THE EFFECT OF CIRCADIAN MISALIGNMENT ON CARDIOMETABOLIC PARAMETERS IN A RAT MODEL OF ESTROGEN DEFICIENCY

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Declaration

I, Refentshe Amandu's Nthlane, declare that this dissertation is my own work. It is being submitted for the degree of Master of Science of Medicine, in the Faculty of Health Sciences, University of Witwatersrand, Johannesburg, South Africa. The work contained in this thesis, has not been submitted for any degree or examination in this university, or any other university.

Refentshe Amandu's Nthlane _____

Signed on the 27th day of May 2024

I certify that the studies contained in this dissertation have the approval by the Animal Ethics Screening Committee of the University of the Witwatersrand, Johannesburg. The ethics approval number is 2017/11/76/C

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Refentshe Amandu's Nthlane

Signed on the 27th day of May 2024

.....	.....
Frederic Michel (supervisor)	Karine Scheuermaier (co-supervisor)
Date.....	Date.....

To the loving memory of my late dad

Phillemon Mthombeni

1976 - 2022

Conference presentations

Data presented in this dissertation has been presented at the 1st annual Research day of the School of Physiology held at the University of Witwatersrand Bohaleng Block (Education Campus), 13 September 2023, and at the 2nd Southern African Hypertension Society NextGen Virtual Spring School held on Zoom video conferencing platform, 12 October 2023. The title of the oral presentations was “The absence of circadian misalignment effect on cardiometabolic parameters in a rat model of estrogen deficiency”.

"*Life is a journey, not a destination*."

Ralph Waldo Emerson

Abstract

Menopausal women engaging in shift work are at an increased risk of cardiometabolic disorders (CMD). The increased risk is due to the dual impact of menopause-associated estrogen withdrawal and the shiftwork-induced irregular light exposure, which disrupts circadian rhythms, leading to chronic circadian misalignment. However, the combination of circadian misalignment related to shift work and menopause on cardiometabolic health remains poorly understood. Therefore, the present study aimed to determine whether circadian misalignment worsens cardiometabolic parameters in estrogen-deficient female spontaneously hypertensive rats (SHR). Circadian misalignment was induced by a 10-week chronic phase shift (CPS) protocol, and estrogen deficiency was induced by ovariectomy. Thirty-six female SHR were ovariectomized or sham-operated 7 weeks after birth, and then subjected to a CPS or control lighting (ctr light) schedule (n=9 per group). Body mass, food and water intake, blood pressure and fasting blood glucose concentrations were measured throughout the 10-week protocol. An oral glucose tolerance test was performed 3 days before the end of the 10-week protocol, while ventricular systolic and diastolic function were assessed by echocardiography at the end of the 10-week protocol. Organ mass was measured, and LDL concentration was determined from collected serum with ELISA. Ovariectomized rats were heavier than sham-operated rats overtime (211 ± 38 vs. 169 ± 22g; $p < 0.0001$). Food intake and organ masses were greater in ovariectomized rats compared to the sham-operated rats. When normalized to body mass, the food intake and organ masses were lower than in the sham-operated rats. Ovariectomized rats had greater left ventricular (LV) end-diastolic diameter (5.1 ± 0.4 vs. 4.6 ± 0.6mm; $p = 0.03$) and LV end-systolic diameter (3.0 ± 0.5 vs. 2.4 ± 0.4mm; $p = 0.004$) than sham-operated rats. The ovariectomized rats also had reduced endocardial fractional shortening (41 ± 8 vs. 49 ± 4%) and LV ejection fraction (71 ± 10 vs. 80 ± 4%) (both $p = 0.007$) than sham-operated rats. The cardiometabolic parameters measured were similar between the CPS and control lighting rats, except for a greater water intake overtime (CPS: 29 ± 7.1 vs. ctr light: 26 ± 3.2ml/day; $p = 0.048$) and a reduced liver mass (CPS: 7.2 ± 0.6 vs. ctr light: 7.6 ± 0.8g; $p = 0.03$) in CPS rats. When normalized to body mass, sham-operated rats had a greater water intake than the ovariectomized rats. No interaction between ovariectomy and CPS was demonstrated. In conclusion, estrogen deficiency impairs systolic function in female SHR. However, circadian misalignment does not worsen the cardiometabolic parameters in female SHR. Because of their genetic predispositions, female SHR may already have abnormal circadian rhythms, illustrating the complex and multifaceted circadian regulation within this rat model.

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List of abbreviations and symbols

A	Late diastole mitral peak flow velocity
a'	Late diastole mitral peak tissue lengthening velocity
ACE	Angiotensin converting enzyme
ADH	antidiuretic hormone
AIDS	Acquired immunodeficiency syndrome
ANG I	Angiotensin I
ANG II	Angiotensin II
ANOVA	Analysis of variance
ANS	Autonomic nervous system
AREC	Animal Research Ethics Committee
AUC	Area under the curve
β1	Beta1
BMAL1	Brain and muscle aryl hydrocarbon receptor nuclear translocator-like protein 1
BMI	Body Mass Index
BP	Blood pressure
CLOCK	Circadian locomotor outputs kaput
cm	centimetre
CMD	Cardiometabolic disorders
cm/s	centimetre per second
CO	Cardiac output
CPS	Chronic Phase Shift
Ctr light	Control lighting conditions
Cry	Cryptochrome
CV	Cardiovascular
CVD	Cardiovascular disease

°C	degree Celsius
DBP	Diastolic blood pressure
E	Early diastole mitral peak flow velocity
e'	Early diastole mitral peak tissue lengthening velocity
e'/a'	Ratio of early to late mitral annular diastolic tissue lengthening velocity
E/e'	Index of left ventricular filling pressure
E/A	Index of left ventricular relaxation
E1	Estrone
E2	Estradiol
E3	Estriol
EDTA	Ethylenediamine tetraacetic acid
EF	Ejection fraction
ELISA	Enzyme linked immunosorbent assay
eNOS	Endothelial nitric oxide synthase
ERT	Estrogen replacement therapy
FSend	Endocardial fractional shortening
FSH	Follicular stimulating hormone
FSmid	Midwall fractional shortening
g	grams
GABA	Gamma-aminobutyric acid
GLUT4	Glucose transporter 4
HDL	High-density lipoprotein
HIV	Human immunodeficiency virus
HPA	Hypothalamic-pituitary-adrenal axis
IDL	Intermediate density lipoproteins
IML	Intermediolateral nucleus

ipRGCs	Intrinsically photosensitive retinal ganglion cells
IVS	Interventricular septal wall thickness
l	litre
LDL	Low-density lipoprotein
Lux	The unit of illumination or light intensity
LV	Left ventricle or left ventricular
LVDD	Left ventricular diastolic dysfunction
LVEDd	Left ventricular end diastolic internal chamber diameter
LVEDs	Left ventricular end systolic internal chamber diameter
LVEDV	Left ventricular end-diastole volume
LVESV	Left ventricular end-systole volume
LVH	Left ventricular hypertrophy
LVPWTd	Left ventricular posterior wall thickness at the end of diastole
LVPWTs	Left ventricular posterior wall thickness at the end of systole
LVSD	Left ventricular systolic dysfunction
ml	millilitre
mmHg	millimetre mercury
mmol/L	millimole per litre
µL	microliter
NE	Norepinephrine
NIBP	Non-invasive blood pressure
NIH	National Institutes of Health
NO	Nitric oxide
OGTT	Oral glucose tolerance test
Ova	Ovariectomized
%	percentage

PCOS	Polycystic ovary syndrome
Per	Period
POI	Primary ovarian insufficiency
PVN	Paraventricular nuclei
RAAS	Renin angiotensin aldosterone system
rpm	Revolutions per minute
RWT	Relative wall thickness
s'	Peak systolic velocity at the mitral annulus
SBP	Systolic blood pressure
SCN	Suprachiasmatic nucleus
SCG	Superior cervical ganglion
SD	Standard deviation
SHR	Spontaneously hypertensive rats
SNS	Sympathetic nervous system
STE	Speckle Tracking Echocardiography
SV	Stroke volume
Sx	Pre-surgery
2D	two-dimensional
3D	three-dimensional
TDI	Tissue Doppler Imaging
VLDL	Very low-density lipoproteins
WHO	World Health Organization
WKY	Wistar Kyoto
WRAF	Witwatersrand Research Animal Facility

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Chapter 1: Introduction

1.1 Background

Cardiometabolic disorders (CMD) such as type 2 diabetes and cardiovascular disease (CVD), along with their related factors like hypertension, dyslipidemia, hyperglycemia, insulin resistance, and obesity, significantly contribute to morbidity and mortality in both men and women (Sowers *et al*., 2001; Russo *et al*., 2015; Petrie *et al*., 2018; Powell-Wiley *et al*., 2021). However, the progression of cardiometabolic risk varies for men and women over the course of a lifetime. Men are significantly at a greater risk for CMD from a young age compared to women of the same age (Machi *et al*., 2016; Jeong and Park, 2022; Nakaya *et al*., 2022; Meloni *et al*., 2023). However, the female advantage gradually disappears with aging, such that after menopause (average, 51 years) the prevalence of CMD rises higher in women than men (Fortepiani *et al*., 2002; Peng *et al*., 2003; El Khoudary *et al*., 2020, Maas *et al*., 2021). The substantial decrease in estrogen levels and alterations in the estrogen-to-androgens ratio observed during the menopausal transition have been proposed as the primary factor contributing to an elevated risk of CMD in postmenopausal women (Cignarella *et al*., 2010; El Khoudary *et al*., 2020; Roa-Diaz *et al*., 2021). Additionally, a decline in estrogen levels is linked to detrimental left ventricular (LV) remodelling and dysfunction, as well as reduced cardiac function in postmenopausal women and rodents (Fortepiani *et al*., 2002; Clark *et al*., 2004; Machi *et al*., 2016).

The already heightened susceptibility to CMD in menopausal women may be exacerbated by additional factors like shift work. Shift work is characterized by alternating work with inconsistent schedules (8-hour shifts – morning, afternoon, night- or 12-hour shifts – day vs. night) (Boivin and Boudreau, 2014; Jørgensen *et al*., 2017; Hemmer *et al*., 2021). Shift work exposes individuals to light at unusual circadian times, leading to a misalignment between the sleep/wake cycle and endogenous circadian rhythms (Haus and Smolensky, 2006; Boivin and Boudreau, 2014, Hemmer *et al*., 2021). This disruption of circadian rhythms in both human and animal studies, has been associated with an increased risk of CMD (Martino *et al*., 2007; Holst *et al*., 2019; Zitting *et al*., 2022; Dicom *et al*., 2023). Therefore, the literature relevant to the present dissertation will be reviewed in this chapter. This will include an overview of menopause and cardiometabolic risk profile, sex hormonal influence on the cardiometabolic health, the typical cardiovascular (CV) and metabolic responses to declined estrogen levels, cardiac physiology (including LV structure and function assessment), and the possible effects of misaligned circadian rhythms on cardiometabolic health of estrogen-deficient individuals. The review will contribute to understanding of the complex interplay between hormonal influences, circadian rhythms, and cardiometabolic health.

1.2 Menopause and Cardiometabolic Risk

Menopause (non-surgical) describes the biological process in a woman's life when the menstrual cycles cease permanently at an average age of 51 years (Greendale and Sowers, 1997; Sherman, 2005; Gracia *et al*., 2005; Burger, 2006; Davis *et al*., 2015). Menopause signifies the end of a woman's reproductive years (Greendale and Sowers, 1997; Sherman, 2005; Maas *et al*., 2021). Menopause can be divided into three phases, perimenopause, menopause, and postmenopause (Greendale and Sowers, 1997; Greendale *et al*., 1999). Perimenopause is the transitional phase before menopause, characterized by hormonal fluctuations and various symptoms (Greendale *et al*., 1999; Sherman, 2005; McNamara *et al*., 2015). These symptoms, include but are not limited to, hot flashes, mood disturbances, vaginal atrophy, osteoporosis, and urinary tract infections (Greendale *et al*., 1999; McNamara *et al*., 2015; Santoro, 2016). The duration of perimenopause may last 5 years, with symptoms potentially starting 8 years before menopause (McNamara *et al*., 2015). Menopause is diagnosed when a woman has not experienced a menstrual cycle for 12 consecutive months (Greendale *et al*., 1999; Sherman, 2005). The postmenopausal stage begins after this point, signalling the end of the reproductive years (Greendale *et al*., 1999; Sherman, 2005). Postmenopause can be defined as the period equal to or greater than 12 months of amenorrhea (absence of menstruation) (Gracia *et al*., 2005; Sherman, 2005). Postmenopausal women have an increased cardiometabolic risk (Figueiredo Neto *et al*., 2010; de Marchi *et al*., 2017; Pu *et al*., 2017). The increased cardiometabolic risk profile is characterized by the emergence or worsening of several risk factors (Ford *et al*., 1998), discussed in the following paragraph.

Numerous studies have shown that changes in body mass that coincides with alterations in body composition are related to menopause (Siemińska *et al*., 2006; Sowers *et al*., 2007; Franklin *et al*., 2009; Van Pelt *et al*., 2015). The changes in body composition include redistribution of body fat to the skin, increased fat mass and subcutaneous adipose tissue, decreased lean tissue mass and loss of skeletal muscle (Clark and Tarttelin, 1982; Svendsen *et al*., 1995; Davis *et al*., 2012; Nishio *et al*., 2019). A shift toward an increased fat mass and redistribution, especially abdominal fat accumulation leads to abdominal obesity (Toth *et al*., 2000; Flegal *et al*., 2010; Davis *et al*., 2012; Wietlisbach *et al*., 2013). Abdominal obesity is associated with increased subcutaneous fat (beneath the skin) and visceral fat (around internal organs) depot size (Wajchenberg, 2000). Moreover, abdominal obesity correlates closely with dyslipidemia (Wietlisbach *et al*., 2013). Dyslipidemia often manifests as increased levels of triglycerides, total cholesterol, and low-density lipoprotein (LDL), coupled with decreased levels of high-density lipoprotein (HDL) in the bloodstream (Jensen *et al*., 1990; Swiger *et al*., 1990; Wietlisbach *et al*., 2013; Fonseca *et al*., 2017). In addition to

altered lipid profiles, postmenopausal women commonly exhibit reduced glucose tolerance (Proudler *et al*., 1992; Kirtikar *et al*., 2020) and impaired insulin sensitivity (Walton *et al*., 1993; Chedraui *et al*., 2014; Schmiegelow *et al*., 2015). Furthermore, these metabolic abnormalities are among potential contributors that can influence the onset of high blood pressure (hypertension) (Grundy *et al*., 2005; Alberti *et al*., 2006; Stanciu *et al*., 2022). A cluster of at least three of these risk factors is known as metabolic syndrome (Grundy *et al*., 2005; Alberti *et al*., 2006). Metabolic syndrome has been associated with both diastolic and systolic dysfunction (Grundy, 2006; von Bibra and St John Sutton, 2010). However, the adverse cardiac structure and functions may also be observed without metabolic syndrome (Wang *et al*., 2015). Menopause has been linked to concentric remodelling of the LV (Pines *et al*., 1992; Hinderliter *et al*., 2002), and reduced systolic and diastolic function (Kangro *et al*., 1995; Schillaci *et al*., 1998; Hayward *et al*., 2000; Düzenli *et al*., 2007). Taken together, menopause may contribute to the emergence or worsening of cardiometabolic risk factors like alterations of the body composition, the lipid profile, blood glucose regulation, blood pressure (BP), as well as cardiac structure and function.

