is that we can't just focus on one aspect of the biological sex to define it or to determine if it can change or not. We cannot just state that because we have predetermined sex of chromosomes that we must conform to the binary sex, female or male. They are many other aspects that contribute to biological sex."

This was accompanied by students that paired this new found complexity and its malleability. One student wrote:

"One of the main points that I took away from Emilio's lecture was that biological sex has a degree of natural plasticity, as even gene activation/influence isn't set in stone. It's really interesting to me that reductionists who claim some scientific deference to the role of genes in biological sex are really ignoring actual science pointing to the contrary. Another key takeaway for me was the incredible harm that reductionism can do, particularly with regards to people who don't fit into neat categories."

When teaching this subject it is fascinating to me how some students conflate sex with gender as being one and the same or in the least determinant of one another. For example:

"One takeaway from Emilio's lecture on Biological Reductionism is that when talking about if biological sex can be changed, many of us bring gender into the discussion. Personally, when we were asking if biological sex can be changed, I did think about gender which I now realize--through Emilio's lecture-- that we should not do that and it is a different topic of discussion."

Given that my intent is just showing student how sex, gender, and sexuality can or not relate depending on how the person in question identifies and relates them in their own identity. One student noted this spectrum as something new to them and wrote:

"One takeaway from Emilio's lecture was that we have to be mindful not to confuse or interchangeably use biological sex with gender identity, because even though they are related, ultimately they're not the same. Biological sex may influence gender identity, but they are still their own concept. The image that was used in the lecture slides was a really helpful visualization because it shows how these are spectrums, but each one is their own spectrum."

Therefore, it becomes more apparent that some students are not receiving the tools to relate what they are learning in biology to the implications of this knowledge in society.

The third category was the need for diversity in science. Here I compiled comments regarding addressing personal experiences from minorities in science. It is unusual for biological sciences to tap into students' personal experiences or present the relationship between the personal and the political, as I tried to show in my lecture. One student wrote:

"I personally liked this presentation because it touched on topics very sensitive to my own community as well as in the science community, as they are not touched very frequently."

This depersonalized science is a very common complaint from students coming from different backgrounds. Another student wrote:

> *"I think it's very common nowadays, especially in my Latinx household, to get criticized for identifying as something other than heterosexual. Emilio ultimately taught me that it's okay to be different and that his gender norm or sexuality shouldn't affect his career in life because it's important to have diversity in the STEM field. In other words, it's not about who you are it's about what you can bring to the table."*

Exploring my second research question, "What stands out to biological sciences students from my lecture on the social construction of biological sex?", students paid more attention to the aspects of the lecture that would allow them to become better scientists regarding addressing their own biases and understanding the complexity of biological sex. Students also highlighted the impact of taking into account their backgrounds with their multiple acknowledgements of the importance of bringing certain issues to the table such as Latinx and LGBTQ+ experiences. These two at times combined with students that understood it is their duty to understand diversity and be comfortable in the gray areas to better serve their future patients or students. In the next section I will go over the most common questions or interests students had when asked what was missing from the lecture or what they wanted more information on.

4.4.3 Students Post-lecture Responses to the question: "Describe what was missing or unclear in today's lecture."

The last of the two post-lecture questions is relevant to further probe students' interests in the topic, and what areas they want to explore more. In addition, this second question also gave students the chance to share what was not clear and why. This question was optional and only 20 students responded to it. I will bring attention to those student responses that showed resistance to the material because they will highlight some of the positions that oppose *good science* in the biological sciences.

