

Stress and Sex: Characterizing the Distinct Impacts of Various Stressors on Female Reproductive Function

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Thesis Abstract

Most adults experience stress in one form or another, whether it be systemic stress caused by brain injury or psychological stress caused by a perceived environmental threat. Stress of excessive intensity or prolonged duration can adversely impact health, namely by dysregulating the hypothalamic-pituitary-adrenal (HPA) axis. However, while substantial research supports an inhibitory effect of stress on hypothalamic-pituitary-gonadal (HPG) axis function, information is lacking regarding its effects in females, particularly the impact of adulthood stress on estrous cycle regularity.

This PhD thesis includes a set of studies (two systematic reviews and three original studies) aiming to better characterize the impact of various adulthood stressors on HPG axis function and reproductive outputs. The **first** and **second chapters** systematically reviewed the existing evidence on the association between adulthood psychological stress and disruptions to ovarian cyclicity in humans and rodents, respectively. The **first chapter** showed the majority of the 41 included studies to report an association between elevated psychological stress and irregular menstruation in adult women. Similarly, the **second chapter** found that over half of the 32 included papers detected an adverse impact of psychological stress models on the rodent estrous cycle, and shows altered estradiol (E2) levels and high basal corticosterone (CORT) are often observed in tandem with such cycle disruptions. The **third chapter** assessed HPG axis function in female Wistar rats exposed to global cerebral ischemia (GCI). We studied post-ischemic estrous cycle regularity and HPG axis reactivity through expression of estrogen receptor alpha ($ER\alpha$), gonadotropin-inhibitory hormone (GnIH), and kisspeptin (Kiss1), and found a transient interruption of the estrous cycle (persistent diestrus) lasting on average 11 days and enduring dysregulation to HPG neuroendocrine function 25 days post ischemia. The **fourth**

chapter evaluated the impact of social stressors (isolation, crowding, and social instability) in adult female Wistar rats on sexual functioning, fertility, estrous cycling, and all levels of the HPG axis (namely hypothalamic immunofluorescent expression of ER α , GnIH, and Kiss1, serum levels of pituitary and gonadal hormones, ovarian histology). Our data uncovered distinct impairment profiles following social stress exposure in rats, with the isolation condition leading to increased hopping behaviours, alterations to the reproductive organs, and elevated follicle-stimulating hormone (FSH) levels. Exposure to crowding reduced GnIH and ER α immunoreactivity, in addition to decreasing days spent in proestrus, whereas social instability led to reductions in estrous cyclicity, uterine horn weight, and ovarian reserve. The **fifth chapter** expanded on the previous findings by providing an in-depth assessment of the home-cage dynamics during instability and crowding stress, and assessed whether individual differences in play fighting in females were associated with altered stress and reproductive outcomes. This study uncovered distinctive patterns of play fighting in crowded and social instability exposed rats. Additionally, significant differences in adrenal gland weights and days spent in estrus were noted between high and low playing rats, suggesting play fighting to contribute to such physiological outcomes. Together, this series of experiments contributed to characterizing HPG axis dysfunction following different types of adulthood stress and should help future researchers select the most relevant models for studying impacts of stress on female reproductive function.

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Abbreviations

ACTH: Adrenocorticotrophic hormone	HC: Hormonal contraceptive
AMH: Anti-mullerian hormone	HDH: High density housing
AR: Androgen receptors	HPA: Hypothalamic-pituitary-adrenal
ARC: Arcuate nucleus	HPG: Hypothalamic-pituitary-gonadal
AVPV: Anteroventral periventricular nucleus	ir: immunoreactivity
BMI: Body mass index	Kiss1: Kisspeptin
CH: Control Housing	Kiss1R: Kisspeptin receptor
CMS: Chronic Mild Stress	LDH: Low density housing
CORT: Corticosterone	LE: Long Evans
CRH: Corticotropin-releasing hormone	LH: Luteinizing hormone
CRHR1: Corticotropin-releasing hormone receptor type 1	Lhcgr: Luteinizing hormone/choriogonadotropin receptor
CRHR2: Corticotropin-releasing hormone receptor type 2	LMM: Linear mixed-effect model
CSDS: Chronic Social Defeat Stress	MR: Mineralocorticoid receptors
CUMS: Chronic Unpredictable Mild Stress	N/A: Not applicable
CVD: Cardiovascular disease	N/S: Not specified
CVS: Chronic Variable Stress	OFT: Open Field Test
DMH: Dorsomedial nucleus	P4: Progesterone
E2: Estradiol	PBAC: Pictorial Blood Assessment Chart
EPM: Elevated Plus Maze	PBS: Phosphate buffered saline
ER: Estrogen receptor	PFA: Paraformaldehyde
ERα: Estrogen receptor alpha	PCOS: Polycystic ovarian syndrome
ERβ: Estrogen receptor beta	PMS: Premenstrual syndrome
FIGO: International Federation of Gynecology and Obstetrics	POA: Preoptic area
FHA: Functional hypothalamic amenorrhea	PR: Progesterone receptors
FSH: Follicle-stimulating hormone	PRL: Prolactin
FST: Forced Swim Test	PTSD: Post-traumatic stress disorder
GCI: Global cerebral ischemia	PVN: Paraventricular nucleus
GH: Growth hormone	RFRP-3: RFamide related peptide 3
GPα: G protein-coupled estrogen receptor	RH: Regular Housing
GnIH: Gonadotropin-inhibitory hormone	SD: Sprague-Dawley
GnRH: Gonadotropin-releasing hormone	s.c.: Subcutaneous
GR: Glucocorticoid receptors	SIS: Social instability stress
	SPT: Sucrose Preference Test
	T: Testosterone

Study 1 – Stress and Anxiety Scales

BDS: Beirut Distress Scale

CSS: COVID-19 Stress Scale

DASS-21: Depression, Anxiety, and Stress Scale-21

GAD-7: General Anxiety Disorder-7

GHQ: General Health Questionnaire

HADS: Hospital Anxiety Depression Scale

IMPS: Inventory to Measure Psychosocial Stress

JCQ: Job Content Questionnaire

KNHANES: Korea National Health and Nutrition Examination Survey

LAS: Lebanese Anxiety Scale

LEQ: Life Events Questionnaire

PSS: Perceived Stress Scale (Original scale with 14 items)

PSS-4: 4 item Perceived Stress Scale

PSS-7: 7 item Perceived Stress Scale

PSS-10: 10 item Perceived Stress Scale

PSS-10-C: COVID-19 Pandemic-related Perceived Stress Scale

STAI-1: State-Trait Anxiety Inventory-1

STAI-2: State-Trait Anxiety Inventory-2

TICS: Trier Inventory for the Assessment of Chronic Stress

Theoretical Framework

Today's fast-paced society has been marked by an accentuated exposure to stressors (Rao & Indla, 2010). The COVID-19 pandemic further increased the level of perceived psychological stress (Amerio et al., 2021; Fioravanti et al., 2022), effects which were profoundly felt by women (Purvis et al., 2022). At moderate levels, stress can have positive impacts on learning and performance by increasing motivation, promoting efficient cognitive function, and accelerating brain processing (Rudland et al., 2020). However, prolonged stress leads to negative impacts on health, both psychologically and physiologically, by disrupting homeostasis (Yaribeygi et al., 2017) and is associated with increased susceptibility to illness (Salleh, 2008). Importantly, pronounced sex differences have been reported in stress reactivity. Women are more vulnerable to the impacts of stress compared to men, making them particularly at risk of developing mood disorders and stress-related diseases (Helpman, 2023; Verma et al., 2011). Of interest, various types of stress have been linked with an impairment of women's reproduction, including reduced ovarian and sexual function, leading to increased risks of infertility and miscarriage (Hamilton & Meston, 2013; Rooney & Domar, 2018). At present, there is a lack of stress studies focused on female subjects, with some stressors having been particularly understudied. This gap in literature has historically been driven by the preferred use of male rodents in animal research (Zakiniaeiz et al., 2016), despite evidence suggesting that the inclusion of females in animal research does not increase variability (Beery, 2018). In this context, the responses of the female reproductive axis to various types of stress are of renewed interest.

1. Physical and Psychological Consequences of Stress

In broad terms, stress can be defined as the body's response to any event that disrupts homeostasis or poses a perceived threat to this balance (Tran & Gellner, 2023). Depending on its

duration, stress can be characterized as acute (i.e., one brief stressor exposure) or chronic (i.e., repeated exposure over an extended length of time; Atrooz et al., 2021). While stress represents an adaptive response from the body in reaction to a threat and is not implicitly negative, it can have numerous harmful effects when experienced repeatedly, at high intensities, or for prolonged periods. Abundant preclinical and clinical studies highlight an array of poor health outcomes following prolonged stress exposure (Shields & Slavich, 2017). In rodents, both chronic and acute stressors have been shown to increase depressive- and anxiety-like behaviours (Antoniuk et al., 2019; Ménard et al., 2016; Patki et al., 2014) and to reduce cognitive functioning such as learning and memory formation (Alzoubi et al., 2009; Srivareerat et al., 2009). In humans, elevated lifetime stress exposure (such as financial stressors) is associated with heightened risk of mortality (Bourassa, 2021; Kopp & Réthelyi, 2004), increased risk of cardiovascular disease (CVD; Golbidi et al., 2015; Satyjeet et al., 2020), as well as greater prevalence of anxiety and depression (Davis et al., 2017; James et al., 2023). Importantly, adults who report high levels of perceived stress present a greater prevalence of irregular menstruation (Matsumura et al., 2025) and other menstrual problems such as dysmenorrhea (painful menstruation) or premenstrual symptoms (Elah Sadiqi & Salih, 2024; Gollenberg et al., 2010). In female rodents, chronic psychological or physical stress have been shown to alter estrous cycle regularity (Fotsing et al., 2017; Retana-Márquez et al., 2020), decrease oocyte viability and reduce ovarian/uterine health (Casillas et al., 2021; Divyashree & Yajurvedi, 2016; Fu et al., 2018), impair sexual functioning (Geraghty et al., 2015), and lessen fertility (Xi et al., 2022), supporting a wide range of stress-induced reproductive impairments.

As a modifiable risk factor (Alwan et al., 2024), stress represents a highly relevant target variable for the prevention of adverse health outcomes. This thesis therefore aimed to

characterize the consequences of various types of stress on female reproductive functioning, which has historically been understudied. Consequently, understanding the primary signalling molecules within the stress regulating hypothalamic-pituitary-adrenal (HPA) axis and the reproductive regulating hypothalamic-pituitary-gonadal (HPG) axis, as well as the bidirectional functional interactions between the two axes, are of importance.

2. The Hypothalamic-Pituitary-Adrenal Axis

The HPA axis, the neuroendocrine system that coordinates the body's stress response, is required to generate adaptive reactions to the events, internal or external, which threaten homeostasis. Following exposure to a stressor, the parvocellular neurons of the paraventricular nucleus of the hypothalamus (PVN) are activated and produce corticotropin-releasing hormone (CRH), which once released, reaches the anterior pituitary through the hypophyseal portal vasculature (Valsamakis et al., 2019). CRH binds to its type 1 receptors (CRHR1) on the corticotrope cells within the pituitary and causes the secretion of adrenocorticotrophic hormone (ACTH) into the bloodstream, which subsequently binds to its receptors in the adrenal cortex (Oyola & Handa, 2017). Within the adrenal glands, ACTH signalling initiates the synthesis and immediate release of glucocorticoids, notably corticosterone (CORT) in rodents and cortisol in humans (Sheng et al., 2021). Glucocorticoid secretion and subsequent binding to mineralocorticoid receptors (MR) and glucocorticoid receptors (GR) in the brain induces a wide range of physiological responses aiming to regain homeostasis. In situations where overall CORT levels are low, MRs are most active, whereas GRs, which have a comparatively lower binding affinity, are activated when CORT levels rise, such as during the circadian peak or after stress exposure (Joëls, 2008). Ultimately, the HPA axis self-regulates its activity through a number of negative-feedback loops, with CORT acting at the hypothalamic and pituitary levels

to regain homeostasis (Gjerstad et al., 2018). Additionally, CORT acts in the hippocampus by increasing CA1 neuron firing via binding to GRs and MRs, leading to inhibition of PVN-CRH circuitry (Joëls et al., 2009; Keller-Wood, 2015). Prolonged stress has been shown to impair HPA axis negative feedback mechanisms. For instance, chronic stress leads to persistent CORT related activation of GR in the hippocampus, eventually downregulating and altering GR function, further reducing HPA axis inhibitory feedback and leading to an over-activity and hypersecretion of glucocorticoids (Anacker et al., 2011; Conrad, 2008).

3. The Hypothalamic-Pituitary-Gonadal Axis

Reproductive function is primarily controlled by the HPG axis. In rodents, HPG axis activation is initiated upon the secretion of gonadotropin-releasing hormone (GnRH) by neurons located in the preoptic area (POA) and mediobasal hypothalamus, which project to the median eminence to trigger gonadotropin release (Maeda et al., 2007; Moore et al., 2018). Secretion of GnRH occurs in two distinct modes, a pulsatile and a surge mode, the latter being only present in females (Moenter et al., 2003). The slower, pulsatory release of GnRH is observed during the majority of the ovarian cycle and favours follicle-stimulating hormone (FSH) secretion, required for regulating ovarian function, specifically promoting maturation and growth of early follicles (known as folliculogenesis; Hsueh et al., 2000; Smits et al., 2016). The brief GnRH surge mode triggers a large spike in luteinizing hormone (LH), necessary for inducing ovulation, contributing to follicular development, and ensuring follicle luteinization (Clarke et al., 1984; Haisenleder et al., 1991; Toner & Pirtea, 2025). Following release, these gonadotropins activate the ovarian production and secretion of two steroid sex hormones, namely estrogens and progesterone (P4), which then regulate HPG axis processes via positive and negative feedback mechanisms. Estrogens, a class of steroid sex hormones comprised of estrone, estradiol (E2), estriol, and

estrol – of which E2 is the most abundant and physiologically active type – regulate feedback mechanisms by binding to estrogen receptors (ER; Fuentes & Silveyra, 2019). However, due to an absence of expression of the ER alpha (ER α) subtype on GnRH secreting neurons, E2 feedback within the HPG axis relies on signalling from the stimulatory neuropeptide kisspeptin (Kiss1; Acevedo-Rodriguez et al., 2018). Kiss1 cell bodies located in the anteroventral periventricular nucleus (AVPV) and the arcuate nucleus (ARC) of the hypothalamus express all ER types, and respectively regulate positive and negative feedback of ovarian hormones onto GnRH secreting neurons (Oakley et al., 2009; Smith et al., 2005). Further regulation of gonadotropin release occurs via the gonadotropin-inhibitory hormone (GnIH) produced in the dorsomedial nucleus of the hypothalamus (DMH). GnIH, also known as RFamide-related peptide 3; RFRP-3), acts by inhibiting GnRH (Tsutsui et al., 2000; Ubuka et al., 2012). In ewes, approximately 26% of POA-GnRH neurons have been shown innervated by GnIH/RFRP-3 fibres (Rizwan et al., 2012), and a proportion of Kiss1 neurons in the ARC and AVPV of mice also express RFRP-3, which could indicate a modulatory role of GnIH on Kiss1 signalling at these sites (Poling et al., 2013; Rizwan et al., 2012).

Proper signalling within the HPG axis allows for regulation of a panoply of reproductive outputs. The E2 and P4 surges in proestrus act to prime the reproductive system, thus being crucial for the expression of sexual receptivity in many rodent species. Indeed, sexual behaviours can reliably be induced in ovariectomized females by using a subcutaneous injection of ethinylestradiol or estradiol benzoate 24-48 h prior to mating, followed by a P4 injection 4-6 h before testing, acting as hormonal priming (Corlett et al., 2022; Hernández-Munive et al., 2018). If copulation with a male is successful, it can lead to the fertilization of an oocyte, assuming normal gonadotropin release allowed for folliculogenesis and ovulation. Subsequently, if

pregnancy is achieved, P4 and its two receptor isoforms, progesterone receptors (PR)-A or -B, then play a key role in maintaining pregnancy (Grimm et al., 2016).

In a healthy reproductive system, the critical gonadotropins and gonadal hormones fluctuate predictably across the female reproductive cycle. In humans, the menstrual cycle is divided into two main phases (the luteal phase and the follicular phase: Carmichael et al., 2021; Patricio et al., 2018). The estrous cycle in rats is divided into four distinct stages spanning on average 4-5 days (see Figure 1 enabling to compare phases of the human menstrual cycle versus rat estrous cycle). The proestrus stage is characterized by a surge of E2 followed shortly after by a surge in P4, LH, and FSH (Butcher et al., 1974). This induces ovulation, signalling entry into the estrus stage, during which gonadal hormone levels gradually begin to decrease (McLean et al., 2012). By the end of estrus, E2 and P4 levels have reached their lowest point, and rats enter metestrus, a very brief stage during which gonadal hormone concentrations remain relatively low, with the exception of a small P4 surge, likely linked to corpora lutea formation in the ovaries (Hashimoto et al., 1968). Subsequently, diestrus is characterized by a slow rise in gonadal hormone levels until the following proestrus stage, at which point the next pre-ovulatory hormone surges occur (Ajayi & Akhigbe, 2020). Ultimately, the closely regulated interactions between key HPG axis markers allow for the appropriate hormone levels required for regular cycling, sexual functioning, ovulation, fertility, and maintenance of pregnancy (Christian & Moenter, 2010). Disruptions of this homeostatic balance can concordantly result in a variety of reproductive impairments.

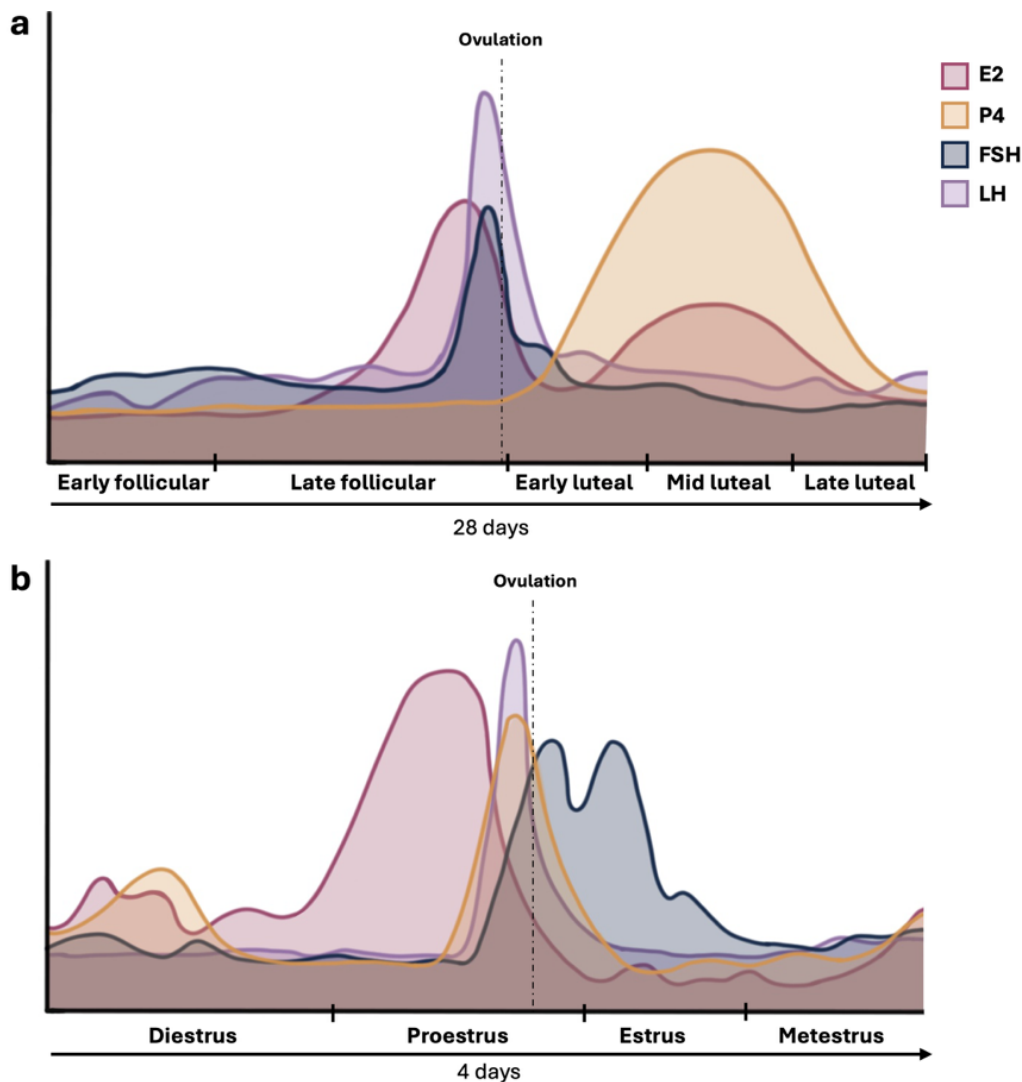


Figure 1. Schematic Representation of Typical HPG Axis Hormone Fluctuations During the Human Menstrual Cycle and the Rat Estrous Cycle.

General overview of the gonadal and pituitary hormone changes occurring during a typical 28-day menstrual cycle in humans (a) and a 4-day estrous cycle in rats (b). Created referencing data presented in Thorneycroft et al. (1971) and Smith et al. (1975), respectively. E2: Estradiol; FSH: Follicle-stimulating hormone; LH: Luteinizing hormone; P4: Progesterone.

4. Bidirectional Interactions Between the HPA and HPG Axes

Persistent HPA axis activation via a variety of stressors has been found to impact all levels of the HPG axis (Chand & Lovejoy, 2011; Toufexis et al., 2014). Importantly, HPA-HPG interactions are bidirectional, with steroid sex hormones having numerous effects on stress

reactivity and female sex hormones contributing to increased vulnerability to stress. Therefore, the evaluation of stress-induced reproductive impairments in females is of particular interest.

4.1 HPG Axis Influence on HPA Axis Reactivity: Female Vulnerability to Stress

Sex differences have been reported in numerous physiological and cerebral processes, with HPA axis activity and the associated stress response being no exception (McCarthy et al., 2012; McEwen & Milner, 2017; McKenzie et al., 2025; Sood et al., 2022). Compared to males, female rodents show an increased ACTH response to stressors, and CORT levels in female rats are higher and stay elevated longer following stress (for review, see Oyola & Handa, 2017). Evidence also suggests reduced CRHR1 internalization in females, due to a deficit in the association between β -arrestin 2 and CRHR1, which might make female neurons more vulnerable and less adaptable to high CRH levels (Valentino et al., 2013). Furthermore, female rats display higher *c-fos* expression in the PVN following exposure to 30 min of restraint, and presented higher colocalization with CRH in these regions compared to males (Babb et al., 2013). Estrogens and their receptors play a key role in modulating the stress response and are believed to be some of the main drivers of observed sex differences in the magnitude of stress reactivity between males and females (Kajantie & Phillips, 2006). Concordantly, ERs are expressed at several levels of the HPA axis, with ER beta (ER β) immunoreactivity having been detected in CRH neurons of the PVN, indicating a contribution of ER β to the CRH transcriptional regulation of stress (Miller et al., 2004). Interestingly, stress reactivity has been shown to fluctuate across the estrous cycle in females, with a clear role of E2 in mediating these effects. Thus, female rats show elevated basal CORT levels in the evening of proestrus (when E2 is highest) compared to all other stages (Atkinson & Waddell, 1997; Viau & Meaney, 1991) and menstrual cycle phase has similarly been shown to influence stress reactivity (for review, see

Klusmann et al., 2023). In gonadectomized male rats, injections of dihydrotestosterone or estradiol benzoate respectively reduced or increased the stress response to a 30 min restraint compared to that observed in intact male rats (Lund et al., 2004). In fact, even inhibitory markers within the HPG axis are susceptible to estrogenic signalling, as a subset of GnIH neurons express ERs, and FOS expression in GnIH neurons is significantly increased 3 h and 6 h following E2 administration (Kriegsfeld et al., 2006).

Abundant evidence shows heightened lifetime risk of experiencing stress-related disorders such as anxiety and depression in women (Helpman, 2023), with prevalence being nearly twice as high as that observed in men (Hallers-Haalboom et al., 2020; Remes et al., 2016). Such risk was further amplified by the COVID-19 pandemic, with a greater negative impact of this unprecedented stressor on women's mental health compared to men's (Purvis et al., 2022). Of note, a large cross-sectional study by Goodwin et al. (2020) showed that anxiety prevalence has significantly increased over the course of the preceding decade in the adult American population, aged 50 and younger, with the most concerning rise being observed in young adults (18-25 years old), whose anxiety prevalence has near doubled, from 7.97% in 2008 to 14.66% in 2018. Seeing as women typically enter menopause around 50 years old (Nelson, 2008), adult women of reproductive age represent a population identified at heightened risk of stress-related adverse health concerns.

4.2 HPA Inhibition of HPG Axis Function: HPA-induced Reproductive Impairments

Concordant with bidirectional interactions between the stress and reproductive axes, direct activation of the HPA axis has been found to impact all HPG axis secretory levels, as shown in Figure 2. Indeed, chronic CORT treatment significantly reduces GnRH mRNA in the POA of male rats (Gore et al., 2006), with roughly 30% of GnRH neurons showing CRHR1 and

CRH receptor type 2 (CRHR2) immunoreactive expression (Jasoni et al., 2006). Administration of a CRHR2 agonist results in a lengthening of the LH pulse intervals, whereas CRHR2 antagonism prevents restraint-stress-induced suppression of LH pulsatility (Li et al., 2005). Similar findings were later observed following CRHR1 antagonism (Li et al., 2006). Chronic administration of high doses of CORT over 6 days significantly reduces LH serum levels in male rats, while a single injection of dexamethasone in females prevents the LH surge (Gore et al., 2006). Furthermore, ovarian levels of LH receptors (*Lhcgr*) are significantly reduced by continuous CORT injections performed over 48 or 55 h periods (Niswender et al., 2000). Concordant with close interactions between GnIH and HPA axis activity, up to 53% of RFRP cells within the DMH of stressed rats are GR positive (Kirby et al., 2009), indicating another mechanism by which HPA axis activation exerts inhibitory effects on HPG axis function. Exogenously induced HPA activation, via administration of CORT or CRH agonists, has, however, been extensively studied compared to the complex neural response occurring in mammals exposed to environmental stressors, for which responses are more strongly influenced by individual variability, such as a subjects' perception of an event (Koolhaas, 2008). The use of stress models which are representative and relevant to real experiences are therefore critical to improving translational research.

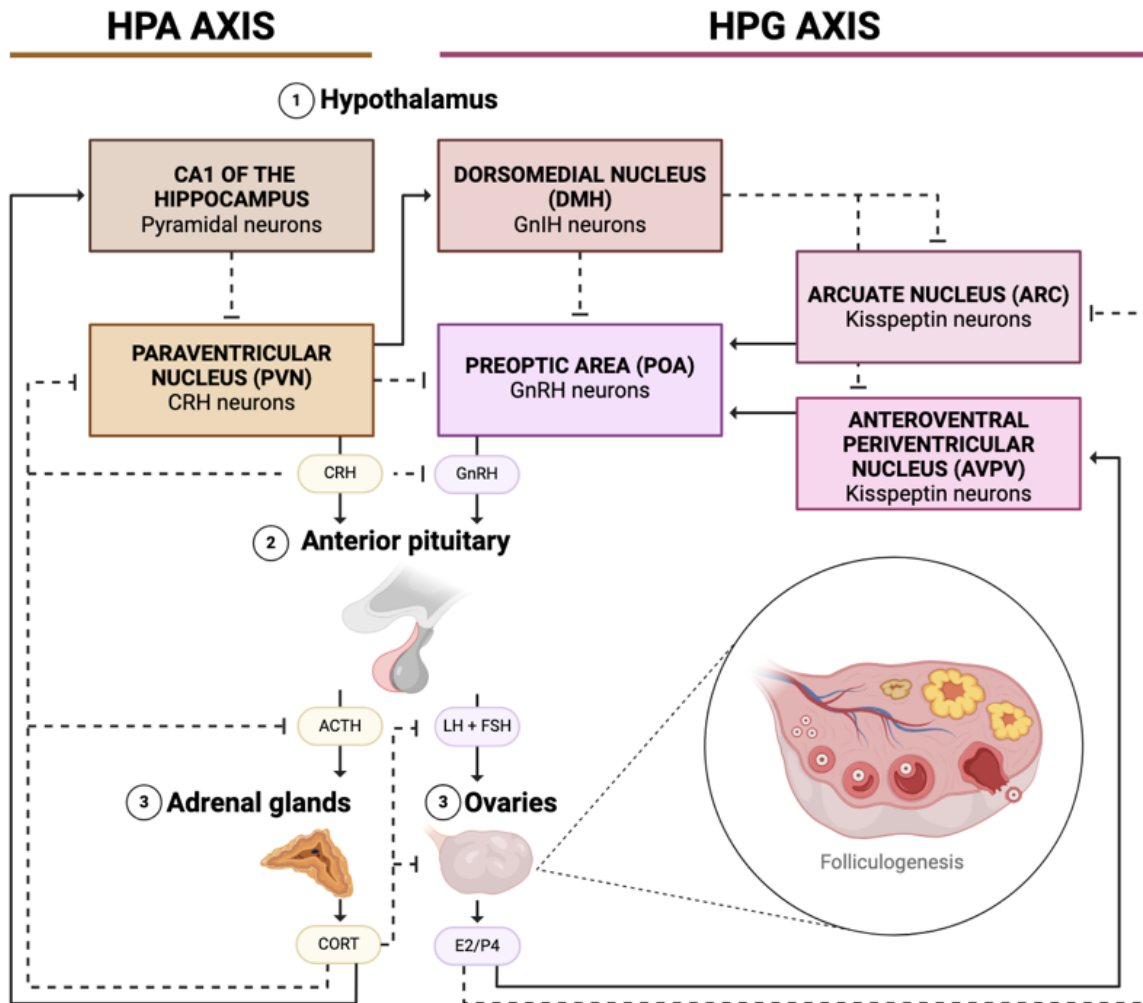


Figure 2. Overview of the HPA and HPG Axes.

The figure shows key structures and substrates of the HPA axis (left) and the HPG axis (right), as well as the main stimulatory (solid lines) and inhibitory (dashed lines) signalling in each axis. Feedback of E2 and P4 within the HPG axis switches from negative to positive during the proestrus stage. The main inhibitory actions of the HPA axis on the HPG axis are depicted. Figure created in BioRender, using information presented in Brunton (2013), Harter et al. (2018), Kauffman (2010), and Padma et al. (2021).

5. Experimental Models for Studying Stress: Clarifying Consequences on Reproduction

Many experimental paradigms have been developed to study the behavioural and biological consequences of stress in rodents. These models can be broadly classified by stressor type, namely 1) systemic stressors, which disrupt physiological/homeostatic regulation, caused

by events such as traumatic brain injury, immune challenge, hemorrhage, or cerebral ischemia (Kagias et al., 2012; Kane & Ismail, 2017; Leigh et al., 2018), 2) physical stressors, which pose a direct threat to bodily health (e.g., pain), such as foot shocks and forced swimming; and 3) psychological stressors, causing anticipation or fear without physical pain, such as immobilization, restraint, loud noises, or exposure to predators (Atrooz et al., 2021). It is noteworthy that precisely defining psychological stress in rodents is difficult and remains contentious, with the term “psychogenic” stress sometimes being preferred. Nevertheless, a variety of stress models are commonly described as psychological stressors, and this thesis will apply such terminology (Atrooz et al., 2021). Social stressors, which utilize alterations to social dynamics, represent a subtype of psychological stress (sometimes named psychosocial stress), and include models using components such as social defeat or social instability (Koolhaas et al., 2016).

Of importance for this thesis, it is well acknowledged that different types of stress have varied behavioural and physiological consequences (Atrooz et al., 2021). Indeed, a comparison of five psychological and systemic stressors (immobilization, hemorrhage, cold exposure, pain, or hypoglycemia) in male rats showed each stressor to have a distinct neurochemical “signature” or stress response, namely through differing ACTH, epinephrine, and norepinephrine plasma levels (Pacák & Palkovits, 2001). Similarly, a comparison of restraint, relocation, crowding, and isolation stress in male rats revealed distinct impacts on CORT levels and anxiety-like behaviours (Adavi et al., 2023). Even conceptually similar stressors can induce distinct ACTH and CORT secretory levels and recovery profiles, such as restraint and immobilization which both prevent movement but use different apparatus to do so (Molina et al., 2023). In fact, while restraint and systemic stress (via liposaccharide injection) both suppress LH pulses in female

rats, CRHR1 antagonism only reverses the inhibitory effect in rats exposed to restraint, not systemic stress, whereas CRHR2 reverses effects of both stressors (Li et al., 2006), suggesting differential HPA mechanisms regulating HPG axis inhibition. Efforts in selecting appropriate and ecologically valid models of stress appear essential for proper interpretation and translation of results to relevant clinical populations. At present, much remains to be determined as to potentially diverging effects of various stressors induced over relatively long delays in female rats, particularly as it relates to reproductive outcomes. It will be part of this thesis's goals to further compare and differentiate such responses to distinctive stress paradigms.

5.1 Systemic Stress Models

With stress representing a physiological response to a change in homeostasis, HPA axis activation can be significantly altered by internal events. Systemic stressors pose a threat to survival by inducing a direct homeostatic challenge at the physiological level, contrasting physical and psychological stressors which arise from an environmental threat (Armario et al., 2020). Systemic stress includes a wide breadth of challenges, including cold, heat, infection, traumatic brain injury, cardiovascular accidents, and hemorrhagic or ischemic stroke, which cause distinct physiological stress responses (Pacák & Palkovits, 2001). Of interest, recent research highlights a heightened risk of experiencing cardiovascular events following a COVID-19 infection (Brooke et al., 2024; Dai et al., 2025; Xie et al., 2022), indicating that more individuals may live with the HPA axis hyperactivity associated with such events. In this context, global cerebral ischemia (GCI) is an animal model that mimics cardiac arrest by interrupting cerebral blood flow. Known for inducing significant systemic stress resulting in persistent changes to HPA axis function (Barra de la Tremblaye & Plamondon, 2016), GCI is associated with altered anxiety-like behaviours in the Elevated Plus Maze (EPM; Morin et al.,

2021, 2022; Poitras, Doiron, et al., 2024; Soares et al., 2013). Accordingly, persistent HPA axis dysfunction is reported post ischemia, with heightened stress-induced CORT levels being observed in ischemic rats compared to sham operated controls (de la Tremblaye et al., 2014; Luo et al., 2019). Furthermore, long-lasting alterations to the HPA axis are detected at the receptor level in the ischemic brain, with CRHR1 and GR immunoreactivity being upregulated in the PVN and downregulated in the CA1 of male Wistar rats 30 days after ischemia (de la Tremblaye et al., 2014). As such, GCI represents an interesting model for studying persistent HPA activation and situations of heightened systemic stress following an acute brain injury, with a particular focus needed on females, which have long been neglected in preclinical ischemia research (DeBow et al., 2003; Ramirez et al., 2017).

5.1.1 Effects of Systemic Stressors on HPG-mediated Responses. The existing literature on the impacts of systemic stressors have shown their ability to alter a variety of HPG axis markers. In adult female rats, chronic exposure to 90 days of heat stress significantly reduced LH and E2 levels while increasing FSH and CORT levels, but did not alter GnRH levels (An et al., 2020). Similarly, immune stress induced via lipopolysaccharide injection reduced hypothalamic Kiss1 mRNA and LH levels in ovariectomized females, while GnRH was unaffected (Iwasa et al., 2008). A 5 min ether exposure immediately increased P4 and CORT levels in naturally cycling and ovariectomized rats, with E2 administration in ovariectomized rats preventing rises in P4 levels, while leaving CORT unaffected (Valdez et al., 2012). These findings further emphasize the complex interplay of peptides and hormones within the HPG axis. Notably, traumatic brain injury in young adult female rats was shown to reduce E2 and LH levels measured one week post injury, with effects on LH secretion perduring at three months, and being accompanied by a lengthening of the estrous cycle through increased non-reproductive

(diestrus) days (Fortress et al., 2019). Importantly, effects of systemic stress via cardiovascular events such as GCI have largely remained unstudied in adult females. Hypoxia-ischemia induced neonatally in female Sprague-Dawley rats has been shown to significantly increase both ER α and ER β measured 30 days after injury (Ezquer et al., 2008). In male rats, adulthood GCI resulted in a drastic decrease in GnRH receptor expression in the CA1 of the hippocampus, with almost no expression being detectable 3 days post injury (Chu et al., 2008), highlighting the potential of this systemic stressor at altering cerebral expression of key HPG axis markers. Concordant with the historical omission of female subjects in cardiovascular research (Ramirez et al., 2017), there are no studies to our knowledge assessing the impact of GCI in adult females on HPG axis function, leaving a significant gap in our understanding of this stressor, particularly as it relates to expression of key HPG axis regulators such as Kiss1 and GnIH.

5.2 Psychological Stress Models

Defining psychological stress in rodents has long been a contentious topic. Although no rodent stress model can perfectly reproduce the complexities of human stress pathophysiology, a variety of stress models have been developed in rodents to attempt to study molecular and behavioural consequences of environmental psychological stress (Italia et al., 2020). Notably, predator exposure, restraint stress, and noise exposure are considered primarily psychological (or psychogenic) in nature, as they do not pose a direct threat to bodily harm, unlike physical stressors such as foot shocks (Atrooz et al., 2021). Forced swimming stress borders these two categories, as it is widely considered to be primarily psychological/emotional in nature despite posing a direct physical threat (de Kloet & Molendijk, 2016; Linthorst et al., 2008). Other models, such as the commonly employed Chronic Unpredictable Mild Stress (CUMS) paradigms, are used to study the pathophysiology of depression by applying a variety of

relatively mild stressors, such as cage tilting, periods of food or water deprivation, overnight illumination, or wet bedding (Papp & Willner, 2023). It is however notable that while CUMS paradigms are typically considered to be psychological stressors (initially developed to mimic daily hassles; Willner, 2005), they often utilize potentially physiologically stressful components (food deprivation, light cycle reversals; Koch et al., 2016), making it challenging to untangle selective contributions of psychological stress to the overall response. Furthermore, considerable variability in the application of these various psychological stress models (e.g., differing lengths, frequency, mild stressors used; Strekalova et al., 2022) participate in making comparisons between studies difficult. Indeed, research has supported relatively minor protocol differences, such as the temperature of the water used for forced swimming, to differentially affect the stress response (Linthorst et al., 2008). At present, research has not enabled reaching a consensus as to these critical variables.

5.2.1 Psychological Stressors Differentially Impact HPG-mediated Function.

Concordant with the broad number of psychological stress models and considerable variability in their application, impacts on HPG signalling and reproductive function have been mixed. Indeed, research has shown that in female mice, FSH and LH are reduced by 28 days of chronic restraint stress (Huang et al., 2022), whereas FSH is increased following 35 days of CUMS (Fu et al., 2018). Acute stress induced via 1 h restraint on the morning of proestrus can reduce LH and P4 surges by the afternoon of proestrus (Donadio et al., 2007), whereas in ovariectomized mice, 180 min of restraint stress significantly reduces LH pulse frequency without impacting pulse amplitude (Yang et al., 2017). The effects of stress on E2 levels also vary widely, both between and within stress models. Indeed, while some studies report CUMS to cause a decrease in E2 levels (Fu et al., 2018; Xu et al., 2020), others found no effects (Grippe et al., 2005), and some

chronic restraint studies have reported increased E2 levels post-stress (Blume et al., 2019). Importantly, one study reported post-CUMS decreases to E2 levels in proestrus rats, but no detectable differences in diestrus rats (Lu et al., 2015). Similarly, predator stress failed to induce changes in diestrus rats but significantly reduced estrus E2 levels (Medubi et al., 2017), suggesting stage-dependent effects of psychological stressors. Of interest for this thesis, the effects of different psychological stress models on estrous cycle regularity have been mixed (see for example the inconsistent effects of restraint stress: Breen et al., 2012; Fotsing et al., 2017; Pansarim et al., 2023; Wagenmaker & Moenter, 2017). As such, the potential of such models for disrupting this key reproductive indicator remains to be clarified.

5.3 Social Stress Models

Many experimental stress models, such as restraint or forced swimming, while effective at activating the HPA axis, have limited ecological validity with stressors that might be experienced by rodents, or by humans, in everyday life (Brunton, 2013; Gądek-Michalska et al., 2016). Thus, social stress models are generally viewed as achieving enhanced translational value, related to a greater resemblance to stressors experienced in modern society (Koolhaas et al., 2016). One commonly used social stress model, known as Chronic Social Defeat Stress (CSDS) or Resident-Intruder Stress, involves introducing an intruder rat into the cage of an aggressive resident rat (Koolhaas et al., 2013). Social defeat stress elevates heart rate and mean arterial pressure in male rats, whether the rat is undergoing CSDS or simply witnessing it (Finnell et al., 2017). Unfortunately, many social stressors commonly used in male rodents are more challenging to implement in females, partly due to reduced aggressivity in the latter group. Indeed, female rats typically display territoriality and aggressivity towards intruders only during the first half of the lactation period (Brunton & Russell, 2010; Kuske & Trainor, 2022).

Concordantly, a systematic review of 318 social defeat studies found that 95% of studies used male rodents (Lyons et al., 2023), leaving a comparatively limited understanding of the efficacy of this model in females.

Alternately, changes to population density represent a significant source of social stress in many species, including humans, with both extremes of the social density spectrum having negative impacts on well-being (Love & Zelikowsky, 2020). On one end of the spectrum, isolation has repeatedly shown a negative impact on well-being and health. In adult female rats, 42 days of chronic social isolation elevates depressive-like behaviours as measured by the Sucrose Preference Test (SPT) and Forced Swim Test (FST), while also lowering body weight and increasing CORT levels (Carnevali, Statello, et al., 2020). Similarly, 14 days of social isolation resulted in increased anxiety- and depressive-like behaviours in the Light Box Test and the SPT in both male and female adult Sprague Dawley rats (Carrier & Kabbaj, 2012). Interestingly, at the other end of the population density spectrum, crowding similarly results in increased anxiety-like behaviours in the Open Field Test (OFT) and EPM, as well as elevated CORT levels in adult male Wistar rats (Adavi et al., 2023). In female rats, effects of crowding have been mixed, with some studies reporting no effects on CORT secretion (do Nascimento et al., 2019) or even lowered CORT levels compared to that observed in isolated females (Brown & Grunberg, 1995). On the other hand, Social Instability Stress (SIS) models, which involve alternating phases of isolation and crowding, often with varied group compositions, were shown to increase stress levels in female rodents (Goñi-Balentziaga et al., 2018). Thus, adult female Wistar rats having undergone SIS for a 15-day period displayed reduced weight gain, heightened CORT levels, and lowered sociability (Baranyi et al., 2005). Other social instability models which solely rely on modifying group compositions rather than increasing housing density have

also been found to have short- and longer-term impacts on stress physiology (For review, see Koert et al., 2021).

5.3.1 Social Stressors' Impact on HPG Function Remains Understudied. Exposure to 28 days of SIS in female Wistar rats was found to increase CORT levels and reduce the number of rats presenting normal estrous cycles (Herzog et al., 2009), indicating a potential for social stress models to be used to study stress-induced changes to reproductive function. However, while this study evaluated prolactin (PRL) and LH, and found significant increases to both hormones post-stress, authors failed to include any other key HPG axis markers, providing a partial understanding of the impact of this stressor. The effects of crowding stress on reproductive parameters also remain to be extensively characterized, although Kim & You (2022) reported reduced FSH and LH levels following crowding stress in C57BL/6 mice, paralleled by a reduction in the number of primordial and primary follicles. Prolonged exposure to isolation during adolescence has been linked with decreased sexual functioning in mice, namely through reduced lordosis intensity, and led to ER α overexpression in the AVPV (Kercmar et al., 2014). Additionally, adult female Sprague-Dawley rats housed in isolation were found to enter constant estrus (i.e., reproductive senescence) on average 2 months earlier than group housed counterparts (LeFevre & McClintock, 1991). As such, while the existing literature supports social stress to disrupt various facets of reproductive function, much remains to be known about how social stressors impact HPG axis signalling in adult females. A lack of information on the expression of critical neuropeptides such as Kiss1 and GnIH is particularly felt. Knowing that social stressors could better mimic the human experience, determining the most effective social stress model to induce reproductive disruptions can significantly benefit translational research.

6. Associations Between Stress and Reproductive Impairments in Humans

Numerous animal studies have demonstrated interactions between stress-related HPA axis activation and inhibition of HPG axis signalling and reproductive function, with evidence supporting similar effects in clinical populations. In women, systemic stress via COVID-19 infection has been found to significantly increase the risk of menstrual cycle irregularities (Tayyaba Rehan et al., 2022). Furthermore, women with congenital heart disease experience sexual dysfunction and poor pregnancy outcomes (e.g., miscarriage, premature labour) at higher rates than healthy counterparts (Haberer & Silversides, 2019). Existing evidence further supports high levels of perceived stress or elevated frequency of stressful life events to be associated with various reproductive impairments in men and women, namely sexual dysfunction, reduced fertility, and menstrual irregularity (Gołyszny et al., 2022). In fact, psychosocial stress is one of three primary causes of functional hypothalamic amenorrhea (FHA), an HPG-axis endocrine disorder relating to disrupted pulsatile secretion of GnRH in the absence of anatomic pathology, characterized by an interruption of menstruation (Saadedine et al., 2023). Compared to healthy controls, individuals with FHA have been found to present significantly higher CRH levels, paralleled by significantly reduced serum Kiss1 concentrations (Podfigurna et al., 2020). Further supporting the contribution of stress to this pathophysiological state, a study of women with FHA found greater recovery of ovarian activity in participants who were treated with cognitive behaviour therapy compared to those assigned to an observation group (Berga et al., 2003).

It is important to acknowledge that reproductive impairments can have various consequences on the lives of the individuals who experience them and are often in and of themselves stressful (Aimagambetova et al., 2020; de Moraes et al., 2025; Park, 2025). Indeed, research shows that women undergoing infertility treatment have an elevated prevalence of

anxiety and depression compared to fertile controls (Pasch et al., 2016), with women being more affected psychologically by infertility compared to male partners (Chen et al., 2004; De Berardis et al., 2014; Rooney & Domar, 2018). In women, childhood trauma, depressive symptoms, and flattened diurnal cortisol slopes represent significant predictors of higher scores on the Decreased Sexual Desire Screener (O’Loughlin et al., 2020). However, participating in sexual activity has been shown to have many health benefits, notably conferring protection against psychological distress during the COVID-19 pandemic (Mollaioli et al., 2021). Furthermore, orgasms, whether experienced with a partner or through masturbation, have both been associated with improved sleep (Lastella et al., 2019), and good sex quality is linked to reduced risk of CVD later in life (Liu et al., 2016). Decreased sexual functioning is concordantly linked with lowered health-related quality of life (Biddle et al., 2009; Mitchell et al., 2013). As such, the importance of understanding the impacts of stress on female reproductive function does not stem solely from possible reductions in the ability to procreate, but also from the known influence such reproductive disruptions can have in the day-to-day lives of the people affected. The vicious cycle wherein stress-induced reproductive impairments generate further stress (Palomba et al., 2018) emphasizes the need to thoroughly characterize these relationships and the underlying HPA-HPG axis interactions.

7. Regular Ovarian Cycles as Key Indicators of Reproductive Function

Multiple studies support estrous-stage dependent changes in behaviour, physiology, and brain function. It is known that the stress response and its associated anxiety-like behaviours fluctuate across the rodent estrous cycle, with studies reporting increased anxiety-like behaviours in proestrus/estrus compared to diestrus on a variety of tests (for review, see Lovick, 2012). In fact, estrous cycle stage has recently been suggested to modulate the deleterious effects of

restraint on a Pavlovian conditioning task (Johnston et al., 2026). At a biochemical level, estrous cycle stage has an impact on key HPG axis markers, with significantly lower ovarian Kiss1 immunostaining density during the diestrus and metestrus phases than expression in estrus and proestrus (Shahed & Young, 2009). GnRH receptor mRNA content has also been found to fluctuate across the estrous cycle, with a peak in GnRH content being observed in the hypothalamus and in the anterior pituitary gland during the afternoon of proestrus (Bauer-Dantoin et al., 1993; Schirman-Hildesheim et al., 2005). In fact, the effects of stress on estrous cycling itself have even been suggested to depend on estrous cycle stage at stressor initiation, with more pronounced cycle alterations being observed when rats were in proestrus or estrus on the first day of predator stress (Medubi et al., 2017). The studies mentioned herein support the relevance of controlling for estrous cycle stage when assessing both behavioural and biochemical parameters in females, as key effects might be missed if all stages are pooled together (Rocks et al., 2022).

Of critical importance, deviations from regular menstruation or normal uterine bleeding can be indicative of underlying health issues. Irregular menstruation is frequently reported by individuals with hypothalamic hypogonadism (Rothman & Wierman, 2008), polycystic ovarian syndrome (PCOS; Witchel et al., 2019), and endometriosis (Gete et al., 2023). In fact, evidence shows that individuals with irregular menstrual cycles are at increased lifetime risk of other diseases, namely CVD, diabetes, or ovarian cancer (Harris et al., 2017; Kiconco et al., 2022; Rostami Dovom et al., 2016; Wang et al., 2022). Concordantly, some researchers consider menstruation and regular ovulation as the “fifth vital sign” in women (Minakshi et al., 2022), further emphasizing the importance of understanding how stress impacts this key indicator of women’s health. Indeed, experiencing psychological stressors such as academic stress

(Yamamoto et al., 2009), incarceration (Allsworth et al., 2007), or exposure to natural disasters (Li et al., 2011) have been correlated with abnormal menstruation. Repeated stress has also been shown to impact estrous cycling in rodents, often resulting in decreased time spent in reproductive stages (e.g., proestrus), a lengthening of the overall cycle, or an acyclic state (typically persistent diestrus or estrus; Casillas et al., 2021; Medubi et al., 2017; Shimamoto et al., 2011). Of note, however, repeated application of unpredictable foot shocks throughout the day over a two-week period showed no impact on estrous cyclicity (Anderson et al., 1996), and when using other stress models, including restraint, results vary widely as to the impact of stress on cycle regularity. The lack of consensus on this question, paired with a minimal understanding of the unique impact of different stress models, actively limits the ability to select the most appropriate stressor to study stress-induced disruptions of cyclicity.

8. Thesis Objectives

While there is no doubt that stress can negatively affect reproduction, several gaps remain in the examination of these effects. An important question pertains to the ability to better differentiate consequences related to exposure of distinctive stressors, including systemic, psychological, and social stressors, on HPG axis signalling and estrous cyclicity. In this context, the ability of psychological models (such as restraint) at altering HPG axis function has been extensively studied, but widely diverging effects prevent achieving a consensus on their efficacy. In contrast, the impact of social and systemic stressors experienced in adulthood, likely to better translate to human experience, remain largely uncharacterized, particularly in females. To our knowledge, although we know systemic stressors such as stroke and cardiac accidents to induce lasting HPA axis dysfunction (de la Tremblaye et al., 2014), associated with reduced GnRH and GnRH receptor expression in the hippocampus of male rats (Chu et al., 2008), there are no

studies having characterized the impact of such stressor on female HPG axis function. Similarly, while Herzog et al. (2009) found that chronic SIS in adulthood reduced the number of rats normally cycling and LH levels, they failed to include other cerebral or gonadal measures of HPG axis function. Seeing as the physiological and behavioural consequences of various other stressors are known to differ (Atrooz et al., 2021), it goes to reason that psychological, systemic, and social stressors likely differentially affect HPG axis function and associated reproductive processes.

The primary aim of this thesis was to characterize how different stressors impact reproductive function. As we initiated this research, we realized the importance of having a comprehensive comparison of previous research having assessed the impact of exposure to psychological stressors on reproductive parameters in humans and rodents. In this spirit, the first two chapters of this thesis aimed to systematically review the existing evidence assessing 1) associations between psychological stress and irregular menstruation in adult women (**Chapter I**), and 2) the impact of psychological stress models on estrous cycling in rodents (**Chapter II**). With the knowledge that regular ovarian cycling is a key predictor of future health and has been considered a relevant vital sign in women (Minakshi et al., 2022), a thorough and comprehensive review of the existing evidence is critical to informing the selection of relevant models when assessing alterations to cycling. Subsequently, we examined mechanisms through which various stressors affect HPG axis function in adult female rats. Specifically, this thesis characterized the effects of systemic and social stressors on reproductive function and HPG axis reactivity in adult female rats. The **third chapter** of this thesis provides the first assessment to our knowledge of the impacts of adulthood GCI on estrous cyclicity and critical hypothalamic regulators of reproduction. The **fourth chapter** presents a comprehensive evaluation of the impact of three

social stressors (isolation, crowding, and instability) on HPG axis signalling, sexual behaviours, fertility, and estrous cycle regularity. Finally, the **fifth chapter** provides an in-depth exploration of the home cage dynamics during crowding and instability stress, aiming to further evaluate how individual differences in behaviour might influence stress and reproductive outcomes after exposure to a social stressor.

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Overview of Chapters

Chapter	Stressor type	Groups	Outcome measures	
			Behavioural & physiological	Biochemical
I) <i>Bloody Stressed! A Systematic Review of the Associations Between Adulthood Psychological Stress and Menstrual Cycle Irregularity</i>	Psychological	N/A (Systematic review of 41 studies)	Menstrual cycle characteristics	None
II) <i>The Cycle of Stress: A Systematic Review of the Impact of Chronic Psychological Stress Models on the Rodent Estrous Cycle</i>	Psychological	N/A (Systematic review of 32 studies)	Estrous cycle regularity	Any HPG or HPA axis markers reported (e.g., LH, E2, CORT)
III) <i>Global Cerebral Ischemia in Adult Female Rats Interrupts Estrous Cyclicity and Induces Lasting Changes in Hypothalamic-Pituitary-Gonadal Axis Signalling Peptides</i>	Systemic	N = 16 Ischemia (n = 8) Sham (n = 8)	Estrous cycle regularity	Immunofluorescence: ER α , Kiss1, GnIH, GR Ovary weights Thionine staining
IV) <i>Chronic Social Isolation, Crowding, and Instability Distinctly Impact Reproductive Function in Adult Female Wistar Rats</i>	Social	N = 112 Isolation (n = 24) Crowding (n = 24) Social instability (n = 24) Control housing (n = 24) Regular housing (n = 16)	Body weight Estrous cycle regularity Fertility parameters Sexual behaviours	Adrenal gland and uterine horn weight ELISA: LH, FSH, E2, P4, CORT Immunofluorescence: ER α , Kiss1, GnIH, GR Ovarian histology
V) <i>Playing or Fighting? Home Cage Dynamics During Crowding or Social Instability Paradigms Contribute to Stress and Reproductive Impairments in Adult Female Wistar Rats</i>	Social	Subgroup analysis of the crowding and instability groups from Chapter IV	Body weight Estrous cycle regularity Home cage behaviours	<i>Analysis of High vs. Low players</i> Adrenal gland and uterine horn weight ELISA: LH, FSH, E2, P4, CORT Immunofluorescence: ER α , Kiss1, GnIH, GR

Chapter I – Bloody Stressed! A Systematic Review of the Associations Between Adulthood**Psychological Stress and Menstrual Cycle Irregularity**

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Authorial Contributions

M.P. designed the research question and search strategy under H.P.'s supervision and performed the database search. Each abstract and full text was independently screened by M.P. and one other trained research assistant (F.S., A.F.Q., or C.B.). Independently from one other research assistant, M.P. performed or verified the data extraction and risk of bias assessment for each article. M.P. completed all data synthesis, visualizations, and prepared the first draft of the manuscript with support from the co-authors (F.S., A.F.Q., and C.B.).

Studies included in this systematic review are identified in the reference list (†)

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Abstract

Regular menstruation is a key indicator of general health and irregular menstrual parameters have been associated with elevated risk of adverse health outcomes. While psychological stress is believed to contribute to abnormal menstruation, little is known of the effects of discrete psychological stressors, including the COVID-19 pandemic, on menstrual function. A systematic database search was performed and studies investigating the relation between psychological stress and menstrual cycle irregularity in otherwise healthy adults were included. Two independent investigators completed abstract and full-text screening, data extraction, and risk of bias assessment. In the 41 included studies, a variety of stressors were assessed, namely COVID-19 pandemic stress, academic stress, and occupational stress. Our review found most studies reported an association between psychological stress and menstrual dysfunction, with the most common disruptions being irregular menstruation and abnormal menstrual flow. Our findings also underlined notable discrepancies in the operational definitions used in the study of menstrual disorders. These observations emphasize the importance of psychological stress as a modifiable risk factor associated with irregular menstruation.

Keywords: menstrual function, stress, menorrhagia, menstrual cycle irregularity, oligomenorrhea, amenorrhea

PRISMA Abstract

Background: Regular menstruation is a key indicator of general health, and irregular menstrual parameters have been associated with elevated risk of adverse health outcomes. While psychological stress is believed to contribute to abnormal menstruation, little is known of the effects of discrete psychological stressors, including the COVID-19 pandemic, on menstrual function. As such, we aimed to systematically review the evidence on the associations between adulthood psychological stress and irregular menstruation.

Methods: A systematic database search of MEDLINE, APA PsycInfo, Embase, Cochrane CENTRAL, CINAHL, and Web of Science was performed on May 18, 2023, and studies investigating the relation between psychological stress and menstrual cycle irregularity in otherwise healthy adults were included (PROSPERO record: CRD42023428005). Two independent investigators completed abstract and full-text screening, data extraction, and risk of bias assessment using the JBI critical appraisal checklist for analytical cross-sectional studies. Findings are summarized using descriptive statistics.

Results: The review included 41 studies, totalizing 39,756 participants, recruited from 30 countries across the world. A variety of stressors were assessed, namely COVID-19 pandemic stress, academic stress, and occupational stress. Our review found most studies to report an association between psychological stress and increased menstrual dysfunction, with the most common disruptions being irregular menstruation and abnormal menstrual flow.

Conclusions: Our review underlined notable discrepancies in the operational definitions used in the study of menstrual disorders. Overall, these observations emphasize the importance of psychological stress as a modifiable risk factor associated with irregular menstruation.

1. Introduction

Several lifestyle and physiological factors have been linked to alterations in the regularity of the menstrual cycle, including smoking, drug use, excessive exercise, high or low body mass index (BMI), lack of sleep, depression, and hormonal contraceptive (HC) use (Bae et al., 2018; Cho et al., 2017; Kim et al., 2018; Zafar, 2020). Importantly, the existence of bidirectional relationships between the hypothalamic-pituitary-adrenal (HPA) and -gonadal (HPG) axes, which respectively modulate the stress response and reproductive functioning, is well acknowledged (Acevedo-Rodriguez et al., 2018; Gołyszny et al., 2022). Concordant with such interactions, clinical and preclinical studies have highlighted an association between increased stress and irregularities of the ovarian cycle (Divyashree & Yajurvedi, 2016; Prokai & Berga, 2016; Retana-Márquez et al., 2020), and a recent systematic review from our group further highlighted the adverse effects of chronic stress on the ovarian cycle in rodents (Poitras, Lebeau & Plamondon, 2024). To our knowledge, no such review has been performed in humans.

Factors affecting female reproductive health are frequently overlooked and concerns regarding menstrual irregularities or severity of menstrual symptoms are often dismissed by medical practitioners (Berkley & McAllister, 2011; Hoffman et al., 2020). Such behaviour generates additional stress, while also delaying the detection of gynecological disorders such as endometriosis (Wren & Mercer, 2022). Regular ovulation is considered a key vital sign of the overall health of the female body (Itriyeva, 2022) and its disruption has important implications for well-being. Evidence from recent studies suggests individuals with irregular or abnormal menstrual cycles to have a heightened risk of developing adverse health outcomes, such as cardiovascular disease, diabetes, or ovarian cancer (Harris et al., 2017; Kiconco et al., 2022; Rostami Dovom et al., 2016; Wang et al., 2022). Of interest, a significantly elevated anxiety

prevalence has been reported in American adults younger than 50 years of age assessed in 2018 compared to a same age cohort assessed one decade earlier (Goodwin et al., 2020), a phenomenon also shown to have been amplified by the pandemic (World Health Organization, 2022). The reported increase in the prevalence of stress, combined with the higher lifetime risk of stress-related disorders, including anxiety and depression, in women compared to men (Hantsoo & Epperson, 2017; Helpman, 2023), supports a need for a systematic review of the evidence on the associations between perceived stress and menstrual cycle regularity, a key indicator of overall health.

Therefore, the main objective of this systematic review was to summarize findings from the existing literature addressing the relationship between psychological stress and abnormal menstruation in otherwise healthy adults. A second objective aimed to identify the most common stressors associated with specific menstrual disorders (irregular cycles, abnormal cycle lengths, and prolonged or heavy bleeding), while also commenting on key methodological aspects of studies covering this research field.