The relationship between menopause and cardiometabolic health is complex. However, the most extensively studied factors connecting menopause and cardiometabolic health involve sex hormonal changes, estrogens and androgens (mainly androstenedione and testosterone), at the onset of menopause (Davis and Burger, 1996; Roa-Díaz *et al*., 2021). During menopause, there is an irreversible stop of ovarian follicle development and function (Naftolin *et al*., 2019; El Khoudary *et al*., 2020), leading to a depletion in ovarian follicular reserves (Kilim and Chandala, 2013; Dosi *et al*., 2014; de Marchi *et al*., 2017). The depletion of ovarian follicles is followed by a decline in circulating ovarian hormones, especially estrogen (Naftolin *et al*., 2019; El Khoudary *et al*., 2020). Following menopause, the levels of circulating estrogen decline significantly and do not rise again later in life (Soules *et al*., 2001; Harlow *et al*., 2012). Premenopausal women have higher estrogen levels than men, whereas postmenopausal women tend to have similar or lower estrogen levels compared to men (Khosla *et al*., 1998; Greendale *et al*., 1999; Leifke *et al*., 2000). Additionally, androgens also change in aging women (Burger, 2002; Roa-Díaz *et al*., 2021). There is a gradual shift towards androgen dominance in the hormonal balance during menopause (Burger *et al*., 2000; Lasley *et al*., 2002). Research indicates that elevated levels of androgens in postmenopausal women are linked to an increased cardiometabolic risk profile (Lambrinoudaki *et al*., 2006; Golden *et al*., 2007; Torréns *et al*., 2009; Wang *et al*., 2012; Das *et al*., 2019). Moreover, investigations on women suffering from polycystic ovarian syndrome (PCOS), a condition associated with ovarian dysfunction and increased androgen production (King, 2006), found that the high levels of

androgens exacerbate CV and metabolic abnormalities (Apridonidze *et al*., 2005; Coviello *et al*., 2006; Cussons *et al*., 2008; Puurunen *et al*., 2009). Thus, the estrogen-to-androgen ratio may provide an alternative predictor of cardiometabolic risk than estrogen levels alone (Zhao *et al*., 2018; Roa-Díaz *et al*., 2021). The combination of estrogen and androgen replacement therapy has shown promise in postmenopausal women (Sarrel, 1998). However, the specific mechanisms through which estrogen and androgens contribute to CMD in menopausal women remain unclear (Spoletini *et al*., 2014; Roa-Díaz *et al*., 2021).

1.3 Estrogen

Research on the cardiometabolic risk profile in menopausal women has primarily focused on the role of estrogens, with limited attention to androgens. Estrogens have been extensively studied primarily due to their protective effect on CV events, particularly in women with pre-existing heart disease (Knopp *et al*., 1994; Dosi *et al*., 2014). Estrogens are a group of sex steroid hormones that are essential for various physiological functions, such as the menstrual cycle regulation, bone formation, the CV system and cognitive functions (ter Horst, 2010). The primary site of estrogen production is in the ovaries, but estrogens are also produced in smaller quantities in the adrenal glands and fat cells (Mendelsohn and Karas, 1999; Cui *et al*., 2013; Fuentes and Silveyra, 2019). The main representatives of this group are estrone (E1), estradiol (E2), and estriol (E3) (Bennink, 2004; ter Horst, 2010; Fuentes and Silveyra, 2019). Estradiol is the predominant form of estrogen (Thomas and Potter, 2013; Fuentes and Silveyra, 2019; Farkas *et al*., 2022), thus for this dissertation "estrogen" will be referring to estradiol.

1.3.1 Cardiometabolic protective effects of estrogen

Estrogen contributes to the maintenance of CV health by regulating various physiological processes and functions (Kalantaridou *et al*., 2006; Menazza and Murphy, 2016; Aryan *et al*., 2020). Indeed, research has indicated that the increased incidence of CVD with age is associated with reduced estrogen levels after menopause (Kok *et al*., 2006; Li *et al*., 2021; Chen *et al*., 2022; Kaminska *et al*., 2023). Furthermore, there is evidence that women with primary ovarian insufficiency (POI) have an increased risk of CVD due to the loss of ovarian activity leading to a decline of estrogen levels (Hu *et al*., 1999; Goldmeier *et al*., 2014; Christ *et al*., 2018, Tsiligiannis *et al*., 2019). Estrogen's cardioprotective effects are suggested to be due to its ability to mitigate risk factors for CVD (Henderson *et al*., 1986; Lobo, 1990; Subbiah, 1998). As mentioned above, the risk factors include dyslipidemia, hyperglycemia, insulin resistance, hypertension, and LV hypertrophy. Estrogen plays a crucial role in maintaining favourable lipid metabolism in the body (Mauvais-

Jarvis *et al*., 2013; Palmisano *et al*., 2017; Zhu *et al*., 2023). Lipids are a class of hydrocarbon-containing organic compounds that are insoluble in water (Fahy *et al*., 2005; Natesan and Kim, 2021). Lipids serve as signalling molecules, structural elements of cell membranes, and reservoirs of energy in living organisms (Fahy *et al*., 2005; Natesan and Kim, 2021). The main forms of lipids include triacylglycerols, phospholipids, steroids (i.e. cholesterol), fatty acids, and lipoproteins (Fahy *et al*., 2005; Natesan and Kim, 2021). Lipid metabolism is facilitated by the synthesis, assembly, and secretion of lipoproteins, which transport lipids in the blood and regulate cellular cholesterol homeostasis (Illingworth, 1993). Lipid metabolism involves three major interconnected pathways, the transport of hepatic or endogenous fat, the transport of dietary or exogenous fat, and the reverse cholesterol transport (Kwiterovich, 2000; Duan *et al*., 2022). These pathways, primarily regulated by the liver, are crucial in delivering lipids to peripheral tissues and excreting lipids from the body (Katz, 1986; Daniels *et al*., 2009; Duan *et al*., 2022). Moreover, estrogen has been proposed to participate in the hepatic fat transport and the reverse cholesterol transport (Parini *et al*., 2000; Palmisan *et al*., 2017; Zhu *et al*., 2018).

Firstly, the hepatic fat transport includes processes involved in the synthesis, storage, and metabolism of fats within the liver (Mashek, 2013; Li *et al*., 2021). Excess carbohydrates (i.e. glucose) can be converted into fatty acids through a process called de novo lipogenesis in the liver. In lipid droplets within hepatocytes (liver cells), the produced fatty acids interact with glycerol to generate triglycerides (the primary form of fat storage). Triglycerides are then packaged into very-low-density lipoproteins (VLDL). VLDL particles transport lipids, including triglycerides, from the liver to other tissues through the bloodstream. Upon reaching the tissues, lipoprotein lipase hydrolyses VLDL into intermediate-density lipoproteins (IDL) and free fatty acids. Further hydrolysis by hepatic lipases transforms IDLs into low-density lipoproteins (LDL). The LDLs are responsible for delivering cholesterol to the cells (Chen *et al*., 2019; Luo *et al*., 2020). Additionally, cholesterol is the primary substrate for the development of atherosclerotic plaques and LDL is an important cholesterol-rich atherogenic lipoprotein. Atherosclerotic plaques are considered abnormal accumulations of fatty materials and other substances that build up inside and on the walls of the arteries (Rafieian-Kopaei *et al*., 2014). The development of atherosclerotic plaques involves the following steps: i) the build-up of LDL in the intimal layer of arteries; ii) LDL oxidation; iii) monocyte-macrophage recruitment; iv) the uptake of oxidized LDL by macrophage scavenger receptors, transforming macrophages into foam cells; and v) the formation of a fibrous cap (Tedgui and Mallat, 1999; Rafieian-Kopaei *et al*., 2014). The fibrous cap contains smooth muscle cells, collagen-rich fiber tissues, macrophages, and T-lymphocytes, all of which form the mature

atherosclerotic plaque. The atherosclerotic plaques enlarge within the arteries due to the growth of adjacent smooth muscle and fibrous tissues (Rafieian-Kopaei *et al*., 2014). Hence, increased levels of serum LDL may contribute to the formation of atherosclerotic plaques (Chen *et al*., 2019; Luo *et al*., 2020; Duan *et al*., 2022). A build-up of atherosclerotic plaques in the arterial walls leads to narrowed and hardened arteries, resulting in a condition called atherosclerosis (Kruth, 2001; Rafieian-Kopaei *et al*., 2014). Atherosclerosis is a major contributor to CVDs, such as coronary artery disease and stroke (Kruth, 2001). Secondly, the reverse cholesterol transport entails the removal of excess cholesterol out of peripheral tissues, delivering the cholesterol into the liver for conversion to bile acids, which are ultimately expelled in the faeces (Brufau *et al*., 2011; Rosenson *et al*., 2012). Excess cholesterol is taken up by high-density lipoproteins (HDL) from the peripheral tissues and transferred to the liver through the bloodstream. Upon reaching the liver, cholesterol is either excreted into bile or converted into bile acids. The bile acids are eventually released from the liver into the small intestine and can be eliminated from the body through faeces (Kruit *et al*., 2006; Rosenson *et al*., 2012; Wang *et al*., 2019). Estrogen exerts its role through these pathways, mainly by increasing HDL and decreasing LDL (Knopp *et al*., 1994; Samaan and Crawford, 1995; Nasr and Breckwoldt, 1998). Studies on estrogen replacement therapy (ERT) also supports that estrogen positively impact lipid levels in postmenopausal women (Walsh *et al*., 1991; Ulloa *et al*., 2002; Ko *et al*., 2005; Nie *et al*., 2022). ERT upregulates LDL receptors, leading to a reduction in LDL levels (Inukai *et al*., 2000). ERT also enhances the uptake of cholesterol by HDL, resulting in increased HDL levels (Schaefer *et al*., 1983; Lamon-Fava *et al*., 2005).

Estrogen also has non-lipid effects, such as reducing insulin resistance (Nasr and Breckwoldt, 1998; Gupte *et al*., 2015; De Paoli *et al*., 2021). Estrogen has been shown to promote the function of pancreatic beta cells and improving insulin sensitivity (Cignarella *et al*., 2010). Insulin sensitivity is a critical factor in maintaining glucose homeostasis, ensuring the regulation of blood glucose levels (Röder *et al*., 2016). Moreover, estrogen improves glucose uptake in muscles by the induction of glucose transporter 4 (GLUT4) expression (Moreno *et al*., 2010; Gorres *et al*., 2011). When blood glucose levels rise, the pancreas releases insulin from the beta cells. Insulin promotes glucose uptake through glucose transporters (i.e. GLUT4) by cells, particularly muscle and adipose cells. In muscle cells, glucose is stored as glycogen for energy reserve, while adipose cells convert glucose into triglycerides for storage. These actions help return blood glucose to normal levels. As insulin actions lower glucose levels, the pancreas reduces insulin secretion. This feedback loop contributes to the overall balance of glucose homeostasis (Herman and Kahn, 2006; Röder *et al*., 2016). Stable narrowly regulated blood glucose levels help preserve the structural integrity and

function of endothelial cells lining the blood vessels, reducing the likelihood of vascular complications such as endothelial dysfunction (Popov, 2010; Kang *et al*., 2017). Endothelial dysfunction is characterized by a pathological imbalance between the secretion of vasoconstrictors and vasodilators acting on the endothelium of blood vessels (Herrera *et al*., 2010; Flammer *et al*., 2012). The endothelium serves an important function in regulating the vascular smooth muscle tone through the production and release of both vasodilator factors (i.e. nitric oxide (NO)) and vasoconstrictor factors (i.e. endothelin and oxygen-derived free radicals) (Rubanyi *et al*., 2002). A key mechanism through which estrogen may exert its anti-hypertensive effect is the upregulation of endothelial NO production (Rubanyi *et al*., 2002). Estrogen upregulates the expression of the enzyme endothelial nitric oxide synthase (eNOS) through various mechanisms, leading to increased production of NO (Kauser and Rubanyi, 1997; Florian *et al*., 2004). Increased NO promotes endothelial-dependent vasodilation and vascular smooth muscle relaxation (Lieberman *et al*., 1994; Gavin *et al*., 2009; Vitale *et al*., 2010). A decline in estrogen levels may result in endothelial dysfunction, accompanied by reductions in NO and increases in endothelin, which contributes to hypertension and CVD (Coylewright *et al*., 2008).

Another mechanism through which estrogen may exert its anti-hypertensive effects is related to the renin-angiotensin-aldosterone system (RAAS) (O'Hagan *et al*., 2012). The RAAS is known for maintaining BP through water and electrolyte balance (Givertz, 2001; O'Donnell *et al*., 2014). Estrogen stimulates the RAAS by augmenting both tissue and circulating levels of angiotensinogen (precursor of angiotensin peptides) and renin (an enzyme for converting angiotensinogen into angiotensin I (ANG I)) (Brosnihan *et al*., 1999; Fischer *et al*., 2002). ANG I is an inactive precursor that the angiotensin-converting enzyme (ACE) uses to transform into angiotensin II (ANG II), a potent vasoconstrictor (Fischer *et al*., 2002; Komukai *et al*., 2010). ANG II can also stimulate the release of aldosterone to promote sodium and water retention in the kidneys for BP regulation (Coylewright *et al*., 2008). When BP rises, estrogen can prevent the production and vasoconstrictive effects of ANG II (Coylewright *et al*., 2008; O'Hagan *et al*., 2012). Thus, a decline in estrogen levels may result in an increased production of ANG II, ultimately leading to vasoconstriction and potentially hypertension (Coylewright *et al*., 2008). Moreover, premenopausal women aged 20 to 40 years have a lower prevalence of hypertension, with a reported rate of 19% (Leuzzi and Modena, 2011; Lima *et al*., 2012). However, in postmenopausal women (aged >55 years), the prevalence of hypertension is high (Dai *et al*., 2008; Zambrana *et al*., 2014; Brahmbhatt *et al*., 2019), with reported rates ranging from 20-44% (Rasi *et al*., 2009) to as high as 75% in the United States (Barton and Meyer, 2009). Hypertension is one of the important risk factors for hypertension-

induced cardiac remodelling and LV dysfunction (Yildoz *et al*., 2019). Furthermore, estrogen has other effects in the CV system such as: i) reducing apoptosis (programmed cell death) of the cardiomyocytes (cardiac muscle cells) (Patten *et al*., 2004; Kim *et al*., 2006; Ma *et al*., 2009); ii) decreasing cardiac contractility (ability of the heart muscle to contract or shorten in response to an electrical stimulus) (Jiang *et al*.,1992; Sitzler *et al*., 1996; Patterson *et al*., 1998); and iii) influencing calcium and potassium handling mechanisms crucial for contraction (Tanabe *et al*., 1999; Chen *et al*., 2011; Yang *et al*., 2012). Collectively, the absence of estrogen and its influence on blood vessels can result in increased arterial stiffness, which likely contributes to greater ventricular afterload and potentially influence the structural changes of the LV (Wang and Fitch, 2004; Nio, 2020). Taken together, when estrogen levels decline during menopause, the cardioprotective effects on the heart diminish.