One lecture will never be enough to address and dismantle the biases people around biological sex. This was show by students that repeated what chromosomal sex was and how it cannot be changed, and thus biological sex is unchangeable. One student wrote:

> *"I do not mean to attack or come off as mean, but we all have a starting point. You are biologically born female or male. What you decide to do after that is completely your choice and should not affect other people in any way or form. Although it was an interesting discussion, I was a bit confused of the end goal with this lecture. Research studies rely on biological sexes like males and females for data, and I do not think changing this would be beneficial in anyway. I know Emilio talked about studies where they only focus on males. But there are plenty of studies that also examine female reproduction and biological processes."*

During the past years of me teaching this lecture there are always students that double down on how biological sex cannot be changed because of chromosomal sex. Undoubtedly, the concept of biological reductionism is also something that some students struggle with. Hence, reinforcing the occurrence of students that are unable to understand the tools or frameworks that produce all the information about biology that they are learning. One student wrote:

"I am still unclear about what biological reductionism means I don't completely agree with how biological sex is not unchangeable because of chromosomal sex—I feel like it depends on one's definition of biological sex regarding this. I think that biological sex is the sex that one is born with, aka their chromosomal sex, and thus this sex is not changeable unless one changes their genes. Sexual differentiation starts from the moment a gamete is fertilized to become either XX or XY, and although other factors can affect the differentiation (e.g. hormone levels), the biological sex remains pre-determined, at least from my perspective and understanding"

Another observation that came up was regarding the lack of inclusion of female animals in science studies. One student wrote:

"In one of my nutritional science classes, I remember the class discussing a specific experiment that focused on female subjects, and the concern was whether hormone fluctuations and the loss of blood during menstruation would change the result of the treatment. I wonder if these are some of the primary reasons why most researchers prefer using male subjects rather than female subjects."

On one hand the tendency of people to see female animals as more variable when they are having hormonal cycles has been questioned and shown to not be true for all measurements, with males being more variable in certain traits as shown by the multiple studies spearheaded by Dr. Irving Zucker. However, this has been a recent challenge that has started to change research practices and is hard to grasp at times given the plethora of past research done with these assumptions.

The two previous examples show that students struggle to process how to interpret the importance of different levels of biological complexity and of bias in scientific research. This shows the potential of incorporating *good science* into the biological sciences classroom. The benefit is for addressing the limitations and benefits of scientific research from the start in the biology classroom. It is this balance between acknowledging the importance of scientific research around biological sex, but being more careful of the scope and role it has in society by balancing with the critiques of feminist critiques of science studies. This balance aims to not dismiss the science entirely, which is implied in some feminist critiquest of science, but to give it the needed context to understand its creation and implications.

Once we have this balance in mind, even if we haven't resolved it entirely, biology students can start to ask questions that can help them understand and demand *good science* in their education. There were some questions or statements made by students that best reflect the kind of reasoning that I associate with *good science*. One student wrote:

"From this lecture, what I want to discuss further is about the fact that science is biased and not inherently objective at times. However, my question is why aren't biologists or scientists discussing about all the other factors that determine or classify our biological sex? Is it simply because of the biased and subjective view they have of the social definitions of biological sex? Is the benefit in biological reductionism simply due to convenience of understanding scientific information for the general public? Or is it because scientists and biologists are also a part of this biased society, so their findings and research are also biased?"

The realization that there is a whole plethora of levels in which biological sex is studied by experts rises questions of why certain science studies simplify sex, while others ignore it. This is even

more alarming when you consider the studies about the impact of not including biological sex as a variable in animal studies.[28] At the same time students thinking about *good science* are also keeping an eye on the social implications of such science. One student wrote:

"What I'm left wondering about is how whatever inherent sex differences may influence health treatment options or outcomes. For example, I think he stated that women face more adverse side effects due to the over-representation of male animals in drug research. There are clearly some areas in which knowledge of differences is important, but I feel like there's the possibility of people misusing this information to deny transgender identities."

In line with students highlighting the usefulness of learning about my personal journey as a queer Puerto Rican in the biological sciences, more students wanted to delve deeper into the power of their stories. However, most expressed some sort of concern regarding how this could read as unprofessional by others. One student wrote:

"As for anything that could still be clarified, I appreciated Emilio's honest transparency about their healing process and personal story, but I was a little unclear on how I can maintain professionalism in the field when sharing my own personal experiences. I completely agree that personal stories can be professional and even political, as Emilio successfully demonstrated through today's great lecture, but I'd like to learn more about a specific framework that I can look to in following Emilio's example."