2. Methodology

2.1 Protocol and Registration

The findings of this review are reported using the Preferred Reporting Items for Systematic Reviews and Meta-Analyses (PRISMA) checklist. Prior to beginning the project, the protocol was registered in accordance with the International Prospective Register of Systematic Reviews (PROSPERO; CRD42023428005).

2.2 Information Sources and Search Strategy

M.P. and H.P. developed the search strategy and consulted a social sciences research librarian with expertise in knowledge syntheses to review the strategy and assist with identifying

relevant databases. The search strategy was designed by consulting various sources, including a systematic review on the impact of psychological stress (Kuo et al., 2019) as well as examining keywords relating to menstruation (Deligeoroglou & Creatsas, 2012; Edozien, 2006; Tayyaba Rehan et al., 2022). The final strategy used relevant keywords as well as database-specific controlled vocabulary relating to menstrual cycles and stress. Following full text selection, the reference lists of the included publications were screened for any additional eligible studies.

The initial database search occurred on May 18, 2023, and we collected all publications up until that date from the following databases: MEDLINE (Ovid), APA PsycInfo (Ovid), Embase (Ovid), Cochrane CENTRAL (Ovid), CINAHL (Ebsco), and Web of Science (Science Citation Index Expanded; Social Sciences Citation Index; Emerging Sources Citation Index). The complete search strategy is available in [Appendix A1](#).

2.3 Eligibility Criteria

2.3.1 Inclusion Criteria

Throughout all stages of the review, we included studies looking at environmental factors acting as psychological stressors (e.g., academic pressure, war, natural disasters, perceived daily stress) and their association with the regularity of the menstrual cycle in otherwise healthy adults of reproductive age. We included original peer-reviewed publications written in English. For the purposes of this review, the International Federation of Gynecology and Obstetrics (FIGO) guidelines for abnormal menstruation were used (Munro et al., 2018), and as such, we included studies having assessed irregular menstruation onset, frequent cycles (also known as short cycles or polymenorrhea), infrequent cycles (also referred to as long cycles, oligomenorrhea, or amenorrhea when menstruation is absent for > 90 days), prolonged menstrual flow or heavy menses (both otherwise known as menorrhagia). However, due to a particular interest in the

methodology used in studies assessing menstruation, studies were included even when the definitions used were not in perfect accordance with the FIGO guidelines, which define a normal menstrual cycle as being 24-38 days long with cycle-to-cycle variance < 10 days, and up to 8 bleeding days with amount of blood loss self-described as normal (Munro et al., 2018, 2022). For example, studies that defined frequent cycles as being < 21 days, rather than < 24 days as per FIGO guidelines were still included, enabling to comment on such discrepancies from the established guidelines, as well as not penalize studies published before such guidelines were made available. Studies that reported a global “menstrual disorders” category which included disruptions not mentioned in the definition above (such as dysmenorrhea or premenstrual syndrome; PMS) were not included.

2.3.2 Exclusion Criteria

Title and Abstract Screening Exclusion. During the title and abstract screening, references were excluded if they met any of the following criteria: 1) Relevant menstrual cycle parameters not reported; 2) No psychological stress assessed; 3) Stressor occurred prenatally or during childhood/adolescence; 4) Sample includes children/adolescents (aged < 17) or menopausal individuals (absence of menses for > 1 year); 5) Entire sample has a pre-existing medical or psychiatric condition that could interfere with menstrual cycle regularity (e.g., endometriosis, polycystic ovarian syndrome, schizophrenia, anorexia); 6) Animal study; 7) Qualitative data only; 8) Not available in English; 9) Not an original peer-reviewed research/study (e.g., protocol registration, reviews or meta-analysis, case report, erratum articles, program evaluations, graduate thesis, book chapter).

Full Text Screening Exclusion. During the full text screening phase, references were excluded if they met any of the following criteria: 1) Menstrual cycle parameters of interest not

reported or data combined into a larger category with other menstrual disorders (e.g., dysmenorrhea, PMS); 2) Primary stressor not a psychological stressor (e.g., metabolic stress from malnutrition, excessive exercise); 3) Sample includes children/adolescents (aged < 17) or menopausal individuals; 4) No appropriate comparison point (e.g., a pre-stress measure, a group reporting low stress levels or unexposed to the stressor, regularly cycling comparison) or no analysis of relation between stress and menstrual regularity; 5) Qualitative data only; 6) Not relevant publication type (conference proceedings, review or book chapter, brief report, PhD thesis, etc.); 7) Not available in English. See Figure 1 for PRISMA diagram of excluded studies at all stages of the review and [Appendix A2](#) for reasons for exclusion during full-text review.

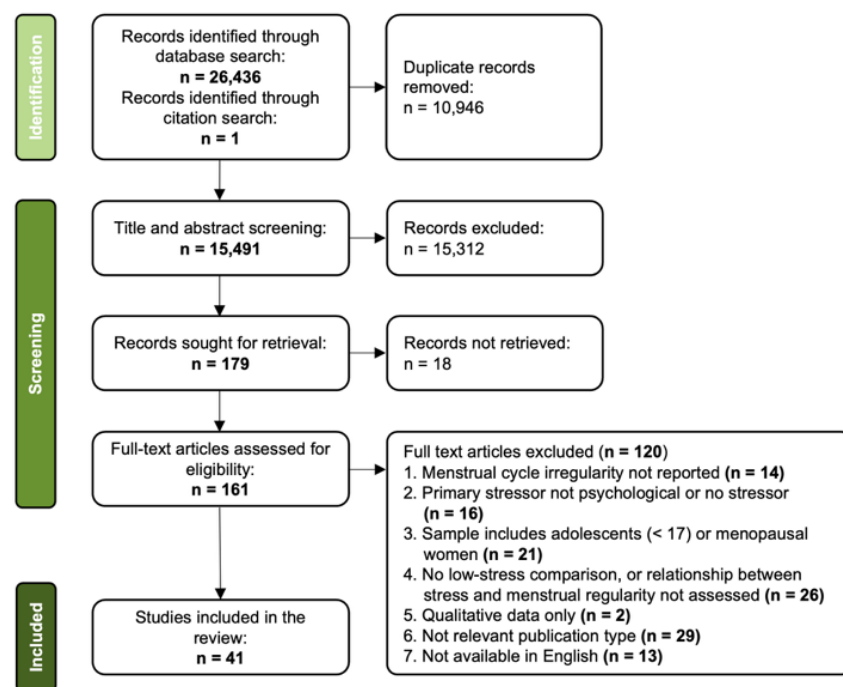


Figure 1. PRISMA Flow Diagram of Studies Included in the Systematic Review.

2.4 Study Records

2.4.1 Data Management

Covidence (Veritas Health Innovation) was used for all steps of the review process (i.e., title, abstract, and full-text screening, data extraction, risk of bias assessment).

2.4.2 Selection Process

Graduate and undergraduate students appropriately trained in using Covidence and applying inclusion/exclusion criteria performed the screening of references. For all stages of screening, each reference was assessed for its relevance by lead author M.P. and one other independent reviewer. In case of disagreement, M.P. reviewed the reference, discussed with the reviewer, and made a final decision.

2.4.3 Data Collection Process

M.P. prepared the data extraction and risk of bias assessment templates in Covidence. Following any required adjustments for the data extraction and risk of bias assessment, senior author H.P. approved changes to the protocol. M.P. trained all reviewers (F.S., A.F.Q., C.B.) to perform data extraction and risk of bias assessment. For each of the included studies, data extraction and risk of bias assessment were performed by a trained reviewer and verified for completeness and accuracy by an independent reviewer, with lead author M.P. performing either the extraction or the verification for each study.

2.5 Data Items

The following information was extracted from the retained publications: a) title of article, lead author name, and year of publication; b) primary aim of the study; c) number of participants; d) any exclusion or inclusion criteria; e) relevant demographic characteristics, such as age, BMI, and age at menarche; f) HC use and presence of other confounds within the sample, namely alcohol consumption, depression, exercise, psychotropic medication use, shift work, sleep duration, and smoking (Liu et al., 2004); g) type of psychological stress and type of measure used to quantify it (e.g., perceived stress scales, anxiety questionnaires); h) method for determining menstrual cycle regularity (e.g., data from period tracking app, self-report); i)

definitions used for each menstrual disorder and prevalence reported in the sample; j) type(s) of statistical analysis(es) performed; k) any results pertaining to associations between stress and abnormal menstruation.

2.6 Risk of Bias in Individual Studies

All studies were assessed for risk of bias using the JBI critical appraisal checklist for analytical cross-sectional studies (Moola et al., 2020), to best capture the most common type of study included in this review.

2.7 Data Synthesis

Following extraction, all data was exported from Covidence into Microsoft Excel. Descriptive statistics were performed using IBM® SPSS statistics 29 (IBM, Armonk, NY, USA) and are presented as median (range) or mean $\pm$ standard deviation. Whenever relevant, we report the proportion of studies presenting specific characteristics (% of studies).

3. Results

Database search yielded 15,490 references after the removal of duplicates, and one additional eligible publication was found via citation searching. In total, this systematic review included 41 studies, published between 1987 and 2023, investigating the associations between psychological stress and menstrual cycle irregularities in otherwise healthy adults.

3.1 General Characteristics of the Included Studies

As shown in Table 1, the included studies sampled a diverse population, with participants having been recruited from 30 countries in total. None of the studies reported individuals identifying as non-binary within their sample, and it should be noted some studies specified ‘identifying as a woman’ as an inclusion criterion (See [Appendix A3](#)). Seeing as all studies reported including only women, this terminology is utilized when discussing the results from the

reviewed studies. Twenty-two studies recruited from the general population (53.7%), 12 from college/university students (29.3%), five from healthcare workers (12.2%), while the last two recruited incarcerated women or US air force employees. A majority of studies recruited independent samples (33 studies), and the remainder used cohort data, with five studies using participants from the Korea National Health and Nutrition Examination Survey (KNHANES). Sample sizes ranged from 41 to 18,076 participants (median: 395), for a total of 39,756 participants across all studies (only counting one of the five KNHANES studies in the total, due to likelihood of overlap in participants). Seventeen studies excluded participants for current HC use, 21 excluded women who were recently or currently pregnant or lactating, and 14 excluded menopausal women. The other studies did not mention these exclusion criteria, although many studies recruited within a specific age range, usually cutting off around 50 years of age, which corresponds with the mean age of menopause transition (See [Appendix A3](#) for other exclusion and inclusion criteria). To determine menstrual cycle characteristics, most studies (34 studies; 82.9%) used data from self-reported questionnaires, 10 of which had a pre-post stress design where participants were asked to recall cyclicity data prior to a stressor (e.g., COVID-19 pandemic, earthquake). Four studies used monthly menstrual diaries (9.8%), two studies used data from a menstrual cycle tracking app (4.9%), and one study used the Pictorial Blood Assessment Chart (PBAC). Of note, two studies that used self-report data also recorded the proportion of participants using a menstrual cycle tracking app (See [Appendix A3](#)), and one required completion of questionnaires on cycle length every two months, reducing the risk of recall error.

Table 1. General Characteristics of the Included Studies.

Study	Country	N	Study population	Stressor type	Cycle determination
Allsworth et al., 2007	USA	446	Incarcerated women	Incarceration	Self-report
Ansong et al., 2019	China	409	Students	Academic stress	Self-report
Anto-Ocrah et al., 2023	USA	354	General population	COVID-19 pandemic	Self-report
Aolymat et al., 2023	Jordan	385	Students	COVID-19 pandemic	Self-report
Bae et al., 2018	South Korea	4788	General population	Daily life stress	Self-report
Chang et al., 2009	Taiwan	1095	Students	Academic stress	Self-report
Chawla et al., 2023	India	306	General population	Daily life stress	Self-report
Demir et al., 2021	Turkey	263	General population	COVID-19 pandemic	Self-report
Fenster et al., 1999	USA	276	General population	Occupational stress	Daily menstrual diary
Gordley et al., 2000	USA	170	US Air Force employees	Occupational stress	Self-report
Haile et al., 2022	Andorra, Australia, Canada, Colombia, Estonia, Germany, Israel, Latvia, Netherlands, Romania, Spain, Sweden, Switzerland, UK, USA	1159	General population	COVID-19 pandemic	Fertility tracker using basal body temperature; period tracking app
Hamid et al., 2022	Iraq	405	General population	COVID-19 pandemic	Self-report
Harlow & Matanoski, 1991	USA	162	Students	Academic stress	Daily menstrual diary
Hatch et al., 1999	USA, Italy	124	Nurses	Occupational stress	Daily menstrual diary and basal body temperature
Hazar et al., 2022	Turkey	522	Nurses	Occupational stress (during COVID-19)	Self-report
Issa et al., 2022	Lebanon	398	General population	COVID-19 pandemic	Self-report
Jung et al., 2018	South Korea	3194	General population	Daily life stress	Self-report
Kim et al., 2018	South Korea	4445	General population	Daily life stress	Self-report
Kollipaka et al., 2013	India	156	Students	Academic stress	Self-report

Kwak & Kim, 2018	South Korea	4731	General population	Occupational stress	Self-report
Kwak et al., 2019	South Korea	4709	General population	Daily life stress	Self-report
Li et al., 2011	China	473	General population	Natural disasters	Self-report
Lin et al., 2007	Taiwan	746	Nurses	Occupational stress	Self-report
Liu et al., 2010	China	170	General population	Natural disasters	Self-report
Maher et al., 2022	Ireland, UK	1035	General population	COVID-19 pandemic	Self-report
Matteo, 1987	USA	41	General population	Occupational stress	Daily menstrual diary
Mittiku et al., 2022	Ethiopia	395	Students	Academic stress	Self-report
Nagma et al., 2015	India	100	Students	Academic stress	PBAC
Nguyen et al., 2021	UK, USA, Sweden, and others	18,076	General population	COVID-19 pandemic	Period tracking app
Nillni et al., 2018	USA, Canada	3346	General population	Daily life stress	Self-report
Ozimek et al., 2022	USA	210	General population	COVID-19 pandemic	Self-report
Palm-Fischbacher & Ehlert, 2014	Switzerland	696	General population	Daily life stress	Self-report
Parveen et al., 2022	Pakistan	150	Nurses (staff and student)	Occupational stress	Self-report
Prabowo et al., 2022	Indonesia	348	General population	COVID-19 pandemic	Self-report
Rafique & Al-Sheikh, 2018	Saudi Arabia	738	Students	Academic stress	Self-report
Romito et al., 2017	Italy	349	Students	Sexual harassment	Self-report
Singh et al., 2015	India	100	Students	Academic stress	Self-report
Takmaz et al., 2021	Turkey	952	Healthcare workers	COVID-19 pandemic	Self-report
Tripathy et al., 2022	India	443	General population	COVID-19 pandemic	Self-report
Yamamoto et al., 2009	Japan	221	Students	Academic stress	Self-report
Zeru et al., 2021	Ethiopia	620	Students	Academic stress	Self-report

Note. PBAC: Pictorial Blood Assessment Chart. UK: United Kingdom; USA: United States of America.

3.2 Menstrual Disorders: Definitions and Prevalence

One objective of this systematic review was to characterize the selected methodologies in studying abnormal menstrual cycle parameters. As such, we collected information regarding the operational definitions provided by authors for the various menstrual cycle abnormalities of interest (i.e., irregular, frequent or infrequent cycles, prolonged or heavy menses). It is important to note that some studies, particularly those investigating the impact of the COVID-19 pandemic, evaluated changes to menstrual cycle characteristics pre- and post-stressor (e.g., comparing mean cycle or menses length pre-post stress), typically failing to provide operational definitions for assessed disorders and/or the proportion of participants presenting these characteristics. While we report both total sample prevalence and low/high stress group prevalences for each menstrual disorder, it is important to note that fewer studies provided sufficient information to determine disorder prevalence in the low/pre-stress groups vs high/post stress groups. Refer to [Appendix A3](#) for complete overview of the information available in each study.

3.2.1 Menstrual Cycle Irregularity: Definitions and Prevalence

As depicted in [Appendix A3](#), the reported prevalence of menstrual cycle irregularity in the total sample varied considerably from study to study, ranging between 12.6% to 58.6% (median: 23.0%) of study participants. When looking specifically at participants reporting low stress or cyclicity data pre-stress exposure, the prevalence of menstrual irregularity ranged from 6.0% to 49.0%, whereas prevalence in the high/post stress groups ranged from 14.6% to 75.0% of study participants. Inconsistencies in definitions used may partly account for this difference. Nine studies defined irregular menstruation somewhat similarly to the FIGO guidelines (wherein > 10 days variability in cycle length is irregular), considering cycles to be irregular when cycle-to-cycle variation was > 4 days (1 study), > 6 days (3 studies), > 7 days (2 studies), > 9 days (1

study), or > 10 days (2 studies). Three studies considered cycles to be irregular when the cycle length fell outside of a given range (i.e., frequent and infrequent cycles), whereas five studies asked participants if their menstrual cycle was regular without specifying additional parameters for this criterion. Four studies used other definitions, such as menstruation without a periodic cycle, or menstrual cycles that were too irregular to accurately determine their length (See [Appendix A3](#)). One study categorized irregular menstruation as a composite variable that included changes to cycle length, duration of menstruation, and heaviness of flow. Finally, five studies did not define what they considered to be irregular cycles.

3.2.2 Abnormal Cycle Length: Definitions and Prevalence

As presented in [Appendix A3](#), the reported total sample prevalence of frequent menstrual cycles (also known as short cycles or polymenorrhea) ranged from 0.3% to 25.9% of study participants (median: 8.3%). In participants reporting low psychological stress, prevalence of frequent cycles ranged from 1.2% to 17.7%, whereas prevalence in the high stress group was reported to vary from 0.0% to 31.3%. On the other hand, infrequent cycles (otherwise referred to as long cycles or oligomenorrhea) in the total sample were reported in 0.5% to 30.4% of study participants (median: 5.5%), while low perceived stress group prevalence ranged from 1.4% to 20.1% and high perceived stress group prevalence of infrequent cycles varied between 0.0% and 39.3% of participants in that category. As shown in Table 2, there was considerable variability in the definitions used to characterize frequent and infrequent cycles, with only seven studies in accordance with the FIGO definition for frequent cycles (< 24 days) and four studies for infrequent cycles (> 38 days; Munro et al., 2018). Amenorrhea was the least frequently measured menstrual disorder, and the reported total sample prevalence ranged from 0.0% to 9.2%. In participants reporting low stress, amenorrhea prevalence ranged from 2.7% to 8.6%, whereas

prevalence varied between 0.0% to 10.3% in those reporting high stress. Most studies defined amenorrhea as > 90 days without menstruation (5 studies; See [Appendix A3](#)).

Table 2. Definitions Used for Abnormal Cycle and Flow Lengths.

Study	Frequent cycles	Infrequent cycles	Prolonged flow
Allsworth et al., 2007	< 21 days	> 35 days	> 8 days of flow
Ansong et al., 2019	≥ 15 periods per year	2-7 periods per year	N/A
Chang et al., 2009	< 24 days	> 35 days	≥ 7 days of flow
Fenster et al., 1999	≤ 24 days	> 35 days	> 7 days of flow
Gordley et al., 2000	< 24 days	> 35 days	> 7 days of flow
Harlow & Matanoski, 1991	< 17 days	> 43 days	Not measured
Hatch et al., 1999	≤ 25 days	≥ 33 days	Not measured
Hazar et al., 2022	< 21 days	> 35 days	N/A
Kolipaka et al., 2013	Not measured	≥ 40 days	≥ 7 days of flow and/or passage of blood clots
Lin et al., 2007	< 24 days	> 35 days	> 7 days of flow
Mittiku et al., 2022	< 21 days	> 35 days	> 8 days flow
Nagma et al., 2015	< 21 days	> 35 days	Bleeding excessive in amount or duration or both
Nguyen et al., 2021	< 24 days	> 38 days	> 8 days of flow
Nillni et al., 2018	< 25 days	≥ 35 days	≥ 5 days of flow
Palm-Fischbacher & Ehlert, 2014	< 24 days	36-89 days	Not measured
Parveen et al., 2022	< 21 days	> 35 days	> 7 days of flow
Rafique & Al-Sheikh, 2018	< 21 days	> 35 days	> 7 days of flow
Singh et al., 2015	< 21 days	> 35 days	> 7 days of flow
Takmaz et al., 2021	< 24 days	> 38 days	> 8 days of flow
Tripathy et al., 2022	< 21 days	> 35 days	> 8 days of flow
Yamamoto et al., 2009	≤ 24 days	≥ 39 days	≥ 8 days of flow
Zeru et al., 2021	< 24 days	> 38 days	> 8 days of flow

Note. For brevity, table excludes studies which had a pre-post design and compared mean lengths pre-post without defining specific disorders, as well as studies which did not provide any definitions for the disorders of interest. N/A: Not applicable because authors compared pre-post lengths without providing cut-offs.

3.2.3 Prolonged and Heavy Menstrual Flow: Definitions and Prevalence

The reported prevalence of prolonged menstrual flow in the total sample of the included studies ranged from 2.3% to 46.3% of study participants (median: 15.0%). In participants

reporting low stress, prolonged menstrual flow was reported in 0.7% to 44.6% of individuals, while this disorder was reported by 1.6% to 58.0% of participants experiencing high psychological stress. Similar to cycle length, some variability was noted in the way authors operationally defined prolonged menstrual flow, although discrepancies were less pronounced than for cycle length (See Table 2). Six studies defined prolonged flow according to FIGO guidelines, wherein > 8 days of flow was considered abnormal (Munro et al., 2018). The median reported prevalence of heavy menstrual bleeding in the total sample was 17.2% (range 0.0% to 63.6%). In studies that provided sufficient information to determine subgroup prevalences, the prevalence of heavy bleeding in low stress participants ranged from 2.7% to 32.9%, whereas prevalence in the high stress participants was reported to vary between 2.2% and 56.7%. For heaviness of menstrual flow, there was considerable discrepancy in the definitions used (See [Appendix A3](#)). Three studies assessed heavy flow by the number of pads/tampons used per day (> 7 pads for two studies, > 4 pads for one study), three studies defined heavy bleeding as > 80 ml of blood loss, five studies used participants' self-ratings of heavy or abnormal flow, and one study categorized heavy flow as > 30 pads/tampons per menses. Such discrepancies likely contributed to the wide range of reported prevalence for heavy flow.

3.3 Relationship Between Elevated Psychological Stress and Menstrual Cycle Irregularity

As shown in Table 3, the most frequently assessed psychological stressor was the COVID 19 pandemic (12 studies; 29.3%) followed by academic stress (10 studies; 24.4%) and occupational stress (8 studies; 19.5%). Seven studies focused on perceived daily life stress (17.1%), while four studies assessed other psychological stressors including natural disasters (2 studies; 4.9%), incarceration (1 study; 2.4%), and sexual harassment (1 study; 2.4%). Of the 16 validated scales used, the Perceived Stress Scale (PSS) and its shorter versions (PSS-4, PSS-7,

and PSS-10) were most frequently selected. The large number of selected scales led to most being only used by one study, limiting direct comparisons of the results between studies.

Importantly, twelve studies used only questionnaires developed by the study authors, which had not been previously externally validated. Three studies did not measure the associations with a stress scale, instead comparing menstrual characteristics pre-post stress. While it was initially intended to review the longer-term impact of stress on menstrual function, limited information was available on this topic, preventing commentary on this aspect of the relationship between stress and abnormal menstruation.

3.3.1 Elevated Psychological Stress and Irregular Menstrual Cycles

Menstrual cycle irregularity was the most assessed menstrual disorder, and 23 of the 26 studies measuring this parameter reported an association between this disorder and elevated stress. Interestingly, only two studies on COVID-19-related stress assessed menstrual cycle irregularity, with both studies finding that high stress on validated stress scales was associated with increased odds of menstrual cycle irregularity (Hamid et al., 2022; Takmaz et al., 2021). Four studies investigating academic stress reported higher stress scores measured using the PSS to be associated with students' menstrual cycle irregularity (Kollipaka et al., 2013; Mittiku et al., 2022; Nagma et al., 2015; Zeru et al., 2021) and such an association was also detected using the Chinese Health Questionnaire (Chang et al., 2009) and the Inventory to Measure Psychosocial Stress (IMPS; Yamamoto et al., 2009). However, Singh et al. (2015) failed to detect an association between stress and menstrual cycle irregularity using their own questionnaire, while Rafique & Al-Sheikh (2018) failed to do so using the PSS. Interestingly, the PSS scores of international students having recently moved to China, although correlated with the odds of experiencing changes to menstruation, were not significantly associated to irregularity of the

menstrual cycle (Ansong et al., 2019). Four studies assessed the relationship between occupational stress and menstrual cycle irregularity. The three studies including nurses reported irregular menstrual cycles to be associated with higher anxiety as measured with the Hospital Anxiety Depression Scale (HADS; Hazar et al., 2022) and stress on the PSS (Parveen et al., 2022), as well as scores from an independently developed perceived job stress questionnaire (Lin et al., 2007). Using the KNHANES data to evaluate the impact of occupational stress, Kwak & Kim (2018) reported an increased likelihood for service/sales workers, manual workers, and unemployed women to report irregular menstruation compared to non-manual workers. In this study, temporary workers and unemployed women were also more likely to experienced irregular cycles than regular workers (Kwak & Kim, 2018). All four studies having used data from the KNHANES cohort reported a significant association between the daily life stress-related questionnaire items and irregular menstruation (Bae et al., 2018; Jung et al., 2018; Kim et al., 2018; Kwak et al., 2019), although it is important to acknowledge considerable overlap in participant pools. Similarly, two studies found an association between elevated PSS scores and irregular menstruation in the general population (Chawla et al., 2023; Nillni et al., 2018), and one study using the Trier Inventory for the Assessment of Chronic Stress (TICS) also reported such an association (Palm-Fischbacher & Ehlert, 2014). Other psychological stressors, such as exposure to the Wenchuan earthquake, were reportedly associated with menstrual cycle irregularities (Liu et al., 2010). These changes were found to be closely linked with the loss of children, property, and social support (Li et al., 2011). A study using a sample of incarcerated women found those having a history of sexual abuse or parents with alcohol problems to be at significantly increased risk of experiencing irregular menstrual cycles (Allsworth et al., 2007). This is concordant with a study of university students which similarly supported menstrual cycle

irregularity to be associated with having experienced sexual harassment, sexual coercion, and cyber harassment (Romito et al., 2017). See Table 3 for an overview of key research findings.

3.3.2 Elevated Psychological Stress and Abnormal Menstrual Cycle Lengths

A relationship between abnormal menstrual cycle length and elevated psychological stress was inconsistently reported, with 6/17 studies finding an association between stress and frequent cycles while 6/18 studies reported an association with infrequent cycles. In particular, some studies reported the COVID-19 pandemic to have contributed to reducing mean menstrual cycle length in women (Haile et al., 2022; Issa et al., 2022), contrasting a significant lengthening of the cycle observed by others (Aolymat et al., 2023; Tripathy et al., 2022). In fact, a study found that COVID-19-related stress increased the risk for abnormal menstrual cycle lengths in either direction (Anto-Ocrah et al., 2023). Surprisingly, Nguyen et al. (2021) rather noted the incidence of abnormal cycle lengths to be attenuated during the pandemic when comparing tracking app data collected pre- and post-pandemic, although no significant relationship was observed with measured perceived stress in this sample. Finally, two studies reported no significant associations between COVID-19-related stress and changes to menstrual cycle length (Hamid et al., 2022; Ozimek et al., 2022), and one noted an absence of association when comparing reported cycle lengths pre- and post-pandemic (Demir et al., 2021).

In students, studies reported no association between abnormal cycle lengths and high perceived stress when measured using the PSS (Ansong et al., 2019; Nagma et al., 2015) or the Chinese Health Questionnaire (Chang et al., 2009), although Rafique & Al-Sheikh (2018) did show an association between the high PSS-10 scores and amenorrhea. Furthermore, performance demands and perceived stress on the PSS-4 in college students were associated with higher risk of having a long segment (> 43 days), while living away from home tended to shorten the cycle

(Harlow & Matanoski, 1991). Analyses of the associations between life stress and cycle length were scarce; one study using KNHANES data found no association between stress and amenorrhea (Kim et al., 2018), although frequent and infrequent cycles were not assessed. One study showed high PSS scores to be associated with short cycle lengths, although estimates remained imprecise due to a small number of women with frequent cycles (Nillni et al., 2018). Interestingly, studying occupational stress, Fenster et al. (1999) reported women with stressful work to be more likely to experience frequent cycles, contrasting Hatch et al.'s (1999) observation of a relationship between high stress scores at work and increased incidence of infrequent cycles in a sample of nurses. Meanwhile, Lin et al. (2007) observed no association between their perceived job stress questionnaire and menstrual cycle length in nurses. Interestingly, while there was no relation between job strain and cycle length in US air force employees, Gordley et al. (2000) found an association between stress from life events and abnormal cycle lengths (note that frequent and infrequent cycles were not analyzed separately). Similarly, regularly experiencing high levels of anxiety and high scores on the Holmes-Rahe Schedule of Recent Life Events were associated with longer menstrual cycles, while there was no significant association with job stress (Matteo, 1987).

3.3.3 Elevated Psychological Stress and Prolonged or Heavy Menstrual Flow

Twelve out of 22 studies reported an association between heavy and/or prolonged menstrual flow and elevated stress levels. As shown in Table 3, the majority of studies reported increased stress related to the COVID-19 pandemic to be significantly associated with these parameters when measured using a variety of validated scales or pre-post stress comparisons (Aolymat et al., 2023; Haile et al., 2022; Hamid et al., 2022; Nguyen et al., 2021; Ozimek et al., 2022). Demir et al. (2021) however noted reduced total period duration and number of daily used

menstrual pads post- compared to pre-pandemic. Anto-Ocrah et al. (2023) reported an association between elevated COVID-19-related stress and both shorter and longer flow durations, suggesting this stressor might broadly impair this menstrual parameter. Issa et al. (2022) and Maher et al. (2022) both reported heaviness of flow to be increased post-pandemic without changes to duration of flow, while Tripathy et al. (2022) found no pre-post pandemic differences for either parameter.

The existing studies were largely consistent in reporting no alterations of menstrual flow parameters associated with academic stress. Indeed, five studies failed to detect any association between heavy bleeding in students and elevated scores using the PSS (Ansong et al., 2019; Kollipaka et al., 2013; Nagma et al., 2015; Rafique & Al-Sheikh, 2018) or an independently developed stress questionnaire (Singh et al., 2015). Only one study, using the Chinese Health Questionnaire, observed a significant association between academic stress and prolonged menstrual flow (Chang et al., 2009). Similarly, of the studies assessing daily life stress, only one using the PSS reported a slightly elevated prevalence of heavy bleeds associated with high stress (Nillni et al., 2018), the other failing to detect association between PSS scores and abnormal flow parameters (Chawla et al., 2023). In nurses, occupational stress was associated with a longer menstrual bleeding duration (Lin et al., 2007), whereas menorrhagia was related to stress from life events but not job strain in US Air Force employees (Gordley et al., 2000). Consistent with observations from the latter study, Fenster et al. (1999) observed no influence of stressful work, defined as high demand in combination with low control, on menses. Notably, heavy bleeding in female university students was strongly associated to sexual coercion, gender harassment, and sexual harassment (Romito et al., 2017).

Table 3. Associations Between Psychological Stress and Menstrual Cycle Irregularity.

Stressor type	Study	Stress measure	Findings
COVID-19	Anto-Ocrah et al., 2023	PSS-10-C	High COVID-19 stress was associated with changes in menstrual cycle length (shorter and longer) and menses length (shorter and longer), with a nonsignificant trend of heavier menstrual flow.
	Aolymat et al., 2023	DASS-21	Significantly increased menstrual cycle length, heavy bleeding days, and heavy bleeding during the pandemic compared to 10 months prior. Total DASS-21, anxiety, and stress scores associated with amenorrhea and heavy bleeding, but not with cycle or menses length.
	Demir et al., 2021	N/A (compared pre-post stressor)	Participants' period duration and pads used per day decreased compared to the recorded data prior to the COVID-19 outbreak. No significant difference between the menstrual cycle length pre-post pandemic.
	Haile et al., 2022	Questionnaire developed by authors	Significant decrease in menstrual cycle length and increase in menstruation length between 2019 and 2020 for women who reported feeling stressed during the pandemic.
	Hamid et al., 2022	PSS-10	Significantly higher proportion of women with high stress scores experienced menstrual cycle irregularity and menorrhagia. No significant association with length of cycle.
	Issa et al., 2022	N/A (compared pre-post stressor)	A significantly shorter cycle length and significantly higher number of pads per day were found after the pandemic compared to before. No change in number of bleeding days.
	Maher et al., 2022	GAD-7	No change in median cycle length or days of menses, but the variability in minimum and maximum cycle length was significantly increased. Significant increase in heavy menstrual bleeding and missed periods compared to pre-pandemic. No association with GAD-7 scores.
	Nguyen et al., 2021	Questionnaire developed by authors	Proportion of users experiencing any abnormal cycle lengths decreased from 28.4% to 26.6%. The proportion experiencing increased menstrual duration increased from 3.5% to 3.9%. No significant association between ratings of stress and abnormal cycle parameters.
	Ozimek et al., 2022	PSS-10	Respondents with high Covid PSS scores were significantly more likely to experience changes in the duration of menses and heavier bleeding. No significant change in length of cycle.
	Prabowo et al., 2022	GHQ	Psychological distress was significantly correlated with the change of menstrual pattern during application of the work from home policy.
Academic	Takmaz et al., 2021	CSS; DASS-21	CSS total score, DASS-21 anxiety, and stress subdimensions were significant predictors of irregular menstruation in healthcare workers. Association with cycle length and menorrhagia were not assessed.
	Tripathy et al., 2022	N/A (compared pre-post stressor)	26.4% of women noticed a change in the menstrual cycle throughout the pandemic. Proportion of women with average menstruation days of 35-45 days increased from 5 to 8%, and >45 days increased from 5.2 to 7.6%. No differences for number of bleeding days or heavy periods.
Academic	Ansong et al., 2019	PSS-10, Questionnaire developed by authors	High stress on the PSS showed a strong association with menstrual cycle changes following arrival to China. However, menstrual cycle irregularity, abnormal menstrual lengths, length of bleeding, and heaviness of flow were not individually associated with stress levels on the PSS.

	Chang et al., 2009	Chinese Health Questionnaire	Students with a score of four or higher in psychiatric stress had significantly higher ORs of having ≥ 7 days in the variability in length of cycles and ≥ 7 days in duration of flow. No significant association between stress and cycle length.
	Harlow & Matanoski, 1991	PSS-4; PERI Life Events Scale; Questionnaire developed by authors	Weekly number of performance demands and perceived stress were positively associated with risk of an extreme menstrual segment (> 43 days). Living away from home and starting college tended to shorten cycle length. No significant association with major life events.
	Kollipaka et al., 2013	PSS-10	Stress scores of students having irregular cycles were significantly higher when compared with those with regular cycles. No association between heavy bleeding and PSS scores.
	Mittiku et al., 2022	PSS-10	Participants who perceived stress were twice more likely to have an irregular menstrual cycle (FIGO definition) compared to those who did not. Associations between stress and specific menstrual disorders (cycle length, bleeding) not analyzed.
	Nagma et al., 2015	PSS-10	There was a significant association between high stress levels (PSS > 20) and irregular menstrual cycles. No association was found with menorrhagia or cycle length.
	Rafique & Al-Sheikh, 2018	PSS-10	A strong positive association between psychological stress and amenorrhea was identified. No association between PSS-10 scores and menstrual cycle regularity or menorrhagia.
	Singh et al., 2015	Questionnaire developed by authors	No association found between high stress levels and irregular cycles or heavy menstrual flow.
	Yamamoto et al., 2009	IMPS	Students with elevated stress scores were more likely to have experienced irregular menstrual cycles. Relationship between stress score and menorrhagia not assessed.
	Zeru et al., 2021	PSS-7	Significant association between menstrual cycle irregularity as per FIGO definitions and perceived stress. Analyses not performed for individual disorders (e.g., prolonged flow).
Occupational	Fenster et al., 1999	JCQ; Questionnaire developed by authors	Women whose work was classified as stressful were twice as likely to experience frequent cycles. Stressful work was not associated with long menses or long cycles. No association was seen between menstrual function and a summary question addressing work stress.
	Gordley et al., 2000	JCQ; LEQ; Questionnaire developed by authors	Only life events stress was significantly associated with hypermenorrhea and abnormal cycle length. There was no association between any of the menstrual outcomes and job strain or non-work activities.
	Hatch et al., 1999	Questionnaire developed by authors	Among nurses who perceived their stress at work to be high, a higher prevalence of infrequent cycles was noted. There was no significant association with frequent cycles.
	Hazar et al., 2022	HADS	During the COVID-19 pandemic, nurses reporting irregular menstruation showed significantly higher anxiety levels compared to those reporting regular menstruation. Association between anxiety and change in cycle length or menstrual bleeding was not assessed.
	Kwak & Kim, 2018	Questionnaire developed by authors	Women with irregular menstruation were more likely to report stress relative to those with regular menstruation. Women who reported irregular menstruation were significantly more likely to be service/sales workers, manual workers, or unemployed than non-manual workers and more likely to be temporary workers or unemployed than regular workers.
	Lin et al., 2007	JCQ	After adjustment for confounds, high level of perceived job stress was significantly associated with irregularity of the menstrual cycle in nurses, as well as a longer menstrual bleeding period. There was no significant association with cycle length.

	Matteo, 1987	Coping checklist, context survey, and stressors checklist; Questionnaire developed by authors	Longer menstrual cycles were best predicted by elevated anxiety levels experienced on a regular basis and high scores on the Holmes-Rahe Schedule of Recent Life Events. Job stress was not significantly associated with altered menstrual cycle length or regularity.
	Parveen et al., 2022	PSS-10	Irregular menstrual cycles were strongly related to elevated perceived stress scores in staff and student nurses. Association between stress and cycle length and menorrhagia was not assessed.
Life stress	Bae et al., 2018	Questionnaire developed by authors	Following the adjustments of modifiable risk factor scores, perceived stress was significantly associated with menstrual cycle irregularity.
	Chawla et al., 2023	PSS-10	Women with irregular menstruation had significantly higher mean stress scores than regularly cycling women. No differences for heavy menses.
	Jung et al., 2018	Questionnaire developed by authors	Women with elevated perceived stress scores had significantly higher ORs of experiencing irregular menstrual cycles.
	Kim et al., 2018	Questionnaire developed by authors	Women with elevated stress had higher ORs of menstrual cycle irregularity, but risk was not increased for severe menstrual cycle irregularity (amenorrhea) in a regression analysis.
	Kwak et al., 2019	Questionnaire developed by authors	Women with irregular menstruation were more likely to display elevated stress levels than those with regular menstruation.
	Nillni et al., 2018	PSS-10	Higher perceived stress was associated with the prevalence of irregular cycles and short cycle lengths (*the number of women with short (< 25 days) cycles was however small). Higher perceived stress was associated with a slightly higher prevalence of heavy bleeds.
	Palm-Fischbacher & Ehlert, 2014	TICS	After controlling for confounding factors, chronic stress was found to be a significant predictor of irregular menstrual cycles. Associations between stress and cycle length were not assessed.
Natural disasters	Li et al., 2011	Questionnaire developed by authors	Stressors linked to the Wenchuan earthquake, namely the loss of children, property, social resources, were associated with self-reported menstrual cycle irregularity.
	Liu et al., 2010	N/A (compared pre-post stressor)	Whereas 22.4% of women had menstruation disorders before, significantly more (51.8%) had such disorders after the earthquake.
Incarceration	Allsworth et al., 2007	Questionnaire developed by authors	Having a parent with a history of alcohol or drug problem was associated with a slightly higher risk of menstrual irregularity. Any history of sexual abuse was significantly associated with menstrual irregularity. Significance was lost when recent HC users were removed.
Harassment	Romito et al., 2017	Sexual Experiences Questionnaire; Questionnaire developed by authors.	Heavy bleeding and irregular cycles were strongly linked to sexual coercion and gender harassment. Irregular cycles were significantly more frequent with exposure to cyber harassment. Significant associations were noted between sexual harassment and menstrual cycle irregularity and heavy bleeding. No significant associations with amenorrhea.

Note. DASS-21: CSS: COVID-19 Stress Scale; Depression, Anxiety, And Stress Scale-2; GAD-7: General Anxiety Disorder-7; GHQ: General Health Questionnaire; HADS: Hospital Anxiety Depression Scale; IMPS: Inventory to Measure Psychosocial Stress; JCQ: Job Content Questionnaire; LEQ: Life Events Questionnaire; OR: Odds ratio; PSS: Perceived Stress Scale; PSS-10-C: COVID-19 Pandemic-related Perceived Stress Scale; TICS: Trier Inventory for the Assessment of Chronic Stress.

3.4 Control of Confounding Variables

Several controllable lifestyle factors and physiological variables are known to impact menstrual cycle characteristics. Therefore, all information pertaining to the control of these variables was extracted (See Table 4). A large proportion of studies failed to collect information regarding age at menarche (23 studies; 56.1%), which has previously been shown to be associated with differences in menstrual cycle characteristics, namely greater odds of irregular cycles for individuals with older menarcheal age (Zurawiecka & Wronka, 2021). Surprisingly, 12 studies (29.3%) failed to measure the sample prevalence of HC users and an additional three (7.3%) reported HC users without controlling for this variable in subsequent statistical analyses. Maher et al. (2022) found that when performing a subgroup analysis of non-HC users, associations between stress and irregular menstruation were more readily detectable. Concordantly, while a strong relationship between elevated stress and irregular menstruation was reported in non-HC users, it was not observed in HC users (Palm-Fischbacher & Ehlert, 2014). Interestingly, when analyses in incarcerated women were limited to those reporting no HC use in the past 3 months, having a history of childhood or sexual abuse or a parent with an alcohol/drug problem were no longer significantly associated to menstrual cycle irregularities (Allsworth et al., 2007). Together, these studies emphasize the need to control for HC use, as the inclusion or exclusion of participants related to this criterion are likely to significantly impact statistically derived conclusions. Furthermore, it is noteworthy that only one study considered psychotropic medication use (Nilini et al., 2018) and no studies reported whether a portion of their participants were currently undergoing psychotherapy interventions, both of which have implications for stress-regulation.

The aftermath of the COVID-19 pandemic has brought additional factors likely to compromise analyses if not controlled. Indeed, irregular menstruation has been associated with COVID-19 infection (Cherenack et al., 2022; Li et al., 2021) and vaccination (Nazir et al., 2022). At present, most COVID-19 studies failed to consider these parameters (68.8% and 81.3% for infection and vaccination, respectively), which prevents fully understanding the contribution of these factors to menstrual disturbances noted post-COVID-19. As Table 4 allows to appreciate, a majority of identified confounding variables were inconsistently collected, and when measured, were not always considered in subsequent analyses. This observation supports a need for improved consistency enabling reliable comparison of study findings.

Table 4. Control of Variables that Confound Assessment of Menstrual Cycle Regularity.

Confounding variable	Measured		Excluded during recruitment	Not measured
	Controlled in analyses	Not controlled in analyses		
Alcohol consumption	11 (26.8)	9 (22.0)	1 (2.4)	20 (48.8)
BMI (underweight)	14 (34.1)	10 (24.4)	1 (2.4)	16 (39.0)
BMI (overweight or obese)	16 (39.0)	10 (24.4)	1 (2.4)	14 (34.1)
COVID-19 infection*	1 (6.3)	4 (25.0)	0	11 (68.8)
COVID-19 vaccine*	1 (6.3)	2 (12.5)	0	13 (81.3)
Depression	7 (17.1)	3 (7.3)	9 (22.0)	22 (53.7)
Excessive exercise	8 (19.5)	11 (26.8)	0	22 (53.7)
HC use	9 (22.0)	3 (7.3)	17 (41.5)	12 (29.3)
Menarche age	6 (14.6)	12 (29.3)	N/A	23 (56.1)
Psychotropic medication	1 (2.4)	0	0	40 (97.6)
Psychotherapy interventions	0	0	0	41 (100)
Shift work	1 (2.4)	4 (9.8)	0	36 (87.8)
Short sleep duration	5 (12.2)	3 (7.3)	0	33 (80.5)
Smoking	16 (39.0)	6 (14.6)	1 (2.4)	18 (43.9)

Note. Data presented as n (%) of studies. * Only applicable to studies for which recruitment was performed as of March 2020 (n = 16). BMI: Body mass index; HC: Hormonal contraceptive.

3.5 Risk of Bias Assessment

Overall, the included studies scored a low or unclear risk in most assessed domains.

Importantly, the highest identified risk stemmed from the measurement of the outcome measures,

which heavily relied on self-reported data for menstrual cycle characteristics (see [Appendix A4](#) for more details). The heterogeneity of the participants' populations and the study designs, which included a considerable variability of stress scales (including many questionnaires developed by study authors), together with inconsistent definitions of menstrual disorders, prevented conducting a meta-analysis.

4. Discussion

To our knowledge, this review is the first to systematically evaluate the existing evidence on the associations between psychological stress and menstrual cycle irregularity in otherwise healthy adults. The compendium of retained studies is characterized by the selection of a wide variety of experimental designs and stress scales, and an absence of consistent use of operational definitions for the measured menstrual cycle abnormalities. Nevertheless, 88.5% of the reviewed studies reported elevated psychological stress to be associated with irregular menstrual cycles, while 54.5% found high stress to be related to abnormal menstrual flow. In contrast, associations between stress and abnormal cycle lengths were not consistently detected in the included papers, although studies assessing the impact of the COVID-19 pandemic more frequently yielded such associations compared to pre-pandemic collected data sets. While a recently published systematic review evaluated the impact of the COVID-19 pandemic on female reproductive function and menstrual health (Tayyaba Rehan et al., 2022), no reviews to our knowledge have compared this unprecedented stressor to other types of psychological stress.

4.1 Associations Between Psychological Stress and Irregular Menstruation

4.1.1 The COVID-19 Pandemic and Menstrual Cycle Changes

The COVID-19 pandemic, declared in March 2020, has been associated with an increased exposure to psychological stressors related to social isolation, financial strain, and

anxiety surrounding possible contraction of the disease (Cooke et al., 2020). A systematic review of studies performed on populations considered most vulnerable showed the majority to report increased stress levels post-pandemic, with students and healthcare workers being highly impacted (Manchia et al., 2022). Unsurprisingly, several studies included in this review have reported the perduring elevated stress experienced during COVID-19 to be associated with altered menstrual cycle length and changes to menses duration or heaviness of flow (Anto-Ocrah et al., 2023; Aolymat et al., 2023; Haile et al., 2022; Hamid et al., 2022; Ozimek et al., 2022; Prabowo et al., 2022; Takmaz et al., 2021). Importantly, collected information in most of these studies was primarily related to changes in discrete characteristics of the menstrual cycle in the pre- and post-pandemic period, often omitting to collect crucial information related to observed changes in menstrual cycle regularity. Such methodology renders the conclusions heavily reliant on retrospective self-report of pre-pandemic menstrual data and prevents a complete understanding of stress-related alterations of menstrual regularity. Furthermore, while several studies reported significant alterations to menstrual characteristics after the pandemic, some failed to include a stress scale (Tripathy et al., 2022) or to analyze the associations between scores obtained on the included stress scales and the menstrual parameters of interest (Demir et al., 2021; Issa et al., 2022). Using digitally collected data from a period tracking app, Nguyen et al. (2021) noted the reduced occurrence of abnormal menstrual lengths observed post-pandemic to be unrelated to measured stress levels, suggesting other factors may contribute to disrupting menstruation. Likely due to the urgency in appreciating the effects of the COVID-19 pandemic, many studies that were published at short intervals post-pandemic are lacking proper methodology, omitting to control for several acknowledged confounding factors, including BMI, smoking, sleep, and depression (See [Appendix A3](#)). Our review highlights that while studies

have reported an association between COVID-19 stress and abnormal menstruation, there is a need for high-quality studies using validated stress scales and including a greater breadth of possible confounding variables into their analyses, as well as menstrual cycle irregularity as a parameter of interest. Importantly, evidence continues to emerge on the associations between COVID-19 pandemic-related stress and irregular menstruation (Calaf et al., 2024; Polese et al., 2024) and much remains to be understood about this relationship.

4.1.2 Academic Stress and Irregular Menstruation

Evidence from previous studies suggests the prevalence of students experiencing stress related to academic demands to be high (Al-Shahrani et al., 2023), and female students to be perceiving significantly elevated academic-related stress compared to male students (Al-Shahrani et al., 2023; Deatherage et al., 2014; Sehlo et al., 2018). Most studies in this review reported adverse effects of academic stress, as shown by the association between perceived stress and irregular menstrual cycles (Chang et al., 2009; Mittiku et al., 2022; Nagma et al., 2015; Yamamoto et al., 2009; Zeru et al., 2021), in the absence of menorrhagia (Ansong et al., 2019; Kollipaka et al., 2013; Nagma et al., 2015; Rafique & Al-Sheikh, 2018; Singh et al., 2015). Such observation is consistent with a reduced prevalence of menorrhagia in young adults, and an increased incidence of developing this condition with age (Ibrahim & Samwel, 2023; Santos et al., 2011). Importantly, recent evidence suggests an association between irregular menstruation and reduced academic performance, with a lower grade point average being observed in students with irregular menstruation (according to the FIGO guidelines) compared to regularly cycling counterparts (Demeke et al., 2023). Further investigation of the relationship between academic stress and menstruation could help identify key factors fostering improved academic performance and overall well-being of students who menstruate.

4.1.3 Occupational Stress and Irregular Menstruation

The existing literature evaluating a possible association between occupational stress and the menstrual cycle is mixed. While some found increased levels of perceived job stress and anxiety in nurses to be associated with a variety of abnormal menstruation parameters (Hatch et al., 1999; Hazar et al., 2022; Lin et al., 2007; Parveen et al., 2022), assessments in other work settings did not yield any association between job stress and menstrual disorders (Gordley et al., 2000; Matteo, 1987). The report of such an association in nurses appears sound considering highly physical and emotional demands of such employment, as well as higher risk of work-related negative health outcomes, illnesses and psychiatric disorders, including anxiety and depression (Damiani & de Carvalho, 2021). In this context, it appears crucial to consider that many nurses work irregular shifts, which itself can disturb multiple endogenous processes (Dall'Ora & Dahlgren, 2020) and has been associated with irregular menstruation and dysmenorrhea (Hu et al., 2023). If not controlled, this factor could act as a confounding variable in assessing the relationship between occupational stress and abnormal menstruation. Other occupational factors worth considering are the degree of manual work, as moderate and high levels of physical activity have been shown to affect menstrual cycle length (Harlow & Campbell, 1994). Concordantly, Kwak & Kim (2018) found that women with irregular cycles were more likely to be manual workers.

Stress-related alterations to menstrual function have significant implications in the workforce. For instance, dysmenorrhea (painful menstruation), shown to affect between 45-95% of people who menstruate, has been associated with reduced work productivity and increased absenteeism (Ponzo et al., 2022), while irregular menstruation has been linked to life dissatisfaction and health-related anxiety, which can diminish the overall quality of life (Attia et

al., 2023). Although data remains limited, abnormal menstruation is related to significant economic costs due to loss of working hours (Côté et al., 2002; Fraser et al., 2009). These findings from the included studies suggest occupational stress may contribute to dysregulation of menstrual function, changes which are susceptible to add social and financial burdens to these individuals' lives.

4.1.4 Life Stress and Irregular Menstruation

Certain environments are recognized as stressful (e.g., academic settings or intensively operating workplaces), but stress can be perceived from a variety of daily life sources. As such, studies assessing the associations between perceived stress and menstruation in a more general setting are essential to our understanding of this relationship. All seven studies having assessed daily life stress reported a significant association between stress and menstrual cycle irregularity (Chawla et al., 2023; Nillni et al., 2018; Palm-Fischbacher & Ehlert, 2014), half of which used KNHANES collected data sets (Bae et al., 2018; Jung et al., 2018; Kim et al., 2018; Kwak et al., 2019). Limited data pertaining to the other menstrual disorders of interest was available. Among life stressors which may increase the risk of menstrual cycle irregularity, experiencing sexual harassment or cyber harassment (Romito et al., 2017), sexual abuse (Allsworth et al., 2007), or exposure to natural disasters such as the Wenchuan earthquake (Li et al., 2011; Liu et al., 2010) were reported to have pronounced impacts. These studies bring forward an interesting facet of the association between stress and menstruation, relating to the impact of stressors which are potentially traumatic in nature. Indeed, sexual assault and natural disasters have both been linked with a high incidence of post-traumatic stress disorder (PTSD; Chivers-Wilson, 2006; Lee et al., 2020). At this time, it remains unclear how PTSD and the associated HPA axis disruptions

(Schumacher et al., 2019) might contribute to enduring alterations to menstruation following stress exposure.

When using data from a large prospective cohort study of American and Canadian women, Nillni et al. (2018) found that conditions of elevated stress or depression combined with use of psychotropic medications represented the tested conditions associated with the highest risk of menstrual cycle irregularities. Interestingly, this was the only study to include psychotropic medication use as a covariate, despite such treatments being highly relevant in the context of stress, anxiety, and depression. In addition, certain psychotropic medications have been associated with the development of reproductive disturbances, such as polycystic ovary syndrome (Ernst & Goldberg, 2002). Importantly, psychotherapy was not considered in the included studies, despite interventions such as cognitive behaviour therapy, psychosocial resilience training, and mindfulness therapy having been found to be effective treatments for a variety of psychological disorders (Burton et al., 2010; Butler et al., 2006; Kolahkaj & Zargar, 2015). In fact, research has shown various psychotherapies to be effective at improving PMS symptoms (Maddineshat et al., 2016; Shariati et al., 2019). Such interventions are not only important to consider while analyzing the relationship between stress and menstrual dysfunction but also present interesting avenues to help reduce stress-related disruptions of menstruation. Notably, Palm-Fischbacher & Ehlert (2014) found that dispositional resilience acted as a significant factor mediating the relationship between stress and menstrual cycle irregularity, with low dispositional resilience increasing the risk of menstrual irregularities when exposed to low and moderate stress levels compared to women showing high dispositional resilience. These studies provide interesting avenues for further investigations into the associations between stress

and menstruation, which could be of particular interest for future studies assessing COVID-19-related stress.

4.2 Measuring Menstruation: Differing Definitions and Reliance on Self-Report

Several inconsistencies in the definitions of the measured menstrual cycle parameters were noted, limiting comparability across studies. In this context, the inconsistent detection of associations between stress and abnormal menstrual cycle length could be due to the overly strict or lenient criteria used to define frequent and infrequent cycles. Most importantly, the majority of studies relied on self-reported recall of menstrual data, although previous research has shown there are notable discrepancies between self-reported recall of cycle length and actual cycle length, with 21% of participants being misclassified when using self-reported cycle length compared to data measured using daily diaries (Small et al., 2007). Perception of heavy menstrual flow has also been reported as highly subjective, with factors such as unemployment, perceived inconvenience, and abdominal pain emerging as significant predictors of complaints of heavy bleeding not meeting clinical criteria for excessive blood loss (Hurskainen et al., 2001). Furthermore, a recent study showed menstrual product absorbance capacity to differ from that advertised by product manufacturers, making estimates of blood loss volumes based on these products likely inaccurate (DeLoughery et al., 2024). In this context, the increasing use of alternative period products, such as menstrual cups, renders the use of a number of pads and tampons as typical metrics for diagnosing heavy menstrual bleeding outdated (DeLoughery et al., 2024). Therefore, studies need to 1) clearly state objective definitions for the various menstrual disorders and follow established criteria (Munro et al., 2022), 2) factor in the type of menstrual product used when assessing heavy bleeding (DeLoughery et al., 2024), 3) select objective measures that do not rely heavily on recall, such as digital tools (app) or menstrual pictograms,

and 4) report and analyze specific menstrual parameters (e.g., infrequent cycles, prolonged flow), rather than composite variables of menstrual disorders, considering that specific abnormal menstrual parameters are associated with distinct adverse health risks (Wang et al., 2022).

4.3 Physiological Correlates

Experiencing chronic stress is associated with concomitant activation of the HPA and HPG axis, leading to alterations of serum hormone levels. For instance, high perceived life stress has been associated with reduced estradiol and luteinizing hormone (LH) levels, and increased follicle-stimulating hormone (FSH) levels (Schliep et al., 2015). Concordantly, Horbatiuk et al. (2019) found that women with amenorrhea which had manifested following return from an armed conflict zone showed significantly elevated FSH and LH secretion levels concomitant with reduced progesterone and estradiol levels. These changes were paralleled to increased secretion of HPA axis signalling molecules, namely adrenocorticotrophic hormone (ACTH) and cortisol. Notably, despite the existence of objective standard measures of elevated stress and its effects on neuroendocrine secretion, none of the reviewed studies included physiological measurements relating to HPG or HPA axis function, making the associations with abnormal menstruation entirely dependent on the validity of the stress scales used. While the collection of saliva or blood samples necessary to detect such biomarkers is not always feasible, it would provide added value beyond subjective measures of perceived stress and significantly benefit the interpretation of the findings.

4.4 Strengths and Limitations

A prevalent limitation relates to the majority of reviewed studies (82.9%) relying on self-reported recall of menstrual cycle characteristics, which is susceptible to inaccuracies (Small et al., 2007). This is particularly concerning for studies using pre-post designs, such as those

collecting data before and after natural disasters or the COVID-19 pandemic. In such situation, self-reports could be influenced or biased by the participants' expectations (Adair, 1972; Coles et al., 2023). Another limitation noted in 12 reviewed studies pertained to the use of non-validated scales to measure stress, which in the absence of properly determined construct validity can affect interpretation of the collected data and study comparisons. Additionally, characterization of study findings based on a primary stressor of interest reported by authors can be problematic as humans are concurrently exposed to multiple types of stress. This is the case for studies having assessed occupational stress during the COVID-19 pandemic (Hazar et al., 2022) or measuring the impact of the COVID-19 pandemic in a population of students (Aolymat et al., 2023). Notably, while investigating the impact of the COVID-19 pandemic, Maher et al. (2022) reported that the most common type of stressor experienced by participants was work-related stress.

In performing this review, some studies were excluded during the screening process due to the data of interest having been collapsed with other menstrual disorders (e.g., dysmenorrhea), preventing relevant information from being considered. Concordantly, some studies focusing on the associations between stress and other menstrual disorders which fall beyond the scope of our review (such as PMS or dysmenorrhea; Aolymat et al., 2022), could present interesting context to the understanding of the relation between stress and menstrual function. Of importance, none of the studies reported participants who identified as non-binary, which is concerning/of interest, as LGBTQ populations might be exposed to unique stressors. For instance, LGBTQ people may have differentially experienced the negative psychological effects of the COVID-19 pandemic and experienced increased mental health problems (Salerno et al., 2020). In fact, LGBTQ people compose 20-40% of the homeless population, and homelessness is associated with increased

mental health problems (Chang et al., 2018; Fraser et al., 2019). Studying the associations between perceived stress and regularity of the menstrual cycle in non-binary and transmasculine people who menstruate could be of interest in evaluating the role of self-identification in influencing such associations. Finally, the fact that this review did not include studies solely focused on depression has restricted the breath of our review, due to an existing overlap in the pathophysiological variables at play in depression and stress (Hammen, 2015).

This review presents several strengths. The use of fairly inclusive selection criteria allowed reviewing studies using multiple experimental design types, namely those allowing comparisons of various stress levels and pre-post stress designs. Our review included studies in which were not perfectly complying with the FIGO guidelines of normal menstruation, which allowed gaining a broader perspective of the issue under study. Finally, studies included have assessed populations across the world, with participants being recruited in 30 countries, allowing a more comprehensive overview of the characterized relationships, susceptible to benefit generalizability of reported outcomes.

5. Conclusion

Overall, this systematic review provides a comprehensive, up to date, and essential summary of the literature surrounding the relationship between high psychological stress and abnormal menstrual parameters. The studies included in this review report various types of psychological stress to influence menstruation, with irregular menstruation and abnormal menstrual flow being most commonly associated with elevated stress. It is known that a high perceived burden from menstruation is associated with worsened quality of life (Bitzer et al., 2013; Weisberg et al., 2016), and that individuals with irregular menstruation are at higher risk of various adverse health outcomes (Harris et al., 2017; Kiconco et al., 2022; Rostami Dovom et

al., 2016; Wang et al., 2022). Therefore, greater attention needs to be paid as to ways in which stress dysregulates menstrual function. Stress indeed represents a modifiable risk factor of interest for interventions aimed at restoring menstrual cyclicity (Berga et al., 2003). Furthermore, long-term effects of stress exposure on menstrual function needs to be closely examined, as limited information exists on the temporal recovery of menstrual cycle regularity after experiencing heightened psychological stress. Investigations are particularly relevant to conduct in the aftermath of the COVID-19 pandemic, which represented a considerable source of stress experienced by people around the world.