1.3.2 The effect of estrogen on the LV

The increased risk of adverse cardiac changes due to effects of declined estrogen levels have been documented in numerous studies. To establish a comprehensive understanding of the forthcoming discussion on cardiac changes, we will first revisit the fundamental aspects of cardiac function and structure, along with the key parameters used for their evaluation. A cardiac cycle consists of two separate phases during one heartbeat: i) the systole, which includes isovolumic contraction and ejection; and ii) the diastole, which includes isovolumic relaxation and filling (Klabunde, 2011; Levick, 2013). The stroke volume (SV) is the total amount of blood ejected during each cardiac cycle. SV is calculated as the difference between the end-diastolic volume (the maximum volume of blood in the LV after filling), and the end-systolic volume (the remaining volume of blood in the LV after ejection) (Levick, 2013). Accordingly, the ratio of SV to end-diastolic volume is referred to as the ejection fraction, given as a percentage. With the beginning of diastole and following systole, the LV begins to lengthen. The cardiac cycle repeats as the next ventricular systole (i.e. isovolumic contraction) begins at the same time as the end of filling phase (Nio, 2020). Heart rate is described as the number of cardiac cycles per minute. The information on heart rate and SV is used to calculate cardiac output (CO), the volume of blood ejected from the LV per minute (Buckberg *et al*., 2001; Buckberg, 2002). In essence, this summarizes the cardiac function. When examining cardiac structure or geometry, attention usually centers on the chambers (atria and ventricles) and the walls (thickness and shape). The specific sizes and measurements of the heart's chambers and walls are what constitute cardiac dimensions (Hoffman and Ritman, 1985). The geometry, systolic and diastolic functioning parameters of the heart can be acquired by echocardiography. Echocardiography is the standard non-invasive diagnostic technique that uses

pulsed ultrasound to assess the size, structure, and function of the heart (Lalani and Lee, 1976; Kleijn *et al*., 2012; Lang *et al*., 2015; Chengode, 2016). It is an essential tool for the diagnosis, prognosis, and management of several CV conditions (Chan *et al*., 2020). We will briefly highlight the key parameters of LV structure and function using echocardiography.

The most frequently used parameters to assess cardiac geometry are the internal LV dimensions (ESD, EDD) and volumes (ESV, EDV) measured at the end of systole and diastole (Lang *et al*., 2015; Magunia *et al*., 2017). The internal dimensions can be obtained using two-dimensional (2D) guided M-mode echocardiography, whereas the volumetric measures obtained by 2D or 3D echocardiography with biplane disk summation (Grossgasteiger *et al*., 2013; Lang *et al*., 2015). Measurements obtained by M-mode echocardiography, including interventricular septal wall thickness (IVS) and posterior wall thickness (PWT), can be utilized to calculate relative wall thickness (RWT). The RWT reflects the proportion of wall thickness relative to the chamber dimensions (Dujardin *et al*., 1997). Subsequently, LV mass can be estimated based on validated formulas employing RWT and chamber dimensions (Li *et al*., 2001). These combined measures provide valuable insights into the presence and extent of LV remodelling (Gaasch, 1979). LV remodelling refers to modifications in the LV, often as a response to various stimuli and stressors, such as hypertension (Gaasch, 1979). These modifications affect the mass, chamber dimensions and shape of the LV (Gaasch, 1979; Patten, 2007). LV remodelling can be categorized as either concentric (decreased chamber sizes, increased RWT and no change in LV mass) or eccentric (increased chamber sizes, no change in RWT and increased LV mass (de Simone, 2004; Mihl *et al*., 2008; Ojji *et al*., 2013). LV remodelling can initially be a compensatory response to maintain cardiac function due to changing physiological states such as during pregnancy or exercise (Wu *et al*., 2017). However, persistent or excessive remodelling can lead to impaired cardiac function and increased risk of CV events (Wu *et al*., 2017).

For the assessment of diastolic function, pulsed wave Doppler echocardiography and tissue Doppler imaging (TDI) are commonly used. Pulsed wave Doppler measures the trans-mitral inflow velocities in early diastole (E) from LV relaxation and in late diastole (A) caused by atrial contraction (Nagueh *et al*., 2016). The ratio of peak E to A wave velocities (E/A) is an index of LV relaxation (Nagueh *et al*., 2016). The peak E velocity is usually greater than the peak A velocity, reflecting efficient LV filling during diastole (Hasegawa *et al*., 2003). However, in conditions reflecting diastolic dysfunction, the opposite of this is observed, reflecting impaired relaxation and reduced LV filling capacity during diastole (Onose *et al*., 1999; Mitter *et al*., 2017). Additionally,

the TDI measures the peak velocities of myocardial tissue lengthening in early (e') and late (a') diastole at the mitral annulus (De Boeck *et al*., 2003; Nikitin and Witte, 2004). The ratio of early to late mitral annular tissue lengthening (e'/a') is considered a measure of LV passive stiffness used to elucidate the functional integrity during diastole (Nikitin and Witte, 2004; Nagueh *et al*., 2016). Combining the mitral inflow during early diastole (E), which is dependent on ventricular relaxation and left atrial pressure, and the mitral annular myocardial lengthening velocity in early diastole (e'), which is dependent solely on LV relaxation, results in the E/e' ratio (Nikitin and Witte, 2004). The E/e' ratio is considered an index of LV filling pressure (Ommen *et al*., 2000; Mitter *et al*., 2017; Silbiger, 2019). Furthermore, TDI can also indirectly assess systolic function by measuring myocardial tissue lengthening at the mitral annulus during systole (s') (Dagianti *et al*., 2000; Ur Rahman *et al*., 2011). Systolic function can also be measured indirectly using the LV internal dimensional and volumetric changes. The measurements are used to calculate measures of contraction such as the SV, LV ejection fraction (EF), endocardial fractional shortening (FSend) and midwall fractional shortening (FSmid) (Lang *et al*., 2015; Chengode, 2016). The FSend and FSmid are measures of the percentage change in the LV internal dimensions during contraction (Borow *et al*., 1982; Clark *et al*., 1991; Muiesan *et al*., 2000). Contraction is influenced by myocardial contractility (the force generated by the cardiac muscle fibers), and the length-tension relationship based on the Frank-Starling mechanism (which dictates the relationship between the LV filling volume and blood ejected per beat) (Babu *et al*., 1988; Shiels and White, 2008). The sympathetic nervous system (SNS) has a major role in mediating myocardial contractility through the release of norepinephrine (NE) (Singh *et al*., 2000; Gordon *et al*., 2015). NE binds to β1-adrenergic receptors, particularly abundant in the midwall region due to richer blood supply (Singh *et al*., 2000).

Research has indicated that estrogen is essential for maintaining cardiac structure and function, with its absence leading to detrimental changes (Cavasin *et al*., 2003; Lu *et al*., 2012; Janicki *et al*., 2013; El Hajj *et al*., 2017). Declined estrogen levels has been linked to adverse myocardial geometry, particularly LV remodelling. Notably, the Framingham study highlights the high prevalence of left ventricular hypertrophy (LVH) among women, with a 67% increase for every 10 years after the age of 60 (Levy *et al*., 1988). LVH is a condition characterized by increased LV mass, attributed to either increased wall thickness or dilated LV cavities, or both (Lorell and Carabello, 2000; Drazner *et al*., 2004; Sayin and Oto, 2022). Most frequently, an overload in pressure induces the LV wall thickening, while an overload in volume induces the chamber dilatation (Mihl *et al*., 2008; Cuspidi *et al*., 2012). LVH is characterized by changes in LV geometry that include increased LV mass,

increased RWT, and decreased end-diastolic and end-systolic dimensions (Carroll *et al*., 1992; Aurigemma *et al*., 1994; Zabalgoitia *et al*., 1998, Nardi *et al*., 2009). LVH is common in older individuals (Levy *et al*., 1988; Hung *et al*., 2011; Seliger *et al*., 2015), with a higher prevalence in postmenopausal women compared to men of the same age (Razandi *et al*., 1996; Leibowitz *et al*., 2010; Yoo *et al*., 2018). LVH can be classified into two main LV remodelling types, concentric hypertrophy and eccentric hypertrophy. Concentric hypertrophy is marked by an increase in the LV wall thickness, but the internal cavity may not significantly enlarge (Grossman *et al*., 1975; Lorell and Carabello, 2000; Müller and Dhalla, 2013). Concentric hypertrophy is often associated with pressure overload conditions, such as hypertension, where the heart has to pump against increased resistance (Grossman *et al*., 1975; Lorell and Carabello, 2000; Müller and Dhalla, 2013). On the other hand, eccentric hypertrophy is marked by an increase in both the LV wall thickness and the dilation of the internal cavity (Grossman *et al*., 1975; Rossi and Carillo, 1991; Lorell and Carabello, 2000). Eccentric hypertrophy is often associated with volume overload conditions, such as chronic valvular regurgitation, where the heart must handle an increased volume of blood (Grossman *et al*., 1975; Rossi and Carillo, 1991; Lorell and Carabello, 2000; Mihl *et al*., 2008). Moreover, the concentric hypertrophy is commonly observed in postmenopausal women (Krumholz *et al*., 1993; Legget *et al*., 1996; Schillaci *et al*., 1998).

Studies also demonstrate that declined estrogen levels worsen the functional integrity of the heart (Pines *et al*., 1992; Cavasin *et al*., 2003; Zile *et al*., 2004). Estrogen is suggested to be an important regulator of diastolic function in women (Li and Gupte, 2017). Such that postmenopausal women have a greater prevalence of LV diastolic dysfunction (LVDD) compared to men of the same age (Voutilainen *et al*., 1993; Gökçe *et al*., 2003). Increasing evidence indicates that estrogen withdrawal leads to LVDD, as indicated by impaired LV relaxation (decreased E/A) and increased filling pressures (E/e') (Prelevic and Beljic, 1994; Kangro *et al*., 1995; Alecrin *et al*., 2004; Düzenli *et al*., 2007), accompanied by reduced or compensated CO (Zile *et al*., 2004). Additionally, LV systolic dysfunction (LVSD) has also been linked to estrogen withdrawal, as indicated by decreased SV, impaired pump function (reduced EF) and impaired contraction (reduced FSend) (Eckstein *et al*., 1993; Prelevic and Beljic, 1994; Schillaci *et al*., 1998). Cardiac function abnormalities (LVDD and LVSD) are strong predictors for the onset of heart failure or other cardiac events (de Leeuw and Kroon, 1998; Lam *et al*., 2011; Farré *et al*., 2018). These abnormalities not only increase the risk of heart failure, but also contribute to the progression of the condition (de Leeuw and Kroon, 1998; Lam *et al*., 2011). Therefore, measures of cardiac function are a crucial aspect of CV risk assessment. Mechanisms through which estrogen withdrawal may affect cardiac function include

the aggravation of mitochondrial dysfunction, inflammation and cardiac remodelling, LVH, inhibition of NO signalling, calcium imbalance, and titin isoform switch (Li and Gupte, 2017). However, these mechanisms will not be explored as this is beyond the scope of this dissertation. It is crucial to note that aging also has effects on the cardiometabolic health of menopausal women (Matthews *et al*., 2001; Davis *et al*., 2012; Thurston *et al*., 2018). Nevertheless, an in-depth discussion on the adverse effects of aging on the cardiometabolic health is beyond the scope of this dissertation.

1.4 Rat Models of Estrogen Deficiency

Similar to postmenopausal women, decline of estrogen levels in rodent models has been linked to adverse cardiometabolic outcomes. Rodent models have been widely used to investigate the influence of estrogen on various physiological systems (Ingberg *et al*., 2012; Koebele and Bimonte-Nelson, 2016). While female rodents do not experience menopause per se, they do undergo transitions from regular estrous cycles to irregular estrous cycles, and eventually to extended and persistent estrous cycles (Clark *et al*., 2004; Ajayi and Akhigbe, 2020). In rodents the estrous cycle is the reproductive cycle, comparable to the menstrual cycle in humans (Ajayi and Akhigbe, 2020). In young female rodents, distinct estrous cycles are characterized by cyclic increases in estrogen levels, whereas adult rodents in persistent estrous cycles exhibit a decline in estrogen levels (Clark *et al*., 2004). Moreover, rats and mice have a short (lasts for 4 to 5 days) and precise length of estrous cycle (Andrews and Ojeda, 1981; Auta and Hassan, 2016). Although rats and mice have similar developmental profiles, female rats begin to undergo sexual maturation at 5 to 6 weeks of age (denoting the beginning of adolescence) (McCutcheon and Marinelli, 2009; Sengupta, 2013). Moreover, female rats experience a decline in reproductive function between 15 and 20 months of age (McCutcheon and Marinelli, 2009). Furthermore, several types of rodent models are used to mimic menopause, including: i) the aging model, where rodents undergo a natural aging process to promote gradual withdrawal of estrogen, similar to natural menopause experienced by women. This model allows for observing the physiological effects of declining estrogen levels associated with the aging process (Bachmann, 2001; Discigil *et al*., 2006; Rocca *et al*., 2011); ii) the ovariectomy model, where the ovaries are surgically removed to induce a state of estrogen deficiency. Although mimicking postmenopausal conditions, this model allows researchers to investigate the consequences of abrupt estrogen deficiency (Rocca *et al*., 2009; Sarrel *et al*., 2016; Zakaria *et al*., 2019*).* Notably, the age at which ovariectomy is performed may influence the alteration of the cardiometabolic profile (Minta *et al*., 2018), as aging itself may also have an effect; which may be compounded by estrogen deficiency and iii) the aromatase inhibitor model, where aromatase

enzyme activity is inhibited by drugs to block the conversion of androgens to estrogens, inducing a gradual decline in estrogen levels. This model provides a gradual and controlled reduction in estrogen levels, allowing researchers to study the effects of long-term estrogen deficiency (Simpson, 2004; Li, 2010; Chan *et al*., 2016).

The ovariectomized rodent model is well-known in the literature for simulating menopause and thereby studying the impact of a deficiency of female sexual hormones on various organs and systems (Farré *et al*., 2018). Studies using the ovariectomized rodent model commonly use sham-operated rodents as controls to account for potential confounding variables (Sniekers *et al*.,2008). Sham-operated rodents undergo the same procedures as the ovariectomized rodents, except for the ovary removal (Sniekers *et al*., 2008; Kruger and Morel, 2016). It is important to include control animals as factors like surgical stress, anesthesia and wound healing may influence the observed outcomes (Kruger and Morel, 2016). Similar to postmenopausal women, ovariectomized rodents present with an increased abdominal mass (Wang *et al*., 2022), increased cholesterol levels (especially LDL) (Van Lenten *et al*., 1983; Lei *et al*., 2021; Zhu *et al*., 2023), impaired glucose tolerance and insulin resistance (Ben-Shmuel *et al*., 2015; Fahmy *et al*., 2018; Lei *et al*., 2021) compared to sham-operated rodents. Similar to postmenopausal women, ovariectomized rodents also show increased BP, in both normotensive (Mercer *et al*., 2002; Clark *et al*., 2004; da Silva *et al*., 2009) and hypertensive rats (Hinojosa-Laborde *et al*., 2000; Hinojosa-Laborde *et al*., 2004; da Silva *et al*., 2009). Furthermore, ovariectomy induced increases in LV mass, wall thickness and chamber sizes in both normotensive (Bhuiyan *et al*., 2007; Felix *et al*., 2015; Jiang *et al*., 2017; Minta *et al*., 2018; Phungphong *et al*., 2020), and hypertensive rats (Dahl salt-sensitive rats and SHR) (Dai *et al*., 2008; da Silva *et al*., 2017; Ludvigsen *et al*., 2018) compared to sham-operated rats. Therefore, estrogen deficiency in rodents may be driving LV remodelling independent of the pressure overload of hypertension. In addition, ovariectomized rodents present with cardiac dysfunction similar to postmenopausal women. Ovariectomized rats presented with decreased SV, decreased FSend, decreased EF and impaired CO compared to sham-operated rats (Felix *et al*., 2015; Minta *et al*., 2018; Bustamante *et al*., 2019; Phungphong *et al*., 2020). Ovariectomized rats also presented with impaired LV ventricular filling and impaired relaxation compared to sham-operated rats (Scheer *et al*., 1987; Alencar *et al*., 2017; Ludvigsen *et al*., 2018).