This concern highlights the lack of inclusion and diversity that students face in science when they come from backgrounds that are underrepresented. Thus highlighting the known need for more robust efforts to not only accept students from diverse backgrounds but to change our institutional practices to help them thrive.[29]

These findings also allowed me to directly respond to my third research question regarding the questions that are presented by students after my lecture on the social construction of biological sex by showing the expected resistance of some students to deviate from a chromosomal view of biological sex. In addition, some students started to pose questions that demonstrate a usage of *good science* by balancing the value of both feminist critiques of science with the findings and implications of biological research.

4.5 To what extent do college biology students describe biological sex as unchangeable?

In the three years that I have given iterations of this lecture I had never received more than the occasional one or two responses from students that thought that biological sex could be changed, with most of the class thinking it could not be changed pre-lecture. Even considering this was only one classroom, it is still an occurrence that provokes careful optimism of the considerations students are balancing with their biological science knowledge. The considerations reported by students who advocate that biological sex can be changed were based on the power of surgery and the right of an individual to identify as they want. In addition, more students brought up the fact that there are reptile and fish species that have dynamic changes to their biological sex depending on the environmental context, or that there are human spectrums of disorders of sexual differentiation, to justify their disagreement regarding biological sex as being unchangeable. It is important to highlight that none of these students even referred to chromosomal sex in their

responses. Although future research will be more systematic in identifying why they choose to not mention chromosomal sex, we can assume that it could be in part for not knowing how it would fit in with their disagreement.

The students' argument that agreed that biological sex cannot be changed remained the same as in other iterations of this lecture. Most students referenced the classical view that chromosomal sex cannot be changed, and thus no matter how humans change their "appearance" they cannot change the "fact" they are male or female. Another argument was that of successful reproduction, stating that no matter what changes are done to the body a male body cannot produce the same reproductive cells or have the same reproductive functions as a female body. Both of these arguments were at times followed with an iteration of these just being facts that cannot be changed, an essentialist view of genetics.[30] These arguments show the promise of providing the view of *good science* to these students to help them consider the possibility of biases in their judgements.

Lastly, we had students who were unsure about how to respond to this statement pre-lecture. These students demonstrated an intention to respect transsexual identities and they recognized the power of surgery. On the other hand, there was confusion about what chromosomal sex contributed to the equation, given that it could not be changed. These student responses reflect the need to be guided or further exposed to a framework that allows them to balance both their knowledge in biology with factors outside of the fields of biology.

The concept of *good science* poses itself as a contender for a framework that can help students balance both their scientific knowledge in conjunction with other factors outside of science. Good examples of this balance regarding biological sex come from the scholarship of Anne Fausto Sterling, Sarah S. Richardson, and Gillian Einstein. The outcome of this balance is better described by Jordan Young, who highlights the *Norm of Reaction*, which is a scientific framework that has a focus on the additive processes that differentiate and create what we call male or female traits.[31] They use this in opposition to an essentialist view that claims one clear blueprint determined in early development, male or female, is later further developed to a complete set of traits. This essentialist view can be observed in the chromosomal view of biological sex that some students focused on when stating that biological sex cannot be changed.

An important caveat of why the concept of *good science* is a promising prospect to explore in students from the biological sciences is that it is carefully crafted to not be *anti-science*. By *anti-science* I am referring to the tendency of critiques of sex science not paving a way for growth, but stay at the level of critique. As stated by Elizabeth A. Wilson in her book "Psychosomatic: Feminism and the Neurological Body", feminist critique often focuses on cultural, social, linguistic, literary and historic perspectives and excludes the domains of biology. Rather than exclude, Elizabeth states that there is room to include the body as a blank canvas for society but that also pushes back with its own materiality and conditions.[32] Thus, to strike a balance between these domains of knowledge to promote *good science* is in line with science, and strives to not to be interpreted as *anti-science*.