6. Acknowledgments

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7. CRediT Authorship Contribution Statement

Marilou Poitras: Conceptualization, Methodology, Project Administration, Investigation, Formal analysis, Data curation, Visualization, Writing – original draft. Fatima Shear zad: Investigation, Writing – original draft. Aliya F. Qureshi: Investigation, Writing – original draft. Cassandra Blackburn: Investigation, Writing – original draft. Hélène Plamondon: Conceptualization, Supervision, Writing – review & editing.

8. Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence this review.

9. Data Availability

All data synthesized for this review is presented in the [appendices](#).

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Chapter II – The Cycle of Stress: A Systematic Review of the Impact of Chronic Psychological Stress Models on the Rodent Estrous Cycle

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Authorial Contributions

M.P. designed the research question and search strategy under H.P.'s supervision and performed the database search. Each abstract and full text was independently screened by M.P. and the co-author (M.L.) or trained research assistants under M.P.'s co-supervision. M.P. performed the data extraction and risk of bias assessment for each included article, which was independently verified for accuracy by the co-author (M.L.). M.P. completed all data synthesis, visualizations, and first draft preparation of the manuscript.

Studies included in this systematic review are identified in the reference list (‡)

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Abstract

Stress is known to impair reproduction through interactions between the hypothalamic-pituitary-adrenal (HPA) and hypothalamic-pituitary-gonadal (HPG) axes. However, while it is well accepted that stress can alter estrous cycle regularity, a key indicator of female HPG axis function, effects of different types of psychological stress have been inconsistent. This systematic review evaluated the impact of rodent models of psychological stress on estrous cyclicity, while reporting biological parameters pertaining to HPA or HPG axis function assessed within these studies. We performed a systematic database search and included articles that implemented a psychological stress model in rodents and reported estrous cyclicity for at least two cycles after initiation of stress. Of the 32 studies included, 62.5% reported post-stress alterations to estrous cyclicity, with Chronic Mild Stress (CMS) models showing the most conclusive effects. Twenty-five studies measured HPG or HPA axis markers, with cycle disruptions being commonly observed in parallel with altered estradiol and increased corticosterone levels. Our review highlights gaps in reporting estrous cyclicity assessments and makes recommendations to improve comparability between studies.

Keywords: Oestrus cycle, ovarian cycle, hypothalamic pituitary ovarian axis, HPO axis, psychological stressor, systematic review

1. Introduction

1.1. The Hypothalamic-Pituitary-Gonadal Axis

The hypothalamic-pituitary-gonadal (HPG) axis regulates a complex array of behavioural and physiological processes which ultimately allow successful reproduction, namely by regulating sexual functioning, fertility, and hormone production. Activity within the HPG axis is initiated by the hypothalamic secretion of the gonadotropin-releasing hormone (GnRH), which has downstream impacts on two gonadotropins within the anterior pituitary gland, the luteinizing hormone (LH) and follicle-stimulating hormone (FSH; Maeda et al., 2007). In females, FSH stimulates the growth and development of ovarian follicles, while LH triggers ovulation and promotes the synthesis and release of steroid sex hormones from the gonads/ovaries, namely estradiol (E2) and progesterone (P4; Acevedo-Rodriguez et al., 2018). The sex hormones produced by the ovaries provide feedback to the hypothalamus and pituitary gland via the actions of kisspeptin (Kiss1; Maeda et al., 2007), influencing the production of GnRH, FSH, and LH. This feedback mechanism, characterized by both inhibitory and stimulatory effects, tightly regulates hormone levels, ensuring hormonal balance and optimal maintenance of reproductive functioning and cyclical hormone release (Christian & Moenter, 2010; Dwyer & Quinton, 2019).

1.2 The Estrous Cycle

Similar to the menstrual cycle of human counterparts, female rodents display a regular pattern of reproductive hormone release, known as the estrous cycle, which comprises four distinct stages lasting on average 4-5 days in total. The proestrus stage, typically lasting approximately 14 h in rats, is preceded by a surge of E2 followed shortly thereafter by a surge in P4. This sequential hormonal release triggers surges in LH and FSH (Butcher et al., 1974), inducing ovulation and indicating entry into the estrus stage, during which gonadal hormone

levels gradually decrease (McLean et al., 2012). At the conclusion of the 24 h estrus, levels of E2 and P4 reach their lowest point, marking the onset of the metestrus stage in rats, a brief phase (~8 h) during which the gonadal hormones remain relatively low. Subsequently is entry into diestrus, the lengthiest phase lasting 48-72 h, characterized by a gradual increase in gonadal hormone levels until the next proestrus stage (Ajayi & Akhigbe, 2020), with a recent study suggesting the E2 surge may occur during diestrus rather than proestrus for mice, contrasting hormone profiles established in rats (Wall et al., 2023). Importantly, regular ovarian cycles are considered to be a key indicator of female health overall, and a history of menstrual cycle irregularity has been associated with a variety of adverse health outcomes in women (Attia et al., 2023), supporting the importance of studying disruptions of ovarian cycles.

1.3 Impact of Stress on Estrous Cycling

Due to the complexity of the reproductive system and the high energy demands of regulating the associated physiological rhythms, several environmental factors can disrupt normal functioning of the reproductive axis (Joseph & Whirledge, 2017; Toufexis et al., 2014). Concordantly, clinical and rodent studies have highlighted an association between increased stress and irregular ovarian cycles (Divyashree & Yajurvedi, 2016; Prokai & Berga, 2016; Retana-Márquez et al., 2020), likely in part related to inhibitory actions of the hypothalamic-pituitary-adrenal (HPA) axis on HPG axis mediated hormonal secretion (Joseph & Whirledge, 2017; Toufexis et al., 2014). Several types of stressors have been linked to disruptions of ovarian cycles. In rodents, daily restraint stress (Huang et al., 2022), chronic cold water immersion (Retana-Márquez et al., 2020), and chronic social instability (Herzog et al., 2009) have all been shown to alter estrous cyclicity. Even stressors that pose a threat at a more systemic level, such as traumatic brain injury or global cerebral ischemia, have been associated with interruptions of

estrous cycling (Fortress et al., 2019; Poitras, Morin, et al., 2024). In spite of observed effects, little is currently known as to whether some stress models are more suitable and reliable tools for studying stress-induced alterations to estrous cycling, nor the extent to which estrous cycle disruptions are reported after stress.

1.4 Psychological Stress Models

Numerous experimental paradigms have been developed to examine the behavioural and biological impacts of stress in rodents, usually categorized as 1) physical stressors, which directly pose a threat to bodily health (e.g., foot shocks, extreme temperatures, forced swimming); or 2) psychological/psychogenic stressors, which evoke anticipation or fear without physical pain (e.g., immobilization, restraint, exposure to loud noises or to predators; Atrooz et al., 2021). Psychological stress also encompasses stressors of a social nature, such as Chronic Social Defeat Stress (CSDS) and Social Instability Stress (SIS), sometimes referred to as psychosocial stressors (Koolhaas et al., 2016). Some stress models include a variety of mild psychological stressors, namely Chronic Mild Stress (CMS), also referred to as Chronic Unpredictable Mild Stress (CUMS) and Chronic Variable Stress (CVS), which are commonly used for studying the pathophysiology of depression (Willner, 2016). Importantly, many such paradigms include stressors such as food deprivation and light cycle reversals, which might confound the assessment of the impact of psychological stress on estrous cyclicity through disruptions of metabolic or circadian processes (Koch et al., 2016). Nevertheless, psychological stress models allow insight into the effects of stress on various physiological and behavioural outcomes, subsequently enabling the study of interventions aiming at reversing such stress-induced changes.

It is well acknowledged that different types of stressors elicit distinctive effects on behavioural and physiological outcomes (Atrooz et al., 2021). Indeed, the response of key parameters of the HPA axis, such as corticosterone (CORT) and adrenocorticotrophic hormone (ACTH), differ in intensity following exposure to physical stress models (i.e., exposure to cold or heat, fasting, crowding; Djordjević et al., 2003). Similarly, exposure to four different psychological stress models led to distinct alterations of anxiety-like behaviours and CORT secretion profiles in male rats (Adavi et al., 2023), supporting stressors of the same category to induce significantly different behavioural and physiological effects. Therefore, selecting a suitable and ecologically valid stress model appears essential to accurately interpreting and translating findings to clinical populations. With anxiety prevalence within the adult American population increasing significantly over the last decade (Goodwin et al., 2020) and regular ovarian cycles being a key indicator of overall health in women (Minakshi et al., 2022), understanding stress-induced alterations to estrous cycles and uncovering the best stress model to do so is of high importance.

1.5 Goals of the Review

The goal of this systematic review was to assess the impact of psychological stress exposure on estrous cycle regularity in rodents. By comparing stress-induced changes, we aim to foster a better understanding of the stress models which are associated with alterations to the estrous cycle and to determine whether different experimental models of stress exert distinct consequences on estrous cyclicity. Whenever possible, to better characterize possible underlying mechanisms of estrous cycle disruptions following stress, this review reports systemic or cerebral changes associated to altered HPG axis functional outcomes and related HPA axis markers.

2. Methodology

2.1 Protocol and Registration

The findings of this review are reported using the Preferred Reporting Items for Systematic Reviews and Meta-Analyses (PRISMA) checklist. Prior to beginning the project, the protocol was registered in accordance with the International Prospective Register of Systematic Reviews (PROSPERO; CRD42023428075).

2.2 Information Sources and Search Strategy

The search strategy used relevant keywords as well as database-specific controlled vocabulary relating to the estrous cycle and rodent stress models (Atrooz et al., 2021; Patchev & Patchev, 2006). The initial database search occurred on May 18th, 2023, and we collected all publications up until that date from MEDLINE (Ovid), APA PsycInfo (Ovid), Embase (Ovid), Cochrane CENTRAL (Ovid), and Web of Science (Science Citation Index Expanded; Social Sciences Citation Index; Emerging Sources Citation Index). The complete search strategy is available in [Appendix B1](#).

2.3 Eligibility Criteria

2.3.1 Inclusion Criteria

We included any study that mentioned an environmental manipulation aiming to induce psychological stress (e.g., restraint stress, CMS, social defeat, social instability; Atrooz et al., 2021) and that assessed the regularity of the estrous cycle during or after stress in otherwise healthy adult female rodents of reproductive age (2-12 months old). We included original peer-reviewed publications written in English.

2.3.2 Exclusion Criteria

Title and Abstract Screening Exclusion. During the title and abstract screening, we excluded publications for the following reasons: 1) Estrous cycle or reproductive function not assessed; 2) No psychological stress model; 3) Stressor occurred prior to adulthood (e.g., neonatal, adolescence); 4) Intervention not relevant (e.g., sleep or food deprivation, pain models, direct HPA axis activation); 5) Irrelevant species (i.e., non-rodents); 6) Not peer-reviewed original research (e.g., reviews or meta-analysis, case report, erratum articles, program evaluations, graduate thesis, book chapter); 7) Not available in English.

Full Text Screening Exclusion. During the full text screening phase, references were excluded if any of the following criteria applied: 1) Estrous cycle not reported for at least 2 full cycles (≥ 8 days) during or post stress exposure; 2) Estrous cyclicity prior to stressor not confirmed; 3) No psychological stress model; 4) Stress occurred prior to adulthood (prior to 2 months of age); 5) No appropriate comparison point (i.e., no-stress control group); 6) Non pertinent publication type (conference proceedings, review or book chapter, brief report, PhD thesis, not peer-reviewed, etc.); 7) Not available in English.

2.4 Study records

2.4.1 Data Management

Covidence (Veritas Health Innovation) was used for all steps of the review process (i.e., title, abstract, and full-text screening, data extraction, risk of bias assessment).

2.4.2 Selection Process

Graduate and undergraduate students appropriately trained in using Covidence and applying inclusion/exclusion criteria performed the screening of references. For each reference at all stages of review, lead author M.P. and one other independent reviewer assessed its relevance. In case of disagreement with the independent reviewer, lead author M.P. reviewed the reference,

discussed with the reviewer, and made a final decision. Reference lists of all included studies were screened for any additional relevant publications, and one additional publication was found with this method. See Figure 1 for references excluded at each stage of the review and [Appendix B2](#) for the exclusion reasons during full-text screening.

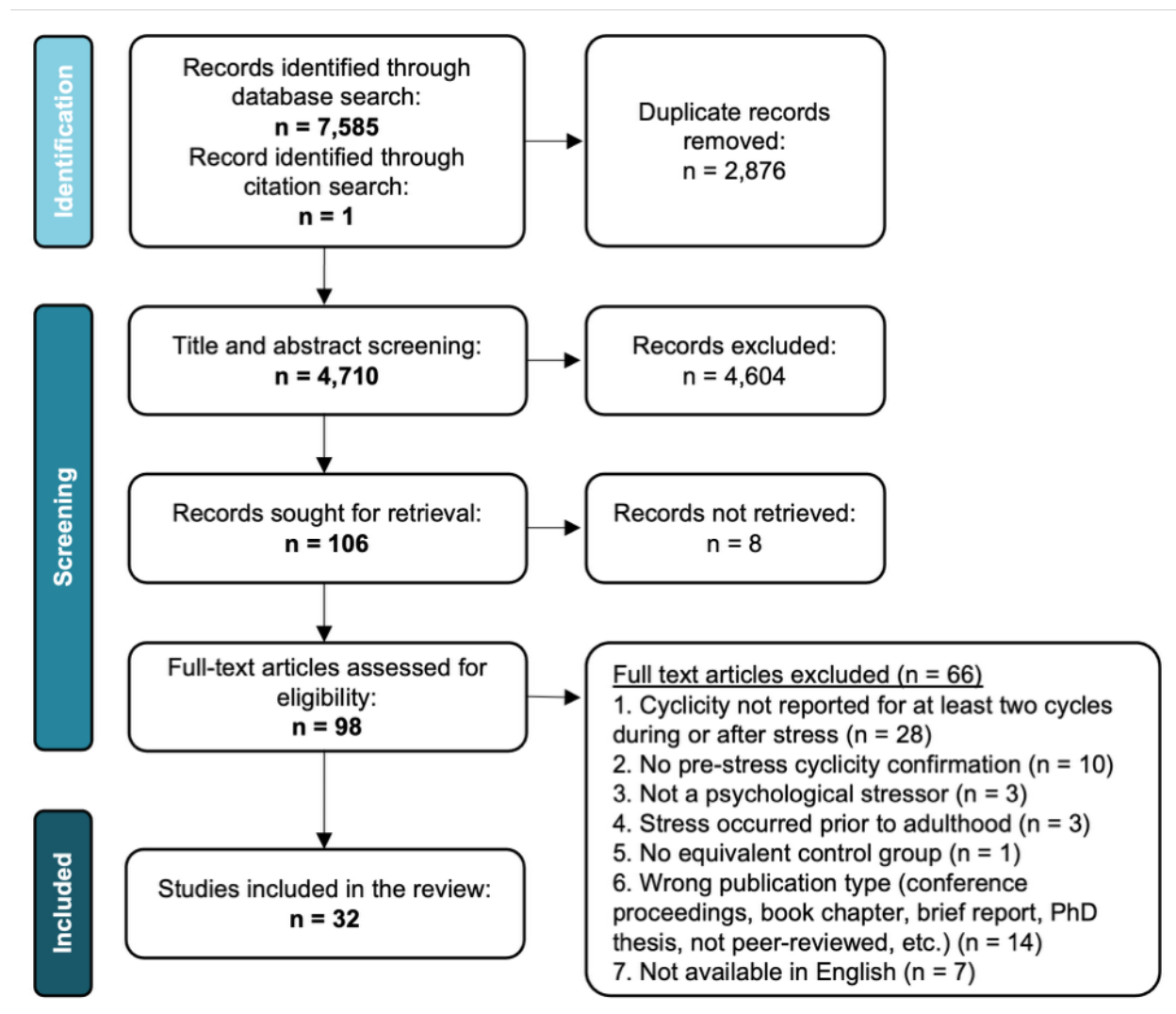


Figure 1. PRISMA Flow Diagram of Studies Included in the Systematic Review.

2.4.3 Data Collection Process

Data extraction and risk of bias assessment for each article was performed by lead author M.P. and independently verified by co-first author M.L. for completeness and accuracy.

2.5 Data Items

The following information was extracted from the included publications: a) title of article, authors' name, and year of publication; b) experimental groups of interest; c) number of subjects per group; d) species and strain; e) age of subjects and body weight at arrival; f) type of stressor used and stress duration/frequency; g) vivarium housing conditions (e.g., light cycle, cage dimensions); h) details of estrous cycle assessment; i) results pertaining to impact of stress on estrous cycle regularity and type of statistical analysis performed; j) if applicable, any measures of HPG axis function (e.g., LH, GnRH, E2) or HPA activation (e.g. CORT, ACTH) and associated results. To retrieve any of the missing methodological information from the included studies, corresponding authors were contacted up to twice. Of the 26 corresponding authors that were contacted, 12 responded to our request, with 10 of them providing some or all missing methodological information.

2.6 Risk of Bias in Individual Studies

Risk of bias was assessed using the SYRCLE risk of bias tool for animal studies (Hooijmans et al., 2014), with slight modifications to better reflect the types of studies included within the review. Notably, the scoring of the 3rd and 5th criteria, relating to allocation concealment and blinding during assessment, were modified to be more lenient because implementation of stress paradigms cannot easily be blinded. All included studies were categorized as having 'Low Risk', 'Unclear Risk', or 'High Risk' for each of the domains.

2.7 Data Synthesis

Following extraction, all data was exported into an Excel document. Descriptive statistics were performed using IBM© SPSS statistics 29 (IBM, Armonk, NY, USA) and are presented as mean $\pm$ standard deviation. While some information was successfully obtained from authors, and

is presented in the tables whenever applicable, descriptive statistics pertaining to the frequency at which methodological information was presented refer specifically to the information readily available in the articles themselves.

3. Results

Of the 4,710 references screened for this review, 32 studies met the inclusion criteria. See [Appendix B3](#) for all data extracted from the methods and results of the included studies, as well as the descriptive statistics performed in the context of this review.

3.1 General Characteristics of the Included Studies

As shown in Table 1, of the 32 studies included in this review, 24 (75.0%) were performed using rats, 7 were done in mice, and one study was done using Golden hamsters. The most common strains were Sprague-Dawley (57.7% of rat studies) and C57BL/6J (71.4% of mice studies). Age at arrival ranged from 2 to 5 months. It should be noted that studies often reported either the age or the body weight at arrival, rarely both, with 15 (46.9%) and 19 (59.4%) studies respectively reporting the rodents' age and body weight at arrival. As can be seen in [Appendix B3](#), most studies did not specify whether or not environmental enrichment (e.g., PVC tubes, nestlets) was present in the cages (96.9%), and very few provided cage dimensions (18.7%), although authors usually provided this information upon request.

Table 1. Study Characteristics and Estrous Cycle Assessment.

Study	Species (strain)	Characteristics at arrival		Light cycle, lights ON	Sampling method	Sampling time	Length of cycle assessment		
		Age	Weight (g)				Pre-stress	During stress	Post-stress
[1] Baker et al., 2006	Rats (SD; LE)	N/S	N/S	12h:12h, N/S	Smears	N/S	7 days	21 days	28 days
[2] Baker & Bielajew, 2007	Rats (SD)	N/S	258-390 g	12h:12h, 7:00 a.m.	Lavage	8-10 h before lights off	21 days	21 days	21 days
[3] Blume et al., 2019	Rats (SD)	~ 9 weeks †	N/S	12h:12h, 7:00 p.m.†	Lavage	0-5 h after lights off	14 days	9 days	N/A
[4] Bowman et al., 2001	Rats (SD)	~ 8 weeks	N/S	14h:10h, 5:00 a.m.	Smears	4-5 h before lights off	14 days	21 days	14 days
[5] Breen et al., 2012	Mice (C57BL/6)	6	N/S	12h:12h, 7:00 a.m.	Lavage	10 h before lights off	18 days	18 days	N/A
[6] Divyashree & Yajurvedi, 2016	Rats (Wistar)	8 weeks †	170-190 g	12h:12h, 7 p.m. †	Smears	3.5 h after lights off †	N/S	28, 56, or 84 days	N/A
[7] Divyashree & Yajurvedi, 2018	Rats (Wistar)	8 weeks †	160-180 g	12h:12h, 7 p.m. †	Smears	3 h after lights off †	N/S	28, 56, or 84 days	28 days
[8] Fotsing et al., 2017	Rats (Wistar)	N/S	N/S	12h:12h, N/S	Lavage	N/S	N/S	28 days	N/A
[9] Fritzsche et al., 2000	Hamster (Golden)	10 weeks	N/S	12h:12h, 7:00 a.m.	Smears	N/S	N/S	35 days	N/A
[10] Fu et al., 2018	Rats (SD)	12 weeks	212-264 g	12h:12h, N/S	Smears	N/S	10 days	35 days	N/A
[11] Geraghty et al., 2015	Rats (SD)	N/S	N/S	12h:12h, 7:00 a.m.	Smears	N/S	12 days	18 days	5 days
[12] Grippo et al., 2005	Rats (SD)	N/S	175-225 g	12h:12h, 7:00 a.m.	Lavage	5-9 h before lights off	21 days	28 days	N/A
[13] Han et al., 2021	Rats (SD)	7-8 weeks	210-230 g	12h:12h, N/S	Lavage	N/S	N/S	14 days	N/A
[14] Herzog et al., 2009	Rats (Wistar)	N/S	180-200 g	12h:12h, 8:00 p.m.	Lavage	5 h after lights off	7 days	28 days	N/A
[15] Huang et al., 2022	Mice (C57BL/6J)	N/S	N/S	12h:12h, N/S	Smears	N/S	10 days	28 days	N/A
[16] Kala & Nivsarkar, 2016	Mice (Swiss Albino)	6-8 weeks	25-30 g	12h:12h, N/S	Smears	N/S	N/S	5 days	16 days

[17] Kerr et al., 1999	Mice (DD/S)	8-16 weeks	N/S	12h:12h†, 6:00 a.m.†	Smears	6-10 h before lights off	18 days	21 days	N/A
[18] Konkle et al., 2003	Rats (SD; LE)	N/S	228-286 g	12h:12h, 7:00 a.m.	Swabs	N/S	N/S	42 days	N/A
[19] Lu et al., 2015	Rats (SD)	8 weeks	220-250 g	12h:12h, 8:00 a.m.	Smears	10-10.5 h before lights off	N/S	21 days	N/A
[20] Mattioli et al., 2009	Rats (Wistar)	N/S	175-225 g	12h:12h, 7:00 a.m.	Swabs	N/S	14 days	42 days	N/A
[21] Medubi et al., 2017	Rats (SD)	N/S	160-180 g	12h:12h, 6:00 a.m.	Smears	9.5-10.5 h before lights off	14 days	14 days	N/A
[22] Nair et al., 2021	Mice (C57BL/6J)	16-20 weeks	20-25 g †	12h:12h, 7:00 a.m. †	Smears	8-10 h before lights off	12-13 days	14 days	N/A
[23] Olave et al., 2022	Rats (SD)	N/S	240-280 g	12h:12h, 7:00 a.m. †	Lavage	9-11 h before lights off	15 days	13-15 days	N/A
[24] Pansarim et al., 2023	Rats (SD)	7 weeks	~ 200 g	12h:12h, 6:00 a.m.†	Smears	8-10 h before lights off	11 days	11 or 22 days	N/A
[25] Rappeneau et al., 2016	Rats (LE)	N/S	200-225 g	12h:12h, N/S (reverse)	Smears	N/S	7 days	21 days	N/A
[26] Rodriguez Echandia et al., 1988	Rats (Holtzman)	12 weeks	N/S	14h:10h, 6:00 a.m.	Smears	N/S	8 days	14 days	10 days
[27] Shimamoto et al., 2011	Rats (LE)	N/S	200-225 g	12h:12h, 8:00 p.m.	Smears	N/S	minimum 14 days	21 days	N/A
[28] Shimamoto et al., 2015	Rats (LE)	N/S	200-225 g	12h:12h, 8:00 p.m.	Smears	N/S	10 days	21 days	N/A
[29] Sun et al., 2017	Rats (SD)	8 weeks	200-240 g	12h:12h, N/S	Smears	N/S	7 days	N/A	35 days
[30] Wagenmaker & Moenter, 2017	Mice (C57BL/6J)	6-23 weeks	20-25g †	14h:10h, 4:00 a.m.	Smears	9.5 h - 10.5 h before lights off	10 days	21 days	10 days
[31] Xu et al., 2020	Rats (SD)	7-8 weeks	190-200 g	12h:12h, 7:00 a.m.	Smears	8 h before lights off	7 days	10 days	N/A
[32] Yohn et al., 2019	Mice (C57BL/6J)	8 weeks	N/S	12h:12h, 6:00 a.m.	Lavage	N/S	14 days	10 days	N/A

Note. Light cycle presented as hours light: hours dark. † indicates information obtained by contacting authors. LE: Long-Evans; N/A: Not applicable; N/S: Not specified; SD: Sprague-Dawley.

3.2 Estrous Cycle Assessments

As shown in Table 1, all studies assessed estrous cycle regularity using vaginal cytology, the most common method for sample collection being vaginal smears (23 studies, 71.9%), with the remaining 9 studies collecting cell samples via vaginal lavage. Of the included studies, only 18 (56.3%) reported the specific times at which they performed estrous cycle assessments, while 3 studies did not specify the time but stated that samples were collected at the same time each day (e.g., mornings; See [Appendix B3](#)). As seen in Table 1, although most studies stated the duration of the light cycle (96.8%), not all reported the time at which light phase switched (46.9% did not provide this information), making it difficult to determine at what time the estrous cycle was assessed relative to the light cycle, reducing possible comparisons between studies. In total, only 13 studies (40.6%) provided all the required information (i.e., time samples were collected, light cycle length, light change time) to determine relative sampling times. In most cases, samples were collected 8-10 h before lights off.

Confirming regularity of the estrous cycle prior to exposure to stress was an inclusion criterion in this review, and variability in the quantity of information reported regarding pre-intervention regularity was noted. Twenty-four studies stated the length of pre-stress cycle assessment (75%), while 2 studies (6.3%) expressed only rats with a set number of regular cycles (e.g., at least 2 regular cycles) were included in the experiments. Six studies gave no information about pre-stress estrous cycling (18.8%), only stating that rats were regularly cycling prior to experiments. Notably, during full-text screening, we excluded 10 studies for not stating that regularity was confirmed prior to stressor start. Further, most authors did not clearly define what they considered a normal estrous cycle (e.g., regular 4-5 day cycles), with only 10 studies (31.3%) clearly stating criteria for regularity (See [Appendix B3](#)).

Of the studies that reported cycling data pre-stress ($n = 24$), the mean length of cycle confirmation was 12.3 ± 4.3 days. The average assessment length during stress was 21.9 ± 8.9 days, with two studies not assessing cyclicity during stress. A total of 9 studies reported post-stress estrous cycling data (i.e., a recovery period), with a mean assessment length of 19.1 ± 10.1 days post-stress.

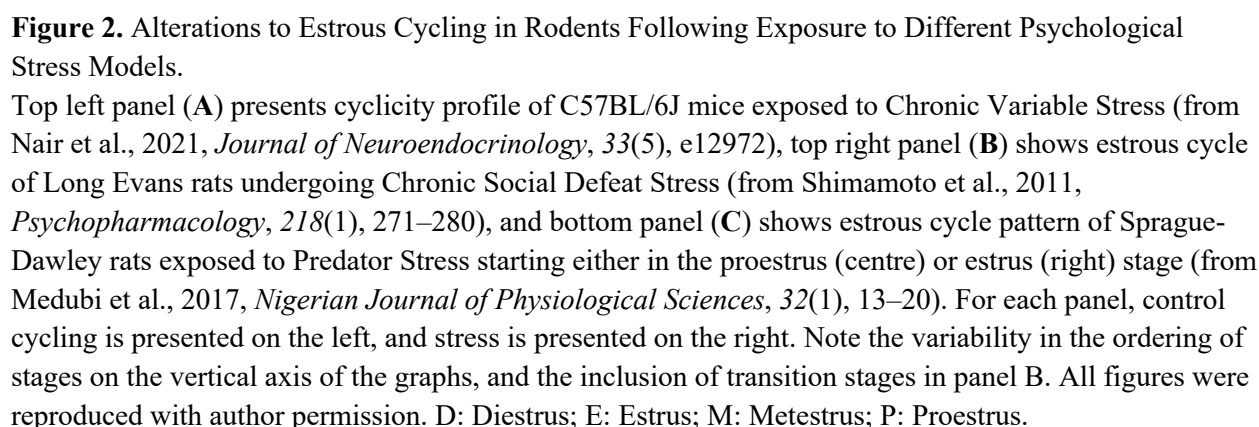
3.3 Psychological Stress Models Used

The most common psychological stress model used by the included studies was CMS (11 studies; 34.4%), with chronic restraint stress models being the second most prevalent (10 studies; 31.3%). Six studies used psychosocial stressors, such as CSDS ($n = 4$) or SIS ($n = 1$). Finally, a total of 5 studies used other psychological stress models, namely predator stress, emotional stress, novelty stress, or mixed stress models using a combination of daily restraint stress and forced swimming. While the duration of the stress protocols varied considerably between studies (ranging from 5 to 84 days), the mean length of the stress exposure periods was 27.1 ± 17.7 days, with an average daily stress exposure duration of 4.4 ± 6.8 h (not including paradigms that had variations to daily stress duration, as is the case with CMS).

3.4 Impact of Psychological Stress on Estrous Cycling

Overall, 20 of the 32 studies reported disruptions of estrous cycling post exposure to psychological stress models (62.5% of studies). See Figure 2 for a visual representation of some of the cycle disturbances reported post stress. Of the studies reporting estrous cycle disruptions, four were performed using mice (57.1% of mice studies) and 16 used rats (66.7% of rat studies). Interestingly, when looking at the different rat strains used, only 53.3% of studies using Sprague-Dawley rats reported estrous cycle disturbances (8/15 studies), contrasting 81.8% of the studies which found estrous cycle disruptions following stress using other rat strains (Wistar, Long-

Surprisingly, although 7 studies failed to detect stress-induced reductions to weight gain, 5 (71.4%) of them similarly indicated changes to the estrous cycle, suggesting weight loss does not represent a reliable predictor of the impact of stress on cyclicity.



3.4.1 Impact of CMS models on Estrous Cycling

Of the 11 studies having used CMS models, 81.8% found this stressor to impact estrous cyclicity. The most frequently reported disruptions were an overall increase in cycle length, typically associated with increased time spent in diestrus. On average, rodents undergoing CMS paradigms were exposed to 35.0 ± 36.6 h per week of stressors which might have a metabolic or circadian stress component (See Table 2), with reversals of the light cycle acting as the longest confounding factor. Of note, the majority of CMS studies included at least one confounding stressor (63.4% of studies).

Table 2. Impact of Chronic Mild Stress Models on Estrous Cyclicity.

Study	Stress duration	Stressors used in model	Confound duration	Impact on cycle
[1] Baker et al., 2006	21 days	Confinement with noise; Pair housing + wet bedding; Water deprivation; Food + water deprivation; Cage tilt + illumination overnight; Light cycle reversal.	22 h food deprivation 16 h overnight illumination 64 h reverse cycle	Yes
[2] Baker & Bielajew, 2007	21 days	Confinement with noise; Stroboscopic light; White noise; Cage tilt; Wet bedding.	None	No
[10] Fu et al., 2018	35 days	Wet bedding; Behavioural restriction; Food deprivation; Water deprivation; Ice water swimming; Light cycle reversal; Noise interference; Tail suspension; Foot shock.	24 h food deprivation	Yes
[12] Grippo et al., 2005	28 days	Illumination overnight; Cage tilt; Pair housing; Wet bedding; Water deprivation; Stroboscopic illumination; White noise.	24 h overnight illumination	Yes
[18] Konkle et al., 2003	42 days	Confinement; Water deprivation; Wet bedding; Novel cage; Food and water deprivation; Food restriction; Overnight illumination + cage tilt; Light cycle reversal.	16 h food deprivation 16 h overnight illumination 48 h reverse light	Yes
[19] Lu et al., 2015	21 days	Wet bedding; Cage tilt; Pair housing; Water deprivation; Stroboscopic illumination; White noise.	None	Yes
[20] Mattioli et al., 2009	42 days	Food deprivation; Water deprivation; Cage tilt; Intermittent illumination; Wet bedding; Overnight illumination; Pair housing; Stroboscopic illumination.	24 h food deprivation 24 h overnight illumination	Yes

[22] Nair et al., 2021	14 days	Shaker stress; Open field exposure; Cold exposure; Restraint; Tail suspension; No bedding; Single housing.	None	Yes
[26] Rodriguez Echandia et al., 1988	14 days	Group housing on mouse scented bedding; White noise; Single housing; Rat blood odor; Cage blows; Intermittent illumination; Group housing stressed rat scented bedding; Placed on platform surrounded by water; Open field exposure.	None	No
[29] Sun et al., 2017	21 days	Food deprivation; Water deprivation; No bedding; Wet bedding; Cage tilt; Tail pinch; Cold stress; Shaker stress; Cold swim; Restraint.	24 h food deprivation 24 h overnight illumination	Yes
[31] Xu et al., 2020	28 days	Food deprivation; Water deprivation; Cage tilt; Wet bedding; Overnight illumination; Restraint; Cold swim; Shaker stress; Empty cage; Tail pinch.	24 h food deprivation 24 h overnight illumination	Yes

Note. Confound duration is presented as number of hours/week.

3.4.2 Impact of Chronic Restraint Stress on Estrous Cycling

Importantly, only 4 of the 10 studies (40%) that used chronic restraint or immobilization stress detected alterations to estrous cycling (see Table 3). Similarly to the impact of CMS, a lengthening of estrous cycle due to increased time in diestrus was most commonly observed (Breen et al., 2012; J. Han et al., 2021), although one study reported either an absence of proestrus or a prolonged cycle (Huang et al., 2022). Surprisingly, another study observed increased time in proestrus (Fotsing et al., 2017). The average length of the restraint protocols used was 18 days $\pm$ 6.8, and the mean daily duration of restraint was 3.3 $\pm$ 2.1 h. The length of restraint, both in number of days and daily hours, did not act as a reliable predictor of altered cyclicity. Similarly, effects of housing conditions (*n* of animals per cage) on cyclicity were not reliably observed.

Table 3. Impact of Chronic Restraint Stress on Estrous Cyclicity.

Study	Species (strain)	Stress duration	Daily exposure	Animals per cage	Impact on cycle
[3] Blume et al., 2019	Rat (SD)	7 days (out of 9)	20 min	3	No
[4] Bowman et al., 2001	Rat (SD)	21 or 28 days	6 h	2	No
[5] Breen et al., 2012	Mice (C57BL/6)	18 days	3 h	1	Yes

[8] Fotsing et al., 2017	Rat (Wistar)	28 days	3 h	N/S	Yes
[11] Geraghty et al., 2015 *	Rat (SD)	18 days	3 h	3	No
[13] Han et al., 2021	Rat (SD)	14 days	6 h	N/S	Yes
[15] Huang et al., 2022	Mice (C57BL/6J)	28 days	1 h	5	Yes
[23] Olave et al., 2022	Rat (SD)	13-15 days	2.5 h	3-4	No
[24] Pansarim et al., 2023	Rat (SD)	11 or 22 days	6 h	2	No
[30] Wagenmaker & Moenter, 2017	Mice (C57BL/6J)	21 days	2 h	2-5 †	No

Note. * indicates study having used immobilization. † indicates information obtained by contacting authors. N/S: Not specified. SD: Sprague-Dawley.

3.4.3 Impact of Social Stressors on Estrous Cycling

As shown in Table 4, of the 6 studies having used social stressors, 3 studies (50%) found the selected stressor to alter estrous cycling, with the most common disruption being increased non-reproductive days (i.e., diestrus and metestrus) following CSDS (Rappeneau et al., 2016; Shimamoto et al., 2011). Interestingly, the other two studies having used CSDS failed to detect changes to estrous cycling (Shimamoto et al., 2015; Yohn et al., 2019). The only study having used SIS found fewer normally cycling rats following stress, although details as to the nature of observed alterations to cycling were not provided (Herzog et al., 2009). Finally, pair-housing stress in Golden Hamsters failed to alter the estrous cycle despite some effects on HPG and HPA axis parameters (Fritzsche et al., 2000).

Table 4. Impact of Social Stressors on Estrous Cyclicity.

Study	Stress model	Control housing	Stress group housing	Stress duration	Daily exposure	Impact on cycle
[9] Fritzsche et al., 2000	Pair housing *	1/cage	2/cage	35 days	24 h	No
[14] Herzog et al., 2009	Social Instability Stress	3/cage	Alternating days 1/cage or 8/cage	28 days	24 h	Yes
[25] Rappeneau et al., 2016	Social Defeat Stress	1/cage	1/cage + aggressive resident	21 days	24 h (1 h direct, 23 h indirect)	Yes
[27] Shimamoto et al., 2011	Social Defeat Stress	1/cage	1/cage + aggressive resident	21 days	24 h (1h direct, 23 h indirect)	Yes

[28] Shimamoto et al., 2015	Social Defeat Stress	1/cage	1/cage + aggressive resident	21 days	24 h (1h direct, 23 h indirect)	No
[32] Yohn et al., 2019	Social Defeat Stress	2/cage, no resident	2/cage + aggressive resident	10 days	24 h (5 min direct, 24 h indirect)	No

Note. * Study performed in Golden Hamsters, for which pair-housing is considered a stressor.

3.4.4 Impact of Mixed and Emotional Stress Models on Estrous Cyclicity

As shown in Table 5, Wistar rats exposed to both 1 h of restraint and a 15 min forced swimming session 4 h later ($n = 2$ studies) presented fewer estrous cycles per month compared to non-stressed controls (Divyashree & Yajurvedi, 2016, 2018). Similarly, the use of a psychological stress model, comprising of 4 h of mild stressors daily, resulted in irregular estrous cycles, with over one third of the stress group becoming acyclic (Kala & Nivsarkar, 2016). In contrast, a novelty stress model failed to alter cyclicity (Kerr et al., 1999). Interestingly, Medubi et al. (2017) showed that exposure to predator stress significantly altered the estrous cycle, and that the characteristics of the alterations depended on estrous cycle stage at first exposure, with rats first exposed in proestrus showing a more pronounced reduction to normal cycling (persistent diestrus for 8-9 days) compared to rats first exposed in estrus which maintained some cyclicity but no longer entered proestrus.

Table 5. Impact of Mixed and Other Psychological Stressors on Estrous Cyclicity.

Study	Stress model	Stress model details	Stress duration	Daily exposure	Impact on cycle
[6] Divyashree & Yajurvedi, 2016	Restraint + Forced swim	Restraint followed by forced swimming after a 4 h gap.	28, 56, or 84 days	1 h restraint, 15 min swim	Yes
[7] Divyashree & Yajurvedi, 2018	Restraint + Forced swim	Restraint followed by forced swimming after a 4 h gap.	28, 56, or 84 days	1 h restraint, 15 min swim	Yes
[16] Kala & Nivsarkar, 2016	Psychological Stress Model	Each day, each stressor applied in random order: Restraint (1 h); slanted cage (1 h); dirty bedding (1 h); isolation (30 min); no bedding (30 min).	5 days	4 h total	Yes
[17] Kerr et al., 1999	Novelty Stress	Brief exposure to varied novel environments: clear plastic container; opaque polypropylene container; cardboard	21 days (5 days/week)	15 min	No

		box divided into compartments; clean standard rodent cage empty of bedding, food, and water.			
[21] Medubi et al., 2017	Predator Stress	Olfactory, visual, and auditory exposure to a cat, without physical contact.	14 days	1 h	Yes

3.4.5 Recovery of Estrous Cyclicity Post-Stress

Nine of the 32 studies included a post-stress cycle assessment period, four of which reported a lingering impact of stress on cyclicity. Divyashree and Yajurvedi (2018) showed daily exposure to restraint + forced swim sessions for 4, 8 or 12 weeks to lead to fewer estrous cycles in the 4 weeks following stress in Wistar rats, regardless of length of exposure. Similarly, Sprague-Dawley and Long-Evans rats continued to display fewer regular estrous cycles in the 4 weeks post-CMS, although there was a gradual recovery of estrous cyclicity over time (Baker et al., 2006). When mice were exposed to a psychological stress model consisting of 4 h of mild stressors daily for 5 days, all mice presented irregular cycles over the following 3 weeks, and 37% were reported to be acyclic (Kala & Nivsarkar, 2016). Finally, in Sprague-Dawley rats, 3 weeks of CMS resulted in increased time spent in diestrus, which persisted up until the end of data collection 5 weeks later (Sun et al., 2017).

3.5 Changes to HPG and HPA Axis Function After Psychological Stress

Of the 32 studies that met inclusion criteria for this review, 7 studies had no biochemical markers of interest, 6 studies included at least one HPA axis signalling molecule, 4 studies included at least one HPG axis marker, and 15 studies included at least one marker for each axis. Biochemical markers were most often collected at euthanasia, usually occurring on the last day of stress (12 studies) or 1-5 days following stress (5 studies). However, a few studies included a stress recovery group, a procedure associated with a more distant collection interval (Divyashree & Yajurvedi, 2018; Sun et al., 2017), and some studies failed to specify when samples were collected (4 studies). Four studies assessed stress-signalling molecules (e.g., CORT) at multiple

timepoints during the stress protocol. Importantly, sample collection intervals appear relevant in the detection of significant alterations to HPA/HPG axis markers, with a greater proportion of studies reporting changes to markers of interest when samples were collected on the last day of stress compared to sample collection occurring 1-5 days post-stress (See [Appendix B3](#)).

3.5.1 Alterations to HPG Axis Function

The average number of HPG axis parameters measured per study was 3.9 ± 2.0 (range 1 to 7 markers). The most commonly assessed marker was E2, for which values were reported in 12 of the studies assessing the HPG axis (63.2%). Table 6 presents differences in expression of HPG axis markers between the stress and control groups, based on whether or not the stress group displayed altered estrous cycles. Of note, altered estrous cycling was noted in all 7 studies reporting reduced E2 levels, and in 3 of the 4 studies showing elevated E2 levels. Both ovarian follicular and corpora lutea counts were significantly reduced by psychological stress exposure, an observation which was concomitant with altered estrous cycling in all these studies. Very few studies included assessment of relevant HPG axis markers within the hypothalamus (e.g., Kiss1, GnRH), making trends in collected data difficult to discuss.

Interestingly, a close examination of findings from studies having assessed biochemical markers at multiple phases of the estrous cycle supports changes in circulatory hormone levels to be less readily detectable in diestrus compared to proestrus (Lu et al., 2015) or estrus (Medubi et al., 2017). As expected, longer stress interventions were more likely to uncover changes to the biochemical markers of interest compared to shorter interventions, with hormonal levels that were unchanged after 28 days of exposure to restraint and forced swimming being altered after 56 and/or 84 days (Divyashree & Yajurvedi, 2016, 2018). Similarly, following 14 days of CMS, Fu et al. (2018) only detected significant changes in anti-Mullerian hormone (AMH) levels,

whereas after 35 days, levels of AMH, GnRH, FSH, and E2 all significantly differed from controls.

Table 6. Changes in HPG Axis Function in Relation to Alterations in Estrous Cycling.

Biochemical marker	Cyclicity altered (n = 15 studies)	Cyclicity not altered (n = 4 studies)
Hypothalamic content		
GnRH	No data	Stress = Controls [11]
RFRP-3 (GnIH)	No data	Stress > Controls [11]
Kiss1	Stress < Controls [15]	Stress = Controls [11]
AR	Stress = Controls [19 ^{p, d, d+p}]	No data
ER α	Stress = Controls [19 ^{p, d, d+p}]	No data
ER β	Stress < Controls [19 ^d] Stress = Controls [19 ^{p, d+p}]	No data
Circulatory hormone levels		
AMH	Stress < Controls [10 ^{14D, 35D}]	No data
GnRH	Stress < Controls [10 ^{35D}] Stress = Controls [10 ^{14D}]	No data
GH	Stress = Controls [22]	No data
LH	Stress < Controls [8, 15, 21 ^e] Stress = Controls [5, 21 ^d , 22, 29] Stress > Controls [14, 31]	Stress = Controls [30]
FSH	Stress < Controls [8, 15, 21 ^e] Stress = Controls [10 ^{14D} , 21 ^d] Stress > Controls [10 ^{35D} , 31]	No data
PRL	Stress = Controls [22] Stress > Controls [14, 15, 21 ^{e, d}]	No data
E2	Stress < Controls [8, 10 ^{35D} , 13, 19 ^{p, d+p} , 21 ^e , 29, 31] Stress = Controls [6 ^{28D} , 7 ^{28D} , 10 ^{14D} , 12, 15, 19 ^d , 21 ^d] Stress > Controls [6 ^{56D, 84D} , 7 ^{56D, 84D}]	Stress > Controls [3]
P4	Stress < Controls [13, 21 ^e , 29, 31] Stress = Controls [5] Stress > Controls [8, 21 ^d]	Stress > Controls [9]
T	Stress < Controls [19 ^{p, d+p}] Stress = Controls [6 ^{28D, 56D} , 7 ^{28D, 56D} , 19 ^d] Stress > Controls [6 ^{84D} , 7 ^{84D}]	No data
Reproductive Organs		
Ovarian weight	Stress < Controls [6 ^{28D, 56D}] Stress > Controls [6 ^{84D}]	Stress = Controls [9]
Ovarian follicular count	Stress < Controls [6 ^{28D} , 10 ^{35D} , 16, 22, 28, 30] Stress = Controls [6 ^{56D}] Stress > Controls [6 ^{84D}]	No data

Ovarian corpora lutea count	Stress < Controls [6, 16, 22, 29]	Stress > Controls [9]
Uterine weight	Stress < Controls [7 ^{28D}] Stress > Controls [7 ^{56D, 84D}]	No data
Uterine follicular count	Stress = Controls [7]	No data

Note. Reference numbers correspond to the numbering used in Tables 1-4. If changes were only detected for specific stressor lengths, protocol length in days (D) is indicated in superscript. For studies having found estrous cycle stage dependent effects, cycle stage is indicated in superscript. AMH: Anti-Mullerian hormone; AR: Androgen receptors; d: diestrus; e: estrus; E2: estradiol; ER: Estrogen receptors; FSH: Follicle-stimulating hormone; GH: Growth hormone; GnRH: Gonadotropin-releasing hormone; Kiss1: Kisspeptin; LH: Luteinizing hormone; p: proestrus; P4: Progesterone; PR: Progesterone receptors; PRL: Prolactin; RFRP-3; RFamide-related peptide; T: Testosterone.

3.5.2 Alterations to HPA Axis Function

As can be expected, the most common HPA axis marker was circulatory CORT, which was reported in 18 of the studies having included HPA axis biochemical parameters (90%; See Table 7). Of these, 12 studies found their stress intervention to increase basal CORT levels, 10 of which reported altered estrous cycling post-stress exposure. Stress resulted in increased adrenal gland weights in all studies reporting this variable, with an even proportion finding and not finding alterations to the estrous cycle. In contrast to the HPG axis, changes to HPA axis function were more readily detectable when samples of the estrous cycle stages were pooled (Lu et al., 2015).

Table 7. Alterations of HPA Axis Function in Relation to Estrous Cyclicity.

Biochemical marker	Cyclicity altered (n = 13 studies)	Cyclicity not altered (n = 7 studies)
Hypothalamic content		
GR	Stress < Controls [19 ^{d, p+d}] Stress = Controls [19 ^p]	No data
MR	Stress < Controls [19 ^{p, p+d}] Stress = Controls [19 ^d]	No data
CRH	Stress = Controls [19 ^{p, d} , 22] Stress > Controls [19 ^{p+d}]	No data
Circulatory hormone levels		
ACTH	Stress = Controls [12]	No data

Basal CORT ‡	Stress = Controls [12, 19 ^{p,d} , 22, 25] Stress > Controls [6, 7, 8, 14, 15, 16, 18, 19 ^{p+d} , 21 ^{e,d} , 27]	Stress = Controls [11, 23, 30] Stress > Controls [4, 32]
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Organs

Adrenal gland weight	Stress > Controls [7, 14, 18]	Stress > Controls [3, 9, 23]
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Note. Reference numbers correspond to the article numbering used in Tables 1-4. For studies with multiple stressor lengths, number of days (D) is specified in superscript next to the reference. For studies having found estrous-stage dependent effect, stage is indicated in superscript. ACTH: Adrenocorticotrophic hormone; CORT: Corticosterone; CRH: Corticotropin-releasing hormone; d: diestrus; estrus; GR: Glucocorticoid receptors; MR: Mineralocorticoid receptors; p: proestrus. ‡ For studies with multiple sampling timepoints, results presented are those closest to stressor end.

3.5 Risk of Bias Assessment

[Appendix B4](#) presents the outcomes for the risk of bias assessment. Most studies had overall low or unclear risk, with the highest risk stemming from the SYRCLE domains pertaining to sequence generation (Domain 1) and random housing in the facilities (Domain 4).

4. Discussion

Of the 32 studies included in this systematic review, the majority (62.5% of studies) reported alterations to estrous cyclicity following psychological stress exposure in rodents, usually involving a lengthening of the cycle driven by increased time spent in diestrus. Of the variety of psychological stress models used (e.g., CMS, restraint, CSDS), CMS was the most effective at inducing estrous cycle disruptions (9 out of 11 studies) and restraint stress the least, with 6 studies out of 10 failing to show significant changes. While stringent inclusion criteria used in systematic reviews limit the number of eligible studies, this systematic review allows a thorough overview of the existing evidence on the impact of psychological stress on estrous cyclicity. Moreover, this review summarizes changes in HPG and HPA axis markers reported in conjunction with estrous cycle disruptions post-stress, allowing insights into possible mechanisms underlying such alterations.

4.1 Is There an Ideal Model for Studying the Impact of Stress on the Estrous Cycle?

4.1.1 The Effectiveness of Chronic Mild Stress and Its Possible Confounds

The various CMS paradigms, characterized by the chronic and often unpredictable application of various mild stressors throughout the day and night (Willner, 2016), were most consistently effective at inducing changes to estrous cyclicity. While CMS models are commonly used to study psychological factors mediating depression (Strekalova et al., 2022), these paradigms often include periods of food deprivation, which may impose a metabolic stress, as well as periods of overnight illumination or light cycle reversals, which pose a circadian stress (Koch et al., 2016). In rodents, estrous cycle disruptions have been reported following chronic food deprivation (Uphouse et al., 2006) and constant light exposure (Anisimov et al., 2004; Guerrero-Vargas et al., 2022; Hardy, 1970). Similarly, irregular menstrual patterns are reported in women restricting food consumption (Montero et al., 1996) or performing shift work (Chang & Chang, 2021). These observations support possible confounding effects when assessing the impact of psychological stress on estrous cyclicity. Interestingly, however, when Baker et al. (2006) controlled for the possible impact of food deprivation within their CMS model by including equivalent periods of food/water deprivation in control counterparts, they found the latter group to cycle normally while cyclicity of the CMS-exposed rats was compromised. Furthermore, evidence suggests Long-Evans and Sprague-Dawley rats to be resistant to mild food deprivation-related alterations to estrous cycling compared to Fisher rats (Tropp & Markus, 2001). On the possible confounding influence of light cycle disturbances, Grippo et al. (2005) argue that the relatively short periods of constant illumination (one or two nights per week) included in CMS protocols appear themselves insufficient to alter cyclicity. Indeed, protocols of constant illumination shown to disrupt cyclicity have largely been chronic in nature (i.e.,

constant illumination over weeks or months). In this context, many of the reviewed CMS studies reported increased time spent in the diestrus stage (Baker et al., 2006; Fu et al., 2018; Lu et al., 2015; Xu et al., 2020), contrasting studies of constant light exposure typically reporting increased time in estrus (Guerrero-Vargas et al., 2022; Hardy, 1970). Thus, while it is noteworthy that the two CMS studies failing to detect changes to estrous cycling included none of the above-mentioned ‘confounding’ stressors (Baker & Bielajew, 2007; Rodriguez Echandia et al., 1988), more research is needed to understand the extent to which the metabolic and circadian alterations commonly related to CMS exposure contribute to noted estrous cycling disruptions.

4.1.2 A Limited Impact of Restraint Stress on Estrous Cycling

Restraint and immobilization stress are amongst the most commonly used stressors in rodent research. This is largely due to the ease of implementation of the paradigm and its validated effects in increasing anxiety-like behaviours and markers of HPA axis activation (For review, see Buynitsky and Mostofsky, 2009). Surprisingly, of 10 studies using such paradigms, only 4 reported estrous cycle disruptions, suggesting effects on cyclicity to be stressor specific and a limited efficacy of models using physical constraints in studying regulators of altered cyclicity. Interestingly, studies that found estrous cycle alterations using restraint (Breen et al., 2012; Fotsing et al., 2017; Han et al., 2021; Huang et al., 2022) failed to show a direct relationship between the duration of the stress protocol (that ranged between 14 and 28 days) or daily physical restraint (1-6 h) and efficacy in altering estrous cyclicity. Concordantly, research has previously shown repeated restraint to lead to habituation of the HPA axis response over time (Benini et al., 2020; Girotti et al., 2006), with exposure to daily restraint inducing a lesser stress response compared to an interrupted restraint paradigm (Zhang et al., 2014). Nevertheless,

two studies reporting estrous cycle disruptions (Fotsing et al., 2017; Huang et al., 2022) demonstrated such changes to be related to elevations of basal CORT secretion, while 3 studies showing no stress effects on the estrous cycle reported no changes in basal CORT from chronic restraint exposure (Geraghty et al., 2015; Olave et al., 2022; Wagenmaker & Moenter, 2017), and one reported stress-induced elevation in CORT secretion without alterations to cycling (Bowman et al., 2001). It is interesting to note that of the 10 studies using restraint stress, 6 have used Sprague-Dawley rats, with only one reporting estrous cycle disruptions post stress (Han et al., 2021). Research has shown that compared to Fischer rats, Sprague-Dawley rats show reduced stress reactivity when exposed to immobilization stress (Porterfield et al., 2011) and greater habituation to restraint (Dhabhar et al., 1997). Concordantly, Konkle et al. (2003) reported that while Sprague-Dawley rats presented some reductions to estrous cyclicity post-CMS exposure, these changes were not statistically different from controls, whereas Long-Evans rats exposed to the same intervention did show significantly reduced estrous cycling compared to controls. At this time, insufficient data is available to determine whether the limited effects of restraint on estrous cycling are due to the stress model itself – and possible habituation to this stressor – or to resistance of the Sprague-Dawley strain to restraint stress induced estrous cycle disruptions.

4.1.3 The Inconsistent Effects of Social Stressors on Estrous Cycling

Of the four studies having used a CSDS model, two studies reported altered estrous cycling post-stress (Rappeneau et al., 2016; Shimamoto et al., 2011) whereas the other two found no alterations to the cycle (Shimamoto et al., 2015; Yohn et al., 2019). Unfortunately, none of the CSDS studies included measured any HPG-axis markers, limiting our understanding of the impact of this stress model on reproductive axis function. On the other hand, an SIS paradigm using alternating phases of isolation and crowding with varied group composition effectively

reduced estrous cycling in Wistar rats while also increasing adrenal gland weights, basal CORT, prolactin (PRL), and LH levels (Herzog et al., 2009). It is noteworthy that social stress models have been proposed to have greater translational potential, due to an increased resemblance to the daily stressors experienced by humans (Solomon, 2017). While this review supports some impact of social stress on estrous cycling, future investigations of such stressors are required, particularly those including thorough assessments of associated HPG-axis dysfunction.

Furthermore, evaluating other social stress models, such as different types of SIS paradigms (Koert et al., 2021), or the individual effects of isolation or crowding, would increase our understanding of how these stressors impact reproductive functioning, which is highly relevant with the COVID-19 pandemic having considerably disrupted the social environment on a global scale (Hosseinzadeh et al., 2022).

4.1.4 The Potential of Other Psychological Stressors for Impacting Estrous Cycling

The majority of the other psychological models included in this systematic review were effective at inducing estrous cycle disruptions, with the exception of a novelty stress paradigm which was likely too mild of a stressor to alter cycling, particularly considering a limited 15 min daily exposure (Kerr et al., 1999). While restraint stress on its own yielded inconsistent findings, the use of restraint followed by forced swimming later in the day reduced the number of estrous cycles per month in two studies by Divyashree and Yajurvedi (2018, 2016). It is interesting to note that these studies both utilized Wistar rats, making it difficult to know if the effectiveness of this stress paradigm at altering estrous cycling stems solely from the addition of forced swimming, or a greater propensity for Wistar rats to display altered cycling post-restraint compared to Sprague-Dawley rats. Indeed, the only study included in this systematic review having assessed restraint stress in Wistar rats did also detect changes to estrous cycling post-

stress (Fotsing et al., 2017). Predator stress, induced via indirect exposure to a cat, proved to be a very effective model for altering estrous cycling in Sprague-Dawley rats, and interestingly, the characteristics of the estrous cycle disruptions were dependent on the stage of the estrous cycle at first exposure to the stressor (Medubi et al., 2017). While disruptions to cycling were detected regardless of stage at first exposure, the subtle differences between first exposure in estrus vs. proestrus (as presented in Figure 2; Medubi et al., 2017) indicate stage at first exposure as a possible variable of interest to detect subtle effects of different stressors.

4.2 Are There Consistent Associations Between Altered HPG or HPA Axis Markers and Disrupted Estrous Cycling?

The negative impact of stress on reproductive function is well acknowledged (Joseph & Whirledge, 2017; Toufexis et al., 2014). This systematic review supports that association, with a higher proportion of the studies having found higher basal CORT levels post-stress also reporting disruptions to estrous cycling (10/12 studies; see Table 7). A relatively small number of HPA markers were assessed within the studies included in this review, limiting the ability to compare disruptions associated with altered estrous cyclicity. Notably, however, increased adrenal gland weight post-stress does not appear to predict altered estrous cycling, with an equal proportion of studies observing effects of stress on this parameter showing cyclicity changes (Divyashree & Yajurvedi, 2018; Herzog et al., 2009; Konkle et al., 2003) as those which found no post-stress changes to the estrous cycle (Blume et al., 2019; Fritzsche et al., 2000; Olave et al., 2022). In addition, studies appeared more likely to detect changes to hypothalamic HPA axis markers when data from proestrus and diestrus stages were pooled for analyses, which could support increased corticotropin-releasing hormone (CRH) and reduced glucocorticoid receptor (GR) and mineralocorticoid receptor (MR) expression to be associated with estrous cycle

disruptions following CMS (Lu et al., 2015). One study reported altered estrous cyclicity post stress to be present in the absence of significant alterations of ACTH levels (Grippe et al., 2005). Additional studies are needed to further investigate the associations between altered HPA axis reactivity and disruptions to estrous cycling.

Notably, included studies assessed a variety of HPG axis markers, although an imbalance in the number of studies having observed stress-related alterations to cycling (15 studies) versus not (4 studies) acted to limit uncovering the relationships between these variables. Nonetheless, altered estrous cycling was most frequently associated with reduced E2 levels (Fotsing et al., 2017; Fu et al., 2018; Han et al., 2021; Sun et al., 2017; Xu et al., 2020), with alterations being more readily detectable in proestrus (Lu et al., 2015) and estrus (Medubi et al., 2017) compared to diestrus. Conversely, elevations in E2 concentrations were noted with exposure to the shortest (9 days; Blume et al., 2019) and longest (56 and 84 days; Divyashree and Yajurvedi, 2018, 2016) stress paradigms, which could support a non-linear relationship of E2 secretion and stress duration. No clear trend was observable between LH concentrations measured at the end of the stress exposure period and altered estrous cycling, with studies reporting LH concentrations to be reduced (Fotsing et al., 2017; Huang et al., 2022; Medubi et al., 2017), increased (Herzog et al., 2009; Xu et al., 2020) or unchanged (Breen et al., 2012; Nair et al., 2021; Sun et al., 2017). Of interest, although Sun et al. (2017) found no changes to LH immediately following 21 days of CMS, they reported levels to be increased 5 weeks following exposure, highlighting possible delayed effects of stress on hormonal signalling. In the same vein, Wagenmaker and Moenter (2017) noted that although the amplitude of the proestrus LH surge did not differ between stress and control mice, the onset of the surge was delayed in stressed mice (i.e., did not consistently occur at lights out), suggesting that acute stress applied the morning of proestrus might act on the

timing of the surge more so than its amplitude. FSH levels were most often decreased in rodents which presented altered estrous cycling post-stress (Fotsing et al., 2017; Huang et al., 2022; Medubi et al., 2017), although some studies reported increased FSH post-stress (Fu et al., 2018; Xu et al., 2020), which may relate to length of exposure. Indeed, Fu et al. (2018) reported no changes in FSH when measured at 14 days of stress, while they detected differences after 35 days of stress exposure. For both FSH and LH, changes appeared to be more readily detectable in the estrus than diestrus stage (Medubi et al., 2017). PRL, which suppresses reproduction and is thought to be linked to the stress response (Levine & Muneyyirci-Delale, 2018), was increased in 3 of the 4 studies reporting estrous cycle disruptions (Herzog et al., 2009; Huang et al., 2022; Medubi et al., 2017), while the other study found no changes to PRL levels post-stress (Nair et al., 2021). Overall, while a variety of HPG axis disruptions were reported in parallel with irregular estrous cycling, there is no single marker that appears to most consistently be associated with such alterations to cyclicity, highlighting the complex interplay between the different facets of the HPG axis.