1.5 Shift work

As we continue to delve into the factors influencing cardiometabolic health in estrogen-deficient individuals, it is essential to explore beyond hormonal considerations. Environmental factors such

as shift work exacerbate the already heightened risk of CMD. In literature, the term "shift work" has many definitions. Widely, shift work is regarded as work patterns in which employees change schedules on a regular basis (Pati *et al*., 2001; Fong, 2022). Shift work can consist of rotating work with irregular schedules, early work starts and compressed workweeks (Boivin and Boudreau, 2014; Hemmer *et al*., 2021). Shift work can also involve a work schedule that requires employees to work beyond the standard 8:00 a.m. to 5:00 p.m. hours (Caruso, 2014; Wickwire *et al*., 2017). Shift work can also be employment between 7 p.m. and 7 a.m., rotating around the clock (changing work times from day to evening/night, and vice versa) (Depner *et al*., 2014; Wohrmann *et al*., 2020; Fong, 2022). The latter definition will be used in the present dissertation to refer to "shift work". Unfortunately, permanent shift work has increased because of the necessity for many industries and services to operate around the clock (Kervezee *et al*., 2020; Fong, 2022). 15 to 20% of the working population are shift workers, and emergency services such as healthcare (i.e. nurses) have a high prevalence of shift workers (Hossain and Shapiro, 1999; Haus and Smolensky, 2006; Mosendane *et al*., 2008; Kervezee *et al*., 2020). Moreover, over 45% of shift workers in the healthcare industry are women (Lajoie *et al*., 2015). Shift workers frequently change their sleep and wake patterns throughout the course of a 24-hour day (Torsvall *et al*., 1989; Penev *et al*., 1998; Boivin and Boudreau, 2014). To compensate for the sleep debt incurred during their work hours, shift workers tend to take longer naps on their non-working days (Tune, 1969; Ruggiero and Redeker, 2014). Hence, shift work disrupts the body's internal biological clock by requiring individuals to work during hours that are conventionally designated for sleep, significantly impacting circadian rhythms (Penev *et al*., 1998; Ruggiero and Redeker, 2014; Boivin *et al*., 2021).

1.5.1 The circadian timing system

Organisms synchronize their behaviours to the daily fluctuations in their surroundings, creating 24-hour cycles such as those related to sleep/wake, fasting/feeding, rest/activity, and light/dark cycles (Thosar *et al*., 2018; Draaijer *et al*., 2022). The endogenous circadian timing system is responsible for regulating the 24-hour daily cycles (Scheer *et al*., 2009; Thosar *et al*., 2018). The circadian timing system in most species creates 24-hour rhythms to guarantee that physiological processes take place at the suitable time of day or night (Czeisler *et al*., 1999; Froy, 2010; Thosar *et al*., 2018). In mammals, the circadian rhythms influence the metabolism, body temperature, and activity of the endocrine, renal, and CV systems (Froy, 2010; Thosar *et al*., 2018). Circadian rhythms comprise biological alterations resulting from circadian clocks with an entrainable oscillation that persists even in the presence of unchanging environmental conditions (Chellappa *et al*., 2019). The circadian rhythms are generated by oscillators found in cell nuclei, which work with numerous

genes to establish a transcriptional-translational feedback system (Haus and Smolensky, 2006). The intracellular feedback loops are governed by a multitude of clock genes found in the suprachiasmatic nucleus (SCN) and in most tissues (Boivin and Boudreau, 2014). The SCN, located in the hypothalamus, is the central circadian clock, and peripheral clocks are located in tissues and vital organs such as the kidneys, liver and heart (Mosendane *et al*., 2008; Scheer *et al*., 2009; Thosar *et al*., 2018). The SCN coordinates circadian rhythms through the modulation of the humoral mediators, the autonomic nervous system (ANS), or other unknown factors (Takeda and Maemura, 2011; Thosar *et al*., 2018). The circadian rhythms are reset and modified by zeitgebers (time givers), mainly light, but also exogenous melatonin, exercise and food timing (Lewy *et al*., 1998; Khalsa *et al*., 2003; Emens *et al*., 2005; Gooley *et al*., 2006; Youngstedt *et al*., 2019). The time cue information is transmitted from the SCN to peripheral tissues by monitoring a variety of outputs such as hormonal, neuronal, behavioural, and temperature signals (Kervezee *et al*., 2020). Moreover, circadian synchronization is influenced by the intensity, duration, wavelength, and the timing of the light exposure (Brainard *et al*., 2001; Khalsa *et al*., 2003; Lockley *et al*., 2003; Zeitzer et al., 2005; St Hilaire *et al*., 2012).

Irregular light exposure, such as that experienced by shift workers, disrupts circadian rhythms through several mechanisms (Cipolla-Neto *et al*., 1999; Beckett and Roden, 2009; Touitou *et al*., 2017). Some of the most studied mechanisms include the suppression of melatonin production and disruption of the SCN and clock genes. Melatonin is the hormone produced by the pineal gland during the biological night and serves as the body's internal biological signal for darkness (Lewy *et al*., 1980; Shanahan and Czeisler, 1991). Melatonin secretion peaks during the dark period and maintains an approximate 24-hour pattern even in the absence of external time cues (Ralph *et al*., 1971; Lockley, 2007; Telles *et al*., 2018). The secretion of melatonin involves a multi-synaptic pathway (de Toledo *et al*., 2023). In the absence of light, the pre-autonomic neurons of the paraventricular nuclei (PVN) receive projections from the SCN. These neurons provide signals to the higher thoracic segments of the intermediolateral nucleus (IML) of the spinal cord. The IML then communicates with superior cervical ganglion (SCG) to release norepinephrine into the pineal gland by a sympathetic postsynaptic fiber, thereby inducing the production of melatonin (Larsen *et al*., 1998; Kalsbeek *et al*., 2006; Amaral and Cipolla-Neto, 2018; de Toledo *et al*., 2023). In the presence of light, light penetrates the cornea and pupil of the eyes. After passing through the lens, it converges on the retina (Mosendane *et al*., 2008; Boivin and Boudreau, 2014; Kervezee *et al*., 2020). Within the retina reside intrinsically photosensitive retinal ganglion cells (ipRGCs), specialized neurons exhibiting heightened sensitivity to light (Pérez-León *et al*., 2006; Pickard and

Sollars, 2012; de Toledo *et al*., 2023). Light is converted into electrical impulses upon stimulating the ipRGCs (de Toledo *et al*., 2023). These impulses travel up the optic nerve to the SCN (Mosendane *et al*., 2008; Boivin and Boudreau, 2014; Kervezee *et al*., 2020). The SCN is activated through the retino-hypothalamic tract (Do, 2019; Mure *et al*., 2019). The SCN interprets the impulse as daytime and consequently releases the neurotransmitter called gamma-aminobutyric acid (GABA). This causes the hypothalamic PVN to receive an increased GABAergic input. In response, the pre-autonomic neurons in the PVN become less active, thus suppressing melatonin production (Bartol *et al*., 1997; Cipolla-Neto *et al*., 1999; de Toledo *et al*., 2023). Finally, the exposure to light acutely suppresses melatonin production and resets the circadian rhythms of melatonin (Lewy *et al*., 1980; Shanahan and Czeisler, 1991).

At the molecular level, a signalling cascade elicited by light stimulation promotes the transcription of core clock proteins, which then initiates a series of transcriptional-translational feedback loops (Takahashi, 2016; Rijo-Ferreira and Takahashi, 2019). Normally, the core transcription factor clock proteins circadian locomotor outputs kaput (CLOCK) and brain-and-muscle aryl hydrocarbon receptor nuclear translocator-like protein 1 (BMAL1) heterodimerize and migrate into the nucleus (Meléndez-Fernández *et al*., 2023). The heterodimer attaches itself to target sequences of the cryptochrome (Cry) and period (Per) proteins (Beckett and Roden, 2009; Bollinger and Schibler, 2014). The resulting gene products accumulate and dimerize in the cytoplasm, and ultimately migrate back to the nucleus where the cycle begun. In the nucleus, the gene products inhibit their own transcription. As a result of this process, the Per and Cry proteins are eventually degraded and lessened leading to disinhibition of their transcription. In the presence of light this loop is disrupted by inhibiting CLOCK/BMAL1 expression, leading to reduced production and degradation of Per and Cry proteins. The Per and Cry proteins are crucial as transcription factors, playing a role in regulating the expression of other genes involved in various physiological processes. Their reduction disrupts the rhythmic expression of these genes in peripheral tissues, leading to desynchronization between the SCN and peripheral clocks. With the SCN and peripheral clocks out of sync, internal rhythms (e.g. sleep/wake cycle) no longer align with the external light/dark cycle (Meléndez-Fernández *et al*., 2023). Therefore, the presence of light at night leads to desynchronization within the circadian clocks, which may be termed circadian misalignment (Haus and Smolensky, 2006; Baron Reid, 2014; Boivin and Boudreau, 2014; Hemmer *et al*., 2021). Circadian misalignment impacts physiological processes such as body temperature, metabolism, and activity of the CV, endocrine and renal systems (Evans and Davidson, 2013; Baron Reid, 2014; Buijs *et al*., 2016).

1.5.2 Circadian misalignment and CMD

Circadian misalignment has been linked to a range of negative health outcomes, including CVD, diabetes, obesity, metabolic syndrome, menstrual abnormalities, cancer and psychiatric disorders (Penev *et al*., 1998; Haus and Smolensky, 2006; Scheer *et al*., 2009; Golombek *et al*., 2013; Baron and Reid, 2014; Boivin and Boudreau, 2014; Hemmer *et al*., 2021). The circadian clocks influence the timing of metabolic pathways, regulating processes such as glucose metabolism, lipid metabolism, and insulin sensitivity (Gooley, 2016; Poggiogalle *et al*., 2018; Serin and Acar Tek, 2019). Evidence shows that circadian misalignment in shift workers is associated with increased blood glucose levels, glucose intolerance, impaired insulin sensitivity and insulin resistance (Leproult *et al*., 2014; Gan *et al*., 2015; Jung *et al*., 2018; Kim *et al*., 2020). Shift workers tend to also have increased levels of triglycerides, LDL and total cholesterol (Gadallah *et al*., 2017; Kim *et al*., 2020; Schettini *et al*., 2023). In addition to this, shift work is associated with abnormal eating habits leading to an imbalance in appetite-regulating hormones (i.e. leptin and ghrelin), potentially contributing to overeating and body mass gain (Wong *et al*., 2010; Pot *et al*., 2014; Strzemecka *et al*., 2014; Kim *et al*., 2020). A combination of these factors may increase the risk of developing metabolic syndrome, obesity, type 2 diabetes, hypertension, and immune system suppression (Karlsson *et al*., 2001; Morikawa *et al*., 2005; De Bacquer *et al*., 2009; Vyas *et al*., 2012). Moreover, studies also observed that shift workers had increased BP (Scheer *et al*., 2009; Morris *et al*., 2016; Morris *et al*., 2017; Park *et al*., 2019; Draaijer *et al*., 2022). Similar to shift workers, circadian misalignment in rodent models has been associated with increased glucose levels, impaired glucose tolerance and insulin resistance (Lamia *et al*., 2008; Salgado-Delgado *et al*., 2013; Qian *et al*., 2013; Zhong *et al*., 2019), as well as increased LDL cholesterol (Schilperoort *et al*., 2020; Figueiro *et al*., 2021; Chalfant *et al*., 2023). Furthermore, research in both humans and rodent models show that circadian misalignment may impact cardiac function, with potential consequences for heart rate variability, contractility, and overall CV performance (Young *et al*., 2014; Chellappa *et al*., 2019). Additionally, rodent models with mutations of circadian clock genes demonstrated metabolic abnormalities (Rudic *et al*., 2004; Turek *et al*., 2005), while frequent repeated shifts in the light/dark cycle resulted in premature death (Penev *et al*., 2009). Thus, further supporting the concept that circadian misalignment may have severe health effects with serious long-term consequences. In rodents, shift work conditions are stimulated by several methods, including reversed light/dark cycles, delayed light/dark cycles, constant light exposure and forced activity during rest phases (Heinrichs and Koob, 1997; Murphy *et al*., 2003; Opperhuizen *et al*., 2015).

1.6 Interaction between estrogen and circadian rhythms

The cardiometabolic health of estrogen-deficient individuals may also be affected by circadian misalignment (Zhang *et al*., 2022). Postmenopausal women exhibit a higher prevalence of sleep disturbances compared to premenopausal women, potentially contributing to this complex interplay between hormonal changes and misaligned circadian rhythms (Woods and Mitchell, 2010; Blümel *et al*., 2012; Zolfaghari *et al*., 2020). Evidence shows that the sleep disturbances reported by postmenopausal women may be influenced by vasomotor symptoms (Pennestri *et al*., 2006; Xu and Lang, 2014; Arnardottir *et al*., 2016). Vasomotor symptoms (i.e. hot flashes) were observed to exhibit a circadian rhythm, characterized by a predominant occurrence in the afternoon hours (Freedman, 2014). Therefore, postmenopausal women who are shift workers already have an increased risk of frequent sleep disturbances due to possible existing circadian misalignment and increased risk for CMD due to a decline in ovarian hormones (as discussed above). The findings of a recent study lend support to this concept. In estrogen-deficient mice exposed to circadian misalignment, the mice presented with an increased body mass, LDL:HDL ratio and systolic BP (Anderson *et al*., 2023). Additionally, circadian misalignment is possibly an explanation for sleep disturbances in aging females and males. Aging is associated with dampened circadian rhythms, whereby the internal clock becomes less precise in regulating sleep/wake cycles. This further contributes to the existing tendency of older adults to sleep and wake at earlier biological times compared to younger individuals and the difficulty of maintaining regular sleep (Duffy *et al*., 2015; Yuan *et al*., 2020; Kim *et al*., 2022; Rahman *et al*., 2023). Thus, as women age, they may experience independent sleep disturbances in addition to circadian misalignment. It has been shown that the combination of both sleep disturbances and circadian misalignment increase the cardiometabolic risk (Buxton *et al*., 2012). Furthermore, circadian rhythms differ in women and men, especially because sleep may occur at a later biological time for women than men, despite maintaining similar bedtimes and wake times, which could be in itself persistent circadian misalignment (Cain *et al*., 2010; Duffy *et al*., 2011).