Looking at students' take-homes points after the lecture we can admire a self-reported malleability given new data. Even though they were not asked, some students reported how they had initially thought biological sex was unchangeable but given the new information they had changed their view. Students often highlighted with horror the surgical procedures or/and mutilations performed by doctors on intersex bodies to fit their ideas of sex instead of the natural world. This did not only encourage students to question binary biological sex but also to question

how objective can we be as scientists or doctors. This is another reason why *good science* serves as a good catalyst to guide students to grow to be better scientists and doctors.

This experiment, as incomplete as it might be, is a good start to consider further research on bringing *good science* around biological sex to earlier stages of students' college education, as opposed to be hidden in specialized and advanced courses in college.[33] Not to mention that this focus on *good science* promises to accomplish or further develop the projected K-12 "nature of science" learning objective of the Next Generation Science Standards and address *intuitive biological* thinking early on in their training. The goal is that students can understand the situated knowledge of science and how science is not the only factor considered in the decision made by society, even by those in the scientific community, their research, their explanations, and their generalizations for the public.

Notes:

1   Charis Thompson uses this term in her book "Good Science" as she explores the intertwining of ethics and science in stem cell research.

2   Fausto Sterling's work on intersex individuals and how doctors performed an "emergency" surgery when the genitalia of a newborn was ambiguous in her book Sexing the Body.

3   It is important to recognize that there is a whole field on sex differences that are relevant. However, the implications of these differences are at times used to justify a male and female sexual binary in other sciences such as psychology. Thus, the importance of good science and Interdisciplinarity are critical to not allow a pseudo-science logic to develop.

4   In addition to Fausto Sterling's merger of biological sex science and feminist critique that highlights both the benefits and the limmitations of such scientific findings we can reffer to Donna Harraway's concept of *situated knowledge*. Situated knowledge of science focuses that scientist do not see the truth but co-construct the truth about their subject of study, in this case biological sex, best expalined in her 1988 article *Situated Knowledges: The Science Question in Feminism and the Privilege of Partial Perspective*. Thus, warning us about how the biases scientists hold are influencing their creation of biological sex. This co-creative process has been further expanded to the materiality it creates by authors like Judith Butler in her book *Bodies that Matter*.

6   The most famous textbook for teaching general biology is Campbell Biology, currently in its 12th edition. This book has a total of 56 chapters that span about thirteen hundred pages. Like any other biology textbook it starts at the level of the basic chemistry of life, followed by the cell and works up the levels of reductionism in biology up to ecology. Out of these pages there is a grand total of nine pages, pages sixteen to twenty four, that explain how scientists collect and use data to test hypotheses. In addition, it highlights how science builds on the work of others. That is the extent to which students in the biological sciences are trained to understand how science works. Which apparently is as objective as collecting data to test hypotheses, and build on past work. No recognition of the situated knowledge of science, history of science, or social impact of science.

7   For a summary of the assumptions made by scientists regarding using male animals over female animals refer to "*Exploring the biological contributions to human health: does sex matter?*" by Wizzemann and Pardue.

8   This is the main argument being made by Annaliese K. Beery and Irving Zucker in their paper Sex Bias in Neuroscience and Biomedical Research by showing how more than 60% of the publications in fields such as pharmacology and endocrinology only use male animals. With fields like immunology and neuroscience not even specifying the sex of the used animals more than 50% of the time. This creates a stark contrast to human research that is almost 80% done in both male and female subjects.

9   To learn about the argument that women should be protected from pharmacological testing, thus most of the testing is done in males read " Foreward: a historical overview of advocacy for research in sex-based biology" by Marts and Keitt in 2014.

10   This in between of being labeled anti-science by scientists or of focusing too much on science by feminist scholars is a commonality in scholars with backgrounds in both science and science studies or/and feminist critiques of science. However this tension is best conveyed by Rebeca M. Jordan-Young in the book Brainstorm: The Flaws in The Sciences of Sex Differences, they state in their notes about the book that "Finally and most emphatically, this is not an anti-science book and it is also not an anti-difference book. Questioning brain organization theory is not the same as rejecting science or biology."