4.3 How Can We Improve Reporting When Studying the Estrous Cycle?

Several excellent resources exist describing the techniques for staging and assessing estrous cycle stages in rodent studies (Ajayi & Akhigbe, 2020; McLean et al., 2012). As such, this section provides recommendations on other methodological considerations, which should be adopted whenever possible to improve comparability between studies.

4.3.1 Estrous Cycle Assessment Details

Seeing as some stages of the estrous cycle last fewer than 24 h, one of the key elements to implement when assessing estrous cyclicity is consistent sampling times (Cora et al., 2015). In this systematic review, we found that several studies did not report sampling times, and we

observed variation in the times at which sampling was performed. Indeed, although most studies reported short sampling windows (typically 1-2 h), some provided considerably wider ranges for daily sampling times (4-8 h long). Variability in sampling time from day to day increases the risk of missing cycle stages (particularly metestrus or proestrus), which might lead to incorrectly characterizing the cycle as irregular. Importantly, to allow better comparison of results, time of sampling and time at which lights turn on in the vivarium should both be reported, so that the sampling time relative to light cycle change is known. Furthermore, we recommend authors explicitly define what cycles will be considered irregular (e.g., cycle lengths outside of a 4-5 day range, irregular progression of stages) to allow a better understanding of the results.

4.3.2 Housing Conditions

Another key factor, which should be considered and reported, is the housing conditions of the stress and control groups. Indeed, isolation has been shown to be stressful (Carnevali, Statello, et al., 2020; Carrier & Kabbaj, 2012), and female rats housed in isolation have been found to enter constant estrus (reproductive senescence) on average 2 months sooner than group housed rats (LeFevre & McClintock, 1991). In fact, the study by Baker and Bielajew (2007) showed that single housing had a more pronounced impact on estrous cyclicity than their CMS model, showcasing the importance of clearly reporting housing conditions. Furthermore, some evidence suggests female rodents to enter persistent estrus when housed in a vivarium without male rodents (McLean et al., 2012). Although this effect has been inconsistent and seems more prevalent in mice than rats (Lohmiller et al., 2020), reporting the presence (or absence) of male rats within the vivarium would improve comparability between studies.

4.3.3 Reporting Specific Characteristics of Estrous Cycling

Finally, when reporting the impact of an intervention on the estrous cycle, the more the better. Namely, we recommend providing data for mean time spent in each cycle stage, in addition to mean cycle length and proportion of rats displaying regular versus irregular cycles. Furthermore, presenting a weekly or biweekly breakdown, rather than collapsing cycling data over several weeks, would allow a better understanding of the singular impact of distinct stressors and the temporal profile of changes to cyclicity over the intervention period. A greater amount of detail will not only improve possible comparisons between studies, but it may also enable detecting subtle effects of interventions. Indeed, the distinction between the increased time in diestrus related to CMS (Baker et al., 2006; Fu et al., 2018; Lu et al., 2015; Xu et al., 2020) and the persistent estrus induced by constant light exposure (Guerrero-Vargas et al., 2022; Hardy, 1970) would have gone unnoticed if the authors of these studies had only reported the proportion of irregularly cycling rodents. In turn, more detailed information will foster a better understanding of the effectiveness of possible treatments aiming at restoring estrous cyclicity. See Box 1 for a summary of the recommendations provided in this review.

Box 1.

Methodological recommendations for reporting estrous cyclicity in the context of stress studies.

Housing

- Light cycle duration and light onset time
- Presence or absence of males within the vivarium room
- Number of animals per cage for all conditions

Cycle assessment

- Time(s) at which vaginal cell samples are collected daily
- Criteria for cycles to be considered regular or irregular (e.g., cycles 4-5 days in length with normal progression between stages were considered regular)

- Length of pre-intervention estrous cycle confirmation and, whenever possible, reporting of pre-intervention cyclicity data (e.g., mean time in each stage, mean cycle length)

Presentation of results

- Provide mean cycle length and mean time in each cycle stage broken down into relatively short time periods (e.g., biweekly), as well as proportion of rodents regularly cycling
- Whenever possible, include a representative example of cycling data for each group

4.4 Limitations of This Review

Since the estrous cycle is often overlooked as an outcome of interest, particularly when it is unchanged by the intervention at hand, it is likely to go unmentioned in the abstract of papers. As such, it is possible that papers in which the estrous cycle was not altered could have gone undetected in our database searching, although efforts were made to mitigate this risk by searching the reference lists of the included studies. It is also important to note that some studies measured the estrous cycle, but did not meet our other inclusion criteria, such as confirming estrous cycling prior to stress or reporting estrous cyclicity for at least two cycles (8 days) post stress initiation, and as such were not reported in this systematic review (these are however listed in [Appendix B2](#)). One notable limitation of this review's findings is the exclusion of irregularly cycling rodents prior to experiments. While confirmation of estrous cycling prior to intervention start is undeniably required, the exclusion of subjects that are not regularly cycling eliminates a distinct group, which may be more vulnerable to environmental disruptions, such as transport stress or subtle stressors within the housing facilities. It could be of interest for future studies to include irregularly cycling subjects as a separate subset of animals to investigate whether adverse effects of stress on estrous cycling or HPG-axis parameters would be potentiated in such rodents (e.g., becoming acyclic).

5. Conclusion

To our knowledge, this systematic review represents the first thorough overview of the existing evidence assessing the impact of psychological stress on estrous cyclicity in rodents. In sum, the findings of this systematic review suggest CMS to be the best available psychological stress model for inducing disruptions to estrous cycling, although further investigations are required to elucidate the contribution of confounding factors, such as circadian stress, to the potency of these effects. Furthermore, restraint stress seemed least effective at doing so, although possible confounding effects from the rat strains used are suggested. Knowing that regular ovarian cyclicity is a key indicator of health in women, understanding which stress models are best to study this issue represents a crucial first step prior to devising treatments or interventions that aim to reverse stress-induced alterations to ovarian cycling.

6. Acknowledgements

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7. CRediT Authorship Contribution Statement

Marilou Poitras: Conceptualization, Methodology, Project Administration, Investigation, Formal analysis, Data curation, Visualization, Writing – original draft. Madison Lebeau: Investigation, Data curation, Writing – original draft. H  l  ne Plamondon: Conceptualization, Supervision, Writing – review & editing.

8. Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence this review.

9. Data Availability

All data synthesized for this systematic review is presented in the [appendices](#).

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Chapter III – Global Cerebral Ischemia in Adult Female Rats Interrupts Estrous Cyclicity and Induces Lasting Changes in Hypothalamic-Pituitary-Gonadal Axis Signalling Peptides

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Authorial Contributions

M.P. designed the research question and protocol under H.P.'s supervision. She performed all vaginal lavages, four-vessel occlusion surgeries, and euthanasia with the help of a graduate student (A.M.). M.P. sliced all the brains and performed all immunohistochemical staining. Research assistants under M.P.'s co-supervision (Makenzie Lauzon, Judy Chékiée) assisted with capturing the immunofluorescent images. M.P. performed the thionine staining and image capture. CA1 neuronal density was independently assessed by M.P. and A.M. Finally, M.P. completed all data analyses, visualizations, and first draft preparation of the manuscript.

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Abstract

Persistent post-ischemic alterations to the hypothalamic-pituitary-adrenal (HPA) axis occur following global cerebral ischemia (GCI) in rodents. However, similar effects on hypothalamic-pituitary-gonadal (HPG) axis activation remain to be determined. Therefore, this study evaluated the effects of GCI in adult female rats (via four-vessel occlusion) on the regularity of the estrous cycle for 24-days post ischemia. A second objective aimed to assess persistent alterations of HPG axis activation through determination of the expression of estrogen receptor alpha (ER α), kisspeptin (Kiss1), and gonadotropin-inhibitory hormone (GnIH/RFamide-related peptide; RFRP-3) in the medial preoptic area (POA), arcuate nucleus (ARC), dorsomedial nucleus (DMH) of the hypothalamus, and CA1 of the hippocampus 25 days post ischemia. Expression of glucocorticoid receptors (GR) in the paraventricular nucleus of the hypothalamus (PVN) and CA1 served as a proxy of altered HPA axis activation. Our findings demonstrated interruption of the estrous cycle in 87.5% of ischemic rats, marked by persistent diestrus, lasting on average 11.86 days. Moreover, compared to sham-operated controls, ischemic female rats showed reduced Kiss1 expression in the hypothalamic ARC and POA, concomitant with elevated ER α in the ARC and increased GnIH in the DMH and CA1. Reduced GR expression in the CA1 was paralleled with increased GR-immunoreactivity in the PVN, indicative of lasting dysregulation of HPA axis activation. Together, these findings demonstrate GCI disruption of female rats' estrous cycle over multiple days, with a lasting impact on HPG axis regulators within the reproductive axis.

Key words: oestrus cycle, estrogen receptor alpha, gonadotropin-inhibitory hormone, kisspeptin, RFamide-related peptide-3, RFRP-3, 4VO

1. Introduction

In recent years, research has supported bidirectional influences between the hypothalamus-pituitary-gonadal (HPG) axis and cardiovascular disease (CVD). Irregular menstrual cycles have been linked with increased risk of coronary heart disease and stroke (Kiconco et al., 2022), with discrete reproductive factors, such as early age of menarche and early menopause, being associated with morbidity from CVD in women (Okoth et al., 2020). Reciprocally, women who have suffered from a stroke are at increased risk of pregnancy complications (van Alebeek et al., 2018) and a systematic review found pituitary dysfunction to be observed in 19 to 82% of stroke survivors (Booij et al., 2018). With emerging evidence showing heightened acute and long-term risk of CVD events following SARS-CoV-2 infection in populations younger than 65 years (Xie et al., 2022), understanding how CVD impacts HPG axis function is increasingly relevant as younger women may be at heightened risk of experiencing CVD in years to come. In this context, the use of global cerebral ischemia (GCI) in rodents, which mimics cardiac arrest, appeared a suitable model to study possible effects of CVD on females' HPG axis functioning.

Beyond the known impact of chronic stress (Divyashree & Yajurvedi, 2016; Fotsing et al., 2017), estrous cycle irregularities have also been noted following traumatic brain injury (Fortress et al., 2019), while neonatal hypoxia/ischemia was associated with early reproductive senescence and overexpression of hypothalamic estrogen receptors α (ER α ; Ezquer et al., 2008), supporting a significant impact of brain injuries on female HPG axis function. Furthermore, lasting dysregulation of the hypothalamic-pituitary-adrenal (HPA) axis has been demonstrated following global ischemia, namely by altering glucocorticoid receptor (GR) expression (de la Tremblaye et al., 2014). Post-ischemic HPA axis alterations could likely have significant effects

on HPG axis regulators, considering well acknowledged interactions between stress exposure and perturbations of reproductive function (Rivier et al., 1986; Rosen et al., 1988).

Knowing that ovarian cyclicity represents a key indicator of overall health in women, we assessed regularity of female rats' estrous cycle for a 24-day period following GCI and determined the expression of two peptides respectively acting as key modulators and inhibitors of the HPG axis, namely kisspeptin (Kiss1; Harter et al., 2018) and gonadotropin-inhibitory hormone (GnIH/RFRP-3, RFamide-related peptide; Tsutsui & Ubuka, 2021). Considering its crucial role in regulating estrous cyclicity, ER α expression was also characterized (Yeo & Herbison, 2014). Finally, assessment of GRs measured the impact of the ischemic insult on HPA axis activation in female rats. Based on hormonal dysregulation in other brain injury models (Fortress et al., 2019) and stress's impact on hormonal cycles, we predicted that GCI would result in irregular estrous cycles and would inhibit Kiss1 while increasing ER α and GnIH peptide expression within relevant hypothalamic nuclei.

2. Methodology

2.1 Subjects

Sixteen adult female Wistar rats (~3 months old, weighing 200-250 g at arrival) were purchased from Charles River Laboratories (Rochefort, Québec, Canada). Rats were single-housed in plexiglass cages with beta chip bedding and a 3.5-inch diameter black polycarbonate tube and kept on a 12 h light:dark cycle (lights on at 7:00 a.m.) in a vivarium housing exclusively female rats. The room was kept at 21-23°C and 60 % relative humidity. Rats had *ad libitum* access to water and standard rat chow (Teklad global 18 % Protein Rodent Diet, Envigo, USA). Experimentation complied with the ARRIVE guidelines and was in accordance with the NIH guide for the care and use of laboratory animals. All procedures were performed in accordance

with the Canadian Council on Animal Care and approved by the Animal Care Committee of the University of Ottawa (PY-3278).

2.2 Assessment of Estrous Cycle

Estrous cycle assessments were performed daily, between 14h00 and 15h00, for 2 weeks to confirm regularity. Only rats with at least 2 consecutive cycles were included (regular cycle defined as entering estrus once every 4-5 days). Following surgery, estrous cycle was monitored for a minimum of 24 days before euthanasia. Briefly, a latex dropper bulb was placed on a 200 μ L pipette tip and filled with 100 μ L of sterile water. The pipette tip was gently inserted into the opening of the vaginal canal (less than 5 mm deep), water was slowly flushed into the canal 2-3 times, then deposited onto a clear slide and examined using a light microscope with a 10 $\times$ objective lens. Estrous cycle stage cell compositions were as follows: proestrus – majority round, nucleated epithelial cells; estrus – majority anucleated, cornified epithelial cells; metestrus – similar amounts of epithelial cells, cornified cells, and leucocytes; and diestrus – majority leucocytes (McLean et al., 2012).

2.3 Global Cerebral Ischemia

The four-vessel occlusion model (Pulsinelli & Brierley, 1979) was used to induce GCI for 10 min. The estrous cycle of each rat was monitored to ensure the second day of surgery coincided with the metestrus phase, during which estrogen levels are low (McLean et al., 2012). As per previously published methods (Morin et al., 2022), vertebral arteries were irreversibly electrocauterized, and carotid arteries isolated with silk thread prior to 10 min GCI on the following day, induced by occluding the carotid arteries with microvascular clips. The absence of a righting reflex and unresponsiveness to light and sound confirmed GCI. Sham-operated rats underwent the same anesthesia and incisions, without electrocauterization or interruption of

blood flow. Analgesia consisted of one dose of buprenorphine HCL injection (0.05 mg/mL, s.c.; Vetergesic© Multidose, Ceva Animal Health, Canada), administered following Day 1 surgery. Rats were kept on surgical heating platforms (Prostation Rodent Workstation, Patterson Scientific, USA) during surgery and transferred to a heated incubator (32°C) post-surgery. Rats received a 5 mL 0.09 % saline injection (s.c.) and were given access to wet rat chow and hydrogel following surgery.

2.4 Tissue Collection

Approximately 25 days after surgery, rats in the diestrus stage were injected with a lethal dose of sodium pentobarbital (60 mg/ml; Euthanyl Bimeda-MTC Canada, Canada) and transcardially perfused (4 % paraformaldehyde; 20 % picric acid). Monitoring continued for rats that were not in diestrus on the 25th day until they entered diestrus, at which point they were euthanized. The rats' ovaries were carefully excised and weighted. Brains were rapidly extracted, incubated in paraformaldehyde for 1 h, 10 % sucrose for 1 h, 10 % sucrose overnight, then 20 % sucrose for 24 h. Brains were frozen with CO₂ and stored at -80°C. Brains were sliced at 14µm using a cryostat (Leica CM1900, Leica Microsystems, Germany) and mounted onto Superfrost Plus Slides (Fisher Scientific, Canada). Areas of interest included the CA1 of the hippocampus (-2.80 to -4.16 mm posterior to Bregma), the medial preoptic area (POA; -1.30 to -1.40 mm), arcuate nucleus (ARC; -2.12 to -3.60 mm), dorsomedial nucleus (DMH; -2.56 to -3.60 mm) and paraventricular nucleus (PVN; -1.30 to -2.12 mm) of the hypothalamus (Paxinos & Watson, 2006).

2.5 CA1 Neuronal Assessment

Neuronal cell death in the hippocampal CA1 pyramidal layer was used to confirm achievement of an ischemic state (Morin et al., 2022). Coronal brain slices were air-dried for 30

min and soaked in the following solutions: dH₂O (30 s), thionin (10 min), 50 %, 70 %, 95 %, and twice in 100 % ethanol (2 min each), followed by Citrisolv (2 × 2 min; Decon Labs, Inc., USA), prior to being coated with Permout mounting medium and coverslipped. For each rat, 6 anatomically matched photomicrographs of the CA1 were acquired under a 20 × objective lens using a Leica DAS microscope attached to a SONY digital camera and Norton Eclipse 6.0 software (Empix Imaging, Mississauga, Ontario, Canada). Using Image J software (National Institute of Health, USA), the number of intact neurons (defined as cells having a clear nuclear area and a cytoplasm within a rounded cell body) in 1 mm linear length of CA1 tissue was quantified by two examiners unaware of the experimental conditions.

2.6 Immunofluorescence Detection

Slides containing the regions of interest were incubated in a phosphate-buffered saline (PBS)–5 % donkey serum–0.2 % triton blocking solution for 30 min at room temperature. Slides were then incubated at 4°C overnight in blocking solution containing one of the following primary antibodies: monoclonal mouse anti-ER α (1:200; MA5-13304, Invitrogen Canada Inc., Canada; Ludwik et al., 2020), polyclonal rabbit anti-Kiss1 (1:300, BS-0749R, Bioss, USA; Liu et al., 2018), rabbit anti-GnIH (PAC123/124; 1:5,000; Provided by Dr. G.E. Bentley; Smith et al., 2008), or monoclonal mouse anti-GR (1:500, MA1510, Invitrogen Canada Inc.; Wisłowska-Stanek et al., 2013). The following day, slides were washed (3 × 5 min) in PBS, then incubated in blocking solution containing fluorescence-conjugated anti-mouse donkey secondary antibody (1:1000; A21203, Invitrogen Canada Inc.) or anti-rabbit donkey secondary antibody (1:1000; A21207, Invitrogen Canada Inc.) in a dark cabinet for 1 h. Slides were PBS-washed (3 × 5 min), incubated 5 min at room temperature in Hoechst adenine-thymine binding dye solution (1:20,000 in PBS; Hoechst 33342, Invitrogen Canada Inc.), and PBS-washed (3 × 5 min). Slides were

covered with 30 μ L of antifade solution, coverslipped, and sealed with nail polish. For all antibodies, pilot studies determined optimal primary antibody concentrations and negative controls (without primary antibodies) validated secondary antibody specificity.

Immunofluorescence was detected using an Olympus DX51 microscope (Center Valley, PA, USA) and images were obtained with a 20 $\times$ objective lens using the ProgRes Capture Pro 2.7.6 software. For each rat, immunoreactivity (ir) was analyzed in 6 anatomically matched photomicrographs using ImageJ, which calculated the percentage of area (representing area covered by immunoreactive staining relative to total microphotograph area), for each selected brain region. All regions and markers were analyzed using the ImageJ algorithm “Triangle”, except GnIH throughout the hypothalamus, and all GR expression, for which the algorithm “Moments” was used.

2.7 Statistical Analyses

Statistical analyses were performed using IBM© SPSS Statistics 27 (IBM, Armonk, NY, USA). Estrous cycle data was divided into 4-day bins and rats having entered the estrus stage once during the previous 4 days were considered normally cycling. Cycle regularity was analyzed using a chi-square test for homogeneity, with Fisher’s exact test being reported. Ovary weights, CA1 neuronal density, and fluorescence-ir were analyzed using independent samples *t*-tests. All data were screened using box plots and outliers replaced by the groups next extreme-most value ± 1 . Shapiro-Wilk test and Levene’s test respectively assessed normality and homogeneity of variance. If normality was not respected, data were square root transformed (ER α expression in CA1, GR expression in the PVN). Welch *t*-test is reported if heterogeneity of variance was detected with Levene’s test. Significance was set at $p < .05$ for all analyses and

effect sizes are presented using Cohen's d with Hedge's correction (g). Graphs were generated using R Studio and display individual data points.

3. Results

3.1 Effects of Ischemia on Estrous Cycle Regularity and Ovary Weights

All rats showed regular estrous cycles prior to surgery. Fisher's exact test showed that GCI and sham rodents differed significantly on post-operative Day 4 ($p < .01$) and 8 ($p < .01$, see Figure 1).

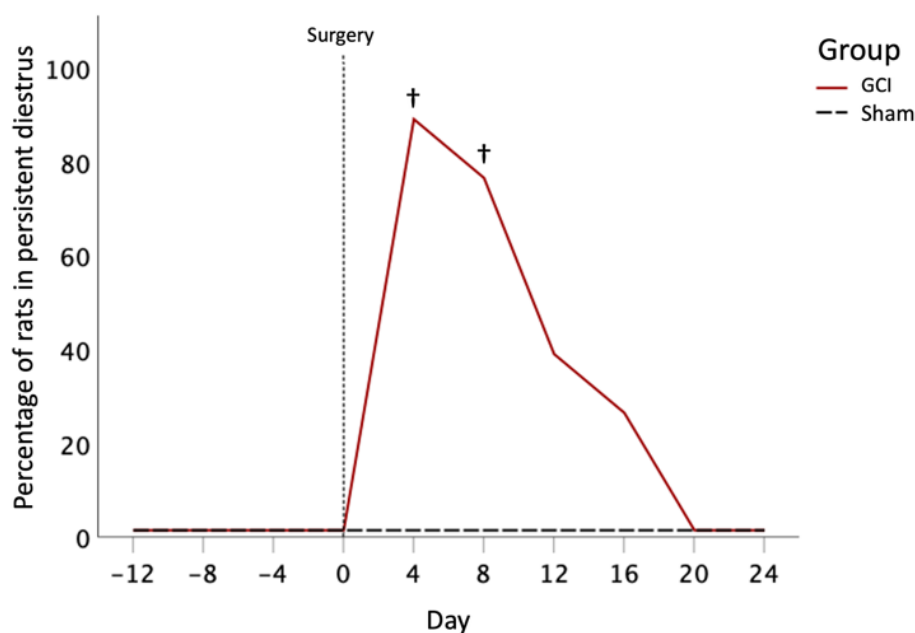


Figure 1. GCI Interrupts Estrous Cyclicity in Female Rats.

Graph presents the percentage of rats displaying persistent diestrus at each timepoint. † indicates statistical significance, $p < .01$.

The surgery showed no significant impact on cycle regularity of the sham-operated rats, with all rats continuing to enter estrus once every 4-5 days. In contrast, 87.5 % of GCI rats showed persistent diestrus, affecting minimum one cycle, which lasted on average 11.86 ± 4.38 days. Figure 2 illustrates the estrous cycle recovery profile of each GCI rat. Please refer to [Appendix C](#), Table C1 for breakdown of number of days spent in each stage. Independent

samples *t*-test found no significant differences between GCI and sham subjects for ovary weights ($p > .05$; data not shown).

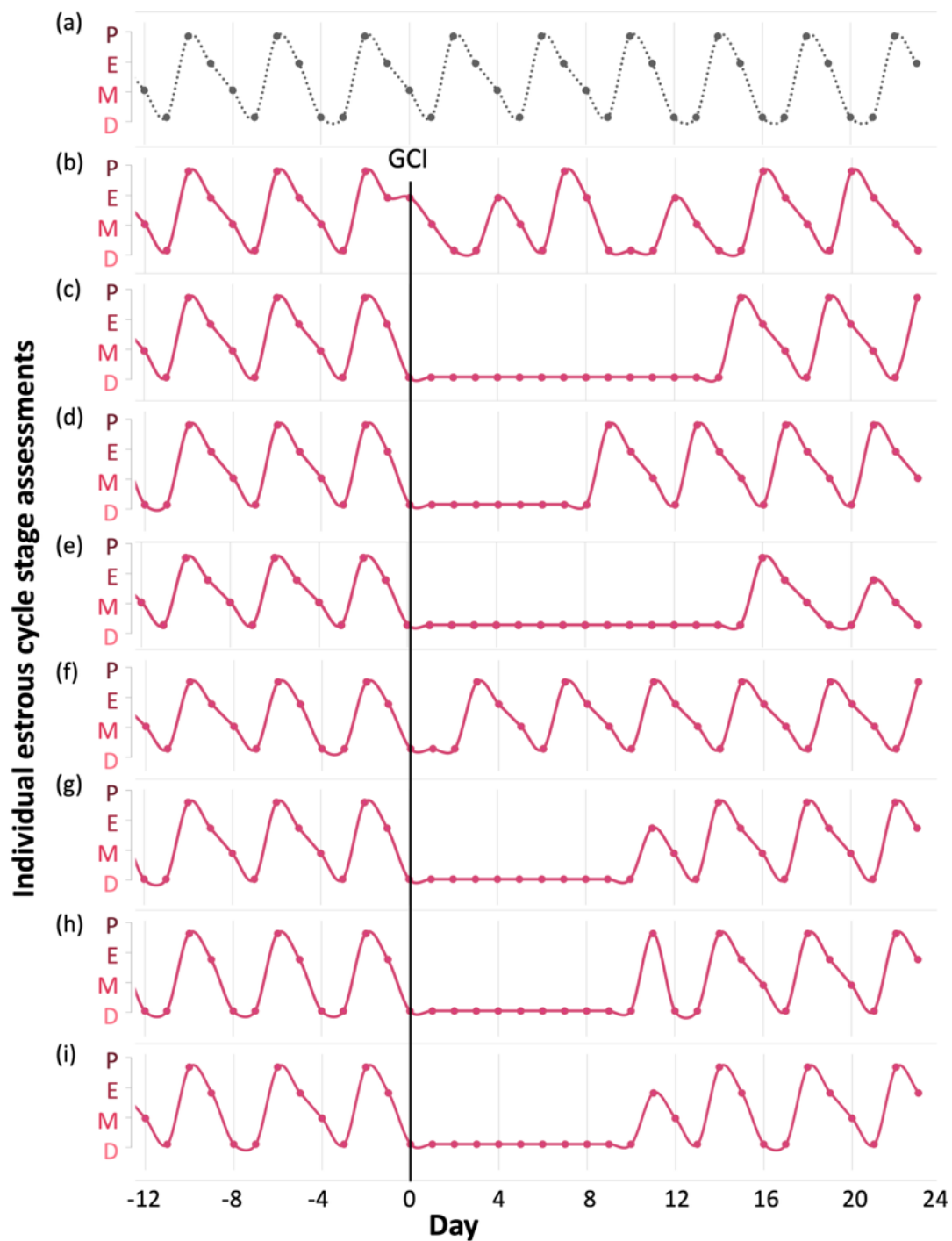


Figure 2. Female Rats Show Distinct Estrous Cycle Recovery Profiles Following GCI.

(2a) Representative estrous cyclicity of a sham subject. (2b–i) Daily estrous cycle stage for each GCI subject. Surgery was performed on Day 0. P: Proestrus; E: Estrus; M: Metestrus; D: Diestrus.

3.2 Hippocampal CA1 Neuronal Cell Death

Independent samples *t*-test indicated a significant difference in mean neuronal densities in the CA1 [$t(14) = 6.157, p < .01, g = 2.910$], with GCI rats having significantly reduced CA1 neuronal density compared to sham-operated rats (see Figure 3). See [Appendix C](#) (Figure C1) for whole coronal view of thionin staining.

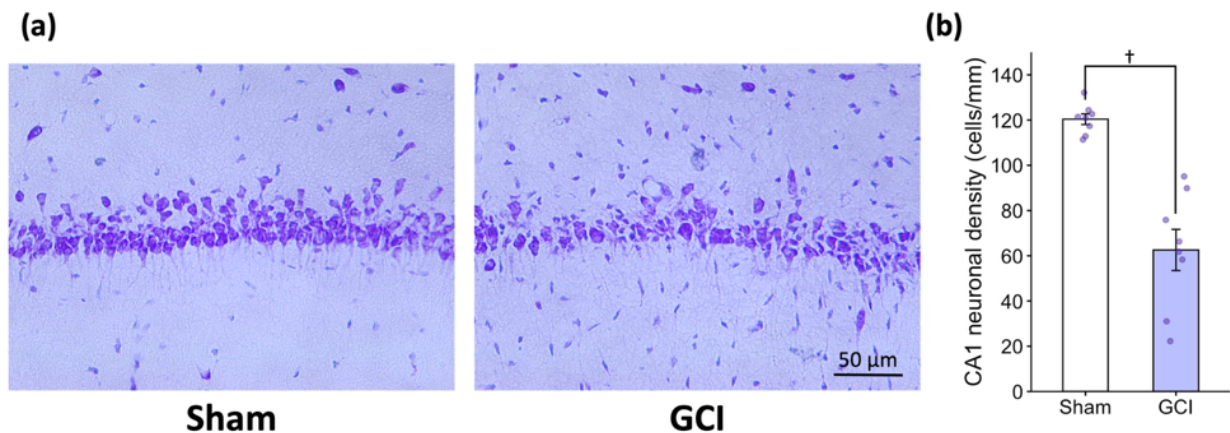


Figure 3. CA1 Neuronal Injury Following GCI.

(3a) Representative photomicrographs of CA1 hippocampal cell density of a sham and GCI subject. (3b) Neuronal cell count per 1mm linear length of the CA1 in sham and GCI rats. Data are presented as mean $\pm$ S.E.M. † indicates statistical significance, $p < .01$.

3.3 Immunofluorescence Detection

3.3.1 *ERα-ir* Expression

In the ARC, independent samples Welch *t*-test revealed a significant difference in *ERα* expression [$t(7.889) = -2.319, p = .049, g = 1.096$, see Figure 4], due to elevated ARC *ERα-ir* in GCI compared to sham rats. No other significant differences were detected (all $p > .05$).

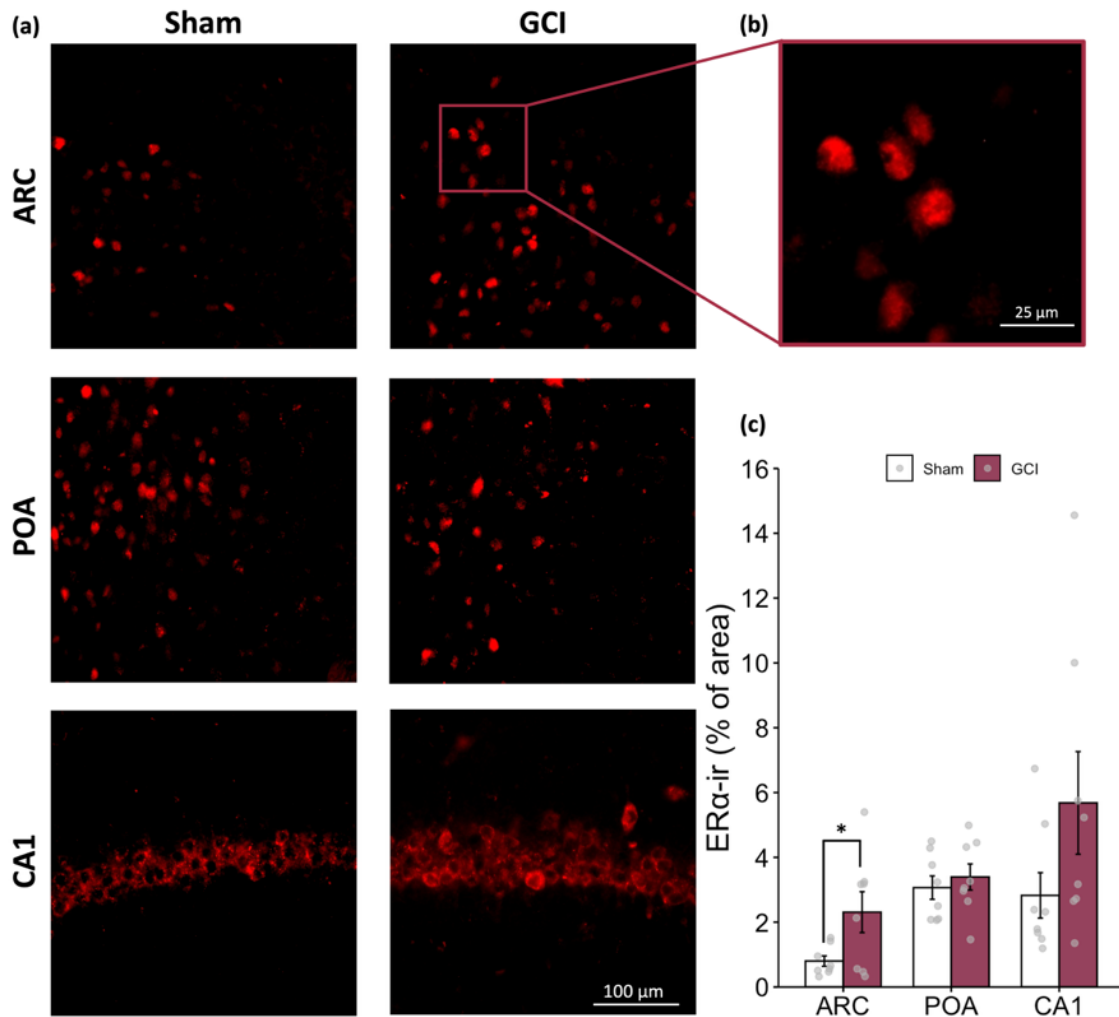


Figure 4. ERα-ir Is Overexpressed in the ARC Following GCI in Female Rats.

(4a) Representative fluorescent immunoreactive staining for estrogen receptors α (ER α) in the ARC, POA, and CA1 of sham and GCI subjects. (4b) Close up of ER α staining in the ARC of a GCI subject. (4c) Histogram of mean ER α -ir in each brain region. Data are presented as mean $\pm$ S.E.M. * indicates statistical significance, $p < .05$.

Kiss1-ir Expression

Independent samples t -test indicated a significant difference in Kiss1-ir in the ARC [$t(14) = 2.417$, $p = .03$, $g = 1.142$, see Figure 5] and the POA [$t(14) = 2.434$, $p = .03$, $g = 1.151$], both attributable to reduced Kiss1-ir in GCI compared to sham-operated rats.

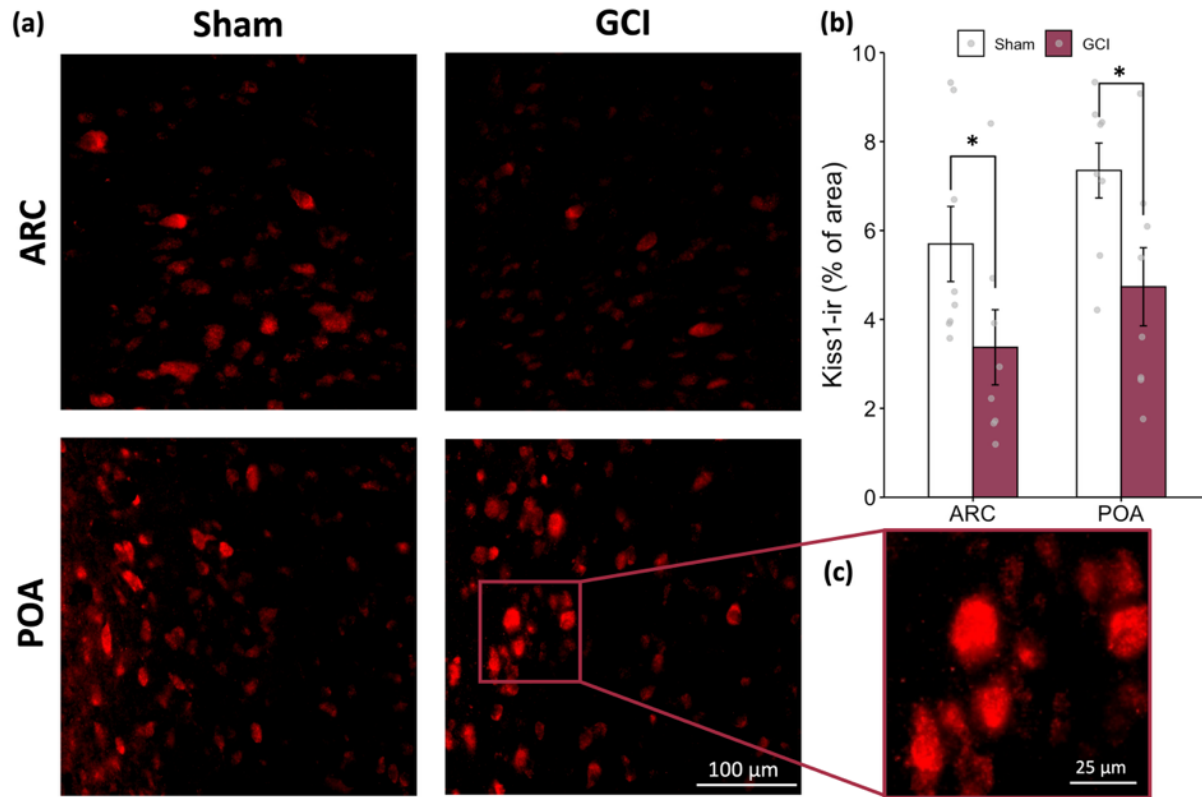


Figure 5. Kiss1-ir Is Reduced in the ARC and POA Following GCI in Female Rats.

(5a) Representative fluorescent-ir for Kiss1 in the ARC and POA of sham and GCI subjects. (5b)

Histogram of mean Kiss1-ir in each brain region. Data are presented as mean $\pm$ S.E.M. (5c) Close up of Kiss1 staining in the POA of a GCI subject. * indicates statistical significance, $p < .05$.

GnIH-ir Expression

Independent samples t -test indicated a significant difference in GnIH-ir in the DMH [$t(13) = -2.477$, $p = .028$, $g = .642$] and in the CA1 [$t(14) = -5.059$, $p < .01$, $g = 2.391$; see Figure 6], both related to elevated GnIH-ir in GCI compared to sham rats. No significant differences were noted in the ARC or POA (both $p > .05$). One GCI rat was excluded from the DMH analysis due to insufficient number of slices of adequate quality for quantification.

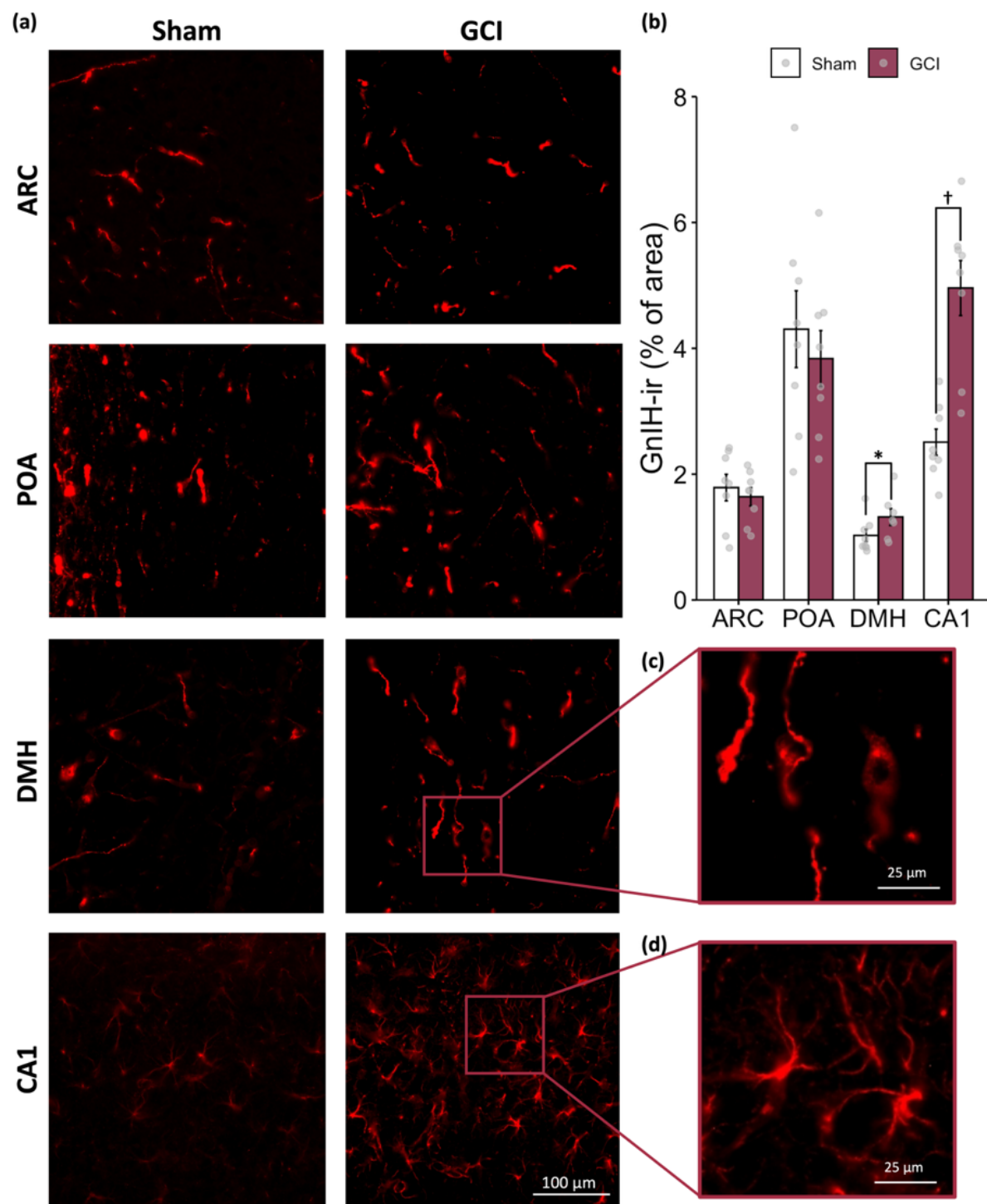


Figure 6. GnIH-ir Is Overexpressed in the DMH and CA1 Following GCI in Female Rats.

(6a) Representative fluorescent immunoreactive staining for GnIH in the ARC, POA, DMH, and CA1 of sham and GCI subjects. (6b) Histogram of mean GnIH-ir in each brain region. Data are presented as mean $\pm$ S.E.M. (6c) Close up of GnIH neuron in the DMH of a GCI rat. (6d) Close up of GnIH projections in the CA1 of a GCI subject. * indicates statistical significance at $p < .05$, † at $p < .01$.

GR-ir Expression

Independent samples *t*-test revealed a significant difference in GR-ir expression in the PVN [$t(13) = -2.554, p = .024, g = -1.376$] and the CA1 [$t(14) = 2.947, p = .011, g = 1.598$; see Figure 7]. In the PVN and CA1, GR-ir was respectively increased and decreased in GCI relative to sham rats. One GCI rat was excluded from the PVN analysis due to insufficient number of slices of adequate quality for quantification. While corticosterone (CORT) levels were not assessed in this study, please refer to the [Appendix C](#) (Figure C2) for basal CORT levels in ischemic male rats as an approximation of the CORT response profile following GCI (de la Tremblaye et al., 2014).

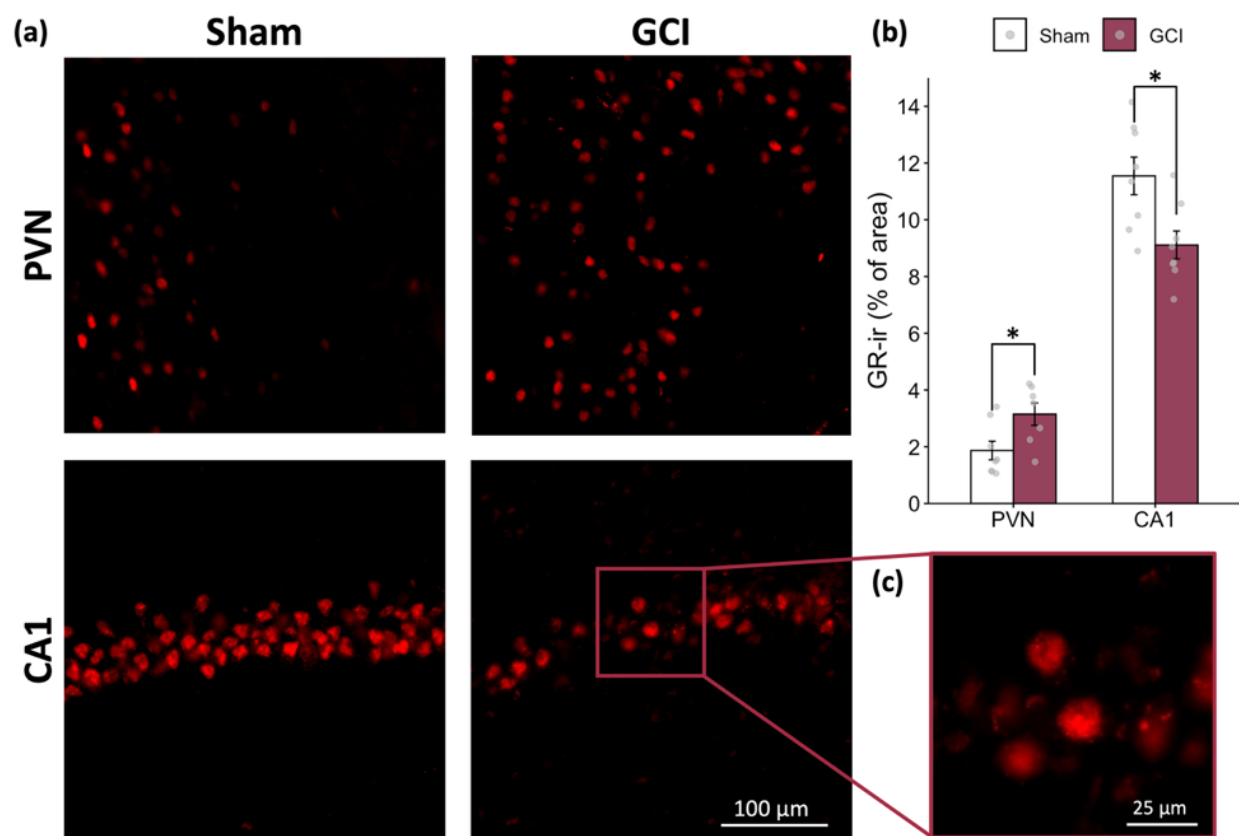


Figure 7. GR-ir Expression Is Increased in the PVN and Inhibited in the CA1 of GCI Female Rats. (7a) Representative fluorescent immunoreactive staining for GR in the PVN and CA1. (7b) Histogram of mean GR-ir in each brain region. Data are presented as mean $\pm$ S.E.M. (7c) Close up of GR-ir in the CA1 of a GCI rat. * indicates statistical significance, $p < .05$.

4. Discussion

To our knowledge, this study is the first to characterize alterations of estrous cyclicity and HPG axis peptidergic signalling following GCI in adult female rats. Our findings indicate significant disruption of estrous cycling affecting 87.5 % of ischemic female rats. This was manifested via persistent diestrus lasting on average 11.86 days. Furthermore, when assessed at a remote 25-day interval, ischemic females showed region-specific overexpression of ER α and GnIH-ir, concomitant with reduced Kiss1-ir, despite recovered estrous cyclicity. Consistent with ischemic male rats (de la Tremblaye et al., 2014), ischemic females showed blunted GR-ir in the CA1 and elevated PVN-GR expression, suggestive of lasting dysregulation of HPA axis activation.

The observation of persistent diestrus in ischemic females indicates a greater impact of GCI on estrous cyclicity than expected, contrasting with studies of chronic stress which report irregular cycles (Divyashree & Yajurvedi, 2016; Fotsing et al., 2017). Interestingly, a study assessing traumatic brain injury in female rats found that in the 8 days following injury, cycling was halted in 28.6 % of rats, with the other 71.4 % displaying irregular cycles (Fortress et al., 2019), supporting a stronger immediate impact of GCI (75 % acyclic in the first 8 days). The singular impact of adulthood GCI is further exemplified by the finding that neonatal hypoxia/ischemia showed no impact on adulthood estrous cycling, despite advancing reproductive senescence (Ezquer et al., 2008). Importantly, the cascade of biochemical events involved in delayed hippocampal neuronal death characteristic of GCI models (Kirino et al., 1984) could contribute to impairing estrous cyclicity in the initial week following adulthood ischemia, as the brain focuses its energetic resources towards recovery and repair. This possibility is supported by substantially elevated circulating CORT concentrations in adult male

rats up to 7 days following ischemia, which returned to concentrations seen in sham operated controls when measured 14 days post ischemia (de la Tremblaye et al., 2014). Although future assessments of CORT levels are required to confirm this recovery profile in females, it seems likely that once cellular homeostasis gradually returns, estrous cyclicity may be reinstated. Importantly, HPG-driven hormonal cycles are known to modulate a variety of behaviours, notably emotionality (Egan et al., 2019) and cognitive abilities (do Nascimento et al., 2019). Consequently, even temporary ovarian cycle disruptions occurring after cardio- or cerebrovascular incidents may have important implications for functional recovery in women.

Knowing that glucocorticoid secretion potentiates GnIH activation (Tsutsui & Ubuka, 2021), the detected upregulation of PVN GR-ir could be linked to post-ischemic increases in GnIH expression in the DMH and CA1, especially considering a study of chronic stress showing up to 53 % of GnIH cells within the DMH to be GR positive (Kirby et al., 2009). Incidentally, elevations of GnIH-ir at the DMH could have exerted downstream inhibitory effects on Kiss1-ir, seeing as a small proportion of Kiss1 neurons in the hypothalamic ARC nucleus express GnIH receptors (Poling et al., 2013). Although the functional impact of these biochemical alterations remains to be ascertained in the context of an ischemic event, a study assessing the impact of chronic restraint stress in female rats demonstrated an increase in GnIH to be associated with reduced mating and pregnancy rates, and increased frequency of embryo resorption, effects which were prevented in GnIH-knockdown rats (Geraghty et al., 2015).

Accordingly, post-ischemic reductions of Kiss1-ir also have implications for sexual functioning, Kiss1-knockout having been found to impair lordosis in female mice (Hellier et al., 2018). Such a proposition is further supported by the post-ischemic overexpression of ER α , the induction of which has previously been associated with reduced fertility in mice (Tomic et al.,

2007). Importantly, the persistent reduction to hypothalamic Kiss1-ir detected herein presents an interesting parallel with the diminished Kiss1 receptor function that precedes ovarian failure in middle-aged rats (Zeydabadi Nejad et al., 2017), indicating that similar to neonatal ischemia/hypoxia (Ezquer et al., 2008), adulthood GCI might precipitate reproductive senescence. As such, the proposed downstream effects of GR and GnIH on Kiss1 and ER α in the post-ischemic brain could significantly impact sexual behaviours and reproductive success in ischemic females.

The secretion of Kiss1 has further been shown to play a critical role in regulating HPG axis activation by enabling the negative feedback of estrogens on gonadotropin-releasing hormone (GnRH) neurons (Harter et al., 2018). In male rats, GCI has been shown to significantly reduce GnRH receptor expression in the CA1 of the hippocampus, with a quasi-complete inhibition being reported at 72 h post-ischemia (Chu et al., 2008). In this context, the significant reduction in Kiss1-ir expression demonstrated in the assessed hypothalamic nuclei might contribute to impairing negative feedback within the HPG axis. It is also possible that the extensive neuronal death observed in the vulnerable CA1 region, known to alter negative feedback regulation within the HPA axis, may further act to accentuate dysregulation of Kiss1 signalling. Interestingly, research has supported distinctive involvement of Kiss1 neurons at the anteroventral periventricular (AVPV) and ARC nuclei, respectively acting to provide positive and negative feedback regulation of estrogen secretion (Lehman et al., 2010). Further exploration supported ER α receptors on ARC Kiss1 neurons to be essential for estrous cyclicity, while ER α receptor deletion in AVPV-Kiss1 neurons failed to affect cyclicity (Wang et al., 2019). Nevertheless, AVPV Kiss1 neurons are key regulators of luteinizing hormone (LH) pulses (Hu et al., 2015; Wang et al., 2019), making this a region of interest in subsequent investigations.

Future studies directly assessing GnRH expression in the ischemic female brain are necessary, as well as timely assessments of circulatory levels of LH throughout recovery, as its secretion closely relates to GnRH function and is essential to estrous cyclicity (Rivier et al., 1986). Indeed, LH acts at the ovaries to stimulate production of estrogens and progesterone, and its' proper function represents a key indicator of ovarian reserves (An et al., 2020). As such, assessment of LH levels in future studies is crucial to confirm possible reductions of fertility in post-ischemic female rats that might occur in association with the downregulation of Kiss1 and upregulation of GnIH and ER α detected in the ischemic brain.

5. Conclusion

To our knowledge, this study represents the first characterization of the impact of GCI on modulators of female reproductive cycles, namely GnIH and Kiss1. Our results highlight a 10-minute ischemic event experienced during adulthood in female rats to be associated with a transient interruption of estrous cyclicity, while disruptions of ER α , GnIH, and Kiss1 expression persisted 25 days post ischemia. These changes were concordant with altered GR expression, possibly contributing to preventing the complete recovery of normal HPG axis reactivity. Our observations highlight the relationship between hypothalamic regulated neuroendocrine systems, emphasizing the impact of physiologically stressful conditions, and bear implications for reproductive function and sexual behaviour post-brain injury.

6. CRediT Authorship Contribution Statement

Marilou Poitras: Conceptualization, Data curation, Formal analysis, Investigation, Methodology, Writing – original draft, Writing – review & editing. Alexandre Morin: Investigation, Writing – review & editing. George E. Bentley: Resources, Writing –

review & editing. Hélène Plamondon: Conceptualization, Supervision, Writing – Reviewing and Editing, Funding acquisition.

7. Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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9. Data Availability

Data will be made available on request.

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Chapter IV – Chronic Social Isolation, Crowding, and Instability Distinctly Impact

Reproductive Function in Adult Female Wistar Rats

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Authorial Contributions

M.P. designed the research question and protocol under H.P.'s supervision. M.P. performed all vaginal lavages, blood samples, behaviour tests, and euthanasia with the assistance of research assistants (A.L., Z.M.I.G.) and graduate students (Emmanuelle Person). M.P. sliced all the brains, performed all immunohistochemical staining, captured all H&E images, and ran all ELISA kits. A group of honours thesis students co-supervised by M.P. captured the immunofluorescent images (A.Q., R.F., A.L.) and performed ovarian follicle counting (R.F., Z.M.I.G.). An honours thesis student performed behavioural scoring for the Paced Mating tests (C.B) under M.P.'s co-supervision. M.P. completed all data analyses, visualizations, and first draft preparation of the manuscript.

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Abstract

Social stressors represent highly relevant stress models which remain understudied in females. Concordantly, little is known of the impact of different types of social stress on female reproductive functioning, despite close interactions between the hypothalamic-pituitary-adrenal (HPA) axis and hypothalamic-pituitary-gonadal (HPG) axis. This study provides a comprehensive assessment of reproductive functioning in female rats following exposure to three social stress paradigms. 112 regularly cycling adult female Wistar rats were exposed to 21 days of isolation, crowding, social instability, control housing (daily cage changes) or regular housing. Rats were then euthanized (in proestrus or diestrus) or underwent paced mating and fertility assessments. Estrous cyclicity, uterine horn weight, hypothalamic expression of kisspeptin (Kiss1), gonadotropin-inhibitory hormone (GnIH), estrogen receptors alpha (ER α), and glucocorticoid receptors (GR), serum luteinizing hormone (LH), follicle-stimulating hormone (FSH), estradiol (E2), progesterone (P4), and corticosterone (CORT), as well as ovarian follicle counts were assessed. Findings show isolation to increase hopping behaviours, decrease early follicle counts, uterine weight, and GnIH, as well as increase FSH levels. In crowded rats, expression of GnIH and ER α were reduced, paralleled by fewer days spent in proestrus and increased hopping behaviours. Following instability, rats presented decreased estrous cyclicity, reduced uterine weight, and diminished ovarian reserve, despite minimal changes at the hypothalamic level. Paced mating exposure increased CORT in all groups except instability and crowding. Overall, social stressors presented distinct consequences on reproduction, highlighting their relevance as stress models and the importance of gaining a better understanding of social stress-induced reproductive dysfunction in females.

Keywords: Estrous cycle, sexual behaviour, hypothalamic-pituitary-ovarian axis, social stress, reproduction

1. Introduction

Changes to social dynamics or housing densities can act as social stressors and have acknowledged consequences on the hypothalamic-pituitary-adrenal (HPA) axis, namely by increasing corticosterone (CORT) levels and altering glucocorticoid receptor (GR) expression (Koert et al., 2021). Importantly, evidence highlights several adverse effects of stress on reproductive function, with a higher number of stressful life events resulting in poorer *in vitro* fertilization outcomes (Ebbesen et al., 2009). In rodents, Herzog et al. (2009) have shown 28 days of social instability in adult female Wistar rats to significantly reduce the number of females displaying regular estrous cycles. Despite known variations in HPA axis activation upon exposure to different stress models (Atrooz et al., 2021), such as distinct physiological stress profiles following four types of systemic stress (Pacák & Palkovits, 2001), there remains a limited understanding of possible differing impacts of social stressors on female reproductive function. Namely, although evidence exists to support that population/housing density significantly influences experienced stress levels (Love & Zelikowsky, 2020), the distinct impact of relevant social stressors, such as isolation or crowding, on reproductive function in adulthood remains limited. Due to their resemblance with the types of stress experienced by women on a daily basis, social stressors are considered to have higher translational potential than physical models (Koolhaas et al., 2016) and consequently are essential to our understanding of stress-related reproductive dysfunction. In humans, the negative consequences emerging from the COVID-19 pandemic, which altered the social environment (Weber et al., 2024), support comparing effects of stressors such as isolation, crowding, and instability.

Several biological processes associated with hypothalamic-pituitary-gonadal (HPG) axis activity regulate ovarian and reproductive function. Thus, hypothalamic release of gonadotropin-

releasing hormone (GnRH) acts on the anterior pituitary to stimulate secretion of luteinizing hormone (LH) and follicle-stimulating hormone (FSH), synergistically acting to promote follicular growth and ovulation (Raju et al., 2013). Under stressful conditions, increased release of gonadotropin-inhibitory hormone (GnIH) inhibits GnRH, significantly reducing overall HPG axis activation (Tsutsui & Ubuka, 2021). Following crowding stress in female mice, reduced FSH and LH levels were observed in parallel with a reduction in the number of follicles (Kim & You, 2022). Studies also support a dysregulation of the stimulatory neuropeptide kisspeptin (Kiss1), known for its role in modulating positive and negative feedback within the HPG axis, in rats exposed to chronic cold water immersion stress (Retana-Márquez et al., 2020). To date, altered expression of hypothalamic Kiss1 and GnIH following adult social stress and its associations with other reproductive processes remain largely unknown.

Sexual function in female rodents depends on gonadotropins reaching the ovaries to stimulate the release of estradiol (E2) and progesterone (P4). Despite the knowledge of female reproductive behaviour being negatively impacted by physical (Retana-Márquez et al., 2020) and psychological stress (Donadio et al., 2007), stress-related health consequences have predominantly been studied in males, and the impact of social stress on sexual behaviours in adult females remains largely unknown. Importantly, the use of hormonally primed ovariectomized rats is common practice in the study of female sexual behaviour, as it allows to homogenize hormonal levels and facilitate the timing of testing. However, the use of natural cycling rodents can enhance translational value of preclinical findings to women of reproductive age (Mondino et al., 2019), for whom stress-induced HPG axis dysregulation likely exerts a strong impact (e.g., menstrual cycle irregularities, reduced fertility). A large cross-sectional study indeed showed a significant increase in anxiety prevalence in the adult American population

younger than 50 years old over the last decade (Goodwin et al., 2020). Seeing as women typically enter menopause around 50 years old, adult women of reproductive age represent a population that might currently be at increased risk of stress-related adverse health concerns. As such, although stress indubitably impacts reproduction, much remains to be known about adulthood social stress and how various models may differ in their impacts on reproduction.

1.1 Goals and Hypotheses

Using a wide range of biochemical and behavioural measures, this study aimed to characterize the impact of three chronic social stressors (isolation, crowding, and instability) experienced in adulthood on estrous cycle regularity, sexual behaviors, fertility, and key HPG axis markers. Notably, we studied these variables in an estrous cycle stage dependent manner (diestrus vs proestrus). Indeed, while estrous cycle stages are often combined for analyses, evidence suggests that collapsing all stages together may increase the risk of masking significant differences (Rocks et al., 2022). We hypothesized that of the three experimental social stress conditions, females would be most strongly affected by social instability (SIS), followed by isolation (low density housing; LDH), and that stress response to crowding (high density housing; HDH) would be of a comparatively lesser intensity (do Nascimento et al., 2019; Herzog et al., 2009). We expected increased stress to cause altered estrous cyclicity, reduced sexual functioning and fertility, and an inhibition of HPG axis function, as well as increased CORT, GnIH, and GR levels.