Evidence indicates that interactions between the circadian timing system and estrogen occur at every level of biological functioning, from gene expression to complex behaviours (Gao and Dahlman-Wright, 2011; Sinchak and Wagner, 2012; Alvord *et al*., 2021). However, there are still a lot of unanswered questions. Particularly in this dissertation, the question of interest is what happens to the cardiometabolic health of hypertensive women when they reach postmenopausal stage. Hypertensive postmenopausal women have a higher risk for developing CMD, yet little emphasis has been given to clinical and experimental studies of this population. Most studies have

previously focused on normotensive rodent models and healthy individuals. Therefore, further studies in individuals already at risk of having a negative cardiometabolic outcomes are required to determine the effects of endogenous circadian rhythms on cardiometabolic risk markers. For investigating such conditions, spontaneously hypertensive rats (SHR) are usually utilized. SHR are a well-established rodent model for human essential hypertension (Kren *et al*., 1997; Pravenec *et al*., 2014; Jama *et al*., 2021). SHR often present with decreased insulin sensitivity and glucose tolerance, as well as altered lipid profiles (Swislocki and Tsuzuki, 1993; Potenza *et al*., 2005; Kwitek, 2019). Moreover, endothelial dysfunction (impaired vasodilation and increased arterial stiffness), LV remodelling, and fibrosis can also be observed in SHR, contributing to reduced LV function (Kokubo *et al*., 2005; Potenza *et al*., 2005; Bernatova *et al*., 2009).

1.7 Research Gap

Historically, studies conducted in female SHR have focused on investigating the effects of sex hormones, especially estrogen, progesterone and follicular stimulating hormone (FSH), on the CV and metabolic health separately. Researchers often isolate the multiple physiological effects of sex hormones on various systems to better understand the underlying mechanisms. Although recent research focused on how changes in estrogen levels may impact the interplay between CV and metabolic parameters, the degree of emphasis on these parameters differs. Moreover, current research findings are contradictory due to the age at which experiments are conducted, the 'menopausal' status, the timing of estrogen administration/treatment, environmental factors (i.e. diet), sample size variations and different measurement techniques. Research has also focused on the impact of shift work, especially night shift work, on various aspects of women's health, including menopause onset or age, worsening of symptoms, and its associations with reproductive and breast health. Several studies have explored sex differences in how shift work affects biomarkers of cardiometabolic health. However, there is a lack of consistency in the findings, which may be attributed to various factors. These include age differences in female participants that is not consistently considered in all studies and varying definitions of shift work among studies. Additionally, the extent of circadian misalignment may be influenced by a wide variety of work schedules, such as the number of night shifts, rotation patterns, and the duration of exposure to shift work. Furthermore, there is limited research on how circadian misalignment worsens the negative effects or the likelihood of developing CMD in the absence of estrogen in females, especially with pre-existing hypertension. In SHR, most of the research has focused on understanding the role of their genetic predisposition and circadian clocks (or clock genes) in relation to cardiometabolic health. Historically, most of the emphasis has been on BP regulation, and the studies are often

conducted in male SHR. There is not enough evidence on the effects of circadian misalignment on various cardiometabolic parameters in SHR. There is limited research on how circadian misalignment may interact with estrogen deficiency in female SHR. Moreover, little is known about the complex interplay between circadian misalignment and an increased cardiometabolic risk profile in estrogen-deficient females, especially those with hypertension. Therefore, it is important to fully comprehend the underlying mechanisms that contribute to the development of CMD in shift workers already susceptible like hypertensive postmenopausal women. In the present study we aimed to determine whether circadian misalignment worsens cardiometabolic parameters in estrogen-deficient female SHR.

1.8 Aim

To determine whether circadian misalignment worsens cardiometabolic parameters in estrogen-deficient female spontaneously hypertensive rats (SHR).

1.9 Objectives

- To develop a rodent model mimicking the cardiometabolic changes related to estrogen deficiency by using ovariectomized female SHR compared to sham-operated female SHR.
- To determine whether shifting the night and day light exposure (using the chronic phase shift protocol) every week for 10 weeks worsens the cardiometabolic parameters in ovariectomized female SHR compared to controls maintained under a standard day-night light exposure.
- The cardiometabolic parameters assessed:
    - Fasting blood glucose concentrations and glucose tolerance measured by a glucose meter.
    - LDL concentrations measured using an Enzyme Linked Immunosorbent Assay (ELISA).
    - Rectal temperature measured using a thermocouple probe.
    - Blood pressure (BP) measured using a tail-cuff technique.
    - Left ventricular (LV) dimensions, systolic and diastolic function assessed by conventional echocardiography.

Justification

The world's leading cause of death and disability is CVD, particularly among elderly women (Yazdanyar *et al*., 2009; Benjamin *et al*., 2017; WHO, 2021). An estimated 17.9 million deaths in 2019 alone were attributed to CVD, making up 32% of all deaths worldwide (WHO, 2021). In South Africa, CVD accounts for up to 40% of adult deaths, making it the second leading cause of death behind HIV/AIDS (BeLue *et al*., 2009). Additionally, the primary risk factors for CMD (obesity, diabetes and hypertension) are already highly prevalent in the majority of women with African ancestry (Alberts *et al*., 2005; Schutte *et al*., 2008; Tibazarwa *et al*., 2009). A substantial percentage of these women are employed in professions that require permanent shift work, such as nursing (Okafor, 2012; Mensah *et al*., 2022; Chikwati *et al*., 2023). Shift work is associated with circadian misalignment, which compromises CV processes and accelerates the onset of CVD (Thosar *et al*., 2018). Studies also demonstrate that following menopause, the risk of metabolic disorders rises (Jeong and Park, 2022). Because of the physiological and hormonal changes associated with menopause, particularly the decline in circulating estrogen, postmenopausal shift workers are more susceptible to CMD. Nonetheless, little is known about how circadian misalignment resulting from shift work affects cardiometabolic markers in hypertensive postmenopausal women. Therefore, understanding the relationship between circadian misalignment and the cardiometabolic risk factors in the already vulnerable postmenopausal women provides insight into heightened susceptibility of these women. This may contribute to the development and implementation of strategies, novel therapies, or recommendations. These may help minimize or prevent the negative impact of circadian misalignment on cardiometabolic risk in this population.

Chapter 2: Materials and Methods

2.1 Animals

Thirty-six (36) five-week-old (peripubertal period in humans) female SHR were obtained from the University of the Witwatersrand Research Animal Facility (WRAF). The rats were housed individually in cages and maintained in temperature-controlled rooms (25 ± 2 °C) with a 12-hour light/dark cycle. They were allowed free access to standard rat chow and plain drinking water. All experimental procedures were approved by the Animal Research Ethics Committee (AREC) of the University of the Witwatersrand (AREC number: 2017/11/76/C).

2.2 Study design

The rats were allowed two weeks of habituation period to acclimatize to environmental conditions and handling. After the habituation period, surgery proceeded on 7-week-old rats (peripubertal period in humans) weighing 104-147g. Ovariectomy was performed by the WRAF veterinarian on half of the rats (n=18) under general anaesthesia (anaket + xylazine, 0.1 ml/100g of the mixture), and metacam (0.1 ml/rat), maintained with 2-4% isoflurane in oxygen. An abdominal incision was made, and then the linea alba was cut and stretched apart so that the ovaries could be seen. The ovaries, arteries, and fallopian tubes were ligatured, and then the ovaries were removed. Under the same conditions as the ovariectomy, the WRAF veterinarian performed a sham operation on the other half of the rats (n=18), where the ovaries were not removed. Using a nylon suture, the surgical incision site was sealed. For postoperative care, the rats were injected with metacam and antisedan (0.1 ml/rat each).

After two weeks of recovery, the rats were synchronized under control lighting conditions (12-hour light: 12-hour dark, lights on at 7 a.m. and lights off at 7 p.m.). The control lighting protocol was adopted from Penev et al. (1998) and Martino et al. (2007). Temperature, humidity, and light intensity were monitored and recorded every 10 seconds with a data logger (HOBO MX1104 Analog/Temp/RH/Light, Onset Computer Corporation, Massachusetts, USA). The light intensity was set at ~ 200 lux for daytime and 0 lux for night-time. At 12 weeks old (emerging adulthood in humans), the rats were randomly assigned into four groups. Two groups of ovariectomized (ova, n=9) and sham-operated rats (sham, n=9) were maintained under control lighting, and two groups of ova (n=9) and sham-operated (n=9) rats were exposed to the chronic phase shift (CPS) protocol. The sample size has been justified in most ovariectomized SHR studies, which utilized six to ten rats per group (Fortepiani *et al*., 2003; Peng *et al*., 2003; Machi *et al*., 2016). The CPS protocol was adopted from Varcoe et al. (2011) (Figure 2.1). During the intervention, the CPS protocol involved manipulating the lighting schedule such that the 12-hour light cycle was reversed every seven days

(lights on at 7 p.m. and lights off at 7 a.m., which was followed again by seven days of control lighting conditions). After 10 weeks of intervention, both control lighting and CPS rats were terminated at 22 weeks old (young adulthood in humans). An outline of the study design is presented in Figure 2.2.

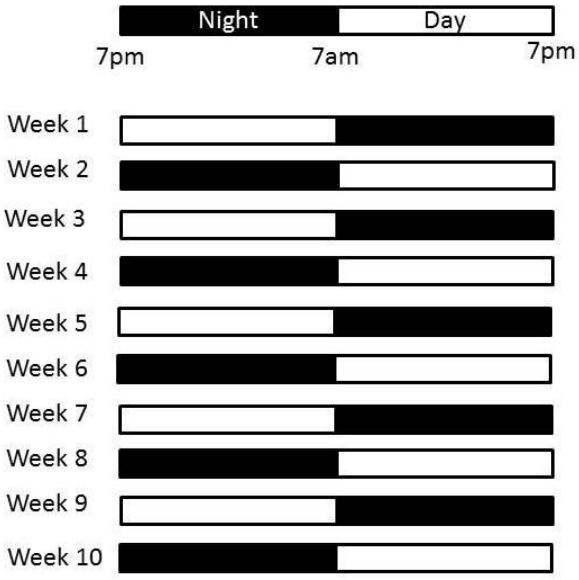

Figure 2.1: The 10-week chronic phase shift (CPS) protocol. The rats assigned to the CPS protocol were exposure to the 12-hour light/dark cycle reversed every 7 days (lights on at 7 p.m. and lights off at 7 a.m., followed by 7 days of control lighting schedule with lights on at 7 a.m. and lights off at 7 p.m.). The black and white bars represent the duration of the periods of darkness (*night*) and light (*day*), as indicated by the bars above the weekly panels.

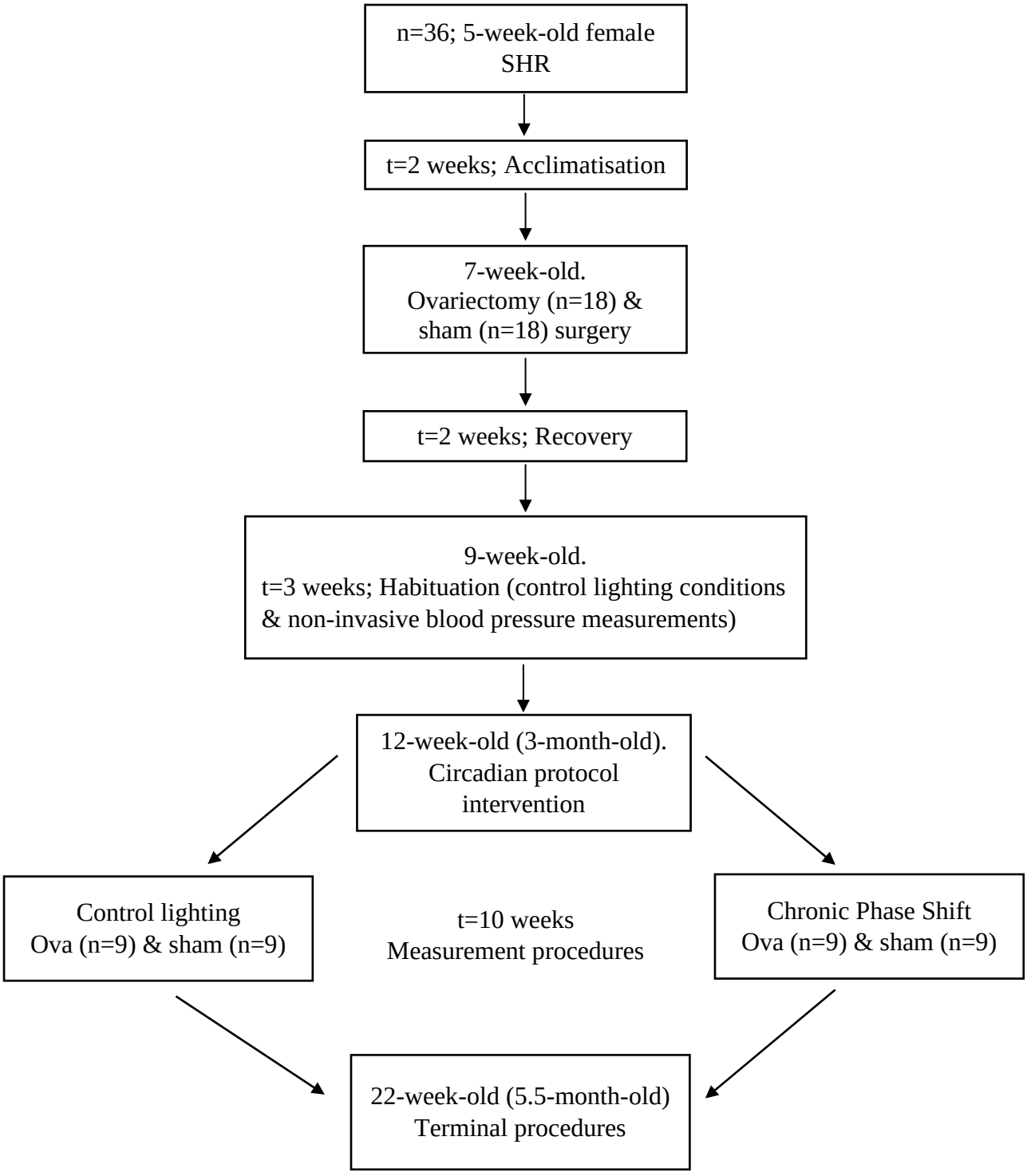

Figure 2.2: An outline of the study design, representing the circadian misalignment intervention timeline and female SHR groupings. 5-week-old female SHR underwent either ovariectomy (n=18) or a sham operation (n=18). At 3-month-old, the rats were allocated to 10 weeks of either control lighting schedule (light on at 7 a.m. and off at 7 p.m. every week) or Chronic Phase Shift (CPS) protocol (lights on at 7 p.m. and off at 7 a.m. for one week, followed again by one week of control lighting schedule). At 5.5-month-old, the rats were terminated. SHR, spontaneously hypertensive rats; t, time; Ova, ovariectomized; Sham, sham-operated

2.3 Measurements and procedures

2.3.1 Body mass, food and water intake

The body mass, food and water intake were weekly measured using a digital scale (LBK6 weighing scale, Adam Equipment S.A. (Pty), Johannesburg, South Africa). These measurements were taken once a week in the morning (10:00-12:00) from the week of surgery to the final week of the CPS intervention. The body mass gain rate was determined using the following formula:

$$\frac{Week_{n+1} - Week_n}{Week_n} x100$$

2.3.2 Rectal temperature

The polyvinyl chloride-insulated thermocouple probe was calibrated to an accuracy of 0.1 °C in a water bath using a high-precision thermometer (Quat 100, Heraeus, Hanau, Germany) across a wide temperature range (35, 36, 37, 38, 39 and 40 °C). The rats were positioned on all four paws on a firm, smooth surface and gently held over their back by hand while the tail was lifted to obtain the rectal temperature. A lubricated Type T copper/constantan thermocouple probe with an exterior diameter of 2 mm was gently inserted 40 mm into the rat's rectum. Core body temperatures in rats have been successfully measured with an insertion depth of 40 mm (Dangarembizi *et al*., 2017). The thermocouple probe was left in the rectum for 40 seconds once inserted to allow temperatures to stabilize and acquire readings. The rectal temperatures were measured once per day for the last 2 weeks of the CPS protocol. The rectal temperature was measured in the morning (10:00-12:00).