11   Stephen G. Bush in his paper "*Should the History of Science be Rated X?*" highlights and makes fun of opponents of history of science being taught in the K-12 biological sciences classroom. These opponents of the *history of science* state that the writings of current science historians can really leave a negative impression of science to the easily impressionable K-12

students. We can see how this argument is implemented when we look at popular books even at the undergraduate level of introduction to biology or general biology that do not spend time in contextualizing science with help of history of science or any critical analysis of science like that provided by feminist critiques of science.

12   It is relevant to highlight the fact that no video has been uploaded regarding Maya Angelou's talk in the biggest undergraduate research conference for minority students. However, Neil deGrasse Tyson also spoke in the same conference, and clips from his talk are easy to find in the YouTube archives of the organizers of the ABRCMS conference. I attribute this to the mindset of "just science". Maya gave her keynote over lunch, as people ate and for the most part ignored her. While Tyson was the keynote speaker for dinner, after everyone had eaten and were giving him full attention.

13   In Puerto Rico, *maricón* is the equivalent of *fag*; the term has been reclaimed by authors, like Paco Vidarte, in his book *Teoria Queer,* and others, to critique the widespread use of the term *queer* as a generalizing term that erases difference among cultures and ethnic groups.

14   During the four years of my undergraduate training in Puerto Rico I was part of the prestigious and competitive undergraduate research programs for minority students Minority Biomedical Research Support - Research Initiative for Scientific Enhancement (MRBS-RISE) and Minority Access to Research Careers (MARC). In addition, I completed three summer research internships programs (SURP) for minority students at Weill Cornell and New York university. I also attended for seven years in a row the Annual Biomedical Research Conference for Minority Students named ABRCMS. My engagement as a research poster judge and representative for the Neuroscience program at UC Berkeley has kept me attending the Annual Biomedical Research Conference for Minority Students (ABRCMS) and Society for Advancement of Chicanos/Hispanics and Native Americans in Science (SACNAS) through graduate school. This positionality informs the push for "just science" in future minority biomedical researchers in STEM.

15   I learned about the nature of science and the struggles to implement it in courses about K-12 teaching at the University of California Berkeley. It was one of the most controversial learning goals of the Next Generation Science Standards. The potential for teachers to use this argumentation to demonize science is the main concern. However, my chapter tries to advocate for good science regarding biological sex to potentially be a gold standard to address the nature of science effectively.

In her book Sexing The Body and later on Biology in a Social World, Fausto Sterling merges History of Science with current knowledge the genetics, hormones, brain differences and psychology of Biological Sex and intersex. Her analysis allows the reader to reflect on the complexity of Biological Sex and the impact of our biases on the science produced. It summarizes key points from her previous book Sexing the Body.

16   In her book Sex and the Brain, Gillian Einstein compiles primary literature on Biological Sex differences. Each unit has an introduction that contextualizes the debates happening as the papers in the unit were published and guiding questions to read the primary literature paired with feminist critiques of biological sex such as those of Anne Fausto Streling.

17   Sari van Anders studies social neuroendocrinology and describes her science as *feminist science* that is about sex and gender. Another scientist that questions the rigid binary of male and female in the brain is Daphna Joel in her 2015 paper "Sex beyond the genitalia:T he human brain mosaic" and her 2021 paper "Beyond the Binary: Rethinking Sex and the Brain".

18   Irving Zucker is a professor emeritus (UC Berkeley) and has been the senior author of various papers with different scientists about the lack of inclusion of female mammalian animals in research. Along with the consequences of such exclusion for biomedical research and medicine.