2. Methodology

2.1 Subjects

Sexually naive adult female Wistar rats ($n = 112$) aged 3 months were obtained from Charles River Laboratories (Rochefort, Québec). Upon arrival, rats were pair-housed in standard

housing conditions: plexiglass cages (45.5 cm x 24 cm x 20.5 cm) containing 1 cm of beta chip bedding and a 3.5-inch diameter polycarbonate tube to rest and hide, with biweekly cage changes. Rats were kept on a 12 h reverse light:dark cycle, with lights off at 10:00 a.m. Room temperature was maintained at 21-23°C with 60% relative humidity. Rats had *ad libitum* access to standard rat chow (2018 Teklad global 18% Protein Rodent Diet, Envigo, United States) and water throughout the experiment. Rats were weighed weekly at estrous cycle assessment. See Figure 1 for experimental timeline.

Proven breeder male Wistar rats, aged 4-5 months at time of testing, were obtained from Charles Rivers Laboratories and used for sexual behaviour tests. Males were single housed in standard conditions in a separate room in the animal vivarium. The week preceding sexual behaviour assessment, to ensure recent sexual experience, stimulus males were given two 30 min un-paced mating sessions with hormonally primed ovariectomized adult female Wistar rats (Cummings & Becker, 2012). All procedures respected ARRIVE guidelines and the Canadian Council on Animal Care and were approved by the Animal Care Committee of the University of Ottawa prior to experiment start (PY-4245).

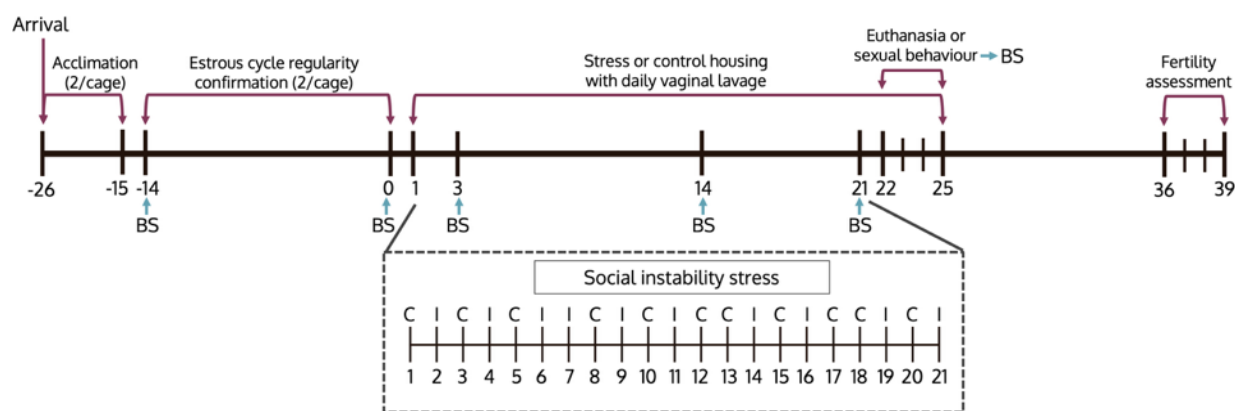


Figure 1. Experimental Timeline.

All rats were pair-housed during acclimation and two weeks of estrous cyclicity confirmation, after which they were randomly assigned to one of five housing conditions for 21 days: high density housing (HDH: n = 8/cage), low density housing (LDH: n = 1/cage), social instability (SIS: alternating days of crowding

and isolation, as shown), control housing ($n = 2/\text{cage}$ with daily cage changes) or regular housing ($n = 2/\text{cage}$). Estrous cycle monitoring occurred daily for the duration of the study. Blood samples (BS) were collected after acclimation (Day -14), prior to stressor initiation (Day 0), on the 3rd, 14th, and 21st days of the stressor, and following sexual behaviour assessment. From days 22 to 25, stress or control conditions were maintained, and rats were either immediately euthanized (in proestrus or diestrus) or underwent paced-mating test and overnight copulation (Proestrus). 14 days after copulation, rats were euthanized to measure fertility parameters. C: Crowding. I: Isolation.

2.2 Estrous Cycle Assessment

After 12 days of acclimation, rats underwent daily vaginal lavage for two weeks to confirm estrous cycle regularity. Only rats with at least two consecutive regular cycles were included. Estrous cycle continued to be monitored daily until the end of the experiment, with sample collection occurring between 8:00 and 10:00 a.m., immediately prior to onset of the dark period (1-2 h before lights off). Vaginal lavages were performed by gently restraining the rat, lifting the base of the tail, and using a 200 μL pipette attached to a latex dropper bulb to slowly flush 100 μL of sterile water into the vaginal canal (Poitras, Morin, et al., 2024). Each sample was deposited onto a clear microscope slide, and a representative photomicrograph of the vaginal cell composition was captured using a Leica DAS microscope attached to a SONY digital camera and Norton Eclipse 6.0 software (Empix Imaging, Mississauga, Ontario, Canada). An independent rater blinded to experimental conditions reviewed all photographs to determine estrous cycle stage and cyclicity. Estrous cycle stage characterization used established criteria (McLean et al., 2012). Cycles were considered irregular if they were condensed (i.e., 3 days or less) or prolonged (i.e., 6 days or more), or if the normal progression of stages was not observed (i.e., 1 proestrus day, 1 estrus day, 2-3 days of metestrus/diestrus).

2.3 Stressors

Following confirmation of estrous cycle regularity, rats were randomly assigned to one of four housing conditions for a 21-day period ($n = 24/\text{group}$) or to a regular housed group ($n = 16$).

All experimental groups were housed in standard plexiglass cages (45.5 cm x 24 cm x 20.5 cm) containing 1 cm of beta chip bedding and a single polycarbonate tube.

Rats assigned to the social isolation condition (low density housing; LDH) were individually housed in standard cages (Carrier & Kabbaj, 2012), with twice per week cage changes. Rats in the crowding condition (high density housing; HDH) were housed 8 rats per standard cage, allowing 136.5 cm² of floor space per rat (Adavi et al., 2023), with daily cage changes. Rats assigned to the social instability stress (SIS) condition were subjected to alternating phases of isolation and crowding, as described above. To increase unpredictability, phase length was randomized to last either 24 h or 48 h, and group composition varied for each new crowding phase (Herzog et al., 2009), with daily cage changes so each phase began in a new cage. Rats in the control housing (CH) condition were pair-housed in standard conditions but underwent daily cage changes during the stress period to reflect the cage change frequency of the HDH and SIS groups. Additionally, a regular housing (RH) group was included (n = 16 rats), in which rats were pair-housed in standard conditions and underwent cage changes twice per week (as per standard procedures within our animal facilities), reflecting the cage change frequency of the LDH group. In addition to providing appropriate housing controls for all stress groups, the inclusion of both a CH and RH group also allowed to evaluate possible contributions of daily cage changes to the stress response of the HDH and SIS groups. Both control groups (CH and RH) were performed at a separate time from the stress groups, to avoid housing in the same vivarium as stressed rats (do Nascimento et al., 2019). Efforts were made to minimize possible confounding effects of running separate cohorts: all experiments (i.e., cohorts) were performed in the same year, housed in the same vivarium room, and all manipulations were performed by the lead researcher (M.P.).

2.4 Blood Sampling

To characterize possible changes in basal HPA axis activation from chronic stress exposure, baseline blood samples were collected at the end of acclimation (Day -14; BS1), prior to start of stress (Day 0; BS2) and on the 3rd (S3), 14th (S14), and 21st (S21) day of the stress period. Before the onset of the dark period, immediately prior to vaginal lavage (1-2 h before lights off), rats were individually brought to an adjacent room and blood collection was performed using the tail nick method (Milot et al., 2012). For rats having undergone sexual behaviour, a blood sample was collected immediately after testing, to detect any group differences in reactivity to first sexual encounter (collected within 2 min of test completion, approximately 3 h after lights off). At least two whole blood drops per rat were deposited onto Whatman Bloodstain Cards (Whatman International Ltd., Maidstone, United Kingdom), after which samples were left to dry overnight and stored at -80°C for subsequent analysis.

2.5 Female Sexual Behaviours

Following 21 days of exposure to stress or control housing conditions, female sexual behaviour was assessed (Day 22-25) in 6-8 rats per experimental group. Vaginal cytology was used to only select rats in proestrus (Retana-Márquez et al., 2020). Females not in proestrus on that day were maintained in the same experimental housing conditions for a maximum of 3 days, until they reached proestrus (mean = 1.51 days). Assessment of sexual behaviour took place during the dark phase (2-4 h after lights off) under red lighting. A two-compartment paced-mating set-up was used (Heijkoop et al., 2018), consisting of a large arena (46 x 78 x 33 cm) with 1 cm of standard bedding, divided in half by a clear plexiglass separator with two holes at the bottom (4.0 cm diameter) through which only the female could cross. The female was placed

in the PM arena and allowed to habituate for 5 min, after which the male was introduced in the opposite compartment and behaviours were recorded for 45 min.

An experimenter blinded to experimental conditions scored the following measures using the Behavioral Observation Research Interactive Software (BORIS; Friard and Gamba, 2016): 1) paracopulatory behaviours, defined as the number of hopping, darting, and ear wiggling; 2) rejection behaviours, namely the number of kicks, squeaks, and defensive postures; 3) receptivity, determined by calculating the lordosis quotient [Lordosis quotient = (number of lordosis/ number of male behaviours) x 100]; 4) lordosis intensity, scored on a scale of 0-3 according to the intensity of dorsiflexion; 5) duration of each lordosis posture (s); and 6) sexual motivation, which included time spent away from the male compartment (s), percentage of exits out of the male compartment (i.e., the likelihood of withdrawal to the other compartment within 10 s of stimulation, scored according to the type of male stimulation [mount, intromission, or ejaculation]) and contact-return latency (i.e., time between exit and the return to the male compartment, scored according to the type of male stimulation). Additionally, the number of mounts, intromissions, and ejaculations performed by the male were scored, as well as the latency (s) to first behaviour (Corlett et al., 2022; Heijkoop et al., 2018).

2.6 Fertility

Following completion of the paced-mating test and collection of post-sexual behaviour blood sample, female and male partner were immediately transferred to a large clean cage (37.5 x 45.5 x 30.5 cm) with access to standard rat chow and water, and allowed to continue copulation overnight. At 8:00 a.m. the following morning, females were moved to a clean cage and single-housed in standard conditions for the remainder of the experiment, with the addition of crinkle paper into the cage to minimize stress. Fourteen days after copulation (D36-39), females were

euthanized using CO₂, uterine horns surgically removed, and embryos excised. Number of rats having achieved pregnancy, number of embryos and resorbed embryos, as well as yolk sac and embryo weights were recorded (Ward et al., 2012). Yolk sac fluid weight was calculated by subtracting the embryo weight from the sac's weight. Finally, whole body and adrenal gland weights of pregnant rats were recorded.

2.7 Euthanasia and Sample Collection

For each experimental condition, 6 proestrus rats and 6 diestrus rats were randomly selected for biochemical analyses (5 per stage for RH rats). Rats sharing a cage were euthanized on the same day to avoid disrupting experimental conditions. As such, since not all rats were in the appropriate estrous cycle stage at the end of the 21-day period, stress conditions were maintained for a maximum of three days until they entered a stage in which sample collection could occur (mean = 1.86 days; Nowacka et al., 2014). Since rats in a given crowding cage were not all in relevant stages on a given day, one additional HDH cage (n = 8 rats) was included to achieve the desired number of animals in each subgroup, while still euthanizing all animals from a single cage on the same day. To minimize any effects from cage-to-cage variability, 6 rats per crowding cage were randomly selected and used in analyses (n = 24 HDH rats included).

Euthanasias took place on days 22-25, between 9:30 a.m. and 12:30 p.m., with all proestrus rats being euthanized within 30 min of lights off (i.e., 9:30-10:30 a.m.). Rats were injected with a lethal dose of sodium pentobarbital (60 mg/ml; Euthanyl Bimeda-MTC Canada, Canada). Upon the loss of reflexes, a minimum of 3 mL of blood was collected via the abdominal aorta, after which rats were transcardially perfused with saline. Uterine horns and adrenal glands were extracted and weighed, and one ovary per rat was collected for subsequent analyses. Visual examination of the uterine horn allowed further confirmation of proestrus stage,

with the horns presenting an engorged appearance. Rats were then perfused using 4% paraformaldehyde (PFA) with 20% picric acid. Brains were extracted, incubated in PFA for 1 h, 10% sucrose overnight, 20% sucrose for 24 h, flash-frozen with CO₂, and stored at -80°C.

Subsequently, 14 µm brain sections were obtained using a cryostat (Leica CM1900, Leica Microsystems, Germany) and mounted onto Superfrost Plus Slides (Fisher Scientific, Canada). Areas of interest included the CA1 of the hippocampus (-2.80 to -4.16 mm posterior to Bregma; GnIH and GR expression), the anteroventral periventricular nucleus (AVPV; - 0.26 to -0.40 mm posterior to Bregma; ERα and Kiss1 expression), arcuate nucleus (ARC; -2.12 to -3.60 mm posterior to Bregma; ERα, Kiss1, and GnIH expression), dorsomedial nucleus (DMH; -2.56 to -3.60 mm posterior to Bregma; GnIH expression), and paraventricular nucleus (PVN; -1.30 to -2.12 mm posterior to Bregma; GR expression) of the hypothalamus (Paxinos & Watson, 2006).

2.8 Immunohistofluorescence

To assess ERα, Kiss1, GnIH, and GR-immunoreactive expression, slides were stained as previously described (Poitras, Morin, et al., 2024) using the following antibodies: monoclonal mouse anti-ERα (1 :400, MA1-80216, RRID: AB_930763, Invitrogen Canada Inc.), polyclonal rabbit anti-Kiss1 (1:300, BS-0749R, RRID: AB_10855680, Bioss, USA), rabbit anti-GnIH (PAC123/124; 1:5,000; RRID: AB_2617160, Provided by Dr. G.E. Bentley), or monoclonal mouse anti-GR (1:500, MA1-510, RRID: AB_325427, Invitrogen Canada Inc.), with secondary antibody being anti-mouse donkey (1:1000; A-21202; Invitrogen Canada Inc.) or anti-rabbit donkey (1:1000; A21207, Invitrogen Canada Inc.). For all antibodies, pilot studies determined optimal primary antibody concentrations and confirmed expression of staining to be specific to expected nuclei, and negative controls validated secondary antibody specificity. Furthermore, all antibodies have previously undergone validation using positive and negative controls and

confirmation of antibody specificity to expected structures and regions: ER α (Mahbub et al., 2022), Kiss1 (Muñoz de la Torre et al., 2022; Risvanli et al., 2020), GnIH (Alonge et al., 2023), and GR (Wisłowska-Stanek et al., 2013). Similar immunofluorescent staining patterns have been reported for ER α (Buqué et al., 2025; Kumar et al., 2015), Kiss1 (Higo et al., 2017; Muñoz de la Torre et al., 2022), GnIH (Smith et al., 2008), and GR (Han et al., 2005).

For each rat, 6 anatomically matched photomicrographs per region of interest were captured using a 20 $\times$ Olympus DX51 microscope objective lens (Center Valley, PA, USA) and the ProgRes Capture Pro 2.7.6 software. The fixed threshold algorithms in the ImageJ software (National Institutes of Health, United States) were used to calculate the percentage of area covered by immunoreactive staining. To select the best algorithm for each marker, a minimum of 50 photos were randomly selected for each relevant brain region (selecting images from all experimental groups/stages) and various automatic thresholds were tested to select the algorithm that most consistently and accurately detected staining. The ImageJ algorithm “Moments” was used to scan GnIH throughout the hypothalamus and all GR expression, while the algorithm “Triangle” was used for all other regions/markers. The percentage of area was determined by calculating the surface covered by immunoreactive staining relative to total photomicrograph area. See [Appendix D](#) (Figure D1) for specific areas where photomicrographs were captured.

2.9 Enzyme-Linked Immunosorbent Assays

Abdominal aorta blood samples collected at euthanasia were left to clot overnight at 4°C, after which they were centrifuged at 9400 rcf for 10 min at 20°C. Supernatant serum was collected and stored at -80°C until use to measure LH (LH ELISA kit, ENZ-KIT107, RRID: AB_2848134, Enzo Life Sciences), FSH (Rat Follicle-Stimulating Hormone ELISA Kit, BSKR60303-96T; RRID: AB_3696445, Bioss Inc., Massachusetts, United States), E2 (17 beta

Estradiol ELISA Kit, ADI-900-008, RRID: AB_3677535, Enzo Life Sciences, Inc., New York, United States), and P4 (Rat Progesterone ELISA Kit, ADI-901-011, RRID: AB_3696444, Enzo Life Sciences). For quantification of E2 levels, liquid-liquid extraction was performed as per manufacturer instructions prior to assaying, and spike/recovery trial confirmed good sample recovery following extraction. CORT levels were analyzed in all animals (n = 112) using the collected whole blood drop samples (Corticosterone ELISA Kit, ADI-900-097, RRID: AB_2307314, Enzo Life Sciences). Using a Gem Hole Puncher (McGill Inc., Marengo, IL, United States), blood collected on the Whatman Bloodstain Cards were hole-punched into 12 x 75 mm glass tubes. Assay buffer was added and each tube sealed with parafilm and placed on a Belly Dancer® orbital shaker (Structure Probe Inc., West Chester, PA, United States) for 24 h, as previously described (Milot et al., 2012). The next day, the blood solution was transferred to a new tube and CORT levels measured. Sensitivity of the assays was 27 pg/mL for CORT, 0.21mIU/mL for FSH, 5.2 mIU/mL for LH, 14 pg/ml for E2, and 8.57 pg/ml for P4.

All kits were performed according to the manufacturer instructions, read with Biotek Powerwave XS2, and analyzed with the Gen 5 V2.0 software. A pooled sample was generated and included on each plate, to confirm stability of readouts between plates. All samples were loaded in duplicates, with samples from each experimental group and estrous stage being included on any given plate. Assay consistency was confirmed by calculating inter- and intra-plate coefficients of variability (See [Appendix D](#) – Table D1 for additional details).

2.10 Ovarian Histological Analysis

One ovary per proestrus rat was randomly selected for histological analysis (Zheng et al., 2022). Following extraction, ovaries were incubated in 4% PFA for 72 h, and stored in 70% ethanol until routine paraffin embedding, sectioning at 4 μ m, and hematoxylin eosin (H&E)

staining. For each rat, 5 ovary slices were collected at 100 μm intervals throughout the ovary. Whole ovary sections were visualized using a 20 $\times$ objective on a Zeiss AxioImager M2 microscope, with tiling and stitching performed in Stereo Investigator software (version 2024.1.2, MBF Bioscience-MicroBrightField, LLC) to reconstruct a continuous large-area image. Two independent raters blinded to experimental conditions counted the number of follicles in each of the 5 slices, which were then summed for each subject. The following follicle types were identified using the criteria by Pedersen & Peters (1968): primordial follicles, primary follicles, secondary follicles, early antral follicles, large antral follicles, and corpora lutea. To avoid double counting, corpora lutea were only scored on each rat's first and last slice. Atretic follicles were identified by the presence and degree of pyknosis and loss of granulosa cell attachment, as well as presence of polymorphonuclear neutrophils or lymphocytes, and pyknotic oocytes in primary follicles (Boyer et al., 2010).

2.11 Statistical Analyses

Data was analyzed using IBM© SPSS Statistics 29 (IBM, Armonk, NY, United States). All data were screened for outliers using boxplots. Data points falling beyond the upper or lower fence (1.5 times the difference between 1st and 3rd quartiles) were winsorized to the next most extreme value in that group ± 1 (Khalil et al., 2024; Yang et al., 2024). Shapiro-Wilk test was used to screen for normality. Levene's test assessed homogeneity variance, with Welch F being reported if assumption was violated. Two-way mixed ANOVAs were used to analyze weight gain and days spent in each estrous cycle stage. For repeated-measures variables with more than two levels, Mauchly's test was used to assess sphericity. When this assumption was violated, Greenhouse-Geisser correction was applied. One-way ANOVA with Bonferroni tests served to analyze sexual behaviour data, fertility parameters, and follicle counts. The proportion of rats

displaying normal cycles during each time period was analyzed using Chi square tests. If the expected count in a cell was less than 5, Fisher's exact test was used instead of the Chi square test, followed by pairwise comparisons using multiple Fisher's exact tests (2 x 2) with Bonferroni correction. A Linear Mixed Model was used to analyze CORT levels over time, using Timepoint and Group as fixed factors, Rat ID as a random factor, and estrous cycle stage as a covariate (proestrus vs all other stages; Atkinson and Waddell, 1997). Significant interaction on the test of fixed effects was followed up with appropriate post hoc tests, maintaining estrous stage as a covariate. All biochemical data was analyzed using two-way ANOVAs with experimental group and estrous cycle stage (proestrus vs diestrus) as between-group factors. When a significant interaction was detected, we conducted simple main effects analyses, testing the effect of experimental group separately within each estrous stage (and vice versa). Effect sizes were estimated using partial eta squared (η^2). Significance was set at $p < .05$ for all analyses, and pair-wise comparisons were corrected for multiple testing (Bonferroni or Games-Howell, if homogeneity was violated). Graphs were generated using R (version R-4.4.3) and R studio (version 2024.09.1) and display individual data points.

3. Results

3.1 Weight Gain

Two-way mixed ANOVA with Greenhouse-Geiser correction found a significant interaction for weight gain over time, $F(8.936, 239.031) = 6.558$, $p < .001$, $\eta^2 = .197$ (See [Appendix D](#) – Figure D2). Groups did not differ significantly at arrival or on D0, immediately prior to implementation of experimental housing conditions (both $p > .05$). Interestingly, the RH group gained significantly less weight than all other groups, with no significant change in body weight between D0 and D7 or between D14 and D21 (both $p > .05$), although weight increased

significantly from D7 to 14 ($p = .001$). For all other groups (CH, HDH, LDH, SIS), there was significant weight gain between each timepoint (all $p < .001$). Furthermore, pairwise comparison with Games-Howell correction showed that RH rats had significantly lower body weight than HDH ($p = .017$), LDH ($p = .022$) and SIS ($p = .025$) on Day 7. Lower body weight in RH rats was also observed on Day 14 compared to SIS rats ($p = .034$), and on Day 21 compared to CH ($p = .021$), HDH ($p = .004$), LDH ($p = .039$) and SIS ($p = .027$) rats.

3.2 Estrous Cycle Regularity

All rats displayed regular 4-5 day cycles at baseline, with the majority displaying exclusively 4-day cycles (93.75% of rats). Cycle characteristics were evaluated for the last 11 days of Baseline (Day -10-D0), the first half of the intervention period (S1; Day 1-11), and the second half of the intervention period (S2; Day 12-22). Mean cycle length was calculated for last two cycles at baseline and for the duration of the intervention (D1-21). Since metestrus was not consistently detected in all rats, it was combined with diestrus for analyses.

3.2.1 Days Spent in each Stage

As shown in Figure 2, two-way mixed ANOVA found a significant time $\times$ group interaction for days spent in proestrus, $F(8,214) = 2.498$, $p = .013$, $\eta^2 = .085$. SIS rats had significantly fewer proestrus days than HDH ($p = .002$) during the first half of the stress period. In contrast, during the second half of the stress period (S2; Day 12-22), HDH had significantly fewer days in proestrus compared to CH rats ($p = .042$). Concordant with this, HDH rats had significantly fewer proestrus days during S2 compared to baseline ($p < .001$) and S1 ($p = .011$), whereas SIS rats had significantly fewer proestrus days during S1 compared to baseline ($p = .015$). Of note, RH rats also had significantly more proestrus days at baseline compared to the first half of the intervention period ($p = .022$), while differences were no longer observed in

the second half ($p > .05$). For number of days spent in diestrus, a significant main effect of time was found [$F(1.893,202.38) = 10.086, p < .001, \eta^2 = .086$], relating to significantly fewer diestrus days at baseline compared to S1 ($p < .001$) and S2 ($p < .001$). There were no significant effects for days spent in estrus (See [Appendix D](#) – Figure D2).

Two-way mixed ANOVA with Greenhouse-Geiser correction found a significant main effect of time on mean estrous cycle length [$F(1,107) = 68.285, p < .001, \eta^2 = .390$], with all rats showing greater mean cycle length during the intervention period (D1 to D21) compared to baseline (D -7 to D0; Data not shown).

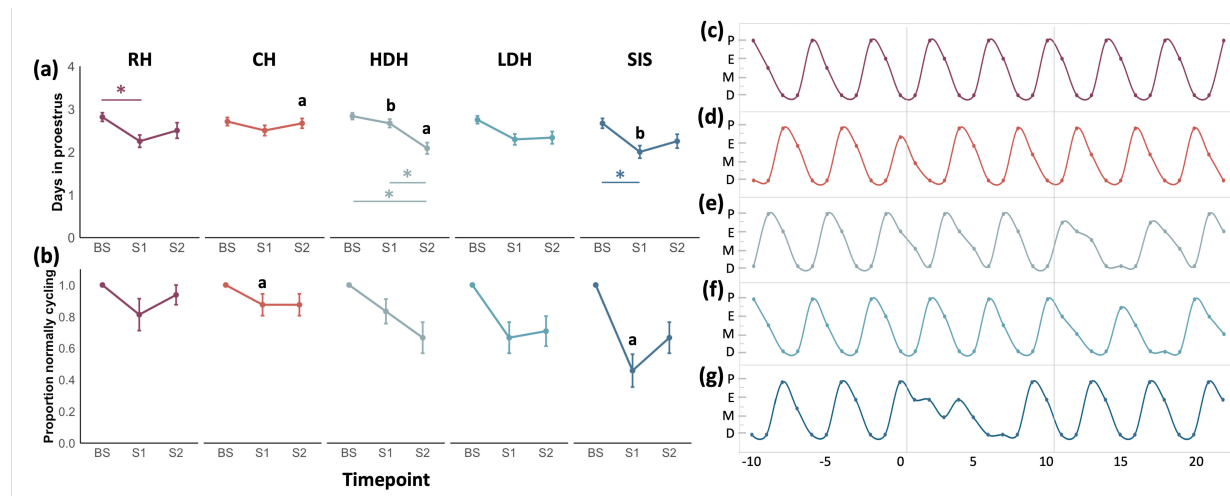


Figure 2. Social Stressors Distinctly Impact Days Spent in Each Estrous Cycle Stage.

Figure shows number of days spent in proestrus (2a) as well proportion of rats normally cycling (2b) during baseline (BS), first half of stress (S1; Days 1-11) and second half of stress (S2; Days 12-22). Two-way mixed ANOVA. Right side of figure presents representative cyclic profiles for RH (2c), CH (2d), HDH (2e), LDH (2f), and SIS (2g) rats. Fisher's exact test. * indicates statistically significant difference between timepoints. Same letters at a given timepoint indicate statistically significant difference between groups. Data presented as mean $\pm$ S.E.M., $p < .05$. P: Proestrus; E: Estrus; M: Metestrus; D: Diestrus.

3.2.2 Estrous Cyclicity

Fisher's exact test found a significant difference between groups during the first half of the intervention, relating to a lower proportion of SIS rats displayed regular cycles compared to CH rats ($p = .005$; See Figure 2). There were no significant group differences in the proportion of

rats regularly cycling at baseline or during the second half of the intervention (both $p > .05$). See Figure 2 for representative estrous cycle profiles. Interestingly, when looking at the proportion of rats having maintained normal cycling for the entire intervention period, 75% of rats in the RH and CH groups maintained regular cycling, whereas 54.16% of rats in the HDH and LDH rats cycled normally throughout, and only 33.33% of SIS rats showed no estrous cycle disruptions.

3.3 Sexual Behaviours

In total, three rats (one from CH, HDH, and LDH) were excluded from sexual behaviour and fertility analyses due to displaying no paracopulatory behaviours and no signs of receptivity during testing. Technical difficulties with the recording equipment prevented analyzing two videos (one HDH and one LDH), but rats were kept for fertility analyses. As shown in Figure 3, one-way ANOVA found a significant effect of stress on the number of hopping behaviours performed [Welch's $F(4,12.438) = 8.758, p = .001, \eta^2 = .488$], with LDH and HDH performing more hops than RH rats ($p = .018$ and $p = .045$, respectively), and LDH performing more hops than the CH ($p = .023$) rats. Notably, time spent away from the male differed significantly between groups [$F(4,12.088) = 6.348, p = .005, \eta^2 = .265$], with LDH rats spending less time away compared to CH rats ($p = .003$). There were no statistically significant differences for any of the lordosis parameters, nor for any of the other behaviours measured (all $p > .05$).

Importantly, the latency to first male behaviour and the total number of sexual stimulations received from the males did not differ between groups (both $p > .05$; Data not shown).

Analysis of blood samples collected immediately following paced-mating testing were compared to samples collected at S21 (as an indicator of basal levels). Two-way mixed ANOVA revealed a significant interaction, $F(4,27) = 4.661, p = .005, \eta^2 = .408$. While groups did not differ significantly in their basal CORT levels on stress Day 21, SIS rats presented significantly

lower CORT levels than RH ($p = .007$) and CH ($p = .042$), while HDH rats had significantly lower CORT than RH ($p = .001$), CH ($p = .005$), and LDH ($p = .019$). Concordantly, a significant CORT increase was noted following sexual behaviour compared to S21 in the RH ($p = .022$), CH ($p = .005$), and LDH ($p = .018$) groups, but not in the SIS or HDH groups (both $p > .05$).

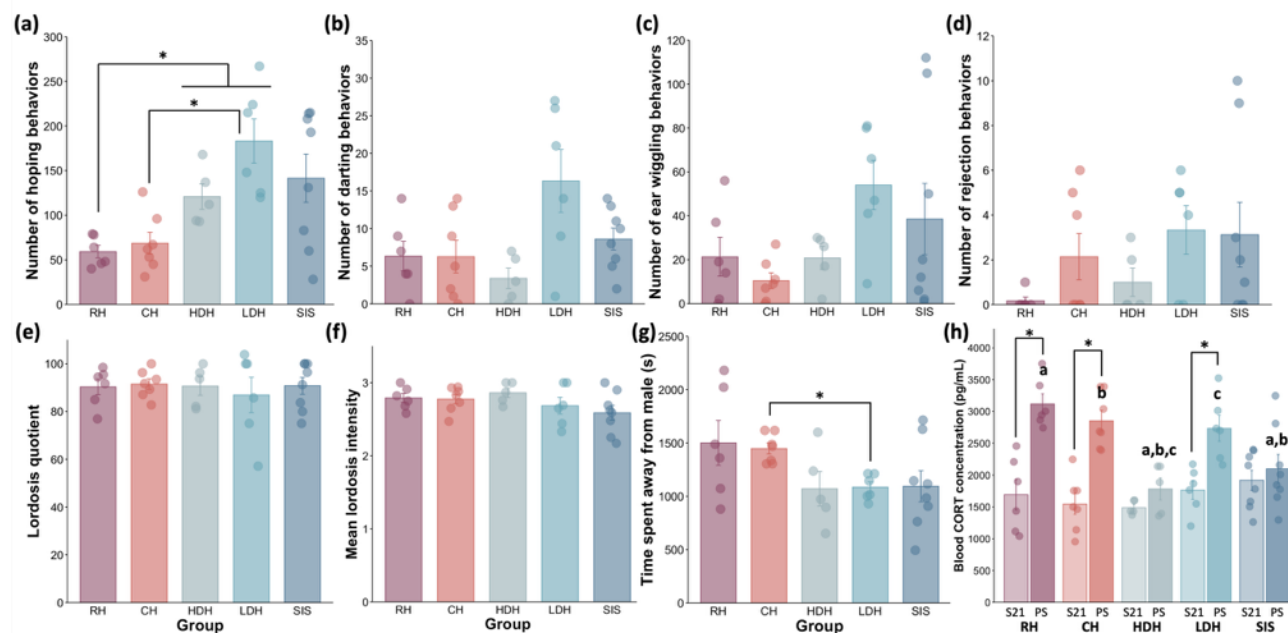


Figure 3. Impact of Social Stress on Sexual Behaviours.

Top half of the figure shows behavioural frequencies for each paracopulatory behaviour measured (**3a–3c**) as well as the number of rejection behaviours (**3d**). Bottom panel shows lordosis quotient (**3e**) and intensity (**3f**), and the total time spent away from the male (**3g**). CORT levels on Stress Day 21 (S21) compared to the blood sample collected immediately post-sexual behaviours (PS) are also shown (**3h**). Group sizes for analyses were as follows: RH = 6, CH = 7, HDH = 5, LDH = 6, SIS = 8. One-way ANOVA (behaviour) or two-way mixed ANOVA (CORT). Data presented as mean $\pm$ S.E.M. Same letters at a given timepoint indicate significant difference between groups, * indicates statistical significance, $p < .05$.

3.4 Fertility Rates and Embryo Counts

All successfully mated rats were pregnant at euthanasia. There were no significant differences for any of the fertility parameters measured (all $p > .05$; See Figure 4 and [Appendix D](#) – Figure D3). Interestingly however, yolk sacs from HDH rats consistently presented a more rounded appearance than those from the SIS group, which were more oblong.

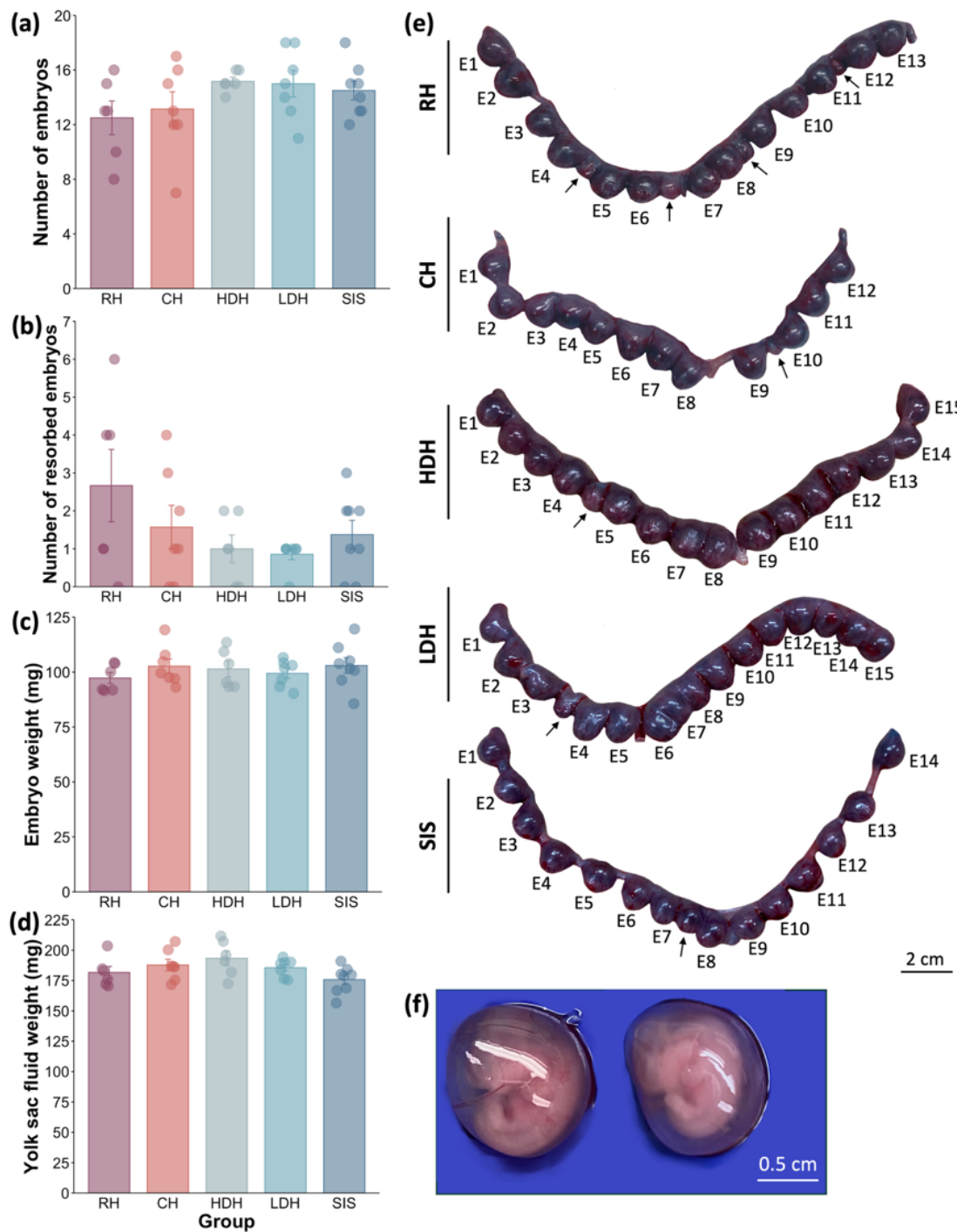


Figure 4. Social Stress Prior to Copulation Has Minimal Impact on Fertility Parameters.

Left side of figure shows the number of embryos (4a), number of resorbed embryos (4b), mean embryo weight (4c), and mean fluid yolk sac weight (4d). Top right (4e) shows representative photographs of a uterine horn for each group, with all embryos numbered and arrows indicating resorbed embryos. Bottom right (4f) shows representative photograph of embryo in yolk sac of an HDH dam (left) and an SIS dam (right). Note the more rounded appearance of the yolk sac from the HDH dam. One-way ANOVA. Data presented as mean $\pm$ S.E.M.

3.5 Hypothalamic Expression of HPG Axis Markers

3.5.1 *ERα-ir*

As shown in Figure 5, two-way ANOVA found a significant main effect of group for $ER\alpha$ expression in the ARC [$F(4,48) = 3.030, p = .026, \eta^2 = .202$]. Pairwise comparisons revealed significantly higher $ER\alpha$ expression for SIS rats compared to LDH rats ($p = .043$). In the AVPV, a significant main effect of group [$F(4,48) = 4.214, p = .005, \eta^2 = .260$], related to significantly elevated AVPV $ER\alpha$ -ir in CH compared to HDH rats ($p = .030$). No other group differences were noted, and estrous stage showed no impact on ir-expression at these two sites (all $p > .05$). For photomicrographs of other brain regions, see [Appendix D](#) – Figure D4.

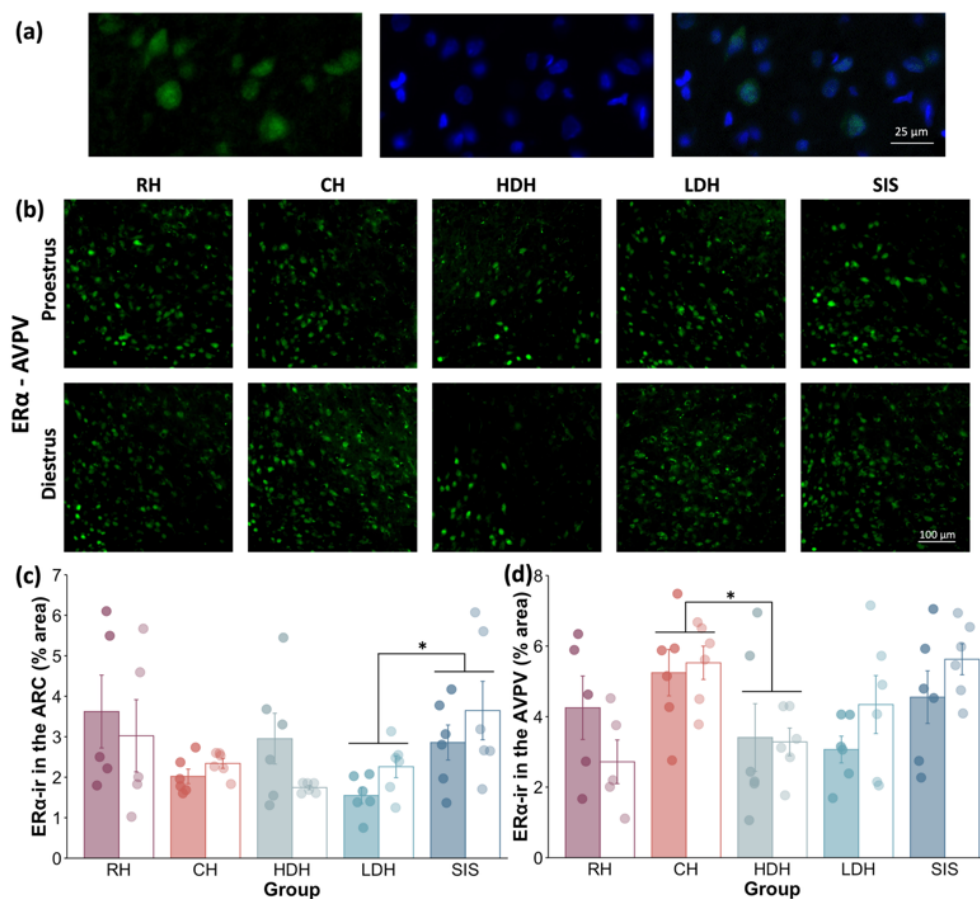


Figure 5. $ER\alpha$ -ir Following Exposure to Social Stressors.

From left to right, top panel (**5a**) of figure shows high magnification photomicrographs of $ER\alpha$ -ir expression, Hoechst staining, and the overlay of the two in the AVPV of a proestrus RH rat. Panel **5b**

shows representative photomicrographs of ER α -ir in the AVPV of proestrus (top row) and diestrus (bottom row) in each experimental group. Bottom half of figure shows mean ER α -ir in the ARC (**5c**) and the AVPV (**5d**). **Proestrus** = filled bars, **Diestrus** = white bars. $n = 6$ per stage for each experimental group, except RH ($n = 5$ per stage). Two-way ANOVA. Data presented as mean $\pm$ S.E.M. * indicates statistical significance, $p < .05$.

3.5.2 *Kiss1-ir*

Two-way ANOVAs assessing Kiss1 percentage of area in the AVPV and in the ARC did not reach statistical significance [respectively $F(4,48) = 2.541$, $p = .052$, $\eta^2 = .175$ and $F(4,48) = 2.366$, $p = .066$, $\eta^2 = .165$]. See Figure 6 and, for photomicrographs of other brain regions, see [Appendix D](#) – Figure D4.

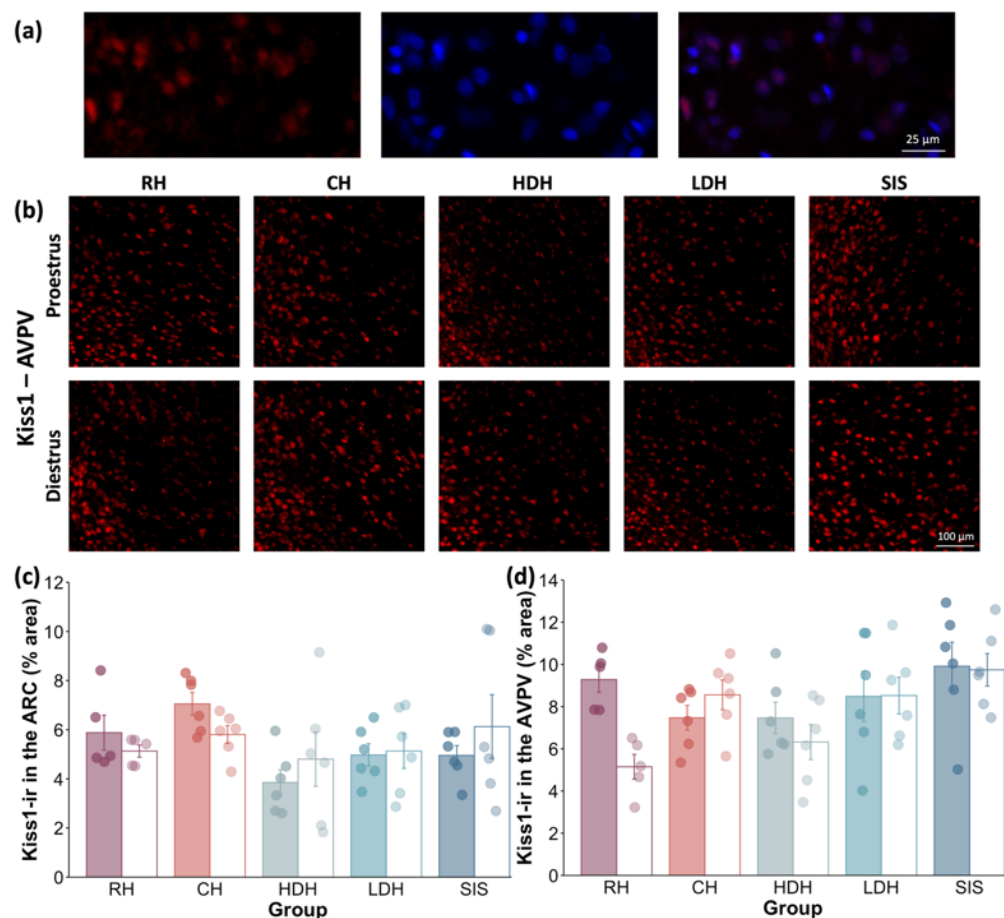


Figure 6. Kiss1-ir Following Exposure to Social Stressors.

From left to right, top panel (**6a**) shows high magnification photomicrographs of Kiss1-ir expression, Hoechst staining, and the overlay of the two in the AVPV of a proestrus RH rat. Panel **6b** shows

representative photomicrographs of Kiss1-ir in the AVPV of proestrus (top row) and diestrus (bottom row) in each experimental group. Bottom half shows mean Kiss1-ir in the ARC (**6c**) and the AVPV (**6d**). **Proestrus** = filled bars, **Diestrus** = white bars. $n = 6/\text{stage}$ for all groups, except RH ($n = 5/\text{stage}$). Two-way ANOVA. Data presented as mean $\pm$ S.E.M. Same letters indicate statistically significant difference between groups at a given stage. * indicates statistical significance, $p < .05$.

3.5.3 GnIH-ir

Two-way ANOVA of DMH-GnIH-ir indicated a significant main effect of group [$F(4,48) = 5.026, p = .002, \eta^2 = .295$], attributable to significantly lower expression in HDH compared to RH ($p = .007$), CH ($p = .043$), and SIS ($p = .003$) rats (See Figure 7). In the ARC, a significant main effect of group [$F(4,48) = 6.782, p < .001, \eta^2 = .361$] was related to significantly higher ARC GnIH levels in RH and CH compared to HDH (both $p = .002$) and LDH ($p = .022$ and $p = .030$, respectively) rats. In the CA1, there was a significant main effect of group [$F(4,48) = 4.407, p = .004, \eta^2 = .269$] and of stage [$F(4,48) = 4.202, p = .046, \eta^2 = .080$]. HDH rats showed significantly reduced CA1 GnIH-ir compared to CH ($p = .007$) and SIS ($p = .008$) rats. The main effect of estrous stage related to significantly higher CA1 GnIH-ir in diestrus compared to proestrus ($p = .046$). There were no other significant differences (all $p > .05$). For photomicrographs of other brain regions, see [Appendix D](#) – Figure D5.

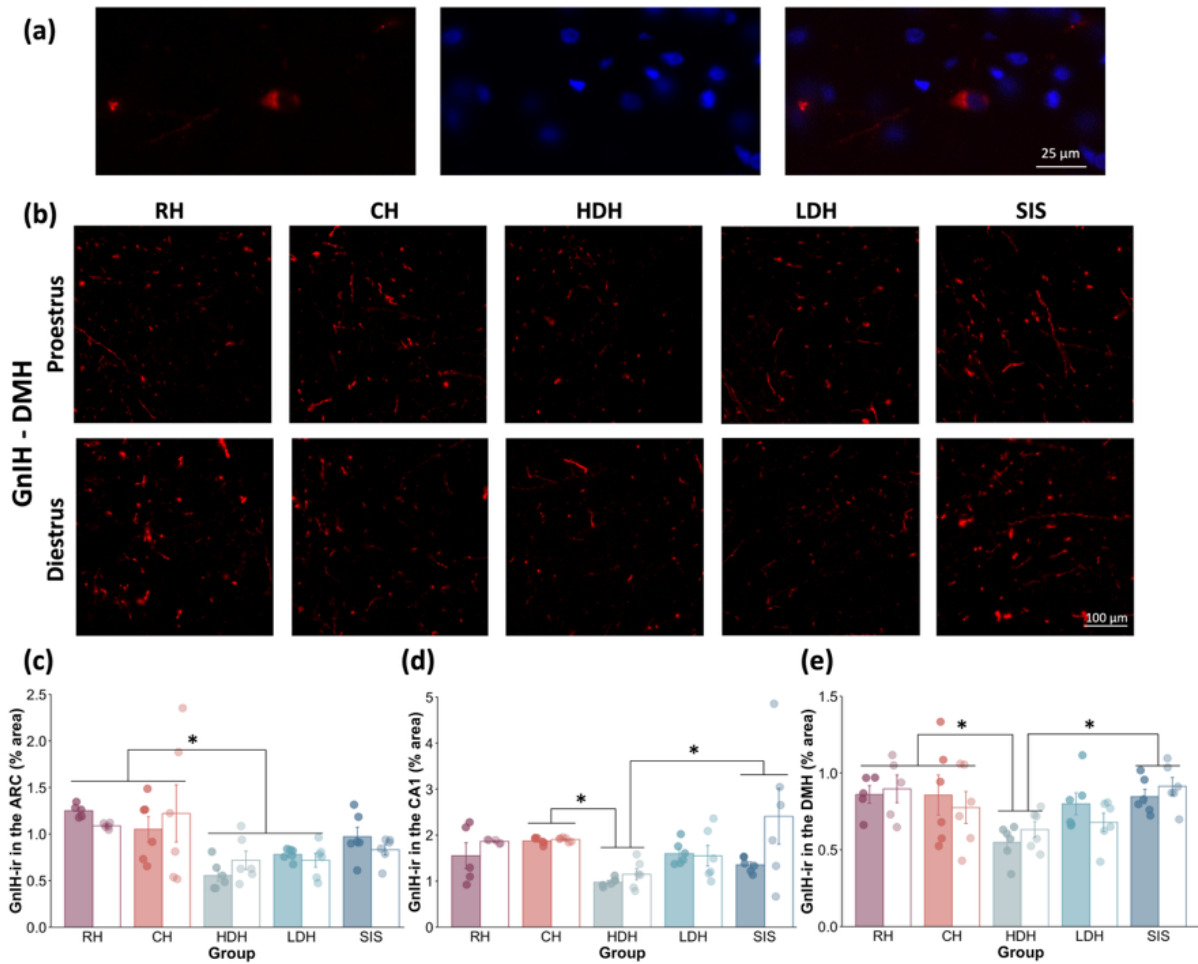


Figure 7. GnIH-ir Following Exposure to Social Stressors.

From left to right, top panel (7a) shows high magnification photomicrographs of GnIH-ir expression, Hoechst staining, and the overlay of the two in the DMH of a proestrous RH rat. Panel 7b shows representative photomicrographs of GnIH-ir in the DMH of proestrous (top row) and diestrus (bottom row) for each experimental group. Bottom half shows mean GnIH-ir in the ARC (7c), the CA1 of the hippocampus (7d), and the DMH (7e). **Proestrus** = filled bars, **Diestrus** = white bars. $n = 6$ rats per stage for each experimental group, except RH ($n = 5$ /stage). Two-way ANOVA. Data presented as mean $\pm$ S.E.M. * indicates statistical significance, $p < .05$.

3.6 Pituitary and Gonadal Hormone Levels

As shown in Figure 8, FSH levels were significantly altered by stress [$F(1,46) = 7.587$, $p < .001$, $\eta_p^2 = .398$], relating to significantly higher FSH levels in LDH compared to RH ($p <$

.001) and SIS ($p < .001$) groups. No significant differences between groups or estrous cycle stages were detected in serum LH concentrations ($p > .05$).

Relating to gonadal hormones, two-way ANOVA showed a significant main effect of stage for E2 levels [$F(1,48) = 4.460$, $p < .001$, $\eta_p^2 = .409$; See Figure 8], with proestrus rats having higher E2 than diestrus counterparts. Stress showed no significant effect on E2, and there were no significant differences in P4 levels (all $p > .05$).

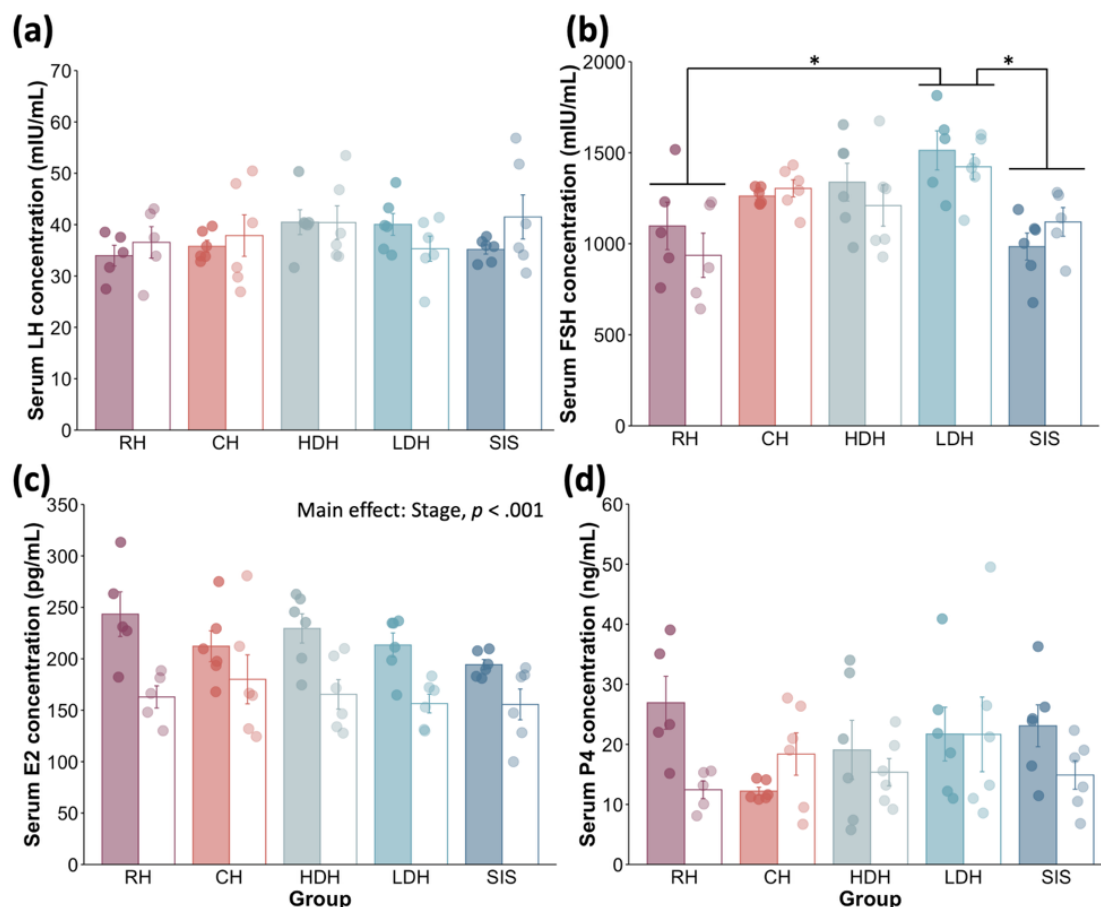


Figure 8. Pituitary and Gonadal Hormone Levels Following Exposure to Social Stress.

Top row shows serum concentration of luteinizing hormone (LH; **8a**) and follicle-stimulating hormone (FSH; **8b**), while bottom row shows serum concentrations of estradiol (E2; **8c**) and progesterone (P4; **8d**). **Proestrus** = filled bars, **Diestrus** = white bars. $n = 6$ per stage for each experimental group, except RH ($n = 5$ per stage). Two rats were excluded from FSH analyses due to insufficient quantity of serum (one SIS, one LDH). Two-way ANOVA. Data presented as mean $\pm$ S.E.M. * indicates statistical significance, $p < .05$.

3.7 Uterine Weight and Ovarian Histology

As shown in Figure 9, two-way ANOVA found a significant main effect of stress on uterine horn weight [$F(4,48) = 3.866, p = 0.008, \eta^2 = .244$]. Post hoc analyses showed higher uterine horn weights in the RH compared to the LDH ($p = .031$) and the SIS ($p = .009$) groups. As expected, there was a main effect of estrous cycle stage [$F(1,50) = 44.835, p < .001, \eta^2 = .483$], with uterine horns being heavier during proestrus than diestrus ($p < .001$).

Relating to ovarian follicular counts, one-way ANOVA found a significant effect of group for the number of primordial follicles [Welch's $F(4,10.857) = 12.173, p < .001, \eta^2 = .413$], primary follicles [$F(4,24) = 4.918, p = .005, \eta^2 = .450$], secondary follicles [$F(4,24) = 4.002, p = .013, \eta^2 = .400$], and early antral follicles [Welch's $F(4,11.358) = 5.447, p = .011, \eta^2 = .069$]. Post hoc analyses revealed significantly fewer primordial follicles in SIS than RH ($p = .004$) and HDH ($p = .041$) rats. Similarly, primary follicle counts were significantly reduced in LDH and SIS compared to RH rats (respectively $p = .015$ and $p = .008$). Finally, early antral follicles were fewer in LDH compared to RH group ($p = .005$). No other significant differences were detected (all $p > .05$; See Figure 9).

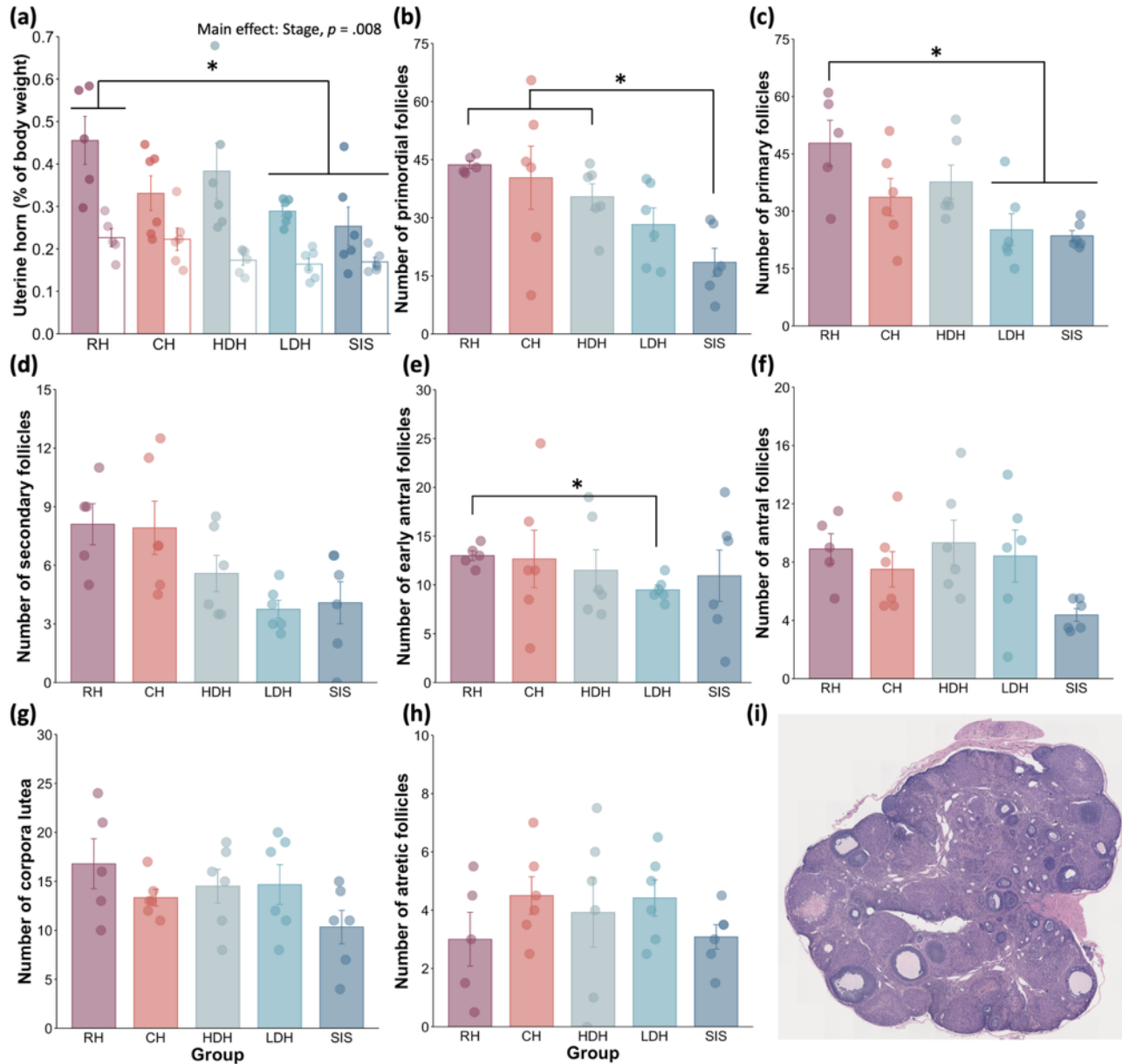


Figure 9. Ovarian Follicular Counts and Uterine Weight Following Social Stress.