2.3.3 Non-invasive blood pressure (NIBP) measurements

Following recovery from surgery (sham vs. ovariectomy), the rats were habituated to the NIBP procedure prior the first measurement to enable them to become acclimated to the procedure. The BP was measured using the NIBP250 BP monitor system (Biopac Systems Inc, Goleta, California, USA) once a week in the morning (10:00-12:00). This procedure was done on a different day of the week than other measurements described. To obtain BP, a conscious rat was placed in a restrainer with the tail outside. The rat was then placed on an electric heating pad (Almohadilla Super Plus 13133, San-UP S.A, San Martín, Argentina). The rat and its tail were allowed to warm to ~32 °C for no more than 30 minutes. The Tail Cuff-Sensor was attached to the rat's tail (between the midpoint and base of the tail). When the rat was relaxed and inactive, systolic, and diastolic BP measurements were recorded at a 200 mmHg cuff pressure. An example of the BP reading is presented in Figure 2.3. A systolic BP of above 150 mmHg (Okamoto and Aoki, 1963; Frohlich *et al*., 1972) and/or diastolic BP of above 90 mmHg (Luo *et al*., 2008) is considered hypertensive in

rats.

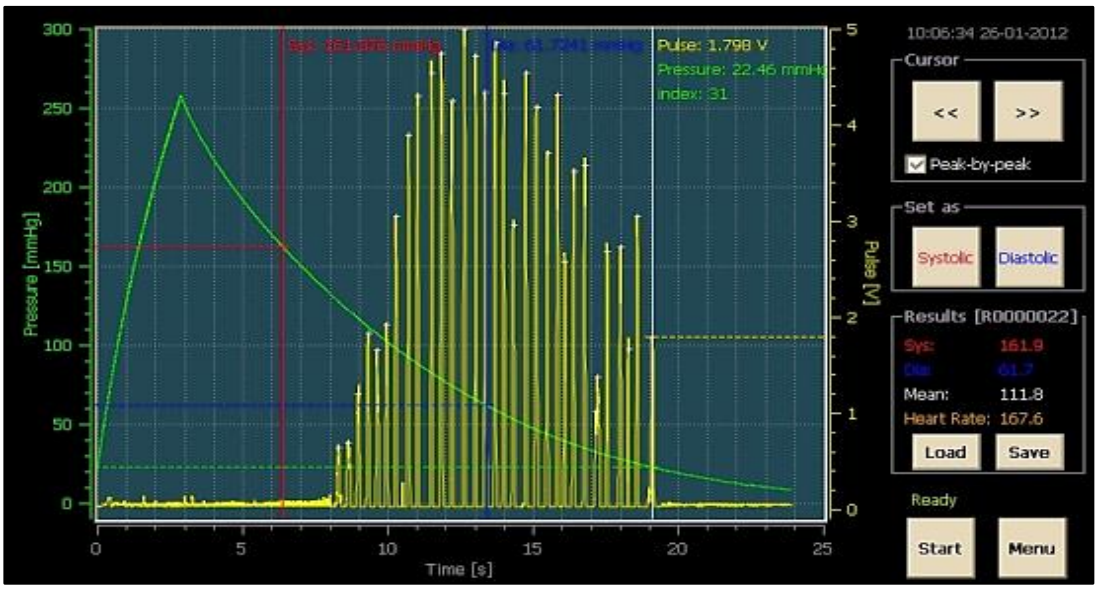

Figure 2.3: An example of BP measurement analysis screen view from NIBP250 BP monitor system. The displayed values are sys: 161.9 mmHg (*red*), dia: 61.7 mmHg (*blue*), and pulse:1.798 V (*yellow*). BP, blood pressure; sys, systolic blood pressure; dia, diastolic blood pressure.

2.3.4 Fasting blood glucose concentration

To determine the fasting blood glucose concentrations, the rats fasted overnight with free access to plain drinking water. In the morning, each rat was placed in a restrainer, then the tail was sterilized with an alcohol swab. The tail vein was pinpricked with a 25-gauge x 15 mm hypodermic needle (Axiology Labs (Pty) Ltd, Meyerton, Vereeniging) to acquire a drop of blood. With a new test strip each time, the drop of blood was collected on a blood glucose test strip (Contour plus, Axiology Labs (Pty) Ltd, Meyerton, Vereeniging) inserted in a calibrated glucose meter (Contour plus meter, Bayer (Pty) Ltd, Isando-Johannesburg, South Africa. The fasting blood glucose concentrations were assessed once a week in the morning (10:00-12:00), on a different day of the week than other measurements. The normal range for the blood glucose concentrations in rats is reported as 2.78 to 7.49 mmol/L (Brăslaşu *et al*., 2007; Hidayaturrahmah *et al*., 2020).

2.3.5 Oral glucose tolerance test (OGTT)

Three days before termination, the rats fasted overnight with free access to plain drinking water. In the morning, the baseline fasting glucose concentration was measured. Thereafter, the rats were gavaged with a sterile glucose solution (D-(+)-Glucose, Merck Chemicals (Pty) Ltd, Johannesburg, South Africa) at a concentration of 2g/1kg of body mass. The blood glucose concentrations were determined at baseline (0), 15, 30, 60, 120 and 180 minutes post gavage. To obtain the measurements, a rat was placed in a restrainer, then the tail was sterilized with an alcohol swab. The tail vein was pinpricked with a 25-gauge x 15 mm hypodermic needle (Axiology Labs (Pty) Ltd, Meyerton, Vereeniging) to acquire a drop of blood. With a new test strip each time, the drop of blood was collected on a blood glucose test strip (Contour plus, Axiology Labs (Pty) Ltd, Meyerton, Vereeniging) inserted in a calibrated glucose meter (Contour plus meter, Bayer (Pty) Ltd, Isando-Johannesburg, South Africa). The areas under the OGTT curve (AUC) and the peak of the OGTT curve for each rat were determined using GraphPad Prism. Blood glucose concentrations of above 8.6 mmol/L 60 minutes post the OGTT is considered impaired glucose tolerance (Perry and Baron, 1999; Kuo *et al*., 2021).

2.3.6 Echocardiography

On the day of termination, rats were anesthetized using 2% isoflurane in oxygen (5% induction). The rats were shaved on the chest, and then placed over a heating pad in the left lateral decubitus position, maintaining body temperature. An experienced observer then performed echocardiography using a non-invasive ultrasound diagnostic machine (Affiniti 70 ultrasound system, Koninklijke Philips N.V., Amsterdam, Netherlands). A high-resolution ultrasonic probe

was placed on the chest of the rat, and the LV was visible in the parasternal long and short-axis views.

The internal dimensions of the cardiac chamber were visualized in vivo using 2D-guided M-mode imaging. The internal dimensions and posterior wall thickness (PWT) of the LV were measured in systole and diastole (Figure 2.4) during three consecutive beats according to the American Society for Echocardiography's leading-edge method (Sahn *et al*., 1978). From the measured dimensions, myocardial systolic functional markers, including LV endocardial fractional shortening (FSend), midwall fractional shortening (FSmid), stroke volume (SV), and the LV ejection fraction (EF) were derived. Moreover, the relative wall thickness (RWT), an index of hypertrophy was also determined. To calculate the SV, LV volumes at the end of diastole (LVEDV) and the end of systole (LVESV), the Teichholz formula (Teichholz *et al*., 1976) was used. The equations below were used to calculate the aforementioned echocardiographic parameters:

Endocardial fractional shortening (FSend) = $\frac{\text{LVEDd} - \text{LVEDs}}{\text{LVEDd}}$

Midwall fractional shortening (FSmid) = $\frac{(\text{LVEDd} + \text{LVPWTd}) - (\text{LVEDs} + \text{LVPWTs})}{\text{LVEDd} + \text{LVPWTd}}$

Left ventricular end-diastole volume (LVEDV) = $\frac{7}{2.4 + \text{LVEDd}}\ x\text{LVEDd}^3$

Left ventricular end-systole volume (LVESV) = $\frac{7}{2.4 + \text{LVEDs}}\ x\text{LVEDs}^3$

Stroke volume (SV) = $\text{LVEDV} - \text{LVESV}$

LV ejection fraction (EF) = $\frac{SV}{\text{LVEDV}}\ x100$

Relative wall thickness (RWT) = $2x\frac{\text{LVPWTd}}{\text{LVEDd}}$

where: LVEDd = left ventricular end-diastolic diameter

LVEDs = left ventricular end-systolic diameter

LVPWTd = left ventricular posterior wall thickness in diastole

LVPWTs = left ventricular posterior wall thickness in systole

LVEDV = left ventricular end-diastole volume

LVESV = left ventricular end-systole volume

SV = stroke volume

To evaluate the LV diastolic function, the mitral valve inflow patterns were obtained using pulse Doppler and tissue Doppler imaging. The images were used to determine the aortic velocity and aortic dimensions. The trans-mitral blood flow velocity in the early (E) and late (A) period of the LV diastolic filling (Figure 2.5), the peak tissue lengthening velocity during the early (e') and late (a') diastole at the mitral annulus and the peak systolic (s') velocity (Figure 2.6) were determined. From these measurements, the diastolic function was assessed using the ratio of early to late diastolic filling velocity (E/A), the ratio of early trans-mitral inflow velocity to early mitral annular tissue lengthening (E/e'), an index of LV filling pressure, and early to late mitral annulus flow velocity (e'/a'), index of LV relaxation. The normal reference values for the M-mode, pulse Doppler and tissue Doppler imaging parameters in rats are presented in Table 2.1.

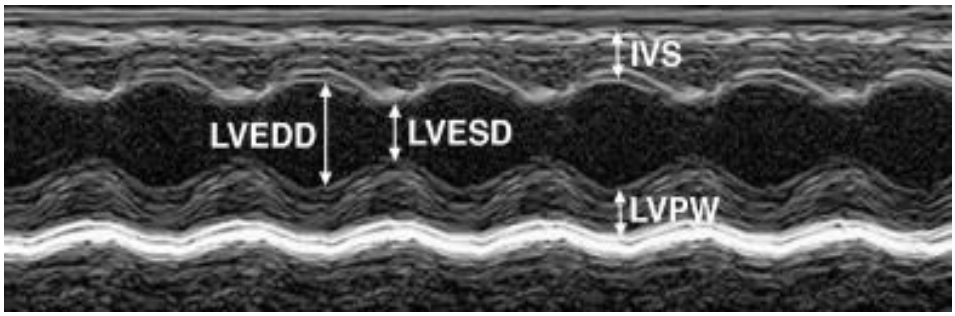

Figure 2.4: M-mode echocardiographic parameters of the left ventricle. The figure shows an example of M-mode echocardiographic tracing obtained in a female SHR. The displayed parameters are the left ventricular end-diastolic diameter (LVEDD), left ventricular end-systolic diameter (LVESD), interventricular septal thickness (IVS) and left ventricular posterior wall thickness (LVPW).

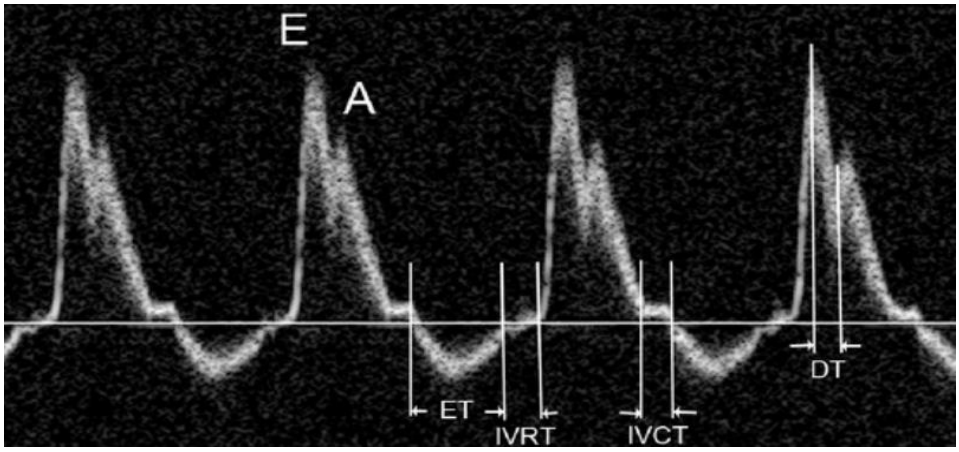

Figure 2.5: An example of pulse wave trans-mitral Doppler imaging parameters. The figure displays the E wave (E) representing early ventricular filling velocity, the A wave (A) representing late filling velocity due to atrial contraction, the ejection time (ET), the isovolumetric relaxation time (IVRT), the isovolumetric contraction time (IVCT) and the deceleration time (DT).

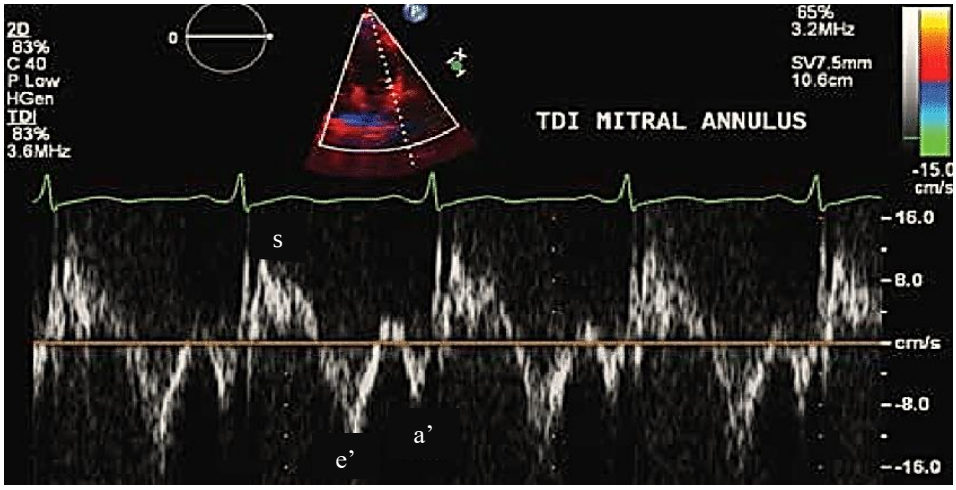

Figure 2.6: An example of tissue Doppler imaging waveforms obtained from the mitral annulus. The figure displays the peak systolic velocity (s'), the early diastolic mitral annulus velocity (e') and the late diastolic mitral annulus velocity (a'), measured in cm/s.