19   MacDougall-Shackleton compiled different categorizations that are described as levels of analysis in behavior research. Mainly supports the distinction made by Baker between ultimate and proximate causation, which asks the why and the how of behavior respectively. Then for the ultimate causation he attributes two levels of analysis of evolution and adaptive value for a species. For the ultimate he attributes the two levels of biological reductionism of evolution/ecology and the evolutionary history of a specific species. For the proximate he attributes the two levels of mechanism and development. In regards to the mechanisms he linked to the biological reductionism of studying molecular, cellular, or physiological mechanisms. While developmental refers to how these mechanisms change as the animal grows older. For more on these categories I suggest MacDougall-Shackleton's "The Levels of Analyses Revisited".

20   For more specifics on neurogenetic essentialism within STEM I suggest reading "Gendered genetics: How reading about the genetic basis of sex differences in biology textbooks could affect beliefs associated with science gender disparities" by Brian M. Donovan and others.

21   See note 4 for an elaboration of Donna Haraway's situated knowledge.

22   Here I do not intend to vilify the medical practitioners of the time. Their reasoning at times was one of "good intentions" regarding how an intersex individual would be judged by society and deprived of a partner or a family. A reasoning that surprisingly acknowledges and defends the fiction of sex and gender norms at the time.

23   Fausto Sterling, Alice Dredger and activists such as the Intersex society of North America acknowledge and distinguish conditions that could impair the health of an individual versus those that are simply threatening the social understanding of Biological Sex.

24   In a 2014 follow up meta-study of 293 research papers by Dr. Irving Zucker and Dr. Brian Prendergast "Female mice liberated for inclusion in neuroscience and biomedical research" show that when accounting and normalizing the reported variation of males and female animals in studies females are not more variable than males.

25   In a follow up study in 2019 Dr. Irving Zuckers and Benjamin Smarr's paper published "Male and female mice show equal variability in food intake across 4-day spans that encompass estrous cycles" highlight this lack of variability between male and female mice in food intake. If anything, he shows the trend of males being more variable in their measurements.

26   In the 2017 paper "Intuitive biological thought: Developmental changes and effects of biology education in late adolescence" the authors state the same prevalence of intuitive thinking between 8th graders and college students of biology. With biology majors showing more intuitive thinking than non-majors. Intuitive thinking is here simplified to three non-exhaustive categories of anthropocentric, teleological, and essentialist. Future research with optimized assessment questions that can be analyzed using the framework of intuitive biological thinking.

27   This methodology was developed based on chapter 1 and 2 of the book Figurative Language by Dancygier and Sweetser.

28   To learn more about the concerns recently spearheaded by Dr. Irving Zucker refer to note 8 of this chapter.

29   Dr. John Matsui has been working on the front of directing and researching novel strategies for diversity and inclusion in biology education for the past decades at UC Berkeley. In his paper ""Outsiders at the Table"—Diversity Lessons from the Biology Scholars Program at the University of California, Berkeley " he highlights the need to focus on listening and understanding

our students to drive institutional change that then eliminates the barriers they face. Instead of selecting the "correct" students from diverse backgrounds and assume the institution has nothing to change about its structure or practices.

30   In the book "Sex Itself: The search for male and female in the human genome" Sara S. Richardson goes over the anthropocentric beginnings of mapping unchangeable "male and female" traits to chromosomes and genes all up to the current research that has been aided by feminist critique of sex science.

31   In the book "Brainstorm: The Flaws in The Sciences of Sex Differences" Rebeca M. Jordan-Young introduces and develops the concept of norm of reaction and describes it as "The norm of reaction is not a new or controversial concept. But it is routinely ignored in favor of the popular, simpler, and wrong model of deterministic, additive factors that influence development".

32   In the book "Psychosomatic: Feminism and the Neurological Body" Elizabeth A. Wilson argues that the neurological body has recorded and is proof of the impact of social pressures exclusive to women that need to be studied and addressed, not dismissed.

33   Both Dr. Anne Fausto Sterling and Dr. Gillian Einstein both have developed their respective books "Sex and Gender: Biology in a Social World" and "Sex and The Brain" are made with the intent of guiding courses in Biology that want to teach biological sex in conjunction with feminist critiques of science. However, a quick search of the courses they teach show specialized courses on biological sex.

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