Uterine horn weights adjusted to total body weight are shown for proestrus (filled bars) vs diestrus (white bars; **9a**). Number of follicles in the five sections assessed for each rat were summed and mean follicle count is shown for primordial (**9b**), primary (**9c**), secondary (**9d**), early antral (**9e**), and antral (**9f**) follicles, as well as for corpora lutea (**9g**) and atretic follicles (**9h**). Representative photomicrograph of ovarian section from an RH rat is shown (**9i**). $n = 6$ per stage for each experimental group, except RH ($n = 5/\text{stage}$); only proestrus rats were included for ovarian follicle counts. Two-way ANOVA (uterine horn) or one-way ANOVA (follicle counts). Data presented as mean $\pm$ S.E.M. * indicates statistical significance, $p < .05$.

3.8 Glucocorticoid Receptors, Corticosterone Levels, and Adrenal Gland Weights

As shown in Figure 10, two-way ANOVA for GR-ir in the PVN revealed a significant main effect of group [$F(4,48) = 3.338, p = .017, \eta^2 = .218$], due to significantly higher GR expression in LDH compared to SIS rats ($p = .013$). In the CA1, there was a significant group $\times$ stage interaction, $F(4,48) = 2.551, p = .048, \eta^2 = .178$. GR-ir was significantly lower during proestrus than diestrus in RH rats ($p = .004$). See [Appendix D](#) (Figure D5) for photomicrographs of other regions.

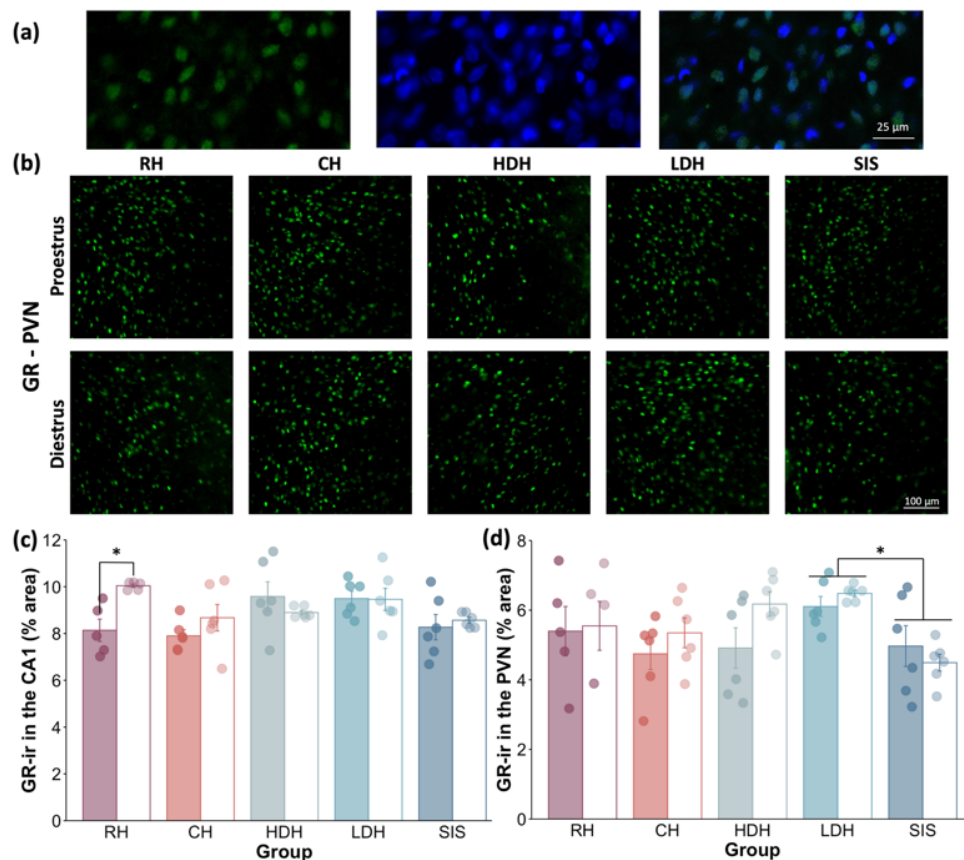


Figure 10. GR-ir Following Exposure to Social Stressors.

From left to right, top panel (10a) shows high magnification photomicrographs of GR-ir expression (green) and Hoechst staining (blue) in the PVN of a proestrus RH rat. Panel 10b shows representative photomicrographs of GR-ir in the PVN for proestrus (top row) and diestrus (bottom row) rats in each experimental group. Bottom half of figure shows mean GR-ir in the CA1 of the hippocampus (10c) and the PVN (10d). **Proestrus** = filled bars, **Diestrus** = white bars. $n = 6$ per stage for each experimental group, except RH ($n = 5$ per stage). Two-way ANOVA. Data presented as mean $\pm$ S.E.M. * indicates statistical significance, $p < .05$.

As shown in Figure 11, when adjusting for estrous cycle stage at each timepoint, linear mixed model analysis of CORT levels revealed a significant group $\times$ time interaction, $F(16,428.014) = 2.373, p = .002$. Over the course of the study, RH rats maintained stable CORT levels, while CH rats showed significantly higher CORT on S21 compared to S3 ($p = .037$). HDH rats had significantly lower CORT at BS2 compared to S3 ($p = .004$). In SIS rats, CORT was significantly elevated at S21 compared to BS1 ($p < .001$), BS2 ($p = .005$), S3 ($p = .002$), and S14 ($p < .001$). Analysis also revealed no significant group differences in baseline measures (BS1 and BS2, both $p > .05$). On S14, SIS rats had significantly reduced CORT than RH ($p = .024$), CH ($p = .003$), HDH ($p = .006$), and LDH ($p = .048$). There were no significant differences in adrenal gland weights ($p > .05$; See [Appendix D](#) – Figure D6).

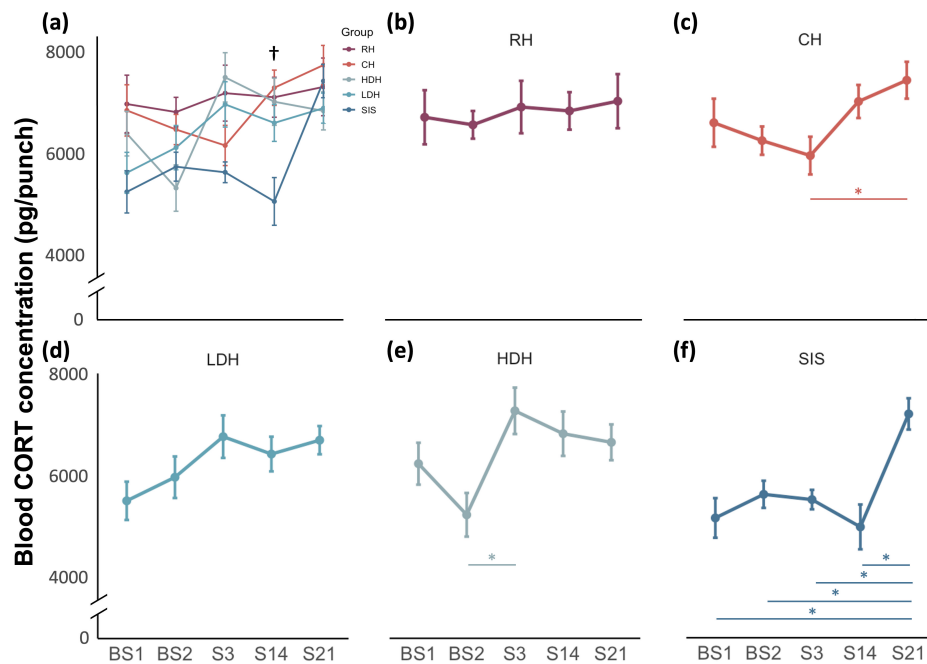


Figure 11. Blood Corticosterone Levels During Social Stress.

Figure shows whole blood CORT levels at baseline 1 (BS1), baseline 2 (BS2), and on stress day 3 (S3), day 14 (S14), and day 21 (S21) for all experimental groups (**11a**). To allow better visualization of within group changes over time, single panels present CORT levels for RH (**11b**), CH (**11c**), LDH (**11d**), HDH (**11e**), and SIS (**11f**) groups separately. * indicates statistically significant difference between timepoints. † indicates significantly higher levels than the SIS group at that timepoint. Linear mixed model. Data presented as mean $\pm$ S.E.M., $p < .05$.

4. Discussion

To our knowledge, this study is the first to include a multifaceted assessment of HPG axis function, namely multiple behavioural and physiological outcomes, in female rats exposed to three types of repeated social stress. Our study revealed these stressors to induce distinct impairment profiles, with restricted disruptions detected at all levels of the HPG axis and its outputs. Importantly, we observed 21-days of LDH to alter hopping behaviours and sexual motivation. LDH also negatively impacted reproductive organs by reducing uterine horn weight and ovarian follicle counts, changes accompanied by decreased GnIH-ir in the ARC and increased FSH levels. On the other hand, HDH in stable groups led to some reductions in days spent in proestrus and the expression of several hypothalamic markers, namely ER α and GnIH, while increasing hopping behaviours. Interestingly, although SIS reduced estrous cycling and decreased the ovarian reserve and uterine horn weights, it failed to significantly alter cerebral HPG axis markers expression compared to controls (See Table 1 for summary of results). Aside minor changes to estrous cyclicity and increasing CORT over time in control groups (RH and CH rats, respectively), there were no significant differences between CH and RH for any of the reproductive variables measured. As such, the two groups are referred to as controls for the ease of discussion. Together, our findings show unexpected impacts of social stress in females, exhibiting distinct alteration profiles between models, as well as key differences from previous literature utilizing physical and psychological stressors.

Table 1. Summary of Results

Outcome Measure	Isolation	Crowding	Instability
Behavioural and Physiological			
Estrous cyclicity	=	↓	↓
Sexual behaviours			
<i>Hopping behaviours</i>	↑	↑	=
<i>Lordosis parameters</i>	=	=	=
<i>Sexual motivation</i>	↑	=	=
Fertility	=	=	=
Cerebral			
GnIH (RFRP-3)			
<i>ARC</i>	↓	↓	=
<i>DMH</i>	=	↓	=
<i>CAI</i>	=	↓	=
Kiss1			
<i>ARC</i>	=	=	=
<i>AVPV</i>	=	=	=
ERα			
<i>ARC</i>	=	=	=
<i>AVPV</i>	=	↓	=
Pituitary			
LH	=	=	=
FSH	↑	=	=
Ovaries and Uterine Horn			
E2	=	=	=
P4	=	=	=
Ovarian follicular count	↓	=	↓
Uterine weight	↓	=	↓

Note. Arrows indicate significantly higher or lower levels than controls (control housed or regular housed groups) at $p < .05$.

4.1 Social Isolation Alters Sexual Functioning and Impairs Early Stage Folliculogenesis

Contrasting previous research reporting chronic physical and psychological stress to decrease paracopulatory behaviours and sexual functioning (Donadio et al., 2007; Retana-Márquez et al., 2020), this study observed increased hopping behaviours in naturally cycling female rats exposed to LDH or HDH. Interestingly, despite elevated CORT following paced-

mating, LDH rats spent more time in the male compartment, which could suggest an increased drive for social interactions despite a perceived aversive/stressful component. This observation is consistent with studies showing longer social contact, paralleled by decreased risk assessments, in female rats exposed to chronic isolation (Shirenova et al., 2023). Of interest, research in naked mole-rats has found ARC GnIH-ir to be increased in reproductively quiescent colony members (Peragine et al., 2017), and chronic administration of GnIH to Syrian hamsters reduced sexual motivation, without impacting lordosis (Piekarski et al., 2013). Therefore, significant decrease in ARC GnIH-ir noted in LDH compared to controls could indicate an upstream GnIH-mediated HPG axis disinhibition, which could contribute to hypersexual responses following social stress. In fact, GnIH receptor (NPFF1R) knockout in mice has been associated with significant increases to FSH levels in adulthood (León et al., 2014), suggesting possible downstream effects of reduced GnIH. Our data indeed support increased FSH concentrations in LDH females compared to RH controls. Concordantly, isolation impacted reproductive organs, significantly reducing both uterine horn weight and early ovarian follicular counts (primary and early antral) compared to control groups. If persistent, the LDH-associated increase in FSH concentrations could lead to internalization of FSH receptors needed for proper follicular maturation. Alternately, such increase could represent a compensatory mechanism to counteract reduced early follicle counts, allowing to recover normal follicular growth at later stages, although further research is required. Seeing as reproductive senescence has been reported to occur earlier in isolated rats than group housed rats (LeFevre & McClintock, 1991), our findings provide insights into potential early effects of adulthood social isolation which may contribute to advancing reproductive senescence later in life.

4.2 Crowding Alters Hypothalamic Signalling and Reduces Days in Proestrus

The current study found several consequences of HDH on reproductive functioning and HPA axis activation in female rats. Our findings show HDH rats to display increased hopping behaviours, as also observed in LDH rats. Interestingly, analyses of circulatory CORT levels suggest differing motivational underpinnings to these behavioural changes under different social stress conditions. Indeed, contrary to LDH and controls, paced mating in HDH and SIS rats failed to alter post-mating CORT secretion, potentially being indicative of a blunted HPA axis response. Interestingly, both crowding and isolation in female rats have been suggested to blunt the HPA axis response to restraint, with only controls showing CORT increases relative to baseline (Viveros et al., 1988). In this study, only rats exposed to crowding (either through HDH or SIS) presented blunted post-mating CORT responses. An alternative explanation is considered, wherein high incidence of social interactions might confer protection against other types of potentially stressful social encounters, consistent with beneficial effects of social buffering on the stress response (Beery & Kaufer, 2015). Therefore, one could presume that HDH rats may be less hesitant to seek out newly introduced male counterparts. In this study, the use of sexually naïve females could have accentuated such effects, as longer latencies to interact with the male are noted in naïve rats compared to experienced counterparts (Arnold et al., 2019). Notably, studies have indicated previous sexual experience to confer protection from restraint stress in females (Arnold et al., 2019). Our findings suggest that the reverse could also be true, with previous exposure to socially dense housing reducing the stress experienced upon first sexual encounter in sexually naïve females. Future studies should consider the inclusion of sexually experienced females to evaluate the contribution of sexual experience to this response. Additionally, inclusion of measures typically associated with sociability, such as oxytocin, which

tightly interplays with the HPA and HPG axes (Hiura & Ophir, 2018; Rao et al., 2011), would be of particular interest to further discern interplay between social stress and alterations to sociability in females.

Contrary to hypotheses, GnIH-ir was reduced in all assessed regions for HDH rats compared to controls, contrasting physical and psychological stress models which typically increase GnIH expression (Iwasa et al., 2018; Kirby et al., 2009), resulting in downstream inhibition of HPG axis markers. Interestingly, previous reports have suggested that crowding in female rats could exert a calming effect compared to isolation, as indicated by lower CORT levels in rats exposed to the former (Brown & Grunberg, 1995), which could provide insight into the reduced GnIH levels in HDH rats. Concordant with beneficial effects of social buffering (Ishii et al., 2016), and typically low levels of aggressivity in females (Oliveira & Bakker, 2022) it is possible that crowding in stable groups might not increase HPA axis activation through manipulations to the social environment. Rather, the elevation in CORT levels immediately following initiation of the housing protocol (Day 3) might represent generally increased levels of excitement or elevated metabolic demands of highly dense housing (Djordjevic et al., 2005; Robinson, 1975). Concordant with such contention, informal observations during daily handling and sampling performed for this study found rats in the HDH group to be highly curious and social toward handlers (personal observations), suggesting potentially beneficial effects of crowding on sociability. Nevertheless, it is important to note that long-term reductions of GnIH may ultimately have detrimental effects, considering that impaired negative feedback within the HPG axis contributes to development of premature ovarian failure and hypergonadotropic hypogonadism (Koysombat et al., 2023). In fact, a possible dissociation was observed between GnIH and ER α signalling, as we found HDH exposure to reduce ER α -ir in the AVPV, in spite of

inhibited GnIH signalling throughout the hypothalamus. Reduced stimulation within the AVPV, through inhibited ER α -ir expression, could dysregulate HPG axis activation in HDH rats and contribute to altering estrous cyclicity. Seeing as HDH showed minimal impact at the pituitary (FSH and LH) and ovarian (E2 and P4) levels, coincidental reduction in stimulatory ER α and inhibitory GnIH signalling responses might partially counteract one another. Further research is required to clarify such interplay.

4.3 Social Instability Decreases Estrous Cycling and Reduces the Ovarian Reserve

Contrary to expectations, the added component of ever-changing group compositions characterizing SIS exposure failed to markedly increase effects on reproductive function compared to those observed in the other groups. However, disruption of estrous cyclicity was most prominent in this group, through an immediate decrease in estrous cyclicity and proestrus days. Of note, SIS did not significantly affect days spent in other stages, differing from other psychological and social stress studies which commonly report lengthening of diestrus or estrus (For review, see; Poitras, Lebeau & Plamondon, 2024). Reported effects of studies using SIS have been mixed, some finding no impact on estrous cycling (Nowacka-Chmielewska et al., 2017) and others observing reduced cyclicity, without providing specifics of the disruptions (Herzog et al., 2009). Interestingly, CORT levels in this study were not reliable indicators of cycle disruptions, as within group increases to CORT did not coincide with cycle alterations. This suggests that in social stress, CORT might not contribute to immediate effects on estrous cyclicity. Consistent with this proposition, a study assessing chronic social defeat in Long Evans rats reported increased non-reproductive estrous cycle days, despite an absence of changes in CORT secretion (Rappeneau et al., 2016). The lack of notable changes to GR expression in all stress groups further supports the contention that while sufficient to induce alterations to

reproductive function and within-group increases to CORT over time, the selected social stressors were relatively mild in comparison to other physical and psychological stress models.

At the hypothalamic levels, SIS had surprisingly minimal impacts. Indeed, the only detected differences were with the LDH group, making interpretation difficult. Furthermore, social stress preceding copulation had minimal impacts on fertility. Interestingly, more oblong yolk sac shapes were generally noted in SIS compared to HDH rats, which is consistent with reduced uterine horn weights post stress and could support a poorer intra-uterine environment. In our study, social stressors applied prior to gestation had no significant impact on fertility outcomes, possibly being too mild (Gotlieb et al., 2022). Nonetheless, the observed reduction in primordial follicles could have implications for the ovarian reserve or affect reproductive senescence, concordant with effects that were reported following isolated housing (LeFevre & McClintock, 1991). Considering that HDH had no detectable impact on ovarian function, it seems reasonable to propose that the isolation component of the SIS procedure contributed to the noted negative effects on early stages of folliculogenesis.

4.4 Contributions of Experimental Manipulations to the Stress Response

Previous research has highlighted a stressful influence of vaginal lavages, with 14 days of sampling in rats leading to increased CORT (Becegato et al., 2021). In this study, the absence of changes from BS1 to BS2 suggests that repeated daily lavages were not in-and-of-themselves sufficient stressors to significantly raise basal CORT levels in adult rats. This finding is further supported by stable CORT levels in the RH group during the whole intervention period. However, CH rats displayed higher CORT at the end of the paradigm compared to S3. This supports daily cage changes to have contributed to the overall stress induced by chronic crowding and instability models, beyond the social component of these models. Nevertheless,

CH rats did not differ from RH on any of the reproductive variables measured, and as such, the consequences of SIS and HDH are unlikely to stem solely (or even mostly) from cage change frequency. Importantly, our study confirms that exposure of sexually naïve females to paced-mating increases CORT levels in controls and LDH rats, increases which were blunted (or prevented) by HDH and SIS. Such post-mating increases are consistent with observations made in females of other species (e.g., quail), which also highlighted the complexity of female sexual responses (Dickens et al., 2012).

4.5 Limitations and Future Considerations

The absence of stage differences for some biochemical markers could be related to time at which sample collection occurred during the day, or the stages selected for analysis. Indeed, estrous-stage dependent changes in ER α expression in norepinephrine neurons of the nucleus tractus solitarii were found in the morning of proestrus relative to diestrus, but not during the afternoon of proestrus (Haywood et al., 1999). Additionally, significant differences have been reported in the proportion of occupied ER α 's between the morning of proestrus and morning of estrus, but not between proestrus and diestrus (Bowlby et al., 2015). The selected collection timepoints and stages might have resulted in missing critical hormone surges during proestrus, and related effects on hypothalamic-driven marker expression, and reducing the possibilities of detecting significant group $\times$ stage interactions. Future projects should consider multiple biochemical sample collection timepoints on the day of proestrus and the inclusion of rats in estrus, to better capture time sensitive changes in the hormones and peptides of interest. Similarly, evaluations of gonadal and pituitary hormones throughout the stress period would allow a greater understanding of possible time-dependent consequences of these stressors and should be considered in future research. Of note, the location of immunofluorescent expression

of Kiss1 on the cells includes both cytoplasmic and nuclear staining patterns. Such pattern in this study coincides with dense immunoreactive expression, possibly relating to a slightly elevated antibody concentration, preventing clear visualisation of cytoplasmic staining with an empty nucleus. Nevertheless, the detected staining was region specific (clear clusters of Kiss1 cells were observed in the AVPV and ARC, as expected), and obtained using an antibody shown to be successful when used by other research groups (Muñoz de la Torre et al., 2022; Risvanli et al., 2020). It is possible that such staining pattern contributed to failure in detecting significant group differences, and future assessments of Kiss1 are needed in the context of social stress to confirm its ability to impact this peptide.

The inclusion of additional HPA axis variables, such as corticotropin releasing hormone (CRH) or adrenocorticotrophic hormone (ACTH) are critical to gain a greater understanding of studies manipulating the social environment, especially in females who might respond more pro-socially and consequently present ‘atypical’ responses compared to those reported in males. Direct evaluations of HPA axis reactivity, through the use of an acute stressor, would further contribute to clarifying effects of these social stressors. It is noteworthy that in this study, immunoreactivity was quantified by measuring the total surface area covered by immunoreactive staining. While this provides information on expression levels, it does not inform on the number of stain-positive cells, which could be a variable of interest in future investigations on this topic. Similarly, measurements provided by immunohistochemical analyses are static, representing a single point in time. The use of real-time monitoring of neuroendocrine secretion using microdialysis would allow greater insight into peptide secretion or accumulation which may occur in response to chronic stress.

5. Conclusion

This study is the first to our knowledge to have comprehensively compared HPG axis function in adult female rats upon exposure to three types of chronic social stress. Our findings show that physiological, behavioural, and endocrine alterations vary between social stressors, and that effects are overall less consistent than impacts from physical stressors. This study underscores the complexity of stress adaptation mechanisms and highlights the limitations of applying research done in males, or using physical stressors, to females exposed to social stress. With the prevalence of anxiety increasing in adult women of reproductive age (Goodwin et al., 2020), a greater understanding of stress-induced reproductive impairment is crucial. Such research has gained further relevance in the context of social disruptions from the COVID-19 pandemic and increasing global population pressures.

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7. CRediT Authorship Contribution Statement

M.P.: Conceptualization, Methodology, Project Administration, Investigation, Formal Analysis, Data curation, Writing – Original draft. A.L.: Investigation, Writing – Original Draft. A.F.Q.: Investigation, Writing – Review and Editing. C.B.: Investigation, Writing – Review and Editing. Z.M.I.G.: Investigation, Writing – Review and Editing. R.F.: Investigation, Writing – Review and Editing. H.P.: Conceptualization, Supervision, Writing – Review and Editing.

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Chapter V – Playing or Fighting? Home Cage Dynamics During Crowding or Social Instability Paradigms Contribute to Stress and Reproductive Impairments in Adult Female Wistar Rats

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Authorial Contributions

M.P. designed the research question and protocol under H.P.'s supervision, with guidance from external collaborator (S.M.P.). M.P. performed all vaginal lavages, blood samples, home cage recordings, and euthanasia (with the assistance of research assistants). See Chapter IV authorial contributions for details on wet lab work. An honours thesis student (É.A.O.) co-supervised by M.P. performed behavioural scoring for home cage recordings, which M.P. independently verified. M.P. completed all data analyses, visualizations, and first draft preparation of the manuscript.

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Abstract

Play fighting represents a key behavioural component in group-housed rats, yet its contribution to the outcomes of social stress paradigms remains unassessed. This study characterized play fighting behaviours over time in two social stress conditions involving crowding. We also evaluated whether play style was associated with changes in stress and reproductive signalling.

Methodology: 56 adult female Wistar rats were exposed to 21 days of high density housing (HDH; 8/cage, stable groups) or social instability (SIS; alternating days of isolation and HDH, unstable groups). Home-cage behaviours and corticosterone (CORT) were determined at discrete timepoints throughout the protocol exposure. Rats (12/stress group) were categorized as high or low players to examine how play profiles influenced stress response and reproductive impairments. **Results:** In both groups, player profiles remained stable throughout the housing period. SIS rats performed more nape contacts and pins than HDH rats but showed shorter pin length. High-playing SIS rats had significantly heavier adrenal glands than low playing SIS rats and high playing HDH rats and spent more days in estrus compared to high playing HDH. CORT secretion profiles were differentiated by an immediate rise in HDH rats compared to delayed increase observed in the SIS group, although player types showed no further associations.

Conclusions: In unstable social environments such as SIS, short, frequent pins may reflect recurrent attempts to establish social hierarchies, with high playing SIS rats showing increased adrenal weights relative to low playing and HDH counterparts. These findings support the hypothesis that play behaviours serve distinct purposes depending on the social context.

Keywords: Crowding, social instability, play fighting, social stress, sociability

1. Introduction

Play fighting, a form of social interaction shown to be rewarding in rodents and many other mammals, comprises competitive behaviours that may mimic aggression but have a cooperative component (Palagi et al., 2016; Pellis & Pellis, 2017). In rats, play fighting involves trying to contact the nape of the partner's neck, which is nuzzled with the tip of the snout if contacted (Pellis & Pellis, 1987; Siviý & Panksepp, 1987). This competition is moderated by cooperation, leading to increased likelihood of role reversals (Pellis et al., 2010). Notably, intrinsic differences exist between individuals in the level of displayed playful behaviour (Pellis & McKenna, 1992), a trait shown to be relatively stable across testing sessions in juvenile rats (Achterberg et al., 2023; Lesscher et al., 2021). Although more frequent in juveniles, play fighting continues into adulthood, and has been proposed to contribute to establishing dominance relationships and subsequent maintenance of social bonds (Kisko et al., 2015). Concordantly, play is generally “rougher” in adulthood, and is more likely to escalate to serious fighting, particularly in males (Foroud & Pellis, 2003). Indeed, elevated frequencies of play behaviours and boldness in juvenile males has been correlated with a dominance position in adulthood (Pellis & McKenna, 1992). At present, such findings have emerged from observation of social dynamics mostly in males (primarily mice and rats; Fulenwider et al., 2022), despite recognized differences between sexes in behavioural response profiles.

Whereas male rats and mice within laboratory settings form linear dominance hierarchies, comparatively little is known of the structure and significance of such relationships in female counterparts (Fulenwider et al., 2022). Interestingly, evidence shows the establishment of consistent and stable social relationships among female mice, through behaviours such as mounting and chasing, as well as less linear, steep, and ‘despotic’ hierarchies compared to those

observed in males (Williamson et al., 2019). Distinctive biochemical response profiles have emerged within these hierarchies, with dominant females spending increased time in estrus, whereas subordinate females show elevated basal corticosterone (CORT) levels and hypothalamic estrogen receptor alpha (ER α) expression (Williamson et al., 2019). In female cuttlefish, dominant individuals present greater levels of gonadotropin-releasing hormone (GnRH) and estradiol (E2) and have higher reproductive success than subordinates (Zhang et al., 2021). These findings emphasize the critical influence of social dynamics on stress-related parameters in the hypothalamic-pituitary-adrenal (HPA) axis, as well as reproductive outcomes regulated by the hypothalamic-pituitary-gonadal (HPG) axis. As such, they indicate an area of potential interest in understanding the outcomes of stressors that are of a social nature. Of further interest, the estrous cycle in female rodents has been shown to influence a variety of social behaviours (Williamson et al., 2019). However, despite such bi-directional interactions between reproduction and social behaviour, the estrous cycle is often omitted from assessments of social dynamics.

Given the critical role of social interactions in wellbeing and social cohesion, studies modifying the social environment, using overcrowding or group instability paradigms, have been found to increase stress in rats and mice (Goñi-Balentziaga et al., 2018; Slezak et al., 2014). Namely, crowding has been suggested to be especially stressful due to uncontrollable and/or unwanted social interactions (Brown & Grunberg, 1995). Moreover, knowing the role of adulthood play fighting in testing and maintaining dominance relationships (Foroud & Pellis, 2003; Kisko et al., 2015), one can foresee play fighting in crowded settings to have a role beyond that of an enjoyable and rewarding behaviour and to be more likely to escalate into serious fighting. In male rats, peripubertal stress exposure has been associated with abnormal forms of

aggression, which were more frequently displayed in high playing rats compared to low playing or non-stressed counterparts (Papilloud et al., 2018). Similarly, in male rats prenatally exposed to ethanol, high frequency of juvenile play-fighting correlates with higher post-pubertal aggression (Royalty, 1990). In other circumstances, including studies where one male rat in a pair is devocalized (preventing use of vocalizations to de-escalate encounters), play fighting more frequently escalates into aggression (Burke et al., 2017). Differences in the social context are therefore highly likely to influence behavioural and biochemical outcomes. Such contributions remain to be thoroughly characterized in female rats.

Various studies have supported the hypothesis that females in many rodent species (including rats and mice) display reduced aggressivity and territoriality compared to male counterparts (Hashikawa et al., 2018; Solomon, 2017). These observations have contributed to reducing inclusion of females in studies assessing effects of social stress exposure (Lyons et al., 2023). Incidentally, there is a limited understanding as to how play fighting might contribute to defining social relations in adult females (although see Hamilton et al., 2020), especially under stressful housing conditions. Some studies using social instability have found increased stress in female rats, even in the absence of elevated levels of aggression (Baranyi et al., 2005; Herzog et al., 2009). However, findings were limited by the sole consideration of overall bite frequency per cage, omitting relevant social factors such as individual differences in play behaviour and structure. As such, the contribution of females' singular play fighting style (and player type) to measured stress responses and associated consequences warrant further consideration, notably effects on reproductive function (Acevedo-Rodriguez et al., 2018). Indeed, despite the existence of bi-directional influences between sex hormones and behaviour (George & Rosvall, 2022), other key markers of HPG axis function remain to be evaluated in the context of social dynamics

between adult females. More detailed assessments, factoring in discrete contributions of home cage dynamics, could allow greater understanding of the relationship between social stress and reproductive impairments.

This study is part of a larger project that assessed the impact of various social stressors on reproductive function in adult female rats in an estrous-stage dependent manner (Poitras, Labonté, et al., 2025). The present investigation aimed to expand on this study by characterizing the contribution of home cage behavioural dynamics during 21-days of crowding (high density housing; HDH) and social instability stress (SIS) on stress and reproductive outcomes. More specifically, we assessed evolution of play fighting behaviours in groups of females over the course of exposure to the two stress paradigms, documented stability of player profiles over time, and assessed if reproductive and stress parameters differed between high and low play fighting rats. Additionally, we controlled for estrous cycle stage in all analyses, due to its' known influence on sociability, stress responses, and reproductive markers (Acevedo-Rodriguez et al., 2018; Williamson et al., 2019). With the unstable social composition induced in the SIS condition, it was hypothesized that play in this group would have a more stressful component than in stable groups, as play fighting might be a key element in establishing and maintaining social hierarchies (Foroud & Pellis, 2003; Kisko et al., 2015). As such, we predicted that play would be rougher in SIS rats, and that aggression (i.e., biting) would be more prevalent. To our knowledge, no studies have evaluated the stability of players' profiles in adult female rats, although studies have shown player type to remain stable over time in juvenile rats (Achterberg et al., 2023). It was further hypothesized that play-fighting in crowded settings would represent a form of unwanted and uncontrollable interaction (Brown & Grunberg, 1995). As such, we predicted that rats that are naturally less inclined to play (i.e., low players) would find such

interactions more aversive, and therefore present elevated stress responses and more reproductive alterations compared to high playing counterparts.

2. Methodology

2.1 Ethical Review

All procedures were in line with ARRIVE guidelines as well as the Canadian Council on Animal Care and had been approved by the Protocol Review Group of the Animal Care Committee of the University of Ottawa prior to experiment start (PY-4245).

2.2 Subjects

Fifty-six adult female Wistar rats (a high playing strain; Himmler et al., 2014; Sivi, 2020) were obtained from Charles River Laboratories (Rocheport, Québec) and arrived at the facilities at 3 months old. Upon arrival, rats were pair-housed in standard plexiglass cages (45.5 cm x 24 cm x 20.5 cm) containing 1 inch of beta chip bedding and a polycarbonate tube, with bi-weekly cage changes. Rats were kept on a 12 h reverse light:dark cycle (off at 10:00 a.m.), with room temperature maintained at 21-23°C, and relative humidity held at 60%. Rats had *ad libitum* access to water and standard rat chow (2018 Teklad global 18% Protein Rodent Diet, Envigo, United States). Weight gain was measured weekly, after vaginal lavage.

2.3 Estrous Cycle Assessment

After 12 days of acclimation to the vivarium, rats underwent daily vaginal lavage for two weeks. Only rats showing a minimum of two consecutive regular cycles were included. Vaginal lavages were performed prior to onset of the dark phase, between 8:00 and 10:00 a.m., and continued to be collected daily for the duration of the experiment. Vaginal lavages were done as previously described (Poitras, Morin, et al., 2024), after which samples were placed on a clear microscope slide and examined using a Leica DAS microscope. Estrous cycle stage

characterization followed established criteria, with rats scored as being in proestrus, estrus, metestrus, or diestrus (Goldman et al., 2007; McLean et al., 2012). Rats were considered to be regularly cycling if they presented 4-5 day cycles with regular progression between stages (i.e., 1 day proestrus, 1 day estrus, 2-3 days metestrus/diestrus).

2.4 Housing Conditions

Following confirmation of estrous cycle regularity, rats were randomly assigned to either HDH ($n = 32$) or SIS ($n = 24$). Rats in the HDH condition were housed 8 rats per standard cage (136.5 cm^2 of floor space per rat; Adavi et al., 2023). Rats assigned to the SIS condition were housed in the same manner as the HDH rats but were subjected to alternating days of isolation ($n = 1$ per cage) and HDH. To increase unpredictability in the SIS group, phase length was randomized to last either 24 or 48 h (see Figure 1; Herzog et al., 2009). Group composition in SIS was modified between crowding phases by changing which individuals were housed in each cage, ensuring no more than 3 familiar cagemates (defined as rats that had been housed together in the previous phase) for each rat in a given session. In both groups, cage changes occurred daily, immediately following vaginal lavage. Note that the larger size of the HDH group relates to the sample collection constraints of the larger project, which required the inclusion of an additional HDH cage (Poitras, Labonté, et al., 2025). To avoid omitting potentially relevant data points, all cages/rats were included in analyses presented herein.

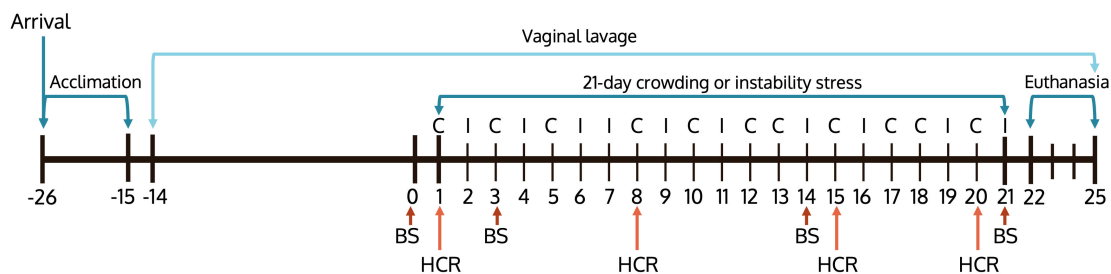


Figure 1. Experimental Timeline.

All rats were pair-housed during acclimation and two weeks of estrous cyclicity confirmation, after which they were randomly assigned to stable high density housing ($n = 8/\text{cage}$) or social instability (SIS);

alternating days of crowding (C) and isolation (I), as shown). Blood samples (BS) were collected at baseline prior to stressor start (Day 26) and on the 3rd, 14th, and 21st day of the stressor. Home cage recordings (HCR) using an overhead camera were performed on the 1st, 8th, 15th, and 20th day of stress during the 30 min immediately preceding lights off.

2.5 Home Cage Recording

Social interactions within the home cage were monitored to characterize the evolution of social dynamics in crowded conditions over the course of the 3-week period. These recordings were performed at the start of Day 1 (Session 1; S1), Day 8 (S2), Day 15 (S3), and Day 20 (S4) of stress. Note the 48 h isolation period preceding S2 in SIS rats, versus the 24 h isolation preceding S3 and S4 (see Figure 1). The first 30 min of crowding after cage change were recorded using an overhead camera (immediately prior to dark phase onset; between 9:30 a.m. and 10:00 a.m.). An experimenter (E.A.O.) blinded to session number scored the behaviours using the Behavioral Observation Research Interactive Software (BORIS; Friard & Gamba, 2016). Subsequently, all videos were reviewed by the lead researcher (M.P.) to confirm accuracy of scoring and identify the rats involved in each behavioural sequence. Different colored non-toxic markers were used to number the rats' tails, allowing differentiation of individual rats. Due to the changing group compositions in the SIS group, it was not possible to maintain blinding to experimental group.

For each rat in the cage, the number of nape contacts, pins, and bites was scored, and, for each occurrence, the rat initiating the behaviour and the recipient of the behaviour were identified. See [Appendix E](#) – Table E1 for detailed description of all behaviours and calculations of proportions (Ham & Pellis, 2024). Mean length of pins and mean duration being pinned were measured, in addition to categorizing rats as using an active or passive defense when being pinned (Pellis et al., 2022). The proportion of attacks defended (calculated from the total number

of nape contacts received), as well as the proportion of different defense tactics utilized (evasion, horizontal rotations, partial rotations, complete rotations; Himmler et al., 2013) were evaluated.

Player types were determined using the method established by Achterberg et al. (2023). For sessions 2, 3, and 4, sum of nape contacts and pins initiated was calculated for each rat. The rat with the highest total was ranked #1, second highest #2, and so on. In the event of a tie (i.e., same number of behaviours for that session), the same rank was attributed to both rats. Ranks were then summed across sessions to establish a final ranking. Ranks were sorted in ascending order, and rats in the top 50% were categorized as high players (12 rats for SIS, 15 rats for HDH), whereas rats in the bottom 50% were considered low players (12 rats for SIS, 17 rats for HDH). The slightly uneven split for HDH is attributable to a tie in final ranks. Due to relatively low behaviour frequency in the HDH group, a medium player type was omitted, as it could not be reliably detected. Consequently, rats were categorized as being high players or low players based on total ranks using a median split, rather than a tertial split as described in juveniles (Achterberg et al., 2023).

2.6 Blood Sampling

Blood samples were collected prior to the start of SIS or HDH conditions (Day 0; Baseline; BS) and on the 3rd, 14th, and 21st day of the housing period, enabling to assess changes in basal circulatory CORT levels. Before the onset of the dark period, immediately prior to vaginal lavage (between 8:00 and 10:00 a.m.), rats were individually brought to an adjacent room and blood collection was rapidly performed using the tail nick method (Milot et al., 2012). A minimum of two blood drops per rat were deposited onto Whatman Bloodstain Cards (Whatman International Ltd., Maidstone, United Kingdom), after which samples were left to dry overnight and stored at - 80°C for subsequent analysis.

2.7 Euthanasia and Sample Collection

As part of the larger project, which evaluated the influence of estrous cycle stage on the outcome measures of interest, 6 proestrus rats and 6 diestrus rats were randomly selected from each group for biochemical analyses (Poitras, Labonté, et al., 2025). To properly target estrous cycle stages, euthanasia took place on Days 48 to 51, between 9:30 a.m. and 12:30 p.m. Since not all rats were in the appropriate estrous cycle stage at the end of the 21-day period, stress conditions were maintained for a maximum of three days until they entered a stage in which sample collection could occur (Nowacka et al., 2014). Rats sharing a cage were euthanized on the same day to avoid disrupting experimental conditions. Rats were injected with a lethal dose of sodium pentobarbital (60 mg/ml; Euthanyl Bimeda-MTC Canada, Canada), and following loss of reflexes, a minimum of 1.5 mL of blood was collected from the abdominal aorta. Rats were transcardially perfused with saline, and uterine horns and adrenal glands were extracted and weighed. Rats were then perfused with 4% paraformaldehyde (PFA) with 20% picric acid. Brains were extracted, incubated in PFA for 1 h, 10% sucrose overnight, 20% sucrose for 24 h, flash frozen with CO₂, and stored at -80°C.

Subsequently, brains were sliced at 14 µm using a cryostat (Leica CM1900, Leica Microsystems, Germany) and mounted onto Superfrost Plus Slides (Fisher Scientific, Canada). To assess HPG and HPA axis function post stress, we measured ERα, kisspeptin (Kiss1), and gonadotropin-inhibitory hormone (GnIH) as critical regulators of reproduction, as well as glucocorticoid receptors (GR) as an indicator of HPA axis activation (Acevedo-Rodriguez et al., 2018). Areas of interest for this study were the CA1 of the hippocampus (-2.80 to -4.16 mm posterior to Bregma; GnIH and GR expression), as well as the arcuate nucleus (ARC; -2.12 to -3.60 mm posterior to Bregma; ERα, Kiss1, and GnIH expression), anteroventral periventricular

nucleus (AVPV; - 0.26 to -0.40 mm posterior to Bregma; ER α and Kiss1 expression), dorsomedial nucleus (DMH; -2.56 to -3.60 mm posterior to Bregma; GnIH), and paraventricular nucleus (PVN; -1.30 to -2.12 mm posterior to Bregma; GR expression) of the hypothalamus (Paxinos & Watson, 2006).

2.8 Immunofluorescence

Immunofluorescent staining was performed as previously described (Poitras, Morin, et al., 2024) using the following antibodies: monoclonal mouse anti-ER α (1:400, MA1-80216, Invitrogen Canada Inc.), rabbit anti-GnIH (PAC123/124; 1:5000; Provided by Dr. G.E. Bentley), polyclonal rabbit anti-Kiss1 (1:300, BS-0749R, Bioss, USA), or monoclonal mouse anti-GR (1:500, MA1-510, Invitrogen Canada Inc.). Briefly, slides containing the regions of interest were incubated for 30 min in blocking solution (phosphate buffered saline [PBS] – 5 % donkey serum – 0.5 % triton), after which they were incubated in blocking solution containing the relevant antibodies overnight at 4°C. The next days, slides were washed in PBS (3 x 5 min), incubated for 1 h in anti-mouse donkey (1:1000; A-21202; Invitrogen Canada Inc.) or anti-rabbit donkey (1:1000; A21207, Invitrogen Canada Inc.) secondary antibodies. Slides were washed, incubated in Hoechst adenine–thymine binding dye solution for 5 min (1:20,000 in PBS; Hoechst 33342, Invitrogen Canada Inc.), washed again, and coverslipped. For all antibodies, pilot studies determined optimal primary antibody concentrations and specificity of staining to expected nuclei, and negative controls validated secondary antibody specificity.

For each rat, 6 anatomically matched photomicrographs were captured in each region of interest using a 20 $\times$ objective lens on an Olympus DX51 microscope (Center Valley, PA, USA) with the ProgRes Capture Pro 2.7.6 software. The ImageJ software (National Institutes of Health, United States) was used to calculate the percentage of area (i.e., surface of immunoreactive

staining relative to total microphotograph area), for each selected brain region. The ImageJ algorithm “Moments” was used to scan GnIH in the hypothalamus and all GR expression, whereas the algorithm “Triangle” was used for hippocampal GnIH as well as ER α and Kiss1 expression.

2.9 Enzyme-Linked Immunosorbent Assays

Abdominal aorta blood samples collected at euthanasia were left to clot overnight at 4°C, centrifuged at 9400 rcf for 10 min at 20°C (de la Tremblaye et al., 2016), and stored at -80°C until use to assess pituitary and gonadal HPG axis function through luteinizing hormone (LH; LH ELISA kit, ENZ-KIT107, Enzo Life Sciences), follicle-stimulating hormone (FSH; Rat Follicle-Stimulating Hormone ELISA Kit, BSKR60303-96T, Bioss Inc., Massachusetts, United States), estradiol (E2; 17 beta Estradiol ELISA Kit, ab108667), and progesterone (P4; Rat Progesterone ELISA Kit, NBP2-60127, Novus Biologicals). Using the whole blood drop samples, CORT concentrations were analyzed in all animals (Corticosterone ELISA Kit, ADI-900-097, Enzo Life Sciences, Inc., Farmingdale, United States) as previously described (Morin et al., 2022). All kits were completed according to manufacturer instructions, read with Biotek Powerwave XS2, and analyzed with the Gen 5 V2.0 software. All samples were loaded in duplicates and inter- and intra-assay consistency was confirmed.

2.10 Statistical Analyses

Statistical analyses were performed using R (version R–4.4.3) and R studio (version 2024.09.1). Data were screened for outliers using boxplots, and outliers that fell beyond the upper or lower fence (1.5 times the difference between 1st and 3rd quartiles) were winsorized to the next extreme most value in that group ± 1 . Where applicable, normality, homogeneity of variance, and homogeneity of regression slopes were confirmed. For all behavioural analyses,

linear mixed-effect models (LMM) were fitted using a Gaussian error distribution and an identity link function (R package lmerTest; Kuznetsova et al., 2017). LMM were selected as they are robust against non-normal data (Schielzeth et al., 2020), flexible with missing data points, and allowed control for estrous cycle stage at each timepoint. If inspection of model residuals indicated substantial deviation from normality, the dependent variable was log-transformed prior to analysis to better meet model assumptions. Marginal and conditional R^2 effect sizes (R^2_m and R^2_c , respectively; Nakagawa & Schielzeth, 2013) were computed using the *r.squaredGLMM* function from the *MuMIn* package (Bartoń, 2023). To compare the home cage behaviours of the two groups (HDH vs. SIS) over the four recording sessions, LMM was used, with estrous cycle stage as a covariate, and individuals and cage assignment as random effects. For each group, LMM was then used to compare social behaviours between player types (High vs. Low) over time, using the same covariate and random factors. For CORT analyses, LMM included player type, group, and timepoint as fixed effects, estrous stage as a covariate, and individual information as a random effect. Estrous cycle characteristics and weight gain were similarly analyzed, without the inclusion of estrous stage as a covariate. Cage assignment was not included as a random factor in the CORT and weight gain analyses, as these measurements did not coincide with a single crowding day (unlike the behaviour recordings), and as such could not be included for SIS rats due to changing group composition. Finally, to evaluate possible associations between player type and the consequences of stress, two-way analysis of covariance (ANCOVA) was conducted to compare effects of group and player type on the biochemical variables collected at euthanasia, maintaining estrous stage as a covariate. In all cases, post hoc analyses of significant effects were performed using estimated marginal means (R package *emmeans*) with Bonferroni correction.

3. Results

3.1 Characteristics of Play Fighting in SIS and HDH Rats

As shown in Figure 2, LMM showed a significant group $\times$ session interaction for nape contact frequency [$F(3,14.995) = 13.201, p < .001, R^2_m = .302, R^2_c = .653$] and pin frequency [$F(3,16.199) = 21.193, p < .001, R^2_m = .397, R^2_c = .557$]. There were no group differences in the frequency of nape contacts during S1, S3, and S4, whereas SIS rats performed significantly more nape contacts during S2 ($p = .004$). HDH rats had the highest frequency of nape contacts during S1, which was significantly higher than S2 ($p = .002$), S3 ($p < .001$), and S4 ($p = .003$). For SIS rats, nape contact frequency only differed significantly between S1 and S2, with more nape contacts at S2 ($p = .01$). Pin frequency did not differ at S1 or S3, but SIS performed significantly more pins than HDH at S2 ($p = .004$) and S4 ($p = .040$). Concordantly, pin frequency remained stable throughout the intervention for HDH rats, with no significant changes over time (all $p > .05$). In contrast, SIS significantly increased pin frequency relative to baseline, with S1 pin frequency being lower compared to S2 ($p < .0001$) and S4 ($p = .010$). However, SIS rats also performed significantly more pins at S2 than S3 ($p = .003$) and S4 ($p = .020$). Significant group $\times$ session interactions were detected for pin length and mean duration pinned [$F(3,62.532) = 4.042, p = .011, R^2_m = .269, R^2_c = .298$ and $F(3,58.698) = 3.918, p = .013, R^2_m = .303, R^2_c = .343$, respectively]. Pin length was significantly shorter for SIS than HDH rats during S2 ($p = .025$). Pin length in SIS rats did not change over time (all $p > .05$), whereas HDH rats presented greater pin length at S2 than S3 ($p = .017$). Similarly, mean pinned duration was significantly shorter for SIS rats at S2 compared to HDH counterparts ($p = .006$). HDH rats presented longer mean duration being pinned during S2 compared to S1 ($p = .003$) and S3 ($p = .002$).

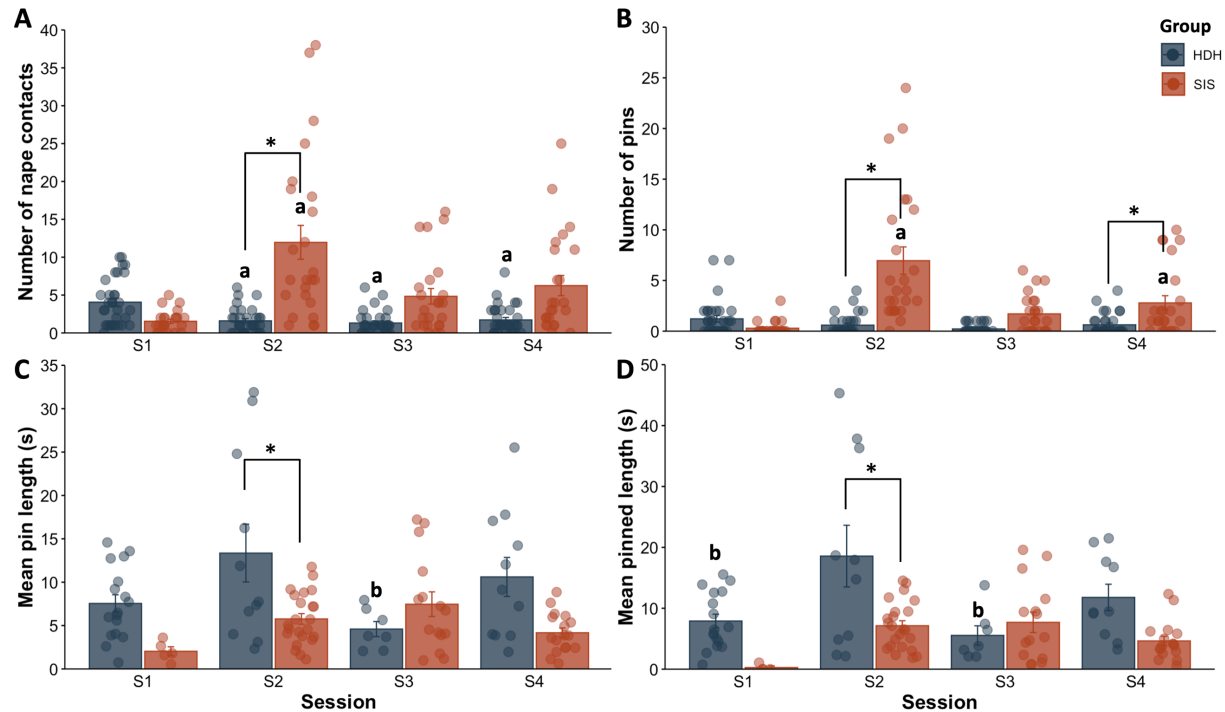


Figure 2. Nape Contacts and Pins During HDH and SIS.

Figure shows the number of nape contacts (A) and pins (B) performed during each home cage recording session, as well as the mean length of pins (C) and mean duration pinned (D) for HDH and SIS rats. Linear mixed model analysis between HDH and SIS over time. Data presented as mean $\pm$ S.E.M. * indicates difference between groups, **a** indicates difference from S1, **b** indicates difference from S2, all $p < .05$.

Bite frequency was low, with a total of 6 bites being detected across all groups and recording sessions. As such, bites were not formally analyzed. However, it is noteworthy that all bites occurred in the SIS group, and that no bites were detected during S1. This supports the hypothesis that play would be rougher in SIS rats. Interestingly, half of the bites were delivered by rats pinning a partner that was actively defending against the pin, and most bites were performed by high playing rats (4 out of 6).

Relating to the percentage of attacks defended, there was a statistical trend for an interaction between group and session, $F(1,184) = 2.234$, $p = .086$, $R^2_m = .111$, $R^2_c = .111$, with SIS rats defending significantly more attacks at S4 than S1 ($p = .043$, See Figure 3). While being pinned, SIS rats were significantly more likely to use a passive defence than HDH rats,

$F(1,49.72) = 8.442, p = .006, R^2_m = .129, R^2_c = .138$. The probability of evading a nape contact changed significantly over time [$F(3,147) = 3.405, p = .019, R^2_m = .132, R^2_c = .132$], with probability of evasion being greater at S1 than S4 ($p = .025$). There were no significant group differences in the frequency of rotations (all $p > .05$). Note the smaller n of rats included for proportion of responses at some timepoints, particularly during S1, due to low occurrence of attacks.

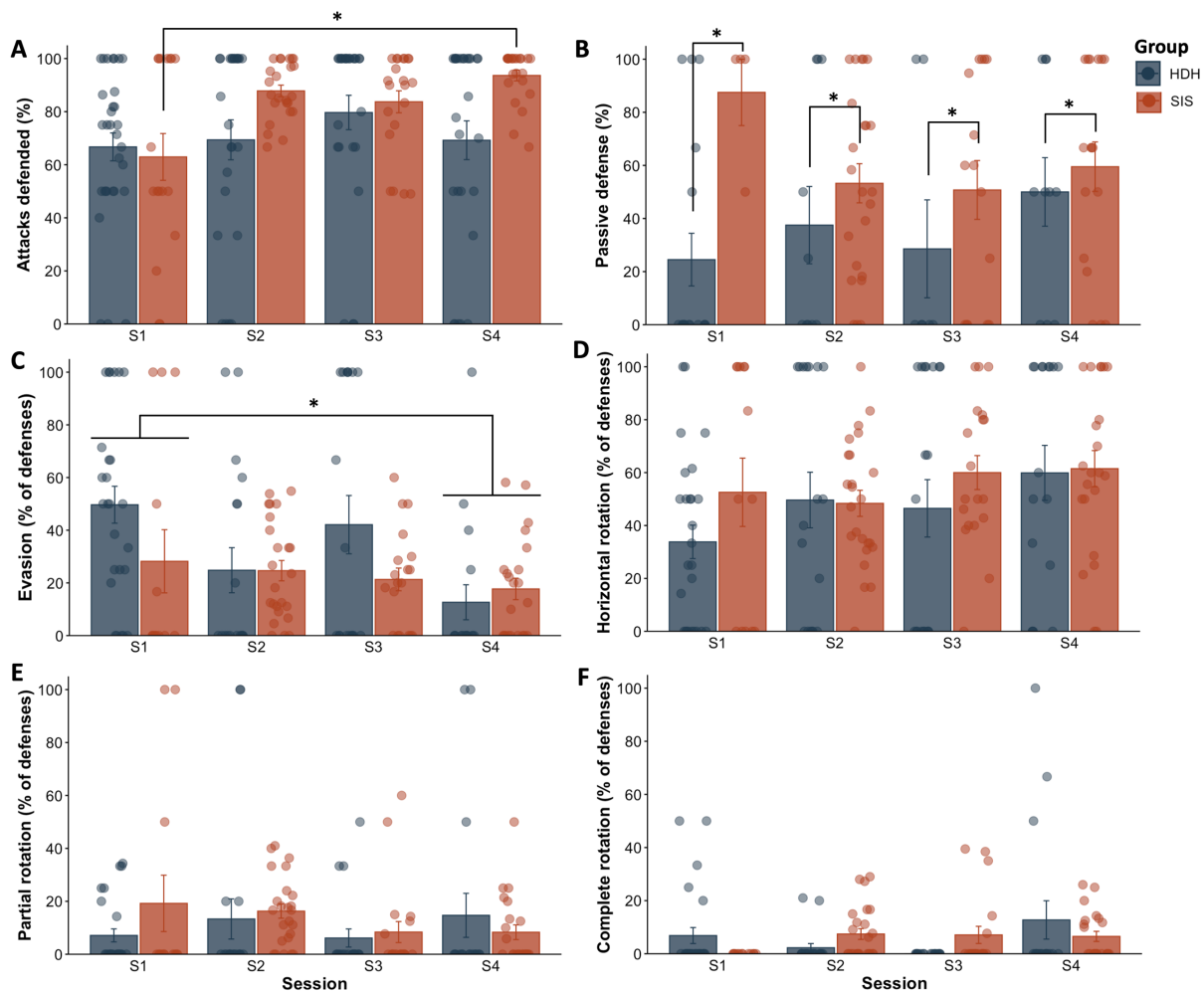


Figure 3. Defence Tactics Used During HDH and SIS.

Figure shows the percentage of attacks which were defended (A), the percentage of pins for which a passive defence was used (B), as well as the preference for different defence tactics in response to a nape contact: evasion (C), horizontal rotation (D), partial rotation (E), and complete rotation (F). Linear mixed model analysis between HDH and SIS over time. Data presented as mean $\pm$ S.E.M. * indicates significant difference between groups, $p < .05$.

3.2 Stability of Individual Player Profiles in Each Group

Figure 4 shows an overview of the frequency of play for all sessions relative to the grand mean for low and high players. Despite relatively low behaviour frequencies in the HDH group, low playing rats more often fell below the grand mean for the number of nape contacts and pins performed, whereas high playing rats usually had 1-2 sessions where their behaviour frequency was above the grand mean. In the SIS group, low playing rats always had a minimum of two sessions where behaviour frequency fell below the grand mean for the number of nape contacts and pins performed, whereas high playing rats usually had 1-2 sessions where their behaviour frequency was above the grand mean.

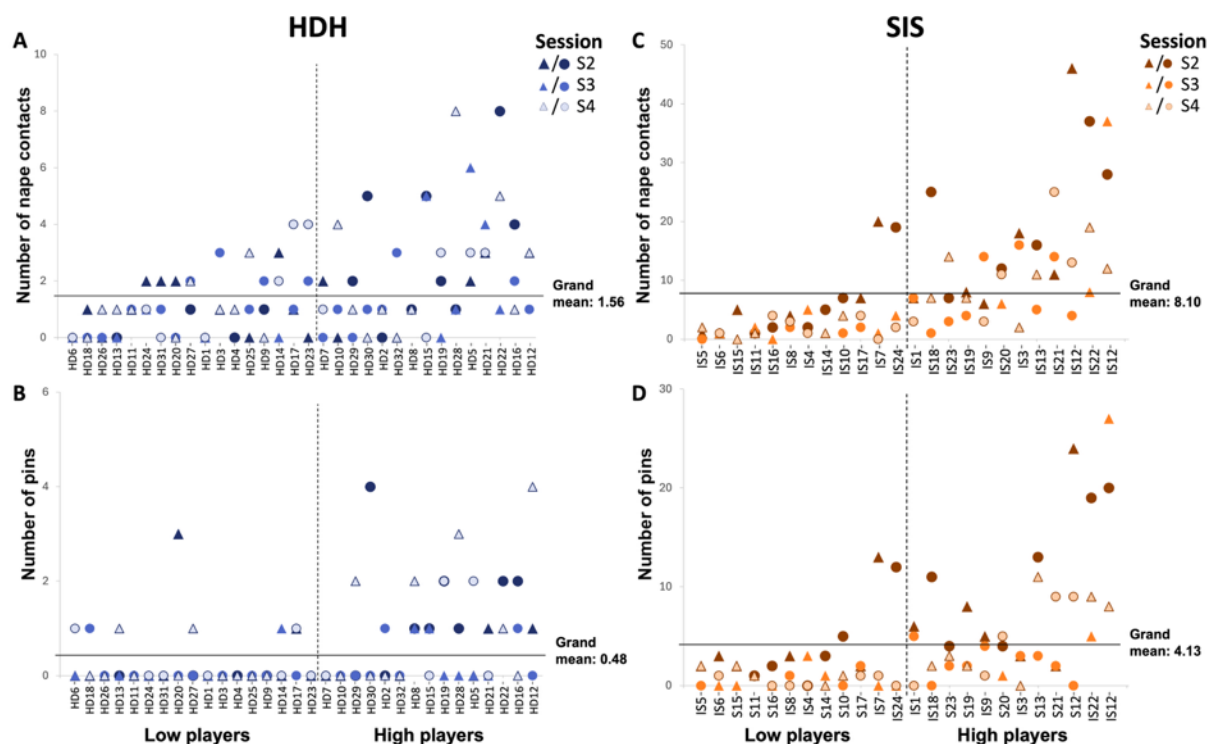


Figure 4. Consistency of Nape Contact and Pin Frequency Across Sessions by Player.