Table 2. 1: Normal reference values for M-mode, tissue Doppler and pulse Doppler echocardiographic parameters in rats.

Echocardiographic parameter	Normal range	Source(s)
M-mode		
IVSTd	0.7 - 2.9 mm	Scheer *et al*., 2012
IVSTs	1.3 - 4.9 mm	Scheer *et al*., 2012
LVEDd	3.5 - 11.4 mm	Scheer *et al*., 2012
LVEDs	2.0 - 7.2 mm	Scheer *et al*., 2012
LVPWTd	0.6 - 4.4 mm	Scheer *et al*., 2012
LVPWTs	1.0 - 5.3 mm	Scheer *et al*., 2012
RWT	< 0.42 - 0.64	Escudero *et al*., 2004; Stolzmann *et al*., 2008
FSend	50 - 80 %	Georgiadis *et al*., 2020
EF	55 - 95 %	Georgiadis *et al*., 2020
SV	0.3 - 0.8 mL/beat	Nakamura *et al*., 1992
Tissue and Pulse Doppler		
e’	10 - 20 cm/s	Sohn *et al*., 1997
a’	5 - 15 cm/s	Sohn *et al*., 1997
s’	< 80 - 150 cm/s	Ismail *et al*., 2018
E	50 - 100 cm/s	Banerjee, 1999; Panesar *et al*., 2017
A	20 - 50 cm/s	Banerjee, 1999; Panesar *et al*., 2017
E/A	0.75 < E/A < 2	Bursi *et al*., 2006
E/e’	< 10	Bursi *et al*., 2006

The normal reference ranges for conventional echocardiographic parameters (with specific units) were obtained from different references, cited in the “Source(s)” column for each parameter. IVSTd, intraventricular septal diameter end diastole; IVSTs, intraventricular septal diameter end systole; LVEDd, left ventricular end-diastolic diameter; LVEDs, left ventricular end-diastolic diameter; LVPWTd, left ventricular posterior wall thickness in diastole; LVPWTs, left ventricular posterior wall thickness in systole; FSend, endocardial fractional shortening; EF, ejection fraction; SV, stroke volume; e’, early peak tissue lengthening velocity at the lateral mitral annulus; a’, late peak tissue lengthening velocity at the lateral mitral annulus; s’, peak systolic velocity; E, maximum early mitral inflow velocity; A, maximum late mitral inflow velocity; E/A, early to late diastolic filling velocity ratio; E/e’, early diastolic mitral inflow velocity to early diastolic mitral annulus velocity.

2.3.7 Organ and blood sampling

After echocardiography, thoracotomy was performed. Blood was obtained by cardiac puncture and collected in EDTA tubes (plasma) and clot activator tubes (serum) between 10:00-13:00. The rats were euthanized by cardiac puncture-induced exsanguination (blood loss) while under anesthesia. The left kidney, liver and heart were harvested from the rats and weighed. The LV was dissected and weighed. The blood was centrifuged at 3000 rpm for 10 minutes. Plasma and serum were collected and stored in a -80 °C freezer until assay.

2.3.8 Low-density lipoprotein concentration

The samples of plasma and serum were centrifuged for 15 minutes at 1000×g at 2–8 °C after being allowed to reach room temperature (18–25 °C). To perform the Enzyme-Linked Immunosorbent Assay (ELISA), the supernatants were collected. To quantify LDL concentrations, the Rat LDL ELISA kit (Elabscience Biotechnology Inc, Houston, Texas, USA) was used for the LDL measurement. The performance characteristics of the kit were: sensitivity of 0.10 μg/mL, detection range of 0.16-10 μg/mL, and < 10% repeatability coefficient of variation. The assay procedure was as follows:

a) Before use, all the reagents were allowed to reach room temperature (18-25 °C).
b) To prepare 750 mL Wash Buffer, 30 mL of Concentrated Wash Buffer was diluted with 720 mL of distilled water.
c) The standard working solution was prepared following the manufacturer's instructions.
d) The Concentrated Biotinylated Detection Ab was centrifuged at 800×g for 1 minute, then the 100× Concentrated Biotinylated Detection Ab was diluted to 1× working solution with Biotinylated Detection Ab Diluent.
e) The Concentrated HRP Conjugate was centrifuged at 800×g for 1 minute, then the 100× Concentrated HRP Conjugate was diluted to 1× working solution with HRP Conjugate Diluent.
f) In duplicate reactions, 100 μL of standard, blank, and sample dilutions were pipetted into the corresponding wells. After sealing the plate, it was incubated for 90 minutes at 37 °C.
g) After decanting the liquid in each well, 100 μL of Biotinylated Detection Ab working solution was pipetted into each well immediately. The plate was then sealed and incubated for 1 hour at 37 °C. Then the solutions were decanted from each well.
h) 350 μL of wash buffer was pipetted into each well and left to soak for one minute. Then the solutions were decanted from each well and dried by patting the plate dry with clean absorbent paper. This washing process was repeated three times.

i) 100 µL of HRP Conjugate working solution was filled to each well. The plate was then sealed and incubated for 30 minutes at 37 °C. The solutions were decanted from each well, and the wash process was repeated as described above.

j) 90 µL of Substrate Reagent was filled to each well. The plate was then sealed and incubated for 15 minutes at 37 °C.

k) Each well was filled with 50 µL of Stop Solution. The Microplate Reader was preheated for about 15 minutes before measuring the optical density (OD). The OD value of each well was determined with a microplate reader set to 450 nm.

l) After averaging the duplicate measurements for each standard and sample, the average OD of the zero standard was subtracted. A four-parameter logistic curve was displayed on the log-log axis, with OD values on the y-axis and standard concentration on the x-axis. The LDL concentrations in the blood samples were calculated by comparing the OD value of the samples to the standard curve. The normal reference range for LDL cholesterol in rats is 38-85 mg/dL (Malik and Sabahelkhier, 2019).

2.3.9 Data Analysis

Data analysis was performed using GraphPad Prism version 9.5 for Windows (GraphPad Software Inc, San Diego, California, USA). The Shapiro-Wilk test was used to confirm the normality of the data. The data was expressed as mean ± standard deviation (SD). A two-way analysis of variance (ANOVA) for the main effect of ovariectomy vs. sham-operated and CPS vs. controlled lighting schedule), followed by a Bonferroni post hoc test, was used to evaluate whether there was a significant difference between the means of the variables for the experimental groups. A repeated measure two-way ANOVA (the main effect of time, ovariectomy vs. sham-operated and CPS vs. controlled lighting schedule), followed by a Tukey post hoc test, was used to evaluate the differences between the means of body mass, body mass gain rate, food and water intake, systolic and diastolic BP, and OGTT between the experimental groups over the 10 weeks of CPS intervention. Furthermore, a mixed-effects model (Residual maximum likelihood), followed by a Tukey post hoc test, was used to determine the fasting blood glucose concentrations between the experimental groups over the ten weeks of CPS intervention. Statistical significance was considered for $p \leq 0.05$.

Chapter 3: Results

3.1 The environmental conditions obtained by the HOBO data logger during the CPS protocol

Figure 3.1 shows the changes in weekly temperature (A) and humidity (B) over 10 weeks. The temperature and humidity were similar between the CPS and the control light schedules. The changes in weekly light exposure over 10 weeks are shown in Figure 3.2. The light intensity was < 200 lux for the light period and 0 lux for the dark period between the CPS and the control light schedules.

3.2 The characteristics of 5-month-old ovariectomized and sham-operated female SHR under CPS or control light

Figure 3.3 (A) shows changes in weekly body mass over time. All the rats significantly grew with time ($p < 0.0001$). The ovariectomized rats were significantly heavier compared to the sham-operated rats from week -2 (i.e. 2 weeks before the start of the CPS vs. control light protocol) ($p < 0.0001$). There was a significant interaction over time with ovariectomized vs. sham-operated status for body mass ($p < 0.0001$). The ovariectomized rats grew faster.

Figure 3.3 (B) shows the changes in weekly body mass gain rate overtime. All the rats significantly gained body mass at a lower rate with time ($p < 0.0001$). The body mass gain rate was greater in ovariectomized rats at week -2 and week -1 ($p < 0.0001$). There was no significant interaction over time with ovariectomized vs. sham-operated status for body mass gain rate.

Figure 3.4 (A) shows changes in weekly food intake overtime. All the rats significantly consumed less food with time ($p < 0.0001$) until week 5. The ovariectomized rats significantly consumed more food compared to the sham-operated rats from week -3 ($p = 0.0001$), except week 8. There was no significant interaction over time with ovariectomized vs. sham-operated status for weekly food intake.

The changes in weekly food intake *normalized to body mass* overtime are shown in Figure 3.4 (B). When normalized to body mass, all the rats significantly consumed less food with time ($p < 0.0001$). The ovariectomized rats significantly consumed less food compared to sham-operated rats when normalized to body mass ($p < 0.0001$). There was no significant interaction over time with ovariectomized vs. sham-operated status for food intake normalized to body mass.

Figure 3.5 (A) shows changes in weekly water intake overtime. All the rats significantly drank less water with time ($p = 0.0003$), except week 4 and week 8. The ovariectomized rats significantly

drank more water compared to the sham-operated rats from week -3 to week 1 ($p = 0.011$), whereas CPS rats significantly drank more water compared to the control light rats from week 4 onwards ($p = 0.048$). There was a significant interaction over time with CPS vs. control light status for water intake ($p = 0.0004$). The amount of water drank by CPS rats decreased less daily.

The changes in weekly water intake *normalized to body mass* overtime are shown in Figure 3.5 (B). When normalized to body mass, all the rats significantly drank less water with time ($p < 0.0001$). The ovariectomized rats significantly drank less water compared to the sham-operated rats when normalized to body mass ($p = 0.014$). There was no significant interaction over time with ovariectomized vs. sham-operated status for normalized water intake.

The changes in weekly systolic blood pressure (SBP) and diastolic blood pressure (DBP) over 10 weeks are shown in Figure 3.6. The blood pressure (SBP and DBP) was similar between the experimental groups, and the data shows no significant interactions over time. Figure 3.7 (A) shows changes in weekly blood glucose concentrations over 10 weeks. The blood glucose concentrations were similar between the experimental groups, and the data shows no significant interactions over time.

The characteristics of the experimental groups are shown in Table 3.1. The rectal temperature was similar between the experimental groups. Figure 3.7 (B) shows changes in oral glucose tolerance test (OGTT) results over 10 weeks. The OGTT results were similar between the experimental groups, and the data shows no significant interactions over time. Moreover, the peak of the OGTT and the area under the curve (AUC) were similar between the experimental groups (Table 3.1).

Table 3.1 also shows the characteristics of experimental groups after termination. There were no significant interactions across these parameters. The heart and the LV were significantly heavier in the ovariectomized rats compared to the sham-operated rats ($p = 0.0006$ and $p < 0.0001$, respectively). When normalized to the body mass, the heart and the LV were significantly lighter in the ovariectomized rats compared to the sham-operated rats ($p < 0.0001$ for both). The liver was significantly heavier in the ovariectomized rats compared to the sham-operated rats ($p < 0.0001$). When normalized to the body mass, the liver was significantly lighter in the ovariectomized rats compared to the sham-operated rats ($p < 0.0001$). Additionally, the liver was significantly lighter in the CPS rats compared to the control light rats ($p = 0.03$). When normalized to body mass, the liver was significantly lighter in the CPS rats compared to the control light rats ($p = 0.01$). The left kidney was significantly heavier in the ovariectomized rats compared to the sham-operated rats ($p = 0.02$).

When normalized to the body mass, the left kidney was significantly lighter in the ovariectomized rats compared to the sham-operated rats ($p < 0.0001$). The LDL concentrations were similar between the experimental groups (Table 3.1).

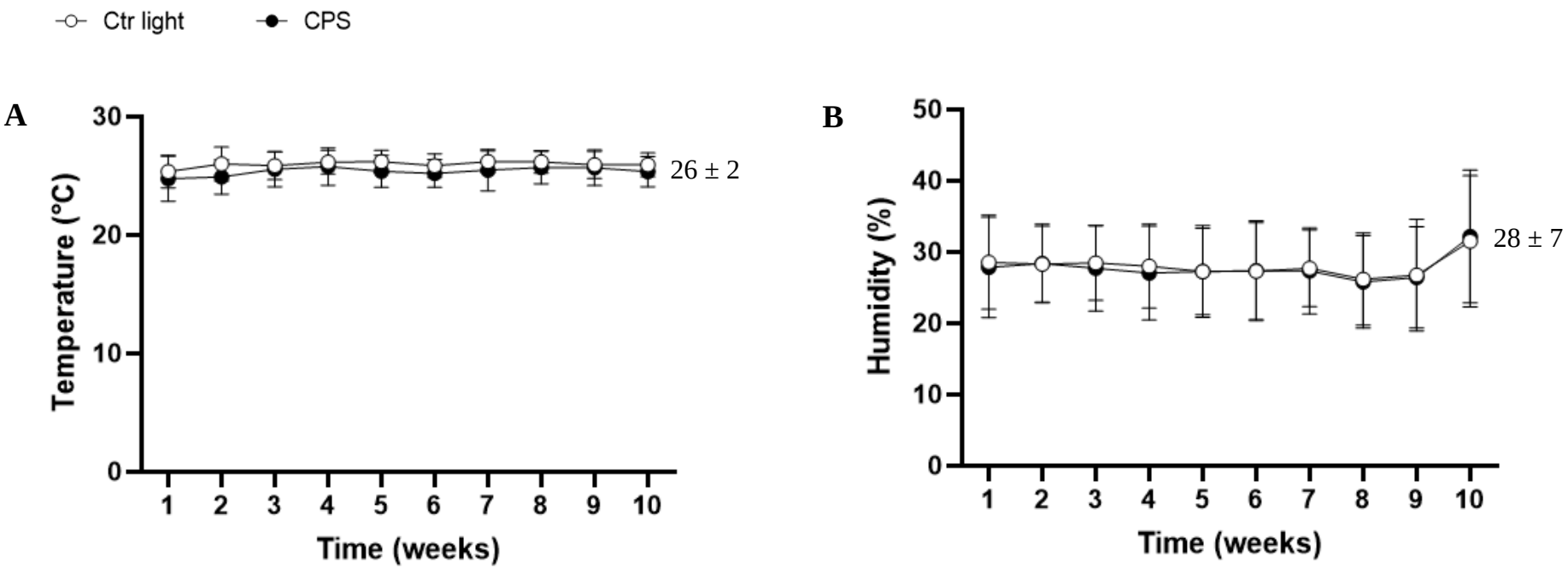

Figure 3.1: Changes in weekly A) temperature and B) humidity during the 10-week CPS intervention. The rats were maintained under controlled conditions, monitored with a HOBO data logger. No significant differences in temperature nor humidity were observed between the CPS and Ctr light groups. Data expressed as mean ± SD. Ctr light, control light; CPS, chronic phase shift.