Left side shows number of nape contacts (A) and pins (B) performed during S2, S3 and S4 for each HDH rat. Right side shows number of nape contacts (C) and pins (D) performed each session for each SIS rat. Δ represents rats in proestrus/estrus, and $\circ$ represents rats in metestrus/diestrus during that recording session.

3.2.1 Player Profiles in the High Density Housing Group

In the HDH group, LMM indicated a significant main effect of player type on probability of nape contacts [$F(1,28.371) = 6.611, p = .016, R^2_m = .128, R^2_c = .170$] and pins [$F(1,87) = 9.576, p < .001, R^2_m = .104, R^2_c = .104$, See Figure 5]. In both cases, high playing HDH rats were more likely to initiate nape contacts and pins than low playing rats ($p = .019$ and $p = .006$, respectively). HDH high playing rats were also more likely to be targeted by an attack than low players, $F(1,28.489) = 6.449, p = .017, R^2_m = .096, R^2_c = .150$. There were no significant differences between player types for the probability of defence ($p > .05$; See Figure 5), or in the tactics used to defend from an attack (all $p > .05$, data not shown).

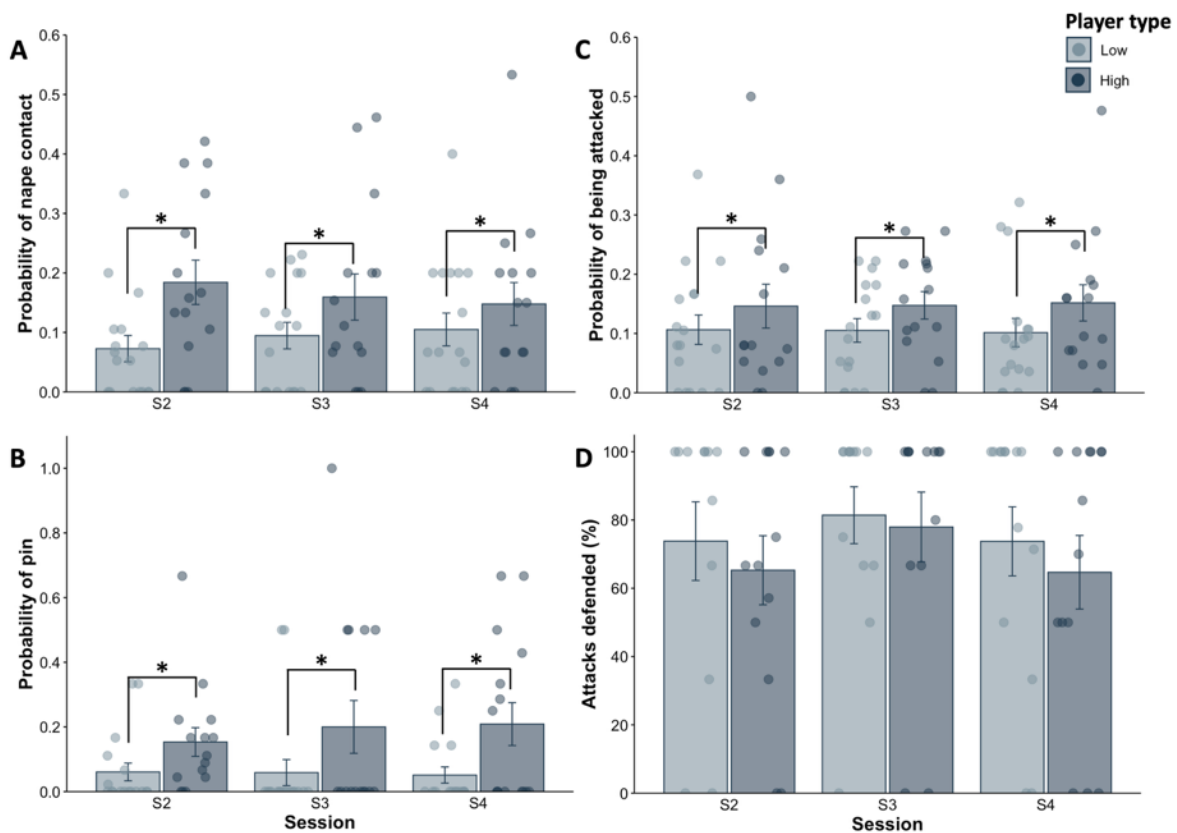


Figure 5. Consistency of Player Type in the HDH Group.

Figure shows the probability of performing a nape contact (A) or a pin (B), as well as the probability of being attacked (C) and the percentage of attacks being defended (D) in low and high playing HDH rats. Linear mixed model analysis between high and low playing HDH rats over time. Data presented as mean $\pm$ S.E.M. * indicates significant difference between groups, $p < .05$.

3.2.2 Player Profiles in the Social Instability Group

Like HDH rats, there was a significant main effect of player type on probability of nape contacts and pins for SIS rats [$F(1,21.614) = 33.458, p < .001, R^2_m = .382, R^2_c = .428$ and $F(1,63) = 23.022, p < .001, R^2_m = .296, R^2_c = .296$, respectively]. High-playing SIS rats were more likely to initiate nape contacts ($p < .001$) and pins ($p < .001$) than low playing counterparts. Similarly, high playing SIS rats were more likely to be targeted by an attack than low players, $F(1,63) = 4.867, p = .037, R^2_m = .183, R^2_c = .276$, See Figure 6. There were no significant differences between player types in the probability of defence or in the different tactics used to defend from an attack (all $p > .05$; data not shown).

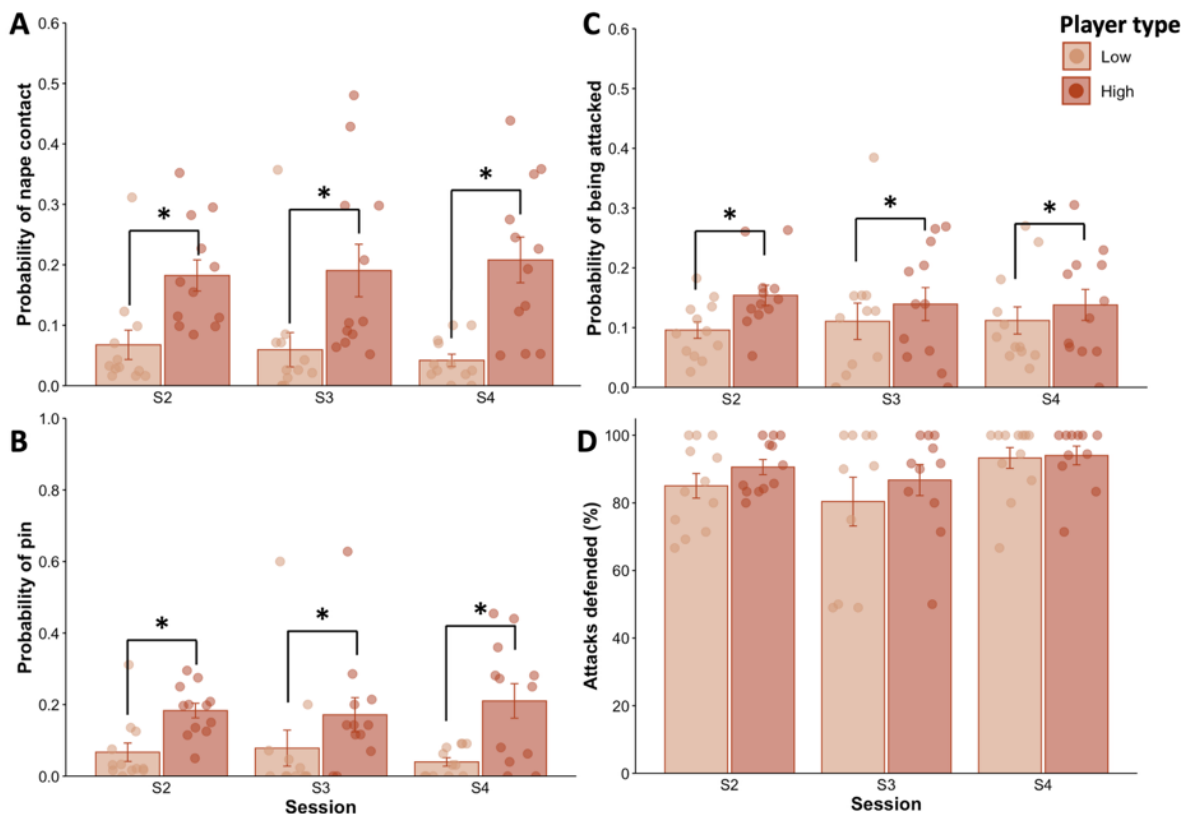


Figure 6. Consistency of Player Type in the SIS Group.

Figure shows the probability of performing a nape contact (A) or a pin (B), as well as the probability of being attacked (C) and the percentage of attacks being defended (D) in low and high playing SIS rats. Linear mixed model analysis between high and low playing SIS rats over time. Data presented as mean $\pm$ S.E.M. * indicates significant difference between groups, $p < .05$.

3.3 Stress and Reproductive Correlates

3.3.1 Hypothalamic-Pituitary-Adrenal Axis

Two-way ANCOVA of GR-ir at the PVN and CA1 did not differ between groups or player type (both $p > .05$; See [Appendix E](#) – Figure E1 for representative photomicrographs for each group). For adrenal gland weights, a significant group $\times$ player type interaction was found, $F(1,19) = 27.192, p < .001, \eta^2 = .59$. Post hoc analysis revealed significantly higher adrenal gland weight in high playing SIS rats compared to low playing counterparts ($p = .007$) and high playing HDH rats ($p < .001$). Conversely, high playing HDH rats had significantly lower adrenal weights compared to low playing HDH rats ($p = .009$).

For CORT levels throughout the study, LMM showed a significant group $\times$ session interaction, $F(3,146.203) = 7.446, p < .001, R^2m = .251, R^2c = .325$. Simple main effect of group was related to significantly higher CORT in HDH compared to SIS rats at S3 ($p = .010$) and S14 ($p = .006$). For simple main effect of time, HDH rats had significantly elevated CORT at S3 ($p < .001$) and S14 ($p = .014$) compared to BS, whereas SIS rats only had higher CORT at S21, relative to BS ($p = .019$), S3 ($p = .010$), and S14 ($p < .001$). Note that two HDH rats were excluded from CORT analyses due to insufficient quantity of samples.

As shown in Figure 7, LMM of weight gain showed a significant group $\times$ time interaction, $F(3,156) = 2.847, p = .040, R^2m = .191, R^2c = .962$, with both groups showing a significant increase in weight as the experiment progressed (all $p < .001$), except between Week 2 and Week 3, where weight gain in SIS rats did not reach significance ($p = .060$). A main effect of player type was also detected [$F(1,52) = 4.466, p = .039, R^2m = .191, R^2c = .962$], with low playing rats having lower body weight than high playing counterparts.

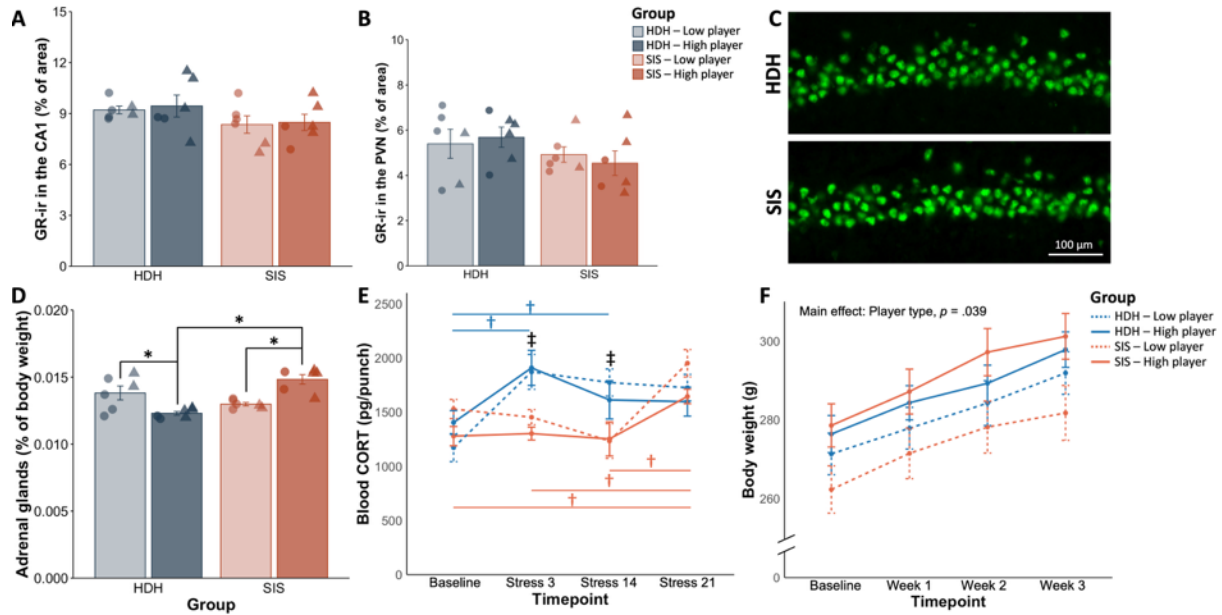


Figure 7. Hypothalamic-Pituitary-Adrenal Axis Function of High and Low Playing Rats After HDH and SIS.

Top half of figure shows mean GR-ir expression in the CA1 (A) and PVN (B) of high and low playing rats for each group. Representative GR immunoreactive staining in the CA1 is shown for a high playing HDH rat and SIS rat (C). Bottom half of figure shows adrenal gland weights at euthanasia (D), whole blood CORT concentrations (E) and weight gain (F) during the study. Note that weight increased significantly between all timepoints for both groups, except Week 2 to Week 3 for the SIS group. ANCOVA of high and low players in the HDH and SIS group, with estrous stage as covariate (A-D), linear mixed model of high and low players in the HDH and SIS groups over time (E-F). Data presented as mean $\pm$ S.E.M., with individual data points representing rats in proestrus (Δ), and diestrus ($\circ$) at euthanasia. * indicates significant difference between groups, † indicates significant difference between timepoints, ‡ indicates significant difference between HDH and SIS groups, all $p < .05$.

3.3.2 Hypothalamic-Pituitary-Gonadal Axis

Two-way ANCOVA found a main effect of group for ER α -ir in the AVPV [$F(1,19) = 6.028, p = .024, \eta^2 = .24$, See Figure 8] with SIS rats displaying higher ER α expression in the AVPV compared to HDH rats. Similarly, a main effect of group for Kiss1-ir in the AVPV [$F(1,19) = 11.599, p = .003, \eta^2 = .38$] was linked to increased expression in SIS rats compared to HDH counterparts. As shown in Figure 8, significant main effects of group were detected for GnIH-ir in the ARC [$F(1,19) = 12.083, p = .003, \eta^2 = .39$], DMH [$F(1,19) = 28.288, p < .001$,

$\eta p^2 = .60$], and CA1 [$F(1,19) = 9.441, p = .006, \eta p^2 = .33$], all of which were due to higher expression in SIS rats compared to HDH rats. There were no significant differences in the other regions of interest (all $p > .05$). See [Appendix E](#) (Figure E2 and Figure E3) for representative photomicrographs for each group.

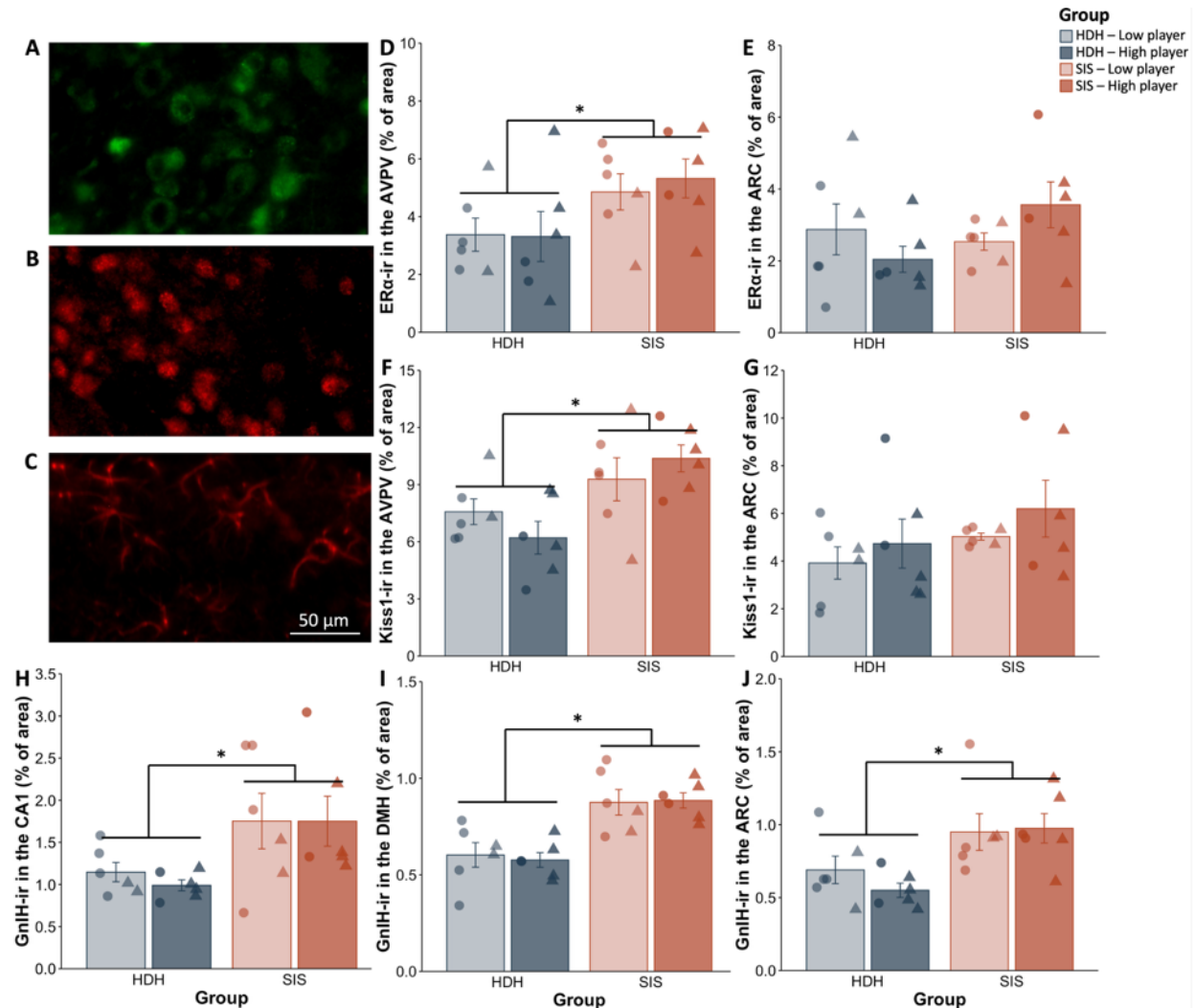


Figure 8. Hypothalamic Expression of HPG Axis Markers in High and Low Playing Rats After HDH and SIS.

Figure shows representative immunoreactive staining from an SIS rat for ERα in the ARC (A), Kiss1 in the AVPV (B), and GnIH in the CA1 (C). Histograms of mean expression are shown for ERα-ir in the AVPV (D) and ARC (E), Kiss1-ir in the AVPV (F) and ARC (G), as well as GnIH-ir in the CA1 (H), DMH (I), and ARC (J). ANCOVA of high and low players in the HDH and SIS group, with estrous stage as covariate. Data presented as mean $\pm$ S.E.M., with individual data points representing rats in proestrus (Δ) and diestrus (○) at euthanasia. * indicates statistically significant difference, $p < .05$

As shown in Figure 9, LMM revealed a significant group $\times$ time interaction for the number of days spent in proestrus, $F(2,104) = 5.631$, $p = .005$, $R^2_m = .183$, $R^2_c = .186$. Although groups did not differ at baseline or the second half of stress, during the first half of the stressor, SIS rats displayed significantly fewer proestrus days than HDH counterparts ($p = .038$). HDH rats displayed a significant decrease in proestrus days during the second half of the stress period relative to baseline ($p < .001$) and the first half of stress ($p = .003$). Conversely, SIS rats spent fewer days in proestrus during the first half of the stressor compared to baseline ($p = .013$). Relating to days spent in estrus, a trend for a group $\times$ player $\times$ time interaction emerged, $F(2,104) = 2.929$, $p = .058$, $R^2_m = .094$, $R^2_c = .105$. Pairwise comparisons at each timepoint revealed no significant differences at baseline or during the second half of stress (all $p > .05$). However, during the first half of stress, high playing SIS rats spent significantly more days in estrus than high playing HDH rats ($p = .007$). No significant differences were detected for days spent in metestrus/diestrus ($p > .05$). For mean cycle length, only a main effect of time emerged, with a significant increase in mean cycle length during stress compared to baseline ($p < .001$; data not shown).

As shown in Figure 9, a significant main effect of group was detected for uterine horn weight, $F(1,19) = 5.924$, $p = .025$, $\eta^2 = .24$], with significantly lower weight in SIS rats relative to HDH rats. There were no significant group differences for pituitary (LH and FSH) or gonadal (E2 and P4) hormone levels (all $p > .05$). Note that one low playing SIS rat was omitted from FSH analysis due to insufficient quantity of serum.

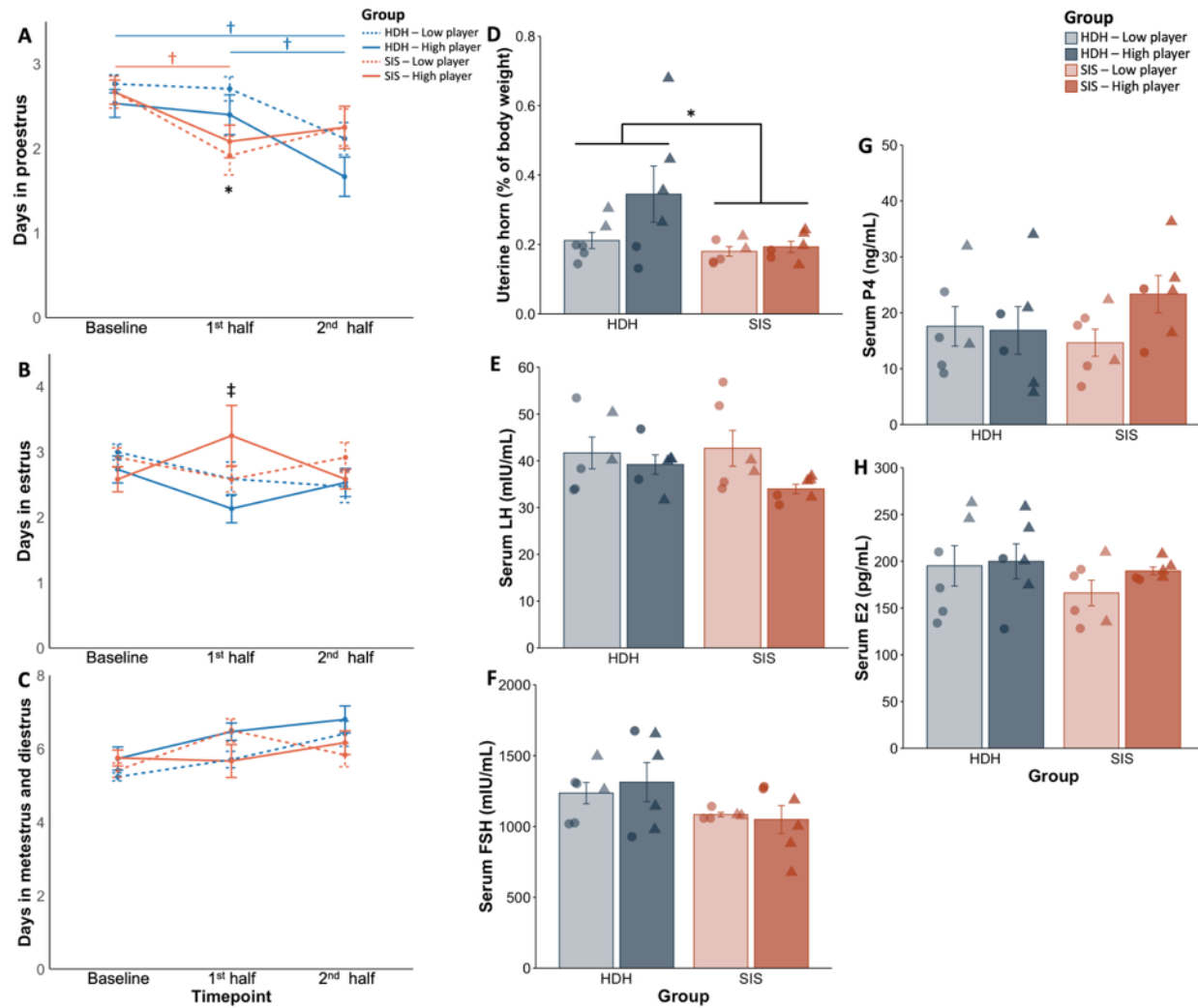


Figure 9. Estrous Cyclicity and Reproductive Hormone Levels in High and Low Playing Rats After HDH and SIS.

Left side of figure shows number of days spent in proestrus (A), estrus (B), and metestrus/diestrus (C) at baseline and during the first and second half of the study period. Right side of figure shows uterine horn weight (D), as well as serum levels of LH (E), FSH (F), P4 (G), and E2 (H) from samples collected at euthanasia. Linear mixed model of high and low players in the HDH and SIS groups over time (A-C), ANCOVA of high and low players in the HDH and SIS group, with estrous stage as covariate (D-H). Data presented as mean $\pm$ S.E.M., with individual data points representing rats in proestrus (Δ) and diestrus ($\circ$). † indicates significant difference between timepoints, ‡ indicates significant difference between high players in the HDH vs. SIS groups, * indicates significant difference between HDH and SIS groups, all $p < .05$.

4. Discussion

This study evaluated the contribution of home cage play fighting in adult female rats exposed to social stress through overcrowding and social instability, with an emphasis on

differences between high and low playing rats. In line with our hypotheses, our findings revealed crowding and social instability to distinctly impact the establishment of home cage dynamics. Notably, exposure to SIS in female rats led to increased occurrence of play behaviour initiations, with shorter pin duration compared to HDH counterparts. As predicted, rats' player profiles (high vs. low players) remained constant across sessions in the two housing conditions, each related to some discrete changes in HPA and HPG axis regulators, although contrary to hypotheses, player type differences were minimal in the assessed biochemical parameters. Overall, our results show active play behaviour to act as a regulating variable in stable and unstable contexts.

4.1 Home Cage Dynamics Evolve Differently in HDH and SIS rats

Although the influence of social stressors on behaviour and physiological responses is well acknowledged (Atrooz et al., 2021), few studies have examined how social dynamics in stringent housing conditions may shape such responses. To our knowledge, no research has closely examined how play fighting evolves within groups exposed to prolonged periods of social stress. Our study finds that play, through both nape contacts and pins, is more frequently expressed in SIS exposed female rats. This is congruent with a proposed role for such behaviours in establishing relationships and hierarchies in frequently changing social groups (Pellis et al., 2022), and a possible influence on social cohesion and stress regulation (Pellis & Pellis, 2017). Conversely, HDH rats demonstrated longer pins and more active resistance, consistent with less aggressive, more affiliative play (Pellis et al., 2024). SIS rats' preference for passive defence could represent a coping strategy aiming to reduce the likelihood of aggression, such as biting, in an environment lacking an established social hierarchy (Takahashi & Lore, 1983). Importantly, it should be noted that passive defence differed significantly between groups during S1, likely due

to low pin frequency limiting the ability to establish clear behavioural patterns in SIS rats during this session. Although these results should be interpreted cautiously, the preference for passive defence in SIS rats aligns with observations showing prior exposure to social defeat in juvenile male rats to lead to more frequent initiation of play behaviour with cage mates, but also to greater propensity to display submissive behaviours (Buwalda et al., 2013). Interestingly, SIS rats showed an increased likelihood of defending themselves from attacks over time. This could suggest constant changes to group composition to accentuate a congener's reactivity to social contact and indicate the presence of social tension amongst rats exposed to instability (Baranyi et al., 2005). Of note, HDH and SIS conditions failed to significantly affect rats' defence tactic preferences in play (e.g., evasion, rotations), although high cage density could have masked subtle group differences, or limit rats from using preferred defence techniques.

In this study, high playing SIS female rats had significantly heavier adrenal glands compared to low playing counterparts and to high playing HDH rats. This finding does not support our hypothesis of accentuated stress responses in low playing rats. Although speculative, such observation may suggest play to act as a tool to reinforce social bonds in a stable but crowded setting (i.e., HDH). In contrast, play fighting within unstable social groups (i.e., SIS) may serve to test and re-establish social hierarchies, therefore imposing a stressful component. Akin to increased adrenal gland weight observed in rodents chronically exposed to social defeat (Brouillard et al., 2016; Wang et al., 2021), establishing dominance in part through elevated play fighting in SIS-exposed female rats likely accentuated stress. This is supported by SIS CORT secretion achieving maximal levels on day 21, contrasting immediate CORT rises in HDH that then plateaued, showing specific paradigm-related CORT alterations over time. In SIS rats, the delayed increase in CORT resembles secretion patterns observed in female rodents exposed to

social defeat, where sustained HPA axis activation is observed upon prolonged exposure (Carnevali, Montano, et al., 2020; van Doeselaar et al., 2021). Thus, accentuated CORT in SIS rats may stem from ongoing instability, which prevents the establishment of a stable hierarchy. In HDH rats, the impact of crowding on CORT secretion may be less persistent, as eventual social stabilization allows levelling of stress over time. Through comparisons of responses in these two paradigms, our findings can contribute to fostering a more complete understanding of differential influences related to distinctive social contexts. Nevertheless, future studies should include a no-stress control group, to further elucidate the contribution of these social housing conditions to CORT secretion profiles.

4.2 Player Profiles Remain Stable Despite Stressful Contexts

Our study suggests that exposure to socially stressful contexts does not override individual differences in play preferences. Our findings indeed show player type to remain constant over time, which expands on the existing literature reporting player type to be relatively stable in both familiar and unfamiliar pairings, as well as when tests are performed in the home cage or in staged dyads (Achterberg et al., 2023; Lesscher et al., 2021). Notably, we observed that in addition to being more likely to initiate play, high playing rats were more likely to be targeted by attacks (i.e., nape contacts), suggesting some degree of reciprocity in play (Pellis & Pellis, 2017), as well as possible play partner preference between high players, even in highly dense environments. Additional research remains required, as studies assessing male rats have reported increased likelihood for subordinates to initiate play with dominant rats than with each other (Pellis et al., 1993). Recently, egocentric network analyses in juvenile male rats have shown that rats often develop partner preferences even when exposed to groups of strangers, and

that preference in such context is not solely driven by proximity of partners, as rats travel across the testing arena to launch attacks against a preferred partner (Ham & Pellis, 2024).

4.3 Differential Paradigms' Impacts on HPG Axis Signalling Molecules Is Not Significantly Influenced by Player Profiles

Considering differences in some key HPA and HPG axis markers related to position in the social hierarchy in mice (Williamson et al., 2019), we had an interest in evaluating if player type influenced endocrine signalling in females exposed to social stress paradigms. Contrary to hypotheses, expression of most examined endocrine markers did not differ between high and low players. However, SIS displayed increased AVPV-Kiss1 and $ER\alpha$, and elevated GnIH in the ARC, DMH and CA1 compared to HDH female rats. Notably, although SIS rats as a group showed reduced days in proestrus and lower uterine horn weights, high playing SIS females spent more days in estrus compared to HDH counterparts during exposure to the initial half of the stress paradigm. This is an interesting finding considering increased estrus days reported in dominant compared to subordinate female mice (Williamson et al., 2019). However, our findings contrast region-specific changes in $ER\alpha$ and P4 receptors noted between dominant and subordinate female mice, in the absence of changes in E2 levels (Williamson et al., 2019). Research in male African cichlid fish also showed that change to social status (i.e., transition from subordinate to dominant) alters E2, testosterone, and progestins. Interestingly, however, endocrine changes were not correlated with specific behaviours associated with the change in rank (Maruska, 2015). In our study, the absence of data from a control group prevents discussing the directionality of changes in SIS vs. HDH rats. However, it appears that the stressful influence of housing, as indicated by changes in CORT levels over time and reduced days in proestrus, does not exclusively stem from the social dynamics within the home cage. Indeed, although

higher behaviour frequencies in SIS rats might contribute to these effects, the absence of pronounced differences between player types suggests other mechanisms being at play beyond dominant/submissive dynamics. Interestingly, subtle social cues, such as passing, have been suggested as mediating variables in establishing hierarchies within colonies of female rats (Ziporyn & McClintock, 1991). However, passing is infrequent in more standard testing environments (Stark & Pellis, 2021) and would be difficult to reliably detect in high density group settings.

The rise in basal CORT levels over time in SIS rats may also reflect the contribution of the isolation component, capable when repeated to accentuate anxiety- and depression-like behaviours (Carnevali, Statello, et al., 2020; Carrier & Kabbaj, 2012). In HDH, stress may arise from different factors, including disrupted circadian rhythms due to persistent overcrowding. Sleep disturbances are linked to social ranking in both male and female rats, with increased sleep duration observed in dominant compared to low status rats (Hurst et al., 1996). These effects could likely be exacerbated in highly dense housing. However, such results must be interpreted cautiously as time bound changes in CORT do not guarantee elevations in CORT relative to a standard housing density control group.

4.4 Limitations and Future Considerations

These data were collected as part of a larger project (Poitras, Labonté, et al., 2025), and some methodological limitations should be noted. Due to limitations of the recording equipment and the challenge of distinguishing individual rats in such highly dense conditions, recordings were not performed in the dark. Given that higher play frequencies are reported during the night phase (Pellis et al., 2022), this might have allowed better characterization of subtle dynamics, especially during the first session and in HDH rats, for which behaviour frequencies were

relatively low. Indeed, the low occurrence of pins during S1 likely contributed to some slight group differences (i.e., passive defence), which made interpretation more difficult. Concordantly, the isolation phases in the selected SIS protocol contributed to increased frequency of play in this group (Vanderschuren et al., 2016), as shown by higher play during S2, which was preceded by a longer isolation period (48 h instead of 24 h). Nevertheless, distinct characteristics of play in HDH and SIS rats (such as alterations to pin duration) suggest that observed differences between groups are unlikely to stem solely from the inclusion of isolation in SIS rats. Future studies with a more balanced experimental design are needed to confirm these observations. Namely, assessing prolonged crowding exposure with changing groups, or paradigms alternating days of HDH and isolation with stable groups, would enable to further understand the contributions of above-mentioned variables to stress outcomes. Difficulties in assessing such conditions pertain to increased aggressivity that may arise through constant unstable housing. In our study, although no bites were observed during the first crowding session, indicating that female rats do not initially resort to overt aggression when introduced to new social partners, biting incidents occurred later in SIS rats, suggesting that aggression emerges with repeated social disruption.

Additionally, the collection of CORT samples immediately pre-post home cage recordings would have allowed a better evaluation of the influence of specific social interactions on the stress response. Similarly, future projects could consider performing behavioural recordings on the same day as euthanasia, to allow more detailed analysis of how frequency and direction of behaviour might influence outcomes. Other methodological considerations, such as the recording of ultrasonic vocalizations or the use of automated scoring, might allow a greater depth of information or longer assessment periods, and could be considered in future assessments. Of note, however, manual scoring remains more prevalent both for social behaviour

scoring and for group housed assessments (Kahnau et al., 2023), as it may allow detection of subtle behaviour changes or abnormalities that may be missed by automated processes.

5. Conclusion

This study shows that female rats display unique social dynamics when exposed to HDH and SIS housing conditions. Importantly, we find that although both social instability and crowding elevated stress levels over time and disrupted certain aspects of reproductive function, their trajectories diverged, suggesting that impacts of stress may depend as much on the social experience of the environment as on the stressor itself. Interestingly, our findings show that, considered in isolation, player type does not account for the variation in biochemical outcomes. Such observation indicates that beyond observable dominance or submission relationships inferred through play fighting, other possibly more subtle factors may play a decisive role in shaping physiological responses under crowding stress. As female social behaviour remains vastly understudied, particularly in group-housed stress models, this work underscores the importance of examining how everyday interactions within the home cage influence stress and reproductive impairment. Further research should examine the complexities of social dynamics and hierarchy formation in females, as stress outcomes do not solely depend on the applied housing conditions, but also on the way individuals navigate social relationships.

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7. Declarations of Interest

The authors have no conflicts of interest to declare.

8. CRediT Authorship Contribution Statement

M.P.: Conceptualization, Methodology, Project Administration, Investigation, Formal Analysis, Data curation, Writing – Original draft. É.A.O.: Investigation, Writing – Original Draft. S.M.P.: Methodology, Writing – Review and Editing. H.P.: Conceptualization, Supervision, Writing – Review and Editing.

9. Data Availability Statement

Data will be made available upon reasonable request.

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Summary of Results

This thesis contributes to our understanding of distinctive impacts of various types of stress on female reproductive function. When systematically reviewing the link between psychological stress and abnormal menstrual parameters in adult women, **Chapter I** found the majority of the 41 included studies to report an association between psychological stress and menstrual dysfunction, with the most common disruptions being irregular menstruation and abnormal menstrual flow (Poitras, Shearзад, et al., 2024). Similarly, systematically reviewing the impact of psychological stress models on rodent estrous cyclicity, as performed in **Chapter II**, revealed that over half of the 32 included papers (62.5% of studies) found an adverse effect of psychological stress models on the rodent estrous cycle, and showed altered E2 levels and elevated basal CORT often occurring in tandem with such cycle disruptions (Poitras, Lebeau & Plamondon, 2024). Importantly, both reviews highlighted several gaps in the literature and methodological limitations in the available databases. In **Chapter III**, we observed persistent diestrus in 87.5% of rats having undergone a systemic stress via global cerebral ischemia, which lasted on average 11 days, while dysregulation to HPG axis neuroendocrine function was noted 25 days post ischemia, through reduced Kiss1, paralleled by heightened expression of ER α and GnIH (Poitras, Morin, et al., 2024). See Table 1 for summary of results and region-specific alterations. In **Chapter IV**, our data uncovered distinct impairment profile following social stress in rats, with the isolation condition leading to increased hopping behaviours, lower uterine horn weight, decreased early follicle counts, and elevated FSH levels. Exposure to crowding reduced GnIH and ER α immunoreactivity, in addition to decreasing days spent in proestrus and increasing hopping behaviours. Social instability led to reductions in estrous cyclicity, uterine horn weight, and ovarian reserve (Poitras, Labonté, et al., 2025). Finally, in **Chapter V**, a

comprehensive behavioural analysis indicated diverging patterns of play fighting in crowded and social instability exposed rats. Additionally, high and low playing rats differed significantly in their adrenal gland weights and days spent in estrus (Poitras, Ouellette, et al., 2025).

Comprehensively, this thesis supports distinct reproductive impairment profiles in adult females following exposure to different stressors.

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Table 1. Summary of Changes in Reproductive Function Across Stressors.

Outcome Measure	GCI (Chapter III)	Isolation (Chapter IV)	Crowding (Chapter IV)	Instability (Chapter IV)
Behavioural and Physiological				
Estrous cyclicity	↓	=	↓	↓
Sexual behaviours				
<i>Paracopulatory behaviours (hop)</i>	N/A	↑	↑	=
<i>Lordosis parameters</i>	N/A	=	=	=
<i>Sexual Motivation</i>	N/A	↑	=	=
Fertility	N/A	=	=	=
Hypothalamic				
GnIH (RFRP-3)				
<i>ARC</i>	=	↓	↓	=
<i>DMH</i>	↑	=	↓	=
<i>POA</i>	=	N/A	N/A	N/A
Kiss1				
<i>ARC</i>	↓	=	=	=
<i>AVPV</i>	N/A	=	=	=
<i>POA</i>	↓	N/A	N/A	N/A
ERα				
<i>ARC</i>	↑	=	=	=
<i>AVPV</i>	N/A	=	↓	=
<i>POA</i>	=	N/A	N/A	N/A
Pituitary				
LH	N/A	=	=	=
FSH	N/A	↑	=	=
Ovaries and Uterine Horn				
E2	N/A	=	=	=
P4	N/A	=	=	=
Ovarian weight	=	N/A	N/A	N/A
Ovarian follicular count	N/A	↓	=	↓
Uterine weight	N/A	↓	=	↓

Note. All differences are relative to controls from each study. For Chapter IV, differences from control housed and regular housed groups are both considered. ARC: Arcuate nucleus; AVPV: anteroventral periventricular nucleus; DMH: Dorsomedial nucleus; E2: estradiol; ERα: Estrogen receptor alpha; FSH: Follicle-stimulating hormone; GCI: Global cerebral ischemia; GnIH: Gonadotropin-inhibitory hormone; Kiss1: Kisspeptin; LH: Luteinizing hormone; N/A: Not applicable; P4: Progesterone; POA: Preoptic nucleus; RFRP-3; RFamide-related peptide.

General Discussion

The overarching objective of this thesis was to further our understanding of the link between stress and female reproductive function, with a particular focus on how various stress models might differ in their impacts. As such, we opted to investigate two stressor types which have historically been understudied in female rodents, namely systemic stress induced through a global ischemic insult, and social stress via isolation, crowding, or instability. The inclusion of two systematic reviews allowed to comment on the existing literature and remark on distinct consequences of psychological stress in both rodents and humans. In discussing collected findings from this thesis, some aspects appear most relevant, notably the clear reproductive impairment profiles which emerged following the included stress models.

1. From Rodent Studies to Clinical Populations: Stress and Ovarian Cycling

The first two studies of this thesis aimed to summarize and contextualize our current understanding of the link between psychological stress and ovarian cycling in women and rodents, respectively. In both reviews, most of the included studies reported a significant association between stress and disruptions to regular cycling (92.7% and 62.5% of studies, respectively). Of note, abnormal menstrual function appeared as the primary focus in most studies included in **Chapter I**, with all but six mentioning menstruation directly in the publication title. Conversely, in **Chapter II**, only four studies included the estrous cycle in their title, suggesting it may often represent a secondary variable in a broader assessment of reproductive function, which might contribute to its inclusion in publications even in the absence of significant effects of stress. As such, the lower frequency at which altered cyclicity is reported in published literature from rodent vs. human research could relate somewhat to publication bias in the latter. Importantly, considerable variability or lack of detail when defining normal cycling

was noted in both reviews, emphasizing a continued need for high-quality studies evaluating stress and reproductive cycles. Of particular interest for future rodent studies would be to evaluate enduring effects of stress on cyclicity (i.e., recovery following the end of the stress period), which was infrequently assessed in the current database (See **Chapter II**). Indeed, adulthood stress is often considered more transient in nature, its effects being more labile and reversible compared to stressor exposure occurring during critical development periods (e.g., gestation, neonatal, pubertal), which have shown enduring effects that differ depending on the targeted period (Chaby et al., 2017; Cotella et al., 2019). Nevertheless, long-lasting consequences of adulthood stress on reproduction have been reported, with estrous cyclicity remaining altered in the weeks following the end of a chronic stressor (See **Chapter II**; Baker et al., 2006; Divyashree & Yajurvedi, 2018; Kala & Nivsarkar, 2016; Sun et al., 2017). In human research, a need is felt not only for studies assessing menstrual function following stress (such as recovery from academic stress after graduation), but for greater attention to the duration of experienced stress. Indeed, very few studies have attempted to selectively characterize the contribution of duration of stressor exposure, considered as a distinctive variable, to the experiences of abnormal menstrual function. Such assessments could promote a more complete understanding of the consequences of stress.

In **Chapter III** and **Chapter IV**, exposure to global ischemia, social instability, or social crowding had distinctive effects on estrous cyclicity, the former systemic stressor inducing a persistent diestrus state. Reflective of the intensity of this stressor, observed impairments were more pronounced than is typically reported during chronic stressors such as cold water immersion and CUMS paradigms, which typically induce lengthening of the estrous cycle through increased diestrus days, without complete acyclicity (Casillas et al., 2021; Fu et al.,

2018). One factor that may contribute to a greater impact of GCI on estrous cyclicity, beyond dysregulation of HPA axis signalling, is post-ischemic weight loss (Faria et al., 2011; Nagaya et al., 2001). In comparison, the impact of social stressors on estrous cyclicity is weaker, leading to reduced number of days in proestrus without alterations in estrus or diestrus days (**Chapter IV**). This could support social stressors being of reduced intensity relative to forced swimming or CUMS paradigms (Atrooz et al., 2021), a proposition aligned with subtle contributions of dominant-subordinate relationships observed in females exposed to crowding and instability (**Chapter V**). Notably, a number of studies evaluating social defeat stress in mice and rats have also failed to detect significant effects on estrous cycling (Newman et al., 2019; Shimamoto et al., 2015; Yohn et al., 2019). Of interest, while most studies reviewed in **Chapter I** reported an association between abnormal menstruation and stress, none observed abnormal menstrual cycling in all participants reporting high perceived stress (average prevalence of irregular menstruation in stress-exposed participants = 36.5%). Such prevalence contrasts rodent studies using psychological stress models such as CUMS, which found irregular estrous cycles in most if not all stress-exposed rodents (91.67% to 100% of rodents; Baker et al., 2006; Mattioli et al., 2009; Sun et al., 2017). With the proposition that social stressors may present greater ecological validity (Lyons et al., 2023) and therefore increased translational potential to human populations (Brunton, 2013; Gądek-Michalska et al., 2016), the lower frequency of irregular cycles following social stress in **Chapter IV** might be more representative of disruption prevalence in humans. Indeed, when looking at prevalence of irregular cycles across the three stress groups at the end of the stress period (HDH, LDH, and SIS), 31.94% of rats were irregularly cycling, further emphasizing the need for a greater focus on social stress-induced reproductive impairments.

It is noteworthy that while not reaching significance, LDH exposure in **Chapter IV** decreased estrous cycling over time (trend for more proestrus days at baseline compared to S1 [$p = .093$] and S2 [$p = .065$]), a surprising contrast to the regular cycles exhibited at baseline in **Chapter III**, for which all rats were single housed. This highlights the limitation of excluding irregularly cycling rodents at baseline, as discussed in **Chapter II**. Indeed, when single housing is initiated at arrival, particularly sensitive individuals would be excluded during baseline assessment, allowing all others to maintain cyclicity for the remainder of the protocol (See sham data, **Chapter III**). In fact, Baker and Bielajew (2007) have previously observed that single housing had a greater effect on cyclicity than their CMS protocol, further emphasizing how seemingly innocuous decisions about housing can impact experimental outcomes. Inclusion of additional manipulations, such as slightly more frequent cage changes (twice vs. once weekly) and collection of blood samples throughout the study period, could have contributed to altering cycling in LDH rats (**Chapter IV**). Importantly, characterization of estrous cyclicity differed between **Chapter III** and **Chapter IV**, with a more detailed approach having been employed in the latter. In **Chapter III**, we opted to focus on the most relevant characteristic in the experimental group (i.e., persistent diestrus), meaning subtle effects of sham surgery may have been missed. Indeed, while all sham rats continued to enter estrus every 4 days, meeting the criteria for regular cycling established for the study, there was a slight decrease in proestrus days post-surgery (See [Appendix C](#), Table C1), suggesting minor surgical procedures might result in skipped or delayed entry into proestrus.

One notable remaining gap in the human and rodent literature pertains to the contribution of individual differences to the impact of stress on ovarian cycling. Over the years, abundant evidence has highlighted individual-to-individual differences in stress reactivity and sociability

(Bales et al., 2023; Sgoifo et al., 2014). When adults are exposed to time pressure in their working environments, individuals with greater reactivity to such constraints (measured via heart rate variability) are more likely to display emotional exhaustion (Schmid et al., 2024). In rodents, baseline differences in freeze response of female rats predict outcomes on a variety of anxiety- and depressive-like behaviour assessments (Smiley et al., 2025). Individual differences decidedly contribute to altering estrous cyclicity post-stress, as not all rats display changes to estrous cycling for the same duration or at the same time during stress exposure (See **Chapter III** and **Chapter IV**). While we found some effects of being a high vs. low player on adrenal weights and estrous cycling in **Chapter V**, many other biochemicals were unaffected by this parameter, despite significant differences between SIS and HDH rats. Of interest, increasing research focuses on the mechanisms underlying social homeostasis, representing the process through which social animals detect, compare, and respond to changes in the quality/quantity of social interactions relative to a ‘set point’ (determined by factors such as sex, species, personal history, etc.; Matthews & Tye, 2019). When deviations from the homeostatic set point are prolonged, a new set point might be established, allowing the animal to adapt to a new expected quantity of social contact (Bales et al., 2023). Such research has shown a rise in pro-social behaviours following an acute period of isolation, whereas prolonged isolation increases aggressive and agonistic behaviours (Lee et al., 2021). This presents interesting parallels to the differences in sociability observed between HDH and SIS exposed rats, with only the latter presenting instances of biting (**Chapter V**), possibly indicative of greater difficulty in regaining homeostatic set point following repeated isolation and unstable housing. While more subjective than quantity, consideration of the quality of interactions is of interest, as it is more likely to be influenced by personal histories, social hierarchy, dominance relationships (Shemesh et al.,

2013). A deeper understanding of individual-level factors which can influence the outcomes of stress paradigms would favour not only greater and more accurate characterization of their consequences but could allow personalization of interventions aiming to reverse stress-induced disruptions to ovarian cycling.

2. Systemic and Social Stressors Have Distinct Consequences on HPG Axis Function

Concordant with research showing increased GnIH expression following exposure to immune (Iwasa et al., 2014) or psychological stress (Geraghty et al., 2015; Kirby et al., 2009), adulthood GCI heightened GnIH-ir in the DMH and the CA1 (**Chapter III**), the latter of which is highly vulnerable to ischemic insults (Neumann et al., 2013). In contrast, social stress in the form of LDH and HDH unexpectedly suppressed GnIH in the ARC, with HDH also reducing levels in the DMH (See **Chapter IV** discussion). GnIH neuron activation has been found to be linked with sociability and aggressivity (Jennings et al., 2016; Teo et al., 2017), as well as anxiety-like behaviours (Kaewwongse et al., 2011), emphasizing possible unique interplay between social stress and GnIH function.

On the other hand, Kiss1 signalling responses were more closely aligned with those reported following other types of chronically experienced stressors. Genetic knockdown studies in mice have supported ARC-Kiss1 function to be essential for estrous cyclicity (Wang et al., 2019). Accordingly, reduced Kiss1-ir in this region was observed following GCI (**Chapter III**). Interestingly, HDH rats, which presented fewer proestrus days in the second half of the stress period showed no such alterations (**Chapter IV**). Of note, all GCI rats had recovered cyclicity by biochemical sample collection at post-ischemic day 24, indicating that while ischemia induces lasting changes to HPG axis hypothalamic signalling, impairments were not so great as to wholly prevent function. Lesion studies of the ARC have been reported to generate near complete

interruption of cyclicity (Abbasi et al., 2025), whereas stress-induced alterations of essential signalling molecules, such as Kiss1, are often paralleled by irregularity rather than acyclicity (Huang et al., 2022; Retana-Márquez et al., 2020). Such contention is supported by the observation that differences in Kiss1-ir in **Chapter IV** did not reach statistical significance ($p = .052$ and $p = .066$ in the AVPV and ARC, respectively), and cyclicity was concordantly not significantly reduced at the end of the stress period. Characterization of hypothalamic signalling molecules earlier in the stress or post-ischemic period, occurring while cyclicity is impaired, might allow greater insight into such HPG axis dysregulation.

Previous literature has found that pituitary hormone levels can be significantly decreased by persistent HPA axis activation, with both FSH and LH being reduced after stress exposure (Fotsing et al., 2017; Huang et al., 2022; Medubi et al., 2017; Retana-Márquez et al., 2020). However, stress does not always inhibit secretion, with some studies reporting increased LH or FSH (**Chapter II**; Fu et al., 2018; Xu et al., 2020), concordant with higher FSH in rats following 21 days of isolation (**Chapter IV**). The presence of compensatory mechanisms, leading to normal or elevated hormone levels, seems likely. Therefore, the absence of detectable changes to pituitary hormones in SIS rats was surprising (**Chapter IV**), as it contrasts increased LH noted following 28 days of SIS exposure (Herzog et al., 2009). It has been suggested that stress contributes more strongly to disrupting the timing of the LH surge, rather than its amplitude (Wagenmaker & Moenter, 2017). Indeed, much of the literature reviewed in **Chapter II** failed to detect significant changes to this hormone (Breen et al., 2012; Nair et al., 2021; Sun et al., 2017), with one study reporting decreased LH restricted to the estrus phase (Medubi et al., 2017). While targeted inhibition of upstream HPG axis signalling (such as Kiss1 antagonism; Sahin et al., 2015; Smith et al., 2011) or activation of HPA axis regulators (such as CRH-PVN agonism: Yu

et al., 2023) may have a more direct impact on LH and FSH secretion, the effects of chronic stressors are more nuanced. Time-dependent changes are presumed to occur, with Fu et al. (2018) finding minimal impacts of 14 days of CMS, but significant alterations in both E2 and FSH after 35 days of exposure. Moreover, E2 and testosterone levels following a combined restraint and forced swim stress protocol were also time-bound, with few changes being detectable at 28 days compared to more distant collection timepoints (56 or 84 days; Divyashree & Yajurvedi, 2016, 2018). Future studies should consider multiple assessments throughout stress exposure and a subsequent recovery period, to better characterize the evolution of pituitary and ovarian hormones during stress. Indeed, considering differing estrous cycle disruption profiles in HDH and SIS rats (**Chapter IV**), which were distinct from the persistent diestrus induced by GCI (**Chapter III**), it seems likely that acute and longer term HPG axis dysfunctions are uniquely mediated by internal and external experiences.

The effects of prolonged exposure to psychological stressors on gonadal hormones have been inconsistent, with the literature reviewed in **Chapter II** suggesting time-related changes in E2 concentrations post-stress. For instance, significantly increased E2 levels after 28 days of SIS in female Wistar rats (Herzog et al., 2009) supports the possibility that this hormone might be highly variable over the course of a stressor, considering that 21 days of SIS, LDH, or HDH failed to alter E2 (**Chapter IV**). Within the ovary, theca cells are primarily responsible for the production of androgens, which are then converted into estrogens by aromatase cytochrome P450 and 17 β -hydroxysteroid dehydrogenase produced in the granulosa cells (Magoffin, 2005). As such, reductions to early follicle counts in SIS and LDH rats could have been expected to lead to altered E2. However, with E2 synthesis also occurring in late-stage follicles and elsewhere in the body (e.g., brain, bones, and adipose tissue; Simpson, 2003), it seems likely that

compensatory mechanisms allowed maintenance of normal E2 production at the 21-day post social stress interval. Interestingly, in mice, crowding has been found to diminish the ovarian reserve (Kim & You, 2022), while ranking within the social hierarchy alters ER α and ER β expression (Williamson et al., 2019). The absence of effects of player type on most biochemical variables in **Chapter V** suggests differing influences of crowding and the underlying social dynamics in rats compared to mice. Nonetheless, both HDH and SIS exerted important effects on reproductive functioning, highlighting a greater need for detailed assessments of the contributions of home cage dynamics on the outcomes of social stress in female rats. In this context, assessing ovarian structure and function in more detail is of particular interest.

In a study evaluating the consequences of long-term isolated housing, Hermes & McClintock (2008) found middle-aged rats (15 months old) to display decreased preovulatory follicles and corpora lutea. With exposure to isolation in young adult rats (aged 3 months) reducing early follicular counts but leaving later stages of folliculogenesis intact (**Chapter IV**), such findings suggest a shift towards late-stage impairments in folliculogenesis when isolation is maintained into late adulthood. Hyperactivation of FSH secretion processes (**Chapter IV**) could ultimately result in FSH receptor internalization at the ovarian level, impairing the later stages of follicular development which are gonadotropin dependent (Gougeon, 2010). Assessments of the relationship between isolation and reproduction throughout adulthood are much needed. This is ecologically relevant as loneliness is an increasing epidemic in young adults (Buecker et al., 2021; Fardghassemi & Joffe, 2021; Pop et al., 2022), which is known to continue into older adulthood (Courtin & Knapp, 2017; Paquet et al., 2023).

3. Stress and Sexual Functioning

Previous studies have reported reduced paracopulatory behaviours or altered lordosis following physical stress induced in naturally cycling rats (Retana-Márquez et al., 2020) or psychological stress in ovariectomized mice and rats (Kercmar et al., 2014; Miryala et al., 2011). Contrastingly, the selected social stressors in **Chapter IV** showed no effects on such sexual responses. In fact, both social isolation and crowding increased paracopulatory (hopping) behaviours, with isolation also increasing time spent with the male, likely relating to differing motivational underpinnings (See **Chapter IV** discussion). Without significant changes to P4 and E2, which are critical for proper lordosis (Corlett et al., 2022; Hernández-Munive et al., 2018), it seems likely that the selected social stressors were not of sufficient intensity (or duration) to reduce sexual functioning itself, rather impacting the more ‘social’ components involved in copulation for rodents. Of note, in male rats, social isolation has been found to increase sexual aggression (Ngala et al., 2025). However, with females being generally more pro-social than males in many species (Christov-Moore et al., 2014), it appears logical that effects of social stress could be nuanced (i.e., not wholly ‘good’ or ‘bad’), further highlighting the complex consequences of social stress models. Indeed, females exposed to social instability exhibit fewer aggressive behaviours towards female intruders (Pittet et al., 2017), and crowding stress has been suggested to have a calming effect compared to isolation in females (Brown & Grunberg, 1995), which could have contributed to decreased GnIH-ir in HDH rats (**Chapter IV**). Isolation in mice induces hyperactivity and increased exploration (Sullens et al., 2021), which could heighten drive to spend time with the male (**Chapter IV**). In rats, HDH and SIS exerted distinct effects on play fighting, and distinctive behavioural profiles emerged between the high and low players (**Chapter V**). Although player type showed minimal impact on biochemical measures, it seems

likely that position in the social hierarchy could influence responses during sexual behaviour testing, as previous studies show that dominant males and females in colonies have higher access to mates and greater reproductive success (McCormick et al., 2017; Murlanova et al., 2022). The low number of rats included in sexual behaviour testing prevented the creation of player subgroups to evaluate this variable in **Chapter V**, but such contributions are worthwhile to consider in future assessments.

The impact of adulthood GCI on sexual behaviours was not evaluated in **Chapter III**. However, evidence from clinical and preclinical research suggest lasting consequences of CVD and ischemia on sexual functioning and libido. In women having suffered myocardial infarction, decreased frequency of intercourse and dissatisfaction with sexual relationship are both commonly reported, along with anxiety surrounding resuming sexual activities (Emami Zeydi et al., 2016). To our knowledge, no studies have assessed the impact of ischemia induced in adulthood on female rodent sexual behaviours. In female rats, however, double perinatal stress via hypoxic-ischemic insult and early maternal separation led to significant decreases in paracopulatory behaviours and lordosis quotient (Ferraz et al., 2023). Conversely, prenatal hypoxia in male rats reduced the latency to first mount and first intromission, increased copulation, and heightened the frequency of homosexual mounting (Ferraz et al., 2015), suggesting hypersexual responses following prenatal ischemia in males. With early developmental phases being critical to organizational and activational effects of sex hormones (Schulz & Sisk, 2016), it is difficult to ascertain if adulthood ischemic insults would have similar impacts. Of note, adulthood GCI in females has been found to promote anxiety- and depressive-like behaviours (Cheng et al., 2013; Panta et al., 2019; Poitras, Doiron, et al., 2024), which could interfere with motivationally driven sexual responses. To this effect, ER α overexpression (Tomic

et al., 2007) and Kiss1-knockout (Hellier et al., 2018) respectively reduce fertility and impair lordosis in female mice. Thus, considering persistent alterations to HPG axis signalling in ischemic versus sham female rats four weeks after injury (**Chapter III**), sexual functioning in the aftermath of adulthood GCI would benefit from further investigation. Future studies evaluating sexual behaviours at multiple timepoints following adulthood GCI would foster a better understanding of possible enduring impacts of this systemic stressor on sexual dysfunction.

4. Women's Reproductive Health: Beyond the Production of Offspring

It is well acknowledged that female health has historically been understudied in preclinical and clinical research (Moley, 2024). Namely, analysis of health research funding in the United States finds that when disease burden primarily affects one gender, funding patterns favour males, either through overfunding of male dominant diseases or underfunding of women-prevalent diseases, such as anxiety and other mental illnesses (Mirin, 2021). Furthermore, obtaining diagnoses for gynecological diseases remains difficult, with the average time between initial endometriosis symptom presentation and formal diagnosis being nearly eight years (Wren & Mercer, 2022). A large contributor delaying gynecological disease diagnosis in individuals who menstruate relates to dismissal of concerns, with painful or abnormal menstruation being minimized or waved off as 'normal' by medical practitioners (Berkley & McAllister, 2011; Ellis et al., 2023). This issue is compounded by recent evidence from a large UK study showing that many women fail to accurately describe their cycle characteristics, highlighting persistent gaps in menstrual awareness (Ali et al., 2026). Further disregard of reproductive issues can occur when patients do not express an immediate interest in getting pregnant. However, as previously discussed, regular menstruation is considered to be reflective of overall health in adolescents and

adults (American Academy of Pediatrics Committee on Adolescence et al., 2006; Minakshi et al., 2022). Irregular menstruation is of concern due to its role as a possible indicator of gynecological disease or subfertility, but also as a parameter which in-and-of-itself exerts a profound influence on daily function and quality of life (Bijloo et al., 2026; Fahs, 2020). Indeed, fluctuations of a large variety of emotional, cognitive, and physiological processes are observed over the course of the menstrual cycle, affecting emotion regulation, pain perception, and sleep processes (Iacovides et al., 2015; Ossewaarde et al., 2010; Rugvedh et al., 2023; Wu et al., 2014). In addition, concerns about abnormal menstruation can increase stress, a vicious cycle which can contribute to further disruptions of reproductive function. A greater focus undeniably needs to be afforded to educating the general population and health care providers on the relevance of the menstrual cycle as an indicator of health in women and people who menstruate.

5. Limitations and Future Considerations

While the most notable limitations of this thesis have been discussed within the studies themselves (See Discussion sections), a few additional considerations are noteworthy. Vaginal lavages have been reported to increase stress in mice (Varol et al., 2022) and might even decrease ARC-Kiss1 neuron synchronization event frequency for up to 3 h post lavage (Vas et al., 2024). In **Chapter IV**, we observed no increase in basal CORT levels after two weeks of vaginal lavages, suggesting that habituation and careful collection by well-trained experimenters minimize the possible stress induced by this technique. Cautious collection of vaginal samples also reduces the risk of pseudopregnancy, which is characterized by a minimum of 14 consecutive days in diestrus (Kaneko et al., 2020) and can occur following cervical stimulation in rodents (McLean et al., 2012). Seeing as no pseudopregnancy was noted in sham rats from **Chapter III** or any of the rats from **Chapter IV**, it is unlikely to have contributed to the

observed cycle disruptions in GCI rats. While visual assessment of the appearance of the vagina has been proposed as a method for evaluating the estrous cycle stage, based on criteria such as coloration, moisture levels, and degree of opening, this technique has primarily been described in mice, and vaginal smears remain the most reliable technique readily available (Ajayi & Akhigbe, 2020).

In **Chapter IV**, due to stress-induced reductions in proestrus days, the selection of rats in specific estrous cycle stages may have had the unintended consequence of limiting the inclusion of most stressed females in the proestrus subgroups (biochemical variables and sexual behaviour testing). This selection criteria may therefore have altered the ability to detect effects of social stress on sexual functioning. Indeed, sexual dysfunction may have been more readily detectable in rats with reduced proestrus days, as these rats might be most vulnerable to stress-induced reproductive dysfunction, but would be less likely to be included in sexual behaviour testing due to failure to enter proestrus at the expected times. This could have particularly occurred in the crowding and SIS conditions where decreased days in proestrus were more commonly observed. Similarly, any rats which displayed reproductive disruptions through a lengthening of the estrus phase would have been less likely to be included in biochemical analyses. Such considerations are critical when opting for a study design which limits the number of estrous cycle stages assessed.

Although efforts were made to comprehensively characterize HPG axis function, constraints in time and resource availability prevented the inclusion of some markers of potential interest. Of note, despite estrous cyclicity being primarily dependent on ER α (Wintermantel et al., 2006), other estrogen receptor types also contribute to E2 signalling (ER β and G protein-coupled estrogen receptor; GPER). ER β antagonism in emotional centres such as the amygdala

has uncovered its role in regulating the adverse behavioural and neuronal consequences of vicarious social stress in female rats (Smiley et al., 2023). Research also shows subfertility in ER β -knockout mice (Krege et al., 1998), although these reproductive phenotypes are less pronounced than in ER α knockouts (Prossnitz & Hathaway, 2015). On the other hand, the role of GPER in regulating reproductive function is minimal in knockout mice (Prossnitz & Hathaway, 2015), although its influence on anxiety and stress responses is supported (Kastenberger & Schwarzer, 2014), making it of possible interest for future studies of stress and reproduction. The consideration of PRL, which plays an inhibitory role in the HPG axis by suppressing GnRH (Levine & Muneyyirci-Delale, 2018), would also be of interest. Research has suggested a close link between stress and PRL secretion, as male rats with induced hypoprolactinemia or hyperprolactinemia both displayed altered HPA axis responses to acute restraint (Jaroenporn et al., 2007). Furthermore, while increasing evidence finds Kiss1 to be critical in acting as the pulse generator within the HPG axis, GnRH signalling is of significant interest (Dungan et al., 2006; Herbison, 2018; Martinez-Corral & Prevot, 2021), although reliable detection of this molecule via immunofluorescence remains difficult. Additionally, assessment of neurotransmitters such as serotonin and dopamine is of interest when evaluating social behaviours and reward processing, with research showing significant correlations between these signalling molecules and altered post-stress sociability (De Michelis Mograbi et al., 2025). More thorough assessments of HPG axis function following adult GCI in females, including pituitary and gonadal hormone measurements, are particularly essential to furthering our understanding of reproductive dysfunction related to potent systemic stressors, including but not restricted to stroke or cerebral ischemia. Other systemic stressors, which are more common in females of reproductive age, such as immune stress, are also of interest for future projects.

Concluding Remarks

Considering the rise in anxiety prevalence reported in the American adult population (50 years and younger; Goodwin et al., 2020), a clear understanding of the impact of adulthood stressors on HPG axis function is essential, as it may have crucial implications for fertility, reproduction, and overall wellbeing. Particularly, with women having an elevated risk of stress-related psychopathology, including anxiety and depression (Bangasser & Valentino, 2014; Helpman, 2023), characterizing how stress impacts female reproductive functioning is of societal interest. Knowing that different physical stressors have varied behavioural and physiological consequences in males (Atrooz et al., 2021), this thesis characterized reproductive impairments following various types of stress and significantly contributes to understanding distinct stress-induced reproductive impairment profiles in females.

It is estimated that 1 in 6 people will experience infertility at some point in their lifetime (World Health Organization, 2023). This prevalence further supports the significant clinical value of studying underlying mechanisms of stress-related alterations to HPG axis functioning. However, the implications of this research reach beyond the clinical population, notably also being of relevance to the reproductive success of endangered species and the production of livestock that provide essential resources for human survival (Ralph et al., 2016). With rapid population growth and increasing environmental changes caused by the rising climate crisis, understanding the impact of stress on reproductive function is essential. Consequently, the outcomes of this thesis further our knowledge of stress-induced HPG axis dysfunction from a wide variety of stressors, improving its applicability to a wider range of human experiences. Ultimately, these findings highlight the unique impact of various stressors on female

reproductive health, with the hope that such knowledge will promote initiatives aiming to help women cope with stress.

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† indicates studies included in the systematic review from Chapter I

‡ indicates studies included in the systematic review from Chapter II

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Supplementary Materials

Appendix A (Chapter I)

A1. Systematic Review Search Strategy

APA Psychinfo (Ovid)

- 1 menstruation/ or menstrual cycle/ or reproductive health/
- 2 menstrual disorders/ or amenorrhea/ or dysmenorrhea/
- 3 (amenorrhea or anovulation or hypomenorrhea or hypermenorrhea or menstua* or menorrhagia or oligomenorrhea or polymenorrhea).ti,ab.
- 4 Minority Stress/ or Chronic Stress/ or Occupational Stress/ or Perceived Stress/ or Social Stress/ or Stress/ or Acute Stress/ or Academic Stress/ or Psychological Stress/ or Environmental Stress/
- 5 caregiver burden/ or financial strain/ or trauma/ or crises/ or disasters/ or distress/ or family crises/ or natural disasters/ or organizational crises/ or homeless/ or housing/
- 6 ((academic or caregiv* or emotion* or financial or job or occupational or work or life or minority or interpersonal or family or marital or pandemic or psycho* or social or environment* or perceiv*) adj4 (strain* or pressure* or burden* or crisis or crises)).ti,ab.
- 7 (war* or homeless* or incarcerat* or "job loss*" or unemploy* or distress or stress* or "natural disasters" or displacement or housing).ti,ab.
- 8 1 or 2 or 3
- 9 4 or 5 or 6 or 7
- 10 8 and 9

Obtained references: 1862

Medline (Ovid)

- 1 menstrual cycle/ or menstruation/
- 2 menstruation disturbances/ or amenorrhea/ or menorrhagia/ or oligomenorrhea/
- 3 (amenorrhea or anovulation or hypomenorrhea or hypermenorrhea or menstua* or menorrhagia or oligomenorrhea or polymenorrhea).ti,ab,kf.
- 4 stress, psychological/ or caregiver burden/ or financial stress/ or occupational stress/
- 5 ((academic or caregiv* or emotion* or financial or job or occupational or work or life or minority or interpersonal or family or marital or pandemic or psycho* or social or environment* or perceiv*) adj4 (strain* or pressure* or burden* or crisis or crises)).ti,ab,kf.
- 6 (war* or homeless* or incarcerat* or "job loss*" or unemploy* or distress or stress* or "natural disasters" or displacement).ti,ab,kf.
- 7 1 or 2 or 3
- 8 4 or 5 or 6
- 9 7 and 8

Obtained references: 5397

Embase (Ovid)

- 1 menstruation/ or menstrual cycle/
- 2 menstruation disorder/ or "amenorrhea and oligomenorrhea"/ or menstrual related disorder/
- 3 (amenorrhea or anovulation or hypomenorrhea or hypermenorrhea or menstrua* or menorrhagia or oligomenorrhea or polymenorrhea).ti,ab,kf.
- 4 mental stress/ or caregiver burden/ or chronic unpredictable stress/ or cognitive stress/ or emotional stress/ or family stress/ or home stress/ or interpersonal stress/ or job stress/ or life stress/ or parental stress/ or school stress/ or social stress/
- 5 ((academic or caregiv* or emotion* or financial or job or occupational or work or life or minority or interpersonal or family or marital or pandemic or psycho* or social or environment* or perceiv*) adj4 (strain* or pressure* or burden* or crisis or crises)).ti,ab,kf.
- 6 (war* or homeless* or incarcerat* or "job loss*" or unemploy* or distress or stress* or "natural disasters" or displacement).ti,ab,kf.
- 7 1 or 2 or 3
- 8 4 or 5 or 6
- 9 7 and 8

Obtained references: 8858

Cochrane CENTRAL (Ovid)

- 1 menstruation/ or menstrual cycle/ or reproductive health/ or menstrual disorders/ or amenorrhea/ or dysmenorrhea/
- 2 (amenorrhea or anovulation or hypomenorrhea or hypermenorrhea or menstrua* or menorrhagia or oligomenorrhea or polymenorrhea).ti,ab.
- 3 Minority Stress/ or Chronic Stress/ or Occupational Stress/ or Perceived Stress/ or Social Stress/ or Stress/ or Acute Stress/ or Academic Stress/ or Psychological Stress/ or Environmental Stress/ or caregiver burden/ or financial strain/ or trauma/ or crises/ or disasters/ or distress/ or family crises/ or natural disasters/ or organizational crises/ or homeless/ or housing/
- 4 ((academic or caregiv* or emotion* or financial or job or occupational or work or life or minority or interpersonal or family or marital or pandemic or psycho* or social or environment* or perceiv*) adj4 (strain* or pressure* or burden* or crisis or crises)).ti,ab.
- 5 (war* or homeless* or incarcerat* or "job loss*" or unemploy* or distress or stress* or "natural disasters" or displacement or housing).ti,ab.
- 6 1 or 2
- 7 3 or 4 or 5
- 8 6 and 7

Obtained references: 1251

CINAHL (Ebsco)

- 1 (MH "Menstrual Cycle") OR (MH "Menstruation")
- 2 (MH "Menstruation Disorders+")
- 3 (MH "Reproductive History") OR (MH "Reproductive Health")
- 4 TI(amenorrhea or anovulation or hypomenorrhea or hypermenorrhea or menstrua* or menorrhagia or oligomenorrhea or polymenorrhea) OR AB(amenorrhea or anovulation or

- hypomenorrhea or hypermenorrhea or menstrua* or menorrhagia or oligomenorrhea or polymenorrhea)
- 5 (MH "Stress+")
- 6 (MH "Natural Disasters") OR (MH "Homelessness") OR (MH "Housing")
- 7 TI((academic or caregiv* or emotion* or financial or job or occupational or work or life or minority or interpersonal or family or marital or pandemic or psycho* or social or environment* or perceiv*) N4 (strain* or pressure* or burden* or crisis or crises)) OR AB((academic or caregiv* or emotion* or financial or job or occupational or work or life or minority or interpersonal or family or marital or pandemic or psycho* or social or environment* or perceiv*) N4 (strain* or pressure* or burden* or crisis or crises))
- 8 TI(war* or homeless* or incarcerat* or "job loss*" or unemploy* or distress or stress* or "natural disasters" or displacement or housing) OR AB(war* or homeless* or incarcerat* or "job loss*" or unemploy* or distress or stress* or "natural disasters" or displacement or housing)
- 9 S1 OR S2 OR S3 OR S4
- 10 S5 OR S6 OR S7 OR S8
- 11 S9 AND S10
- Obtained references: 2540**

Web of Science

- 1 TS=(amenorrhea or anovulation or hypomenorrhea or hypermenorrhea or menstrua* or menorrhagia or oligomenorrhea or polymenorrhea)
- 2 TS=((academic or caregiv* or emotion* or financial or job or occupational or work or life or minority or interpersonal or family or marital or pandemic or psycho* or social or environment* or perceiv*) NEAR/3 (strain* or pressure* or burden* or crisis or crises))
- 3 TS=(war* or homeless* or incarcerat* or "job loss*" or unemploy* or distress or stress* or "natural disasters" or displacement or housing)
- 4 #2 OR #3
- 5 #1 AND #4
- Obtained references: 6495**

A2. References Excluded During Full Text Screening

Appendix A2 can be viewed [here](#) or in the online version of the published article.

A3. Extracted Data

Appendix A3 can be viewed [here](#) or in the online version of the published article.

A4. Risk of Bias

Appendix A4 can be viewed [here](#) or in the online version of the published article.

Appendix B (Chapter II)

B1. Systematic Review Search Strategy

Database search performed on: May 18th, 2023

APA Psychinfo (Ovid)

- 1 estrus/
- 2 sexual reproduction/
- 3 (cyclicity or "cycle irregularity" or "estrous cycl*" or "oestrus cycl*" or "reproductive function*" or "vaginal cytology" or diestrus or estrus or metestrus or proestrus or "hypothalamic–pituitary–gonadal axis" or "HPG axis").ti,ab.
- 4 stress/ or psychological stress/ or social stress/
- 5 (stress* or "Overcrowding" or "Forced swim test" or "Immobilization" or "Isolation" or "Noise exposure" or "Predator" or "Psychogenic" or "Resident-intruder paradigm" or Restraint or "Social defeat" or "Social instability" or "Social isolation").ti,ab.
- 6 rodents/ or gerbils/ or guinea pigs/ or hamsters/ or mice/ or rats/
- 7 rodent*.ti,ab.
- 8 (mouse or mice or "mus musculus").ti,ab.
- 9 (rat or rats or rattus).ti,ab.
- 10 (gerbil or hamster or "guinea pig").ti,ab.
- 11 1 or 2 or 3
- 12 4 or 5
- 13 6 or 7 or 8 or 9 or 10
- 14 11 and 12 and 13

Obtained references: 399

Medline (Ovid)

- 1 estrus cycle/ or anestrus/ or diestrus/ or estrus/ or metestrus/ or proestrus/
- 2 ovarian reserve/
- 3 reproduction/
- 4 (cyclicity or "cycle irregularity" or "estrous cycl*" or "oestrus cycl*" or "reproductive function*" or "vaginal cytology" or diestrus or estrus or metestrus or proestrus or "hypothalamic–pituitary–gonadal axis" or "HPG axis").ti,ab,kf.
- 5 mental stress/ or chronic unpredictable stress/ or social stress/
- 6 acute stress/ or chronic stress/
- 7 (stress* or "Overcrowding" or "Forced swim test" or "Immobilization" or "Isolation" or "Noise exposure" or "Predator" or "Psychogenic" or "Resident-intruder paradigm" or Restraint or "Social defeat" or "Social instability" or "Social isolation").ti,ab,kf.
- 8 rodent/
- 9 rat/ or experimental rat/ or rattus norvegicus/ or rattus rattus/
- 10 mouse/ or experimental mouse/
- 11 (mouse or mice or "mus musculus" or rat or rats or rattus or gerbil or hamster or "guinea pig").ti,ab,kf.
- 12 1 or 2 or 3 or 4

- 13 5 or 6 or 7
- 14 8 or 9 or 10 or 11
- 15 12 and 13 and 14

Obtained references: 1824

Embase (Ovid)

- 1 estrus cycle/ or anestrus/ or diestrus/ or estrus/ or metestrus/ or proestrus/
- 2 ovarian reserve/
- 3 reproduction/
- 4 (cyclicity or "cycle irregularity" or "estrous cycl*" or "oestrus cycl*" or "reproductive function*" or "vaginal cytology" or diestrus or estrus or metestrus or proestrus or "hypothalamic–pituitary–gonadal axis" or "HPG axis").ti,ab,kf.
- 5 mental stress/ or chronic unpredictable stress/ or social stress/
- 6 acute stress/ or chronic stress/
- 7 (stress* or "Overcrowding" or "Forced swim test" or "Immobilization" or "Isolation" or "Noise exposure" or "Predator" or "Psychogenic" or "Resident-intruder paradigm" or Restraint or "Social defeat" or "Social instability" or "Social isolation").ti,ab,kf.
- 8 rodent/
- 9 rat/ or experimental rat/ or rattus norvegicus/ or rattus rattus/
- 10 mouse/ or experimental mouse/
- 11 (mouse or mice or "mus musculus" or rat or rats or rattus or gerbil or hamster or "guinea pig").ti,ab,kf.
- 12 1 or 2 or 3 or 4
- 13 5 or 6 or 7
- 14 8 or 9 or 10 or 11
- 15 12 and 13 and 14

Obtained references: 2934

Cochrane CENTRAL (Ovid)

- 1 estrous cycle.ti,ab.
- 2 reproduction.ti,ab.
- 3 estrus cycle/ or anestrus/ or diestrus/ or estrus/ or metestrus/ or proestrus/
- 4 reproduction/
- 5 (cyclicity or "cycle irregularity" or "estrous cycl*" or "oestrus cycl*" or "reproductive function*" or "vaginal cytology" or diestrus or estrus or metestrus or proestrus or "hypothalamic–pituitary–gonadal axis" or "HPG axis").ti,ab.
- 6 mental stress/ or chronic unpredictable stress/ or social stress/
- 7 stress/
- 8 acute stress/ or chronic stress/
- 9 (stress* or "Overcrowding" or "Forced swim test" or "Immobilization" or "Isolation" or "Noise exposure" or "Predator" or "Psychogenic" or "Resident-intruder paradigm" or Restraint or "Social defeat" or "Social instability" or "Social isolation").ti,ab.
- 10 rodent/
- 11 rat/ or experimental rat/ or rattus norvegicus/ or rattus rattus/
- 12 mouse/ or experimental mouse/

- 13 (mouse or mice or "mus musculus" or rat or rats or rattus or gerbil or hamster or "guinea pig").ti,ab.
- 14 1 or 2 or 3 or 4 or 5
- 15 6 or 7 or 8 or 9
- 16 10 or 11 or 12 or 13
- 17 14 and 15 and 16

Obtained references: 12

Web of Science

- 1 TS=(cyclicity or "cycle irregularity" or "estrous cycl*" or "oestrus cycl*" or "reproductive function*" or "vaginal cytology" or diestrus or estrus or metestrus or proestrus or "hypothalamic–pituitary–gonadal axis" or "HPG axis")
- 2 TS=(stress* or "Overcrowding" or "Forced swim test" or "Immobilization" or "Isolation" or "Noise exposure" or "Predator" or "Psychogenic" or "Resident-intruder paradigm" or Restraint or "Social defeat" or "Social instability" or "Social isolation")
- 3 TS=(mouse or mice or "mus musculus" or rat or rats or rattus or gerbil or hamster or "guinea pig")
- 4 #1 AND #2 AND #3

Obtained references: 2416

B2. References Excluded during Full Text Screening

Appendix B2 can be viewed [here](#) or in the online version of the published article.

B3. Extracted Data

Appendix B3 can be viewed [here](#) or in the online version of the published article.

B4. Risk of Bias

Appendix B4 can be viewed [here](#) or in the online version of the published article.

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Appendix C (Chapter III)

Effects of Ischemia on Estrous Cycle Regularity – Days in Each Stage

Table C1. Number of Days Spent in Each Stage of the Estrous Cycle.

Stage	Pre-surgery (Days -12 to 0)		Post-surgery (Days 1 to 12)		Post-surgery (Days 13 to 24)	
	Sham	GCI	Sham	GCI	Sham	GCI
Proestrus	2.88 ± .125	2.88 ± .125	1.75 ± .366	0.75 ± .366	2.75 ± .164	2.75 ± .164
Estrus	3.00 ± 0.00	3.00 ± 0.00	2.63 ± .183	1.00 ± .378	3.13 ± .125	2.75 ± .164
Metestrus	2.13 ± .398	2.13 ± .350	2.50 ± .189	.625 ± .324	2.13 ± .398	2.50 ± .189
Diestrus	4.00 ± .500	4.00 ± .378	4.88 ± .398	9.63 ± .963	4.00 ± .378	4.00 ± .378

Note. Mean ± S.D. is presented for the 12 days preceding sham or GCI surgery, the first 12 days post-surgery, and the last 12 days post-surgery.

Overview of Thionin Staining

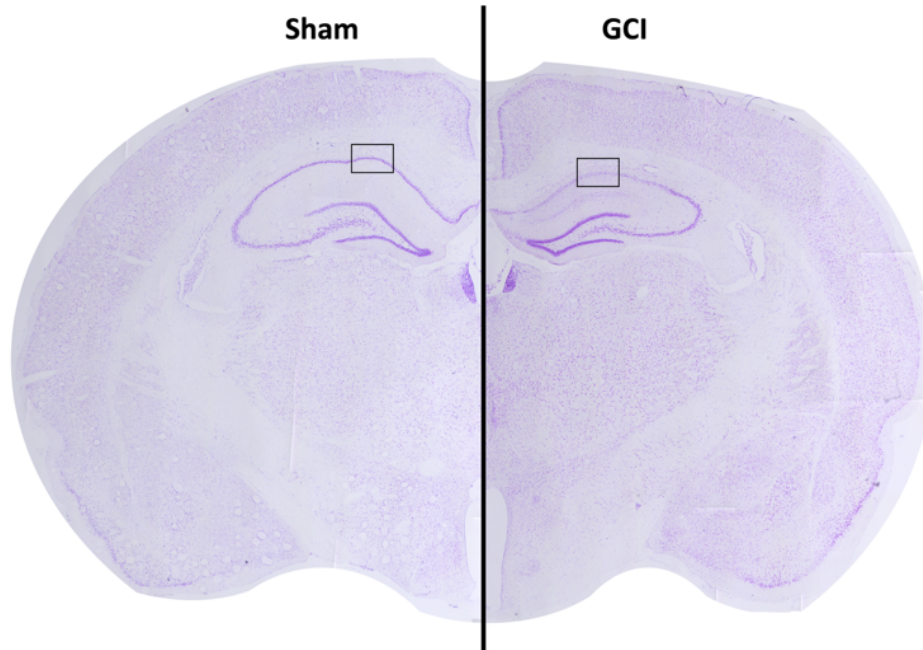


Figure C1. Overall Presentation of Thionin Staining.

Representative photomicrograph of thionin stained whole coronal brain slice for sham rat (left hemisphere) and GCI (right hemisphere). The square area indicates approximately where CA1 neuronal density was assessed (refer to Chapter III – [Figure 3](#)).

Post-Ischemic Basal CORT Levels in Male Rats (de la Tremblaye et al., 2014)

To further contextualize the detected changes in estrous cyclicity, basal CORT level progression following ischemia from a previous study in our laboratory are presented below. This study by de la Tremblaye et al. (2014) used adult male Wistar rats having undergone a 10 min GCI using the same four-vessel occlusion model. While we cannot be certain the CORT recovery profile of the ischemic female rodents used in our study were identical to the males', the similar post-ischemic changes in GR expression in the CA1 and PVN (decreased and increased, respectively) leads us to posit that elevated CORT levels contributed to significant differences in estrous cyclicity persisting up to Day 8 post ischemia in our ischemic female rats.

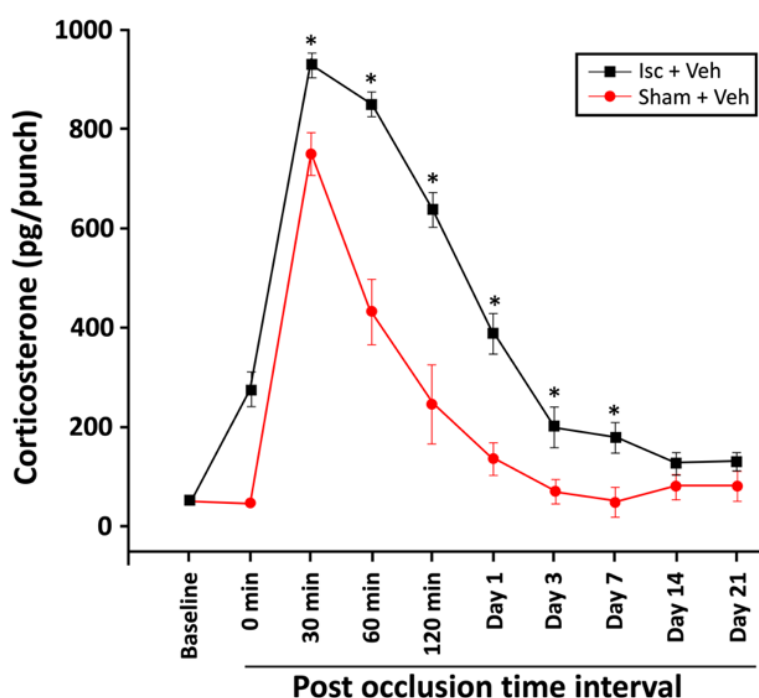


Figure C2. Basal CORT Levels in Male Rats Following 10-min GCI.

CORT levels were significantly elevated in ischemic male rats compared to sham rats up to seven days after ischemia, after which levels no longer differed. * indicates statistically significant difference at $p < .05$. Figure adapted from a previous publication from our laboratory by de la Tremblaye et al. (2014).

Appendix D (Chapter IV)

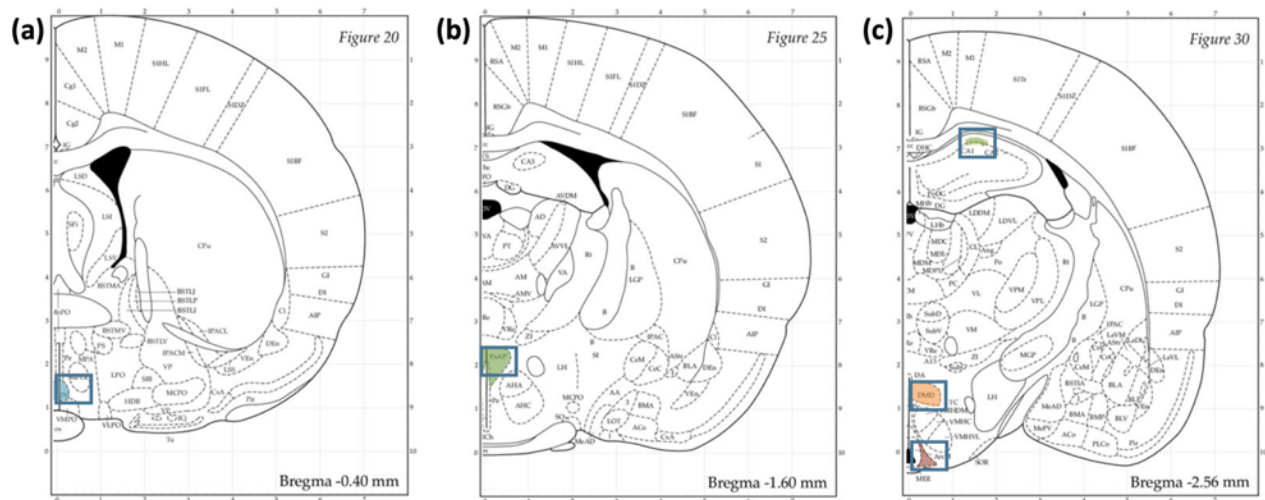


Figure D1. Brain Regions of Interest and Area Captured in Photomicrograph.

Figure shows location of **(a)** the anteroventral periventricular nucleus (AVPV), **(b)** the paraventricular nucleus (PVN), **(c)** the CA1 of the hippocampus (in green), the dorsomedial nucleus (DMH; in orange), and the arcuate nucleus (ARC; in red). Blue squares indicate area where photomicrographs were captured for each region.

Table D1. Hormone Assays.

ELISA plate	Intraassay CV	Interassay CV	n plates	Kit detection range	Antibody specificity and cross reactivity*
LH	6.680 %	12.085 %	2	1.2 – 280 mIU/mL	LH = 100%, FSH < .004%, TSH = .3%, hCG < .004%
FSH	7.174 %	8.863 %	2	0.468 – 30 mIU/mL	No significant cross reactivity reported.
E2	4.172 %	7.258 %	2	15.6 – 3000 pg/mL	17 β -Estradiol = 100 %, Estrone = 17.8%, Estriol = 0.9%, DHT = 0.12%, Ethynylestradiol-7 α = 0.043%, Dehydroisoandrosterone = 0.038%, Estradiol-17 α = 0.032%, Testosterone = 0.01%, Progesterone < 0.001%, Danazol < 0.001%
P4	3.329 %	11.177 %	2	15.62 – 500 pg/mL	Progesterone = 100%, 5 α -Pregnane-3,20-dione = 100%, 17-OH-Progesterone = 3.46%, 5-Pregnen-3 β -o1-20-one = 1.43%, Corticosterone = 0.77%, 4-Androstene-3,17-dione = 0.28%, Deoxycorticosterone = 0.056%, DHEA = 0.013%, 17 β -Estradiol < 0.001%, Estrone < 0.001%, Estriol < 0.001%, Testosterone < 0.001%, Hydrocortisone < 0.001%, 5 α -Pregnane-3 α ,20 α -diol < 0.001%, Danazol < 0.001%
CORT	4.179 %	12.951 %	17	32 – 20,000 pg/mL	Corticosterone = 100%, Deoxycorticosterone = 28.6%, Progesterone = 1.7%, Testosterone = 0.13%, Tetrahydrocorticosterone = 0.28%, Aldosterone = 0.18%, Cortisol = 0.046%, Pregnenolone < 0.03%, β -Estradiol < 0.03%, Cortisone < 0.03%, 11-dehydrocorticosterone acetate < 0.03%

Note. * As specified by kit manufacturer. CORT: Corticosterone; E2: Estradiol; FSH: Follicle-stimulating hormone; LH: Luteinizing hormone; P4: Progesterone.

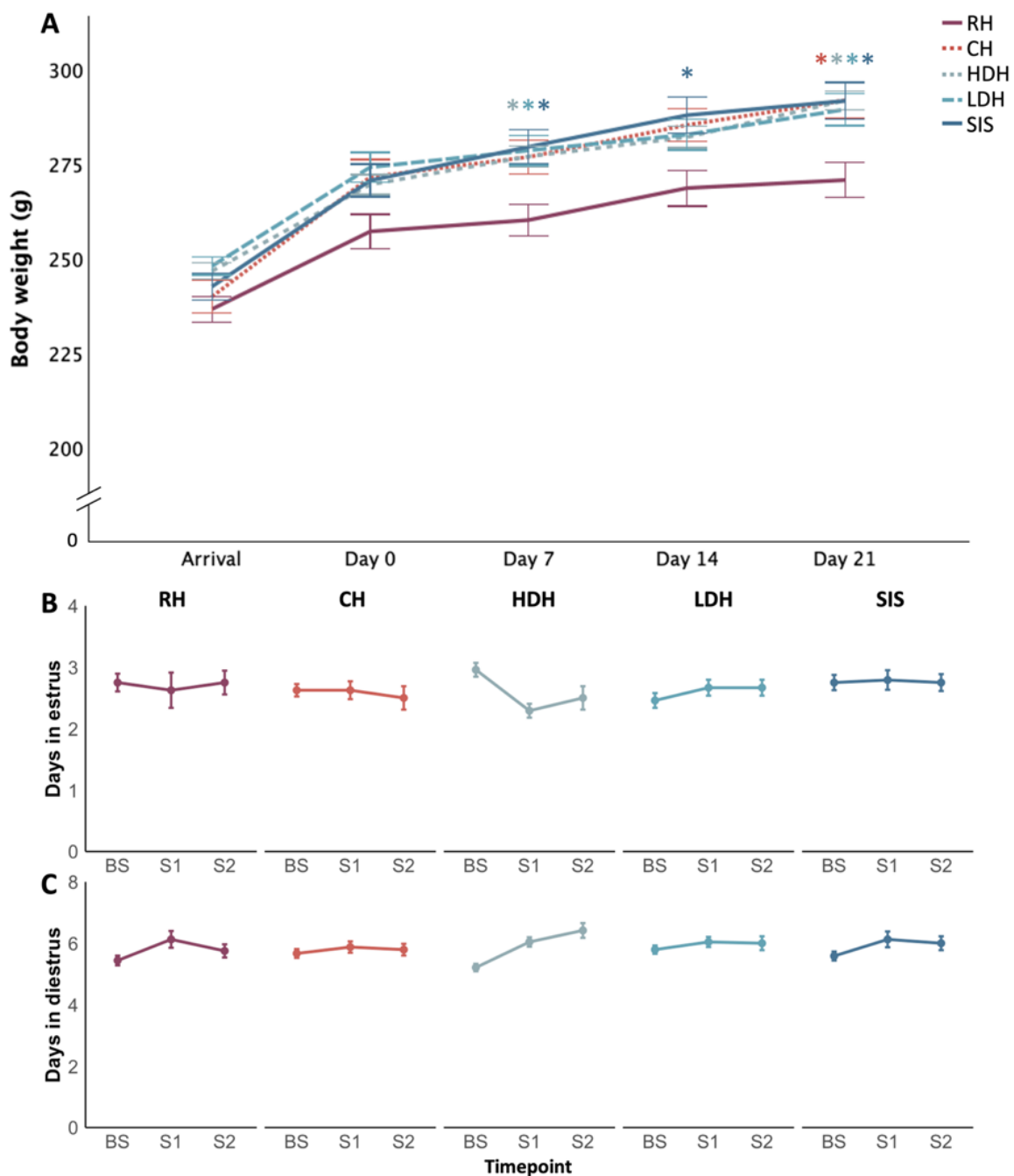


Figure D2. Weight Gain and Estrous Cycling Throughout the Study.

Top half of the figure shows body weight in grams at arrival, baseline (day before stressor start), and throughout the study (A). Asterisk color indicates groups which are significantly different from the RH group. Bottom half shows number of days in estrus (B) and diestrus (C) for each group during baseline (BS), first half of stress (S1; Days 1-11) and second half of stress (S2; Days 12-22). Data presented as mean $\pm$ S.E.M., $p < .05$.

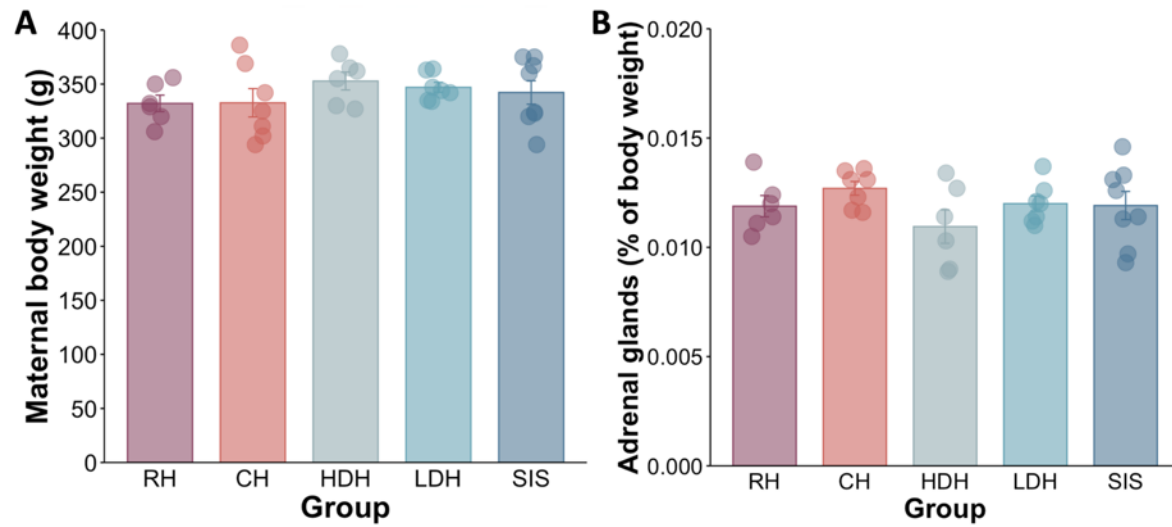


Figure D3. Maternal Body and Adrenal Weight at Gestational Day 14.

Figure shows maternal body weight (**A**) and adrenal gland weights (**B**) of pregnant rats at euthanasia on gestational day 14. Data presented as mean $\pm$ S.E.M.

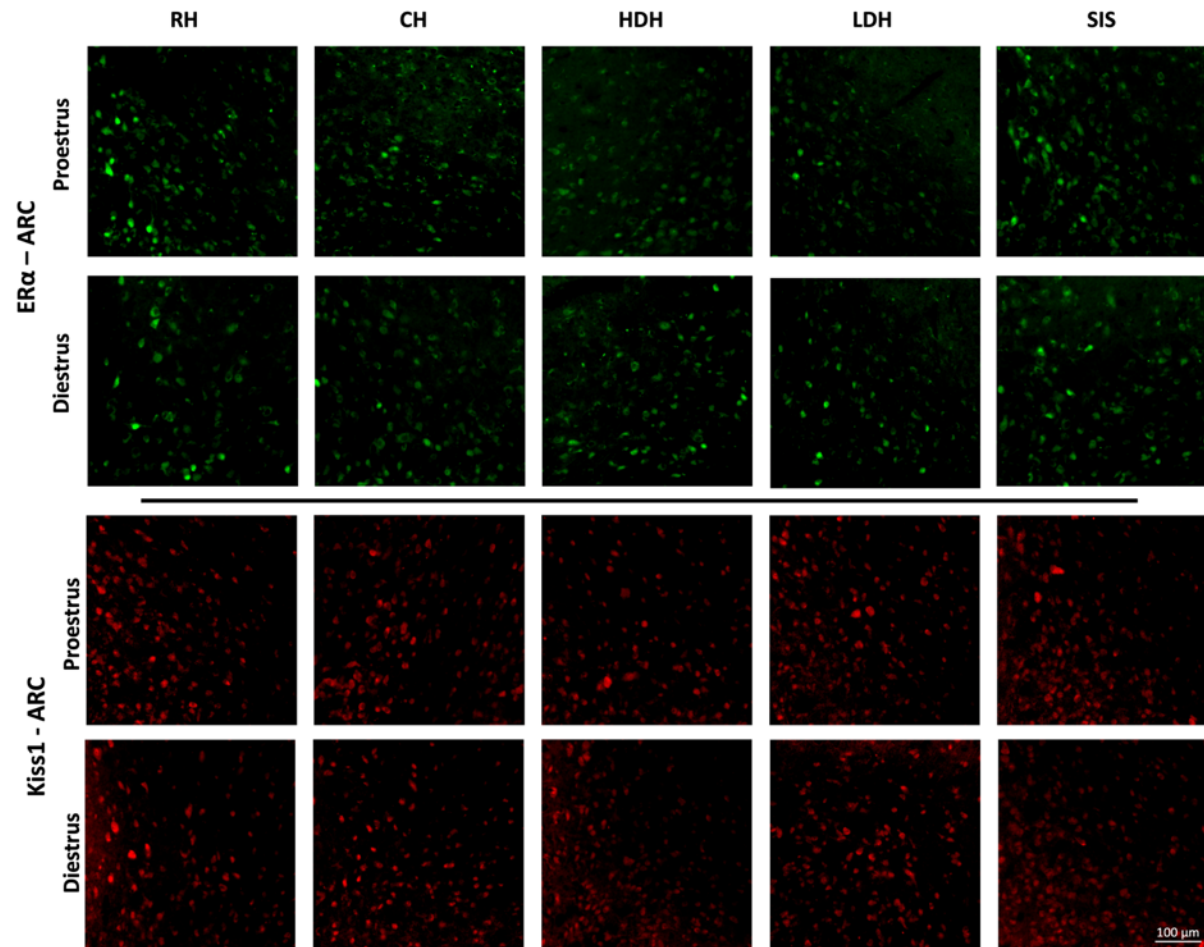


Figure D4. Representative Photomicrographs of ER α and Kiss1-ir.

Top half of figure shows ER α -ir in proestrus (top row) and diestrus (bottom row) for each experimental group. Bottom half of figure shows Kiss1-ir in proestrus (top row) and diestrus (bottom row) for each experimental group.

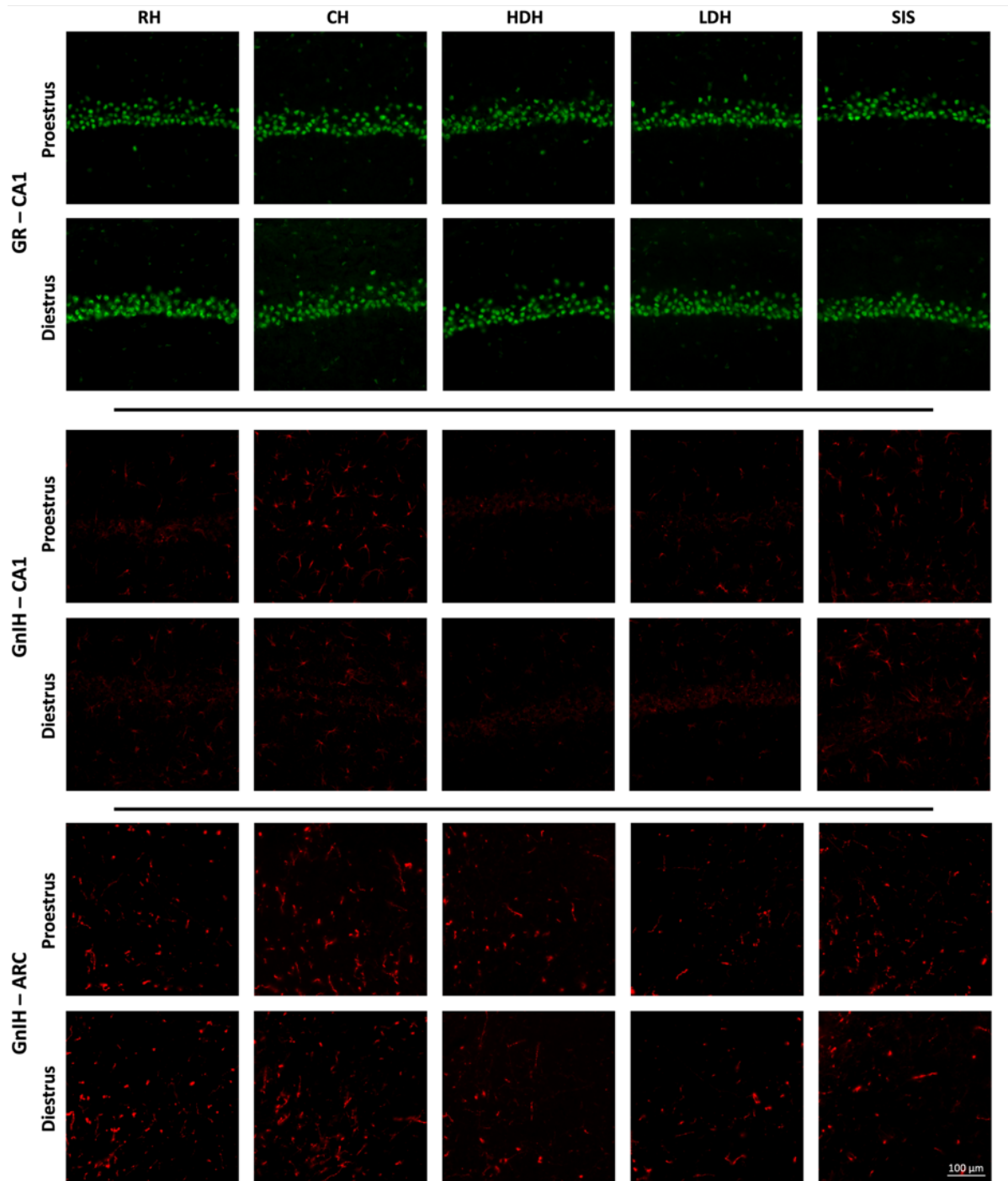


Figure D5. Representative Photomicrographs of GR and GnIH-ir.

Top half of figure shows GR-ir in proestrus (top row) and diestrus (bottom row) in the CA1 for each experimental group. Centre of figure shows GnIH-ir in the CA1 and bottom of figure shows GnIH-ir in the ARC.

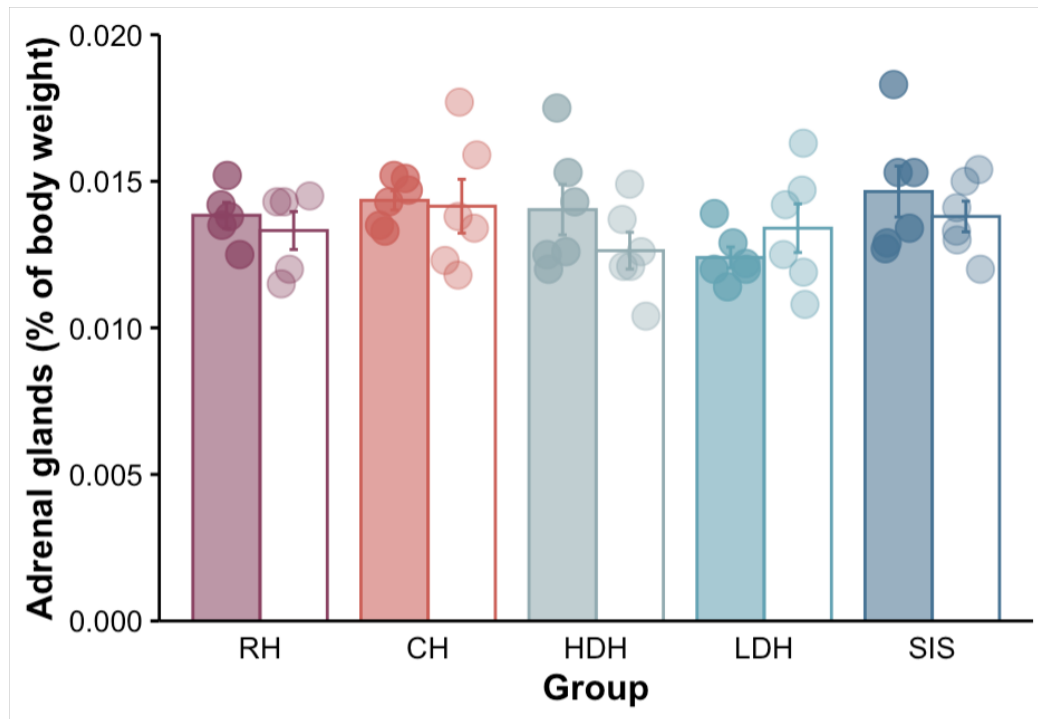


Figure D6. Adrenal Gland Weights Following Social Stress.

Figure shows adrenal gland weights as a percentage of total body weight at euthanasia for proestrus (filled bars) and diestrus (white bars) for each group. Data presented as mean $\pm$ S.E.M.

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Appendix E (Chapter V)

Table E1. Overview of Behaviour Scoring and Score Calculation for Each Individual Rat.

Behaviour	Description	Calculation
Attack		
Nape contact	Rat attempts to contact the nape of a target using its snout, placing its forepaws on either side of the targeted location.	Frequency of nape contacts = sum of all nape contacts initiated Likelihood of nape contact (player type only) = (sum of nape contacts by rat / total number of nape contacts performed in the cage) × 100
Pin	The rat stands over its supine target and moves to block the supine animal's maneuvers, typically also continuing to attempt to contact the nape.	Frequency of pinning = sum of all pins performed Likelihood of pinning (player type only) = (sum of pinning behaviours by rat / total number of pins performed in the cage) × 100 Mean pin length (s) = (total duration spent pinning / total number of times doing a pin behaviour)
Bites	The rat delivers a bite on its target, typically on the lower flanks and rump.	N/A Context of the bite was evaluated (e.g., behaviour of the targeted rat when bite occurred)
Defense		
% defended	In response to a nape contact attempt from another rat, the targeted rat uses one of the defense tactics below.	Attacks defended (%) = [(n attacks received – n attacks not defended) / total attacks received] × 100
% of tactics	Evasion: Rat leaps, jumps, or swerves out of the way to avoid the nape contact. Rotations: Rat completes a rotation to defend the nape, rotating either horizontally or longitudinally (scored as partial or complete, depending on whether all four paws leave the ground; Himmler et al., 2013).	Likelihood of each tactic = (n times defense tactic used / total n of attacks defended) × 100

Pinned	Rat rotates to a supine position and remains relatively immobile (passive defense) or actively attempts to keep the pinning rat off by kicking or returning nape contacts (active defense)	Mean pinned length (s) = (total duration being pinned / total number of times pinned) Likelihood of passive defense = (frequency of passive defense in response to pin / total number of times pinned) $\times$ 100
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Note: Calculations performed for each session.

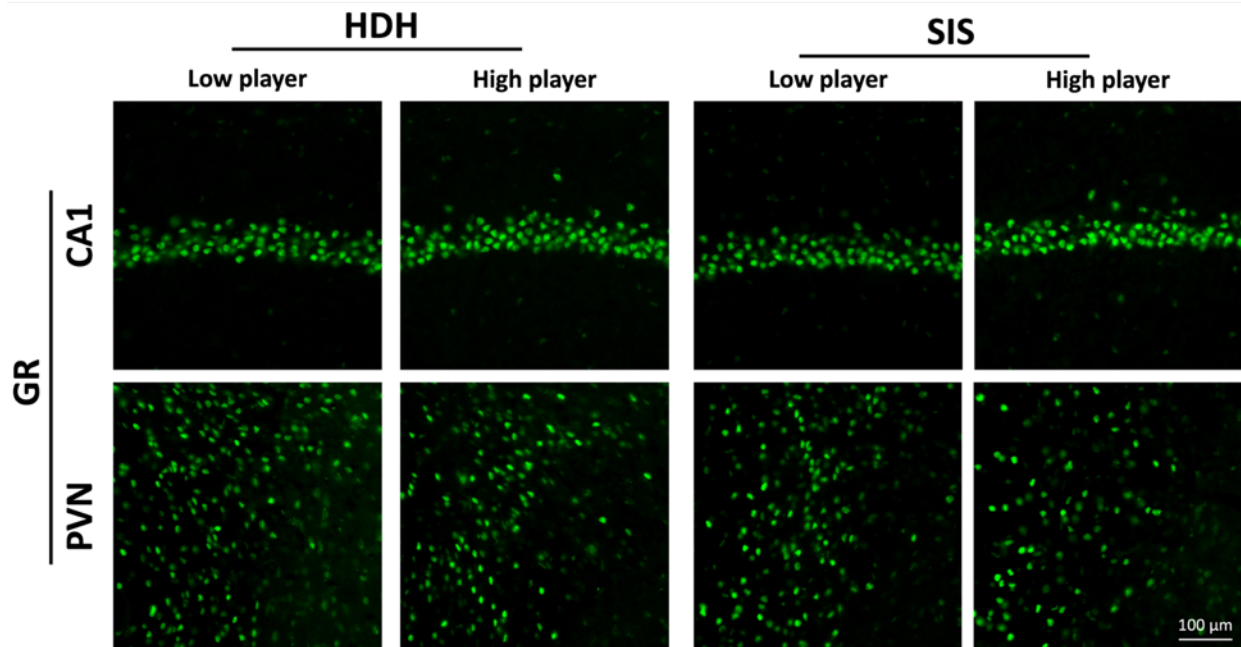


Figure E1. Representative Photomicrographs of GR-ir.

Figure shows GR-ir in the CA1 (top row) and PVN (bottom row) for high and low playing HDH (left) and SIS (right) rats. HDH: High density housing; PVN: Paraventricular nucleus; SIS: Social instability stress.

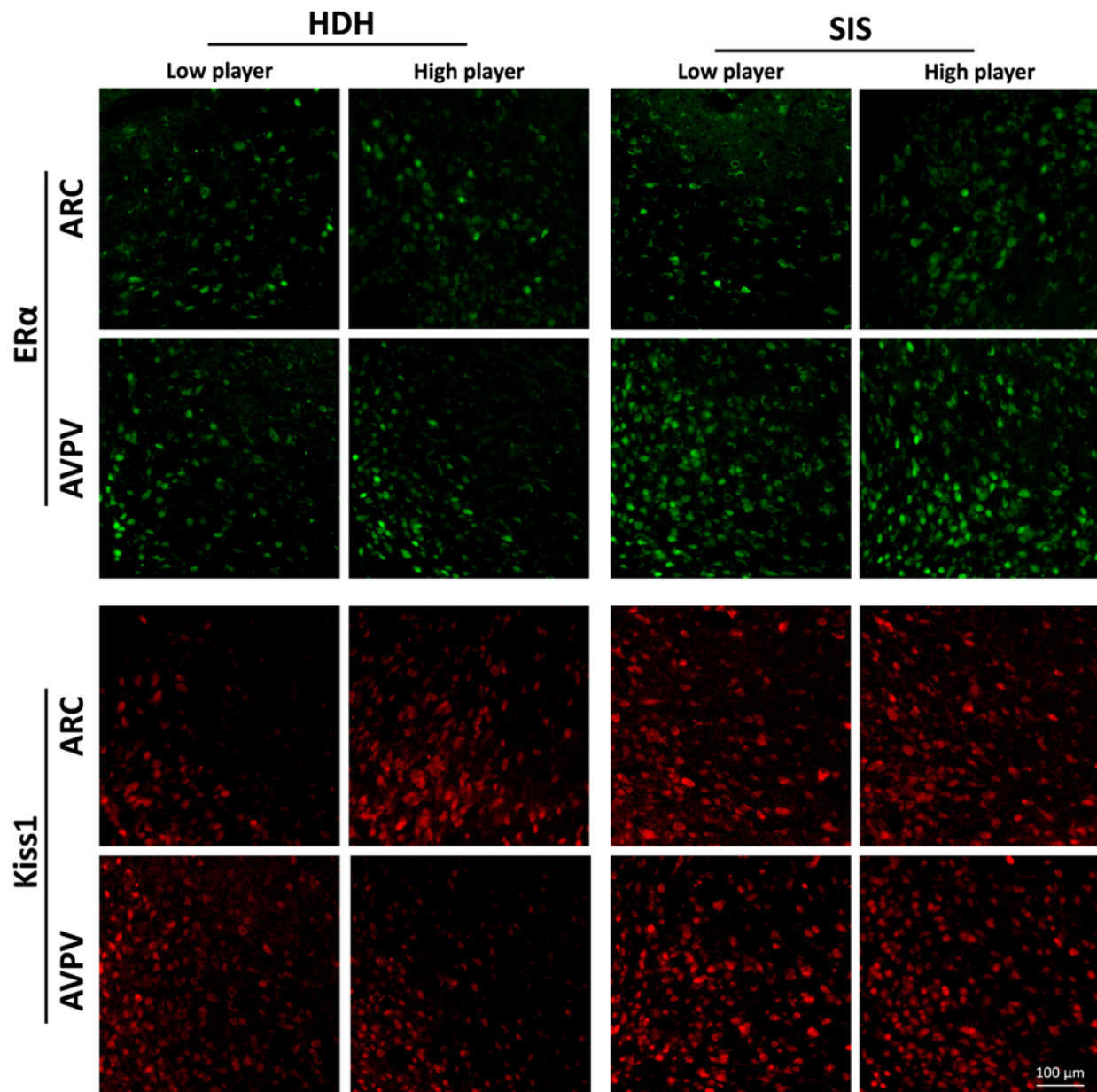


Figure E2. Representative Photomicrographs of ERα and Kiss1-ir.

Top half of figure shows ERα-ir in the ARC (top row) and AVPV (bottom row) for high and low playing HDH (left) and SIS (right) rats. Bottom half of figure shows the same for Kiss1-ir. ARC: arcuate nucleus; AVPV: anteroventral periventricular nucleus; HDH: High density housing; SIS: Social instability stress.

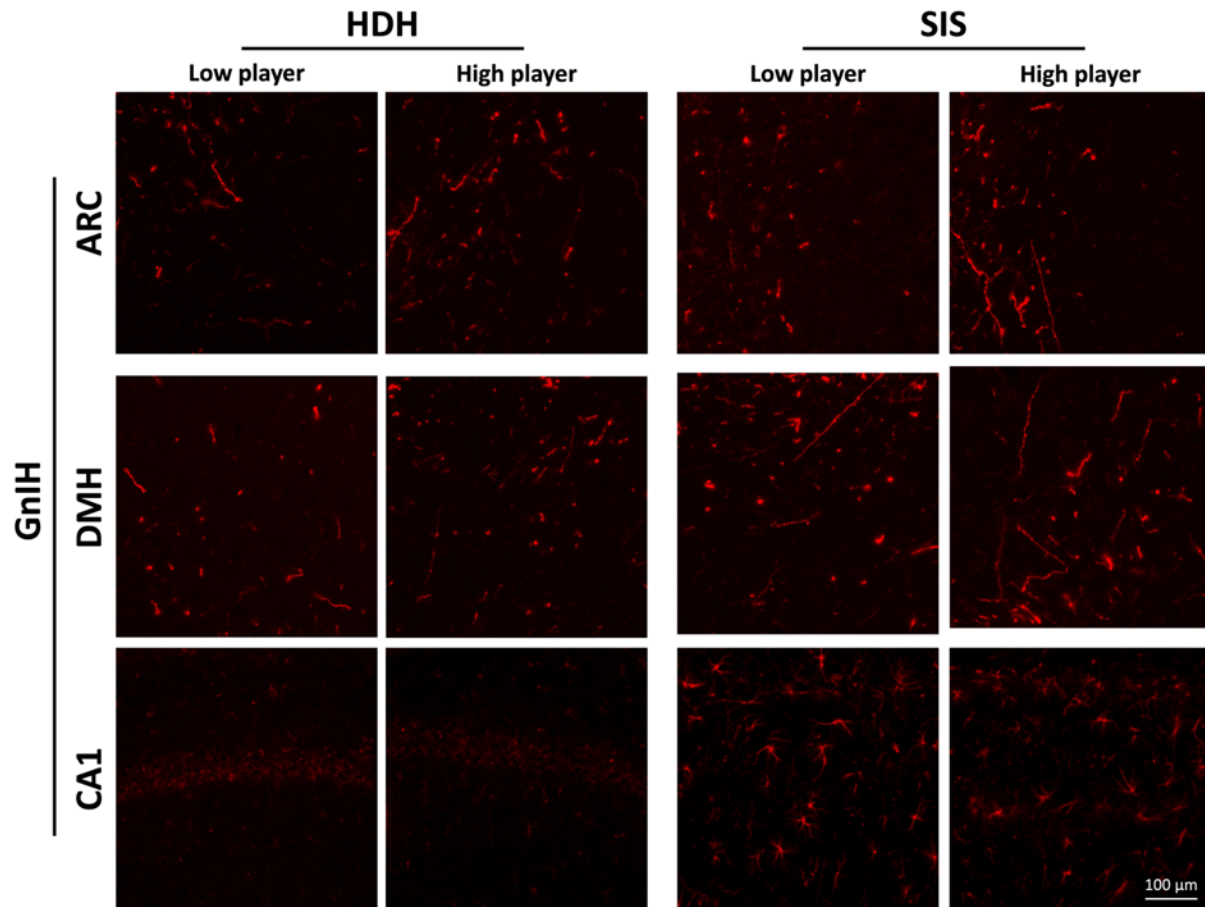


Figure E3. Representative Photomicrographs of GnIH-ir.

Figure shows GnIH-ir in the ARC (top row), DMH (middle row), and CA1 (bottom row) for high and low playing HDH (left) and SIS (right) rats. ARC: arcuate nucleus; DMH: Dorsomedial nucleus; HDH: High density housing; SIS: Social instability stress.

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