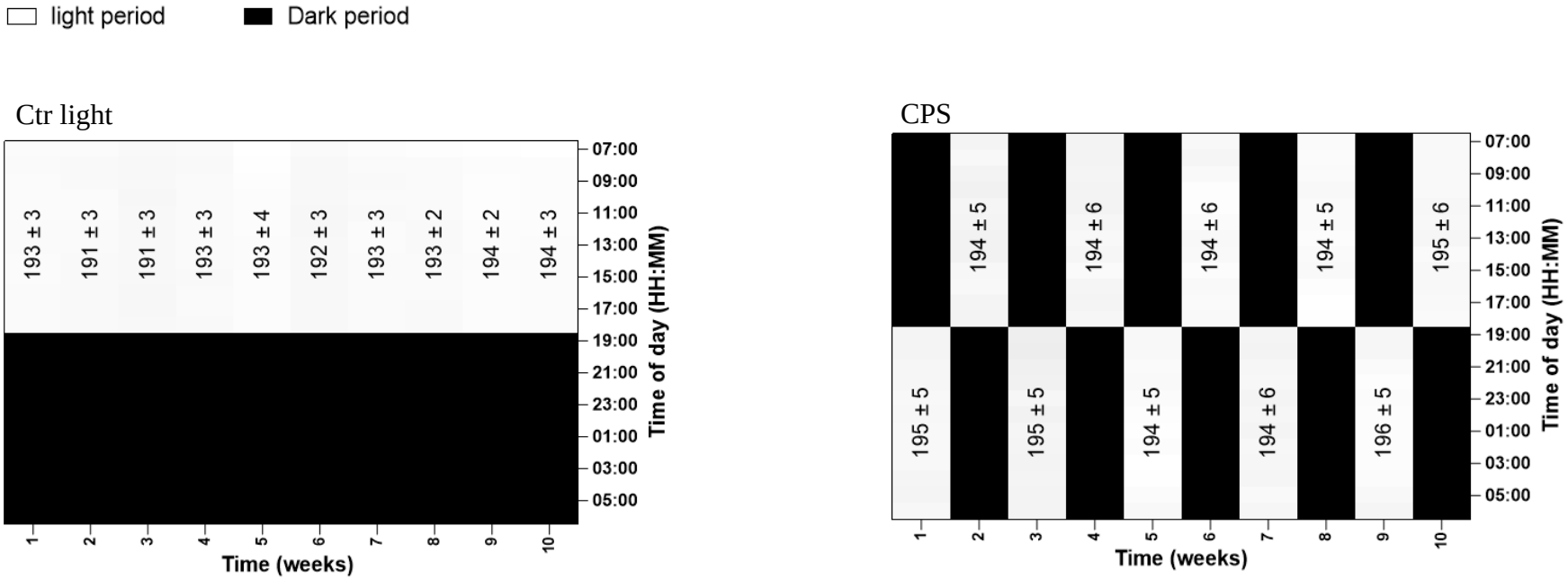

Figure 3.2: Changes in weekly light exposure in the Ctr light and CPS schedules, averaged per hour during the 10-week intervention. The rats were maintained under a controlled 12-hour light/dark cycle, monitored with a HOBO data logger. The black and white bars represent the duration of the periods of light (*day*) and darkness (*night*) as indicated by the key above the heatmaps. The light intensity was <200 lux during the light period and 0 lux during the dark period for both Ctr light and CPS schedules. Data represented as a heat map and expressed as mean ± SD. Ctr light, control light; CPS, chronic phase shift.

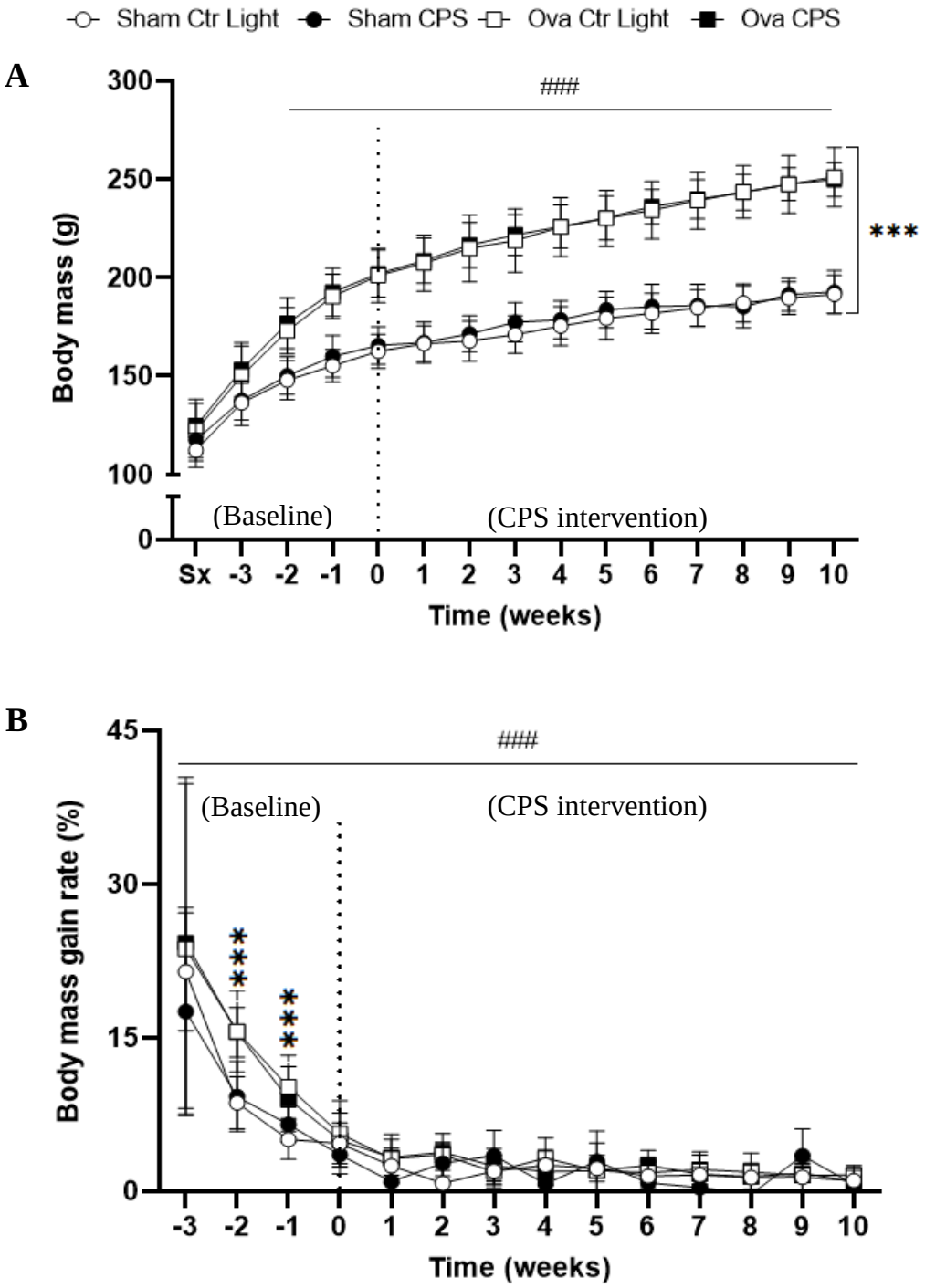

Figure 3.3: Changes in weekly A) body mass and B) body mass gain rate in female SHR. Body mass measurements were taken weekly, starting from the week of surgery (Sx) and continuing through the 10-week intervention period (week 0 to 10). The dotted line at week 0 separates the pre-intervention period (baseline) from the 10-week CPS intervention period. A significant increase in body mass was observed in all groups overtime. The was a significant effect of estrogen deficiency on body mass. Additionally, the body mass gain rate decreased overtime, with a significant effect of estrogen deficiency 2 weeks following Sx. Data expressed as mean ± SD, n=9 per group. Ctr Light, control lighting; CPS, chronic phase shift; Ova, ovariectomy, Sx, surgery. ###, $p < 0.0001$ change overtime; ***, $p < 0.0001$ vs sham-operated groups.

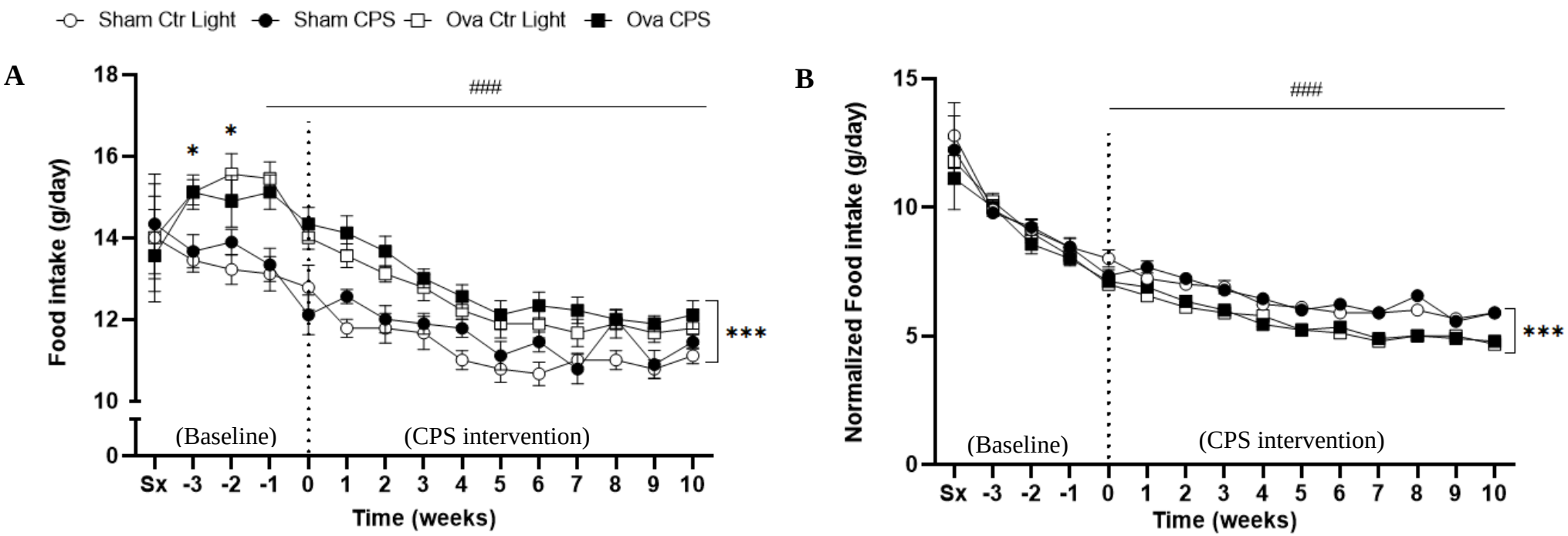

Figure 3.4: Changes in weekly A) food intake and B) *normalized* food intake in female SHR. Food intake was measured weekly from the week of surgery (Sx) and continuing through the 10-week intervention period (week 0 to 10). The dotted line at week 0 separates the pre-intervention period (baseline) from the 10-week CPS intervention period. Both food intake and *normalized* food intake significantly decrease in all groups overtime. Ovariectomized groups had an increased food intake compared to sham-operated groups. However, when normalized to body mass, ovariectomized groups had decreased food intake compared to sham-operated groups. Data expressed as mean ± SD, n=9 per group. Ctr Light, control lighting; CPS, chronic phase shift; Ova, ovariectomy, Sx, surgery. ###, $p < 0.0001$ change overtime; *, $p \leq 0.05$ vs sham-operated groups; ***, $p < 0.0001$ vs sham-operated groups.

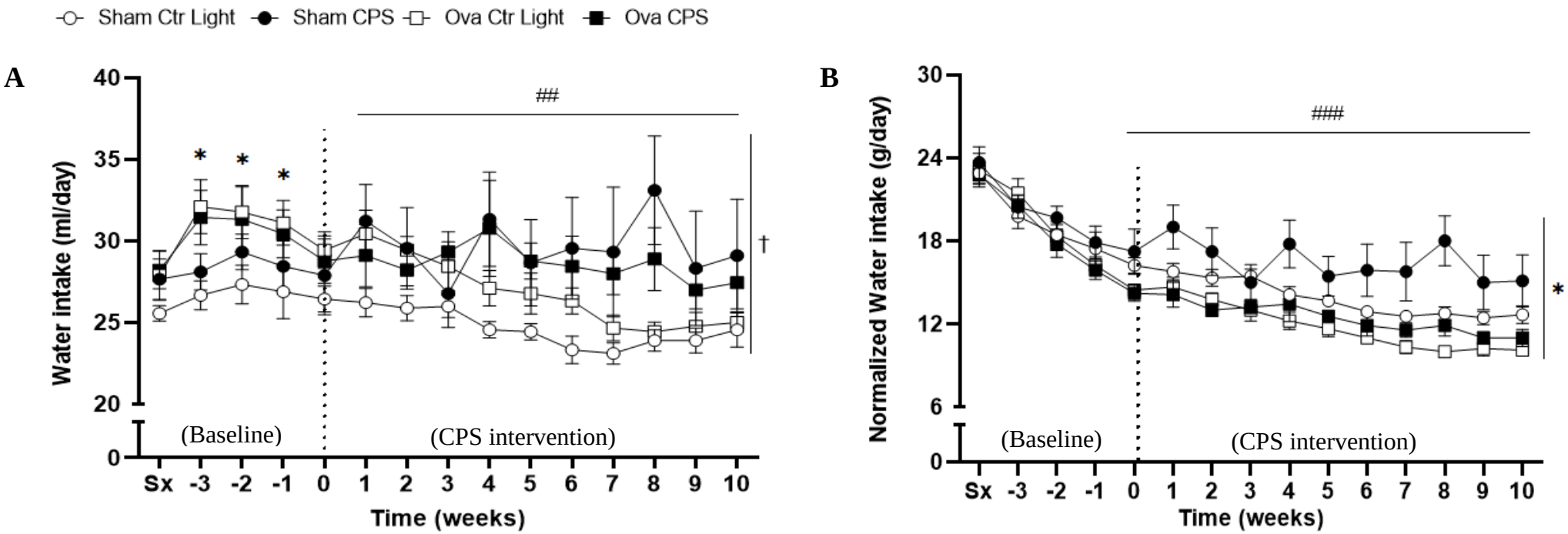

Figure 3.5: Changes in weekly A) water intake and B) *normalized* water intake in female SHR. Water intake was measured weekly from the week of surgery (Sx) and continuing through the 10-week intervention period (week 0 to 10). The dotted line at week 0 separates the pre-intervention period (baseline) from the 10-week CPS intervention period. Both water intake and *normalized* water intake significantly decrease in all groups overtime. Water intake was increased in ovariectomized groups 3 weeks following surgery, and from week 4 of CPS intervention the water intake was increased in the CPS groups compared to the control light groups. However, when normalized to body mass, ovariectomized groups has decreased water intake compared to sham-operated groups. Data expressed as mean ± SD, n=9 per group. Ctr Light, control lighting; CPS, chronic phase shift; Ova, ovariectomy, Sx, surgery. ##, $p < 0.01$ and ###, $p < 0.0001$ change overtime; †, $p < 0.05$ vs CPS groups; *, $p \leq 0.05$ vs sham-operated groups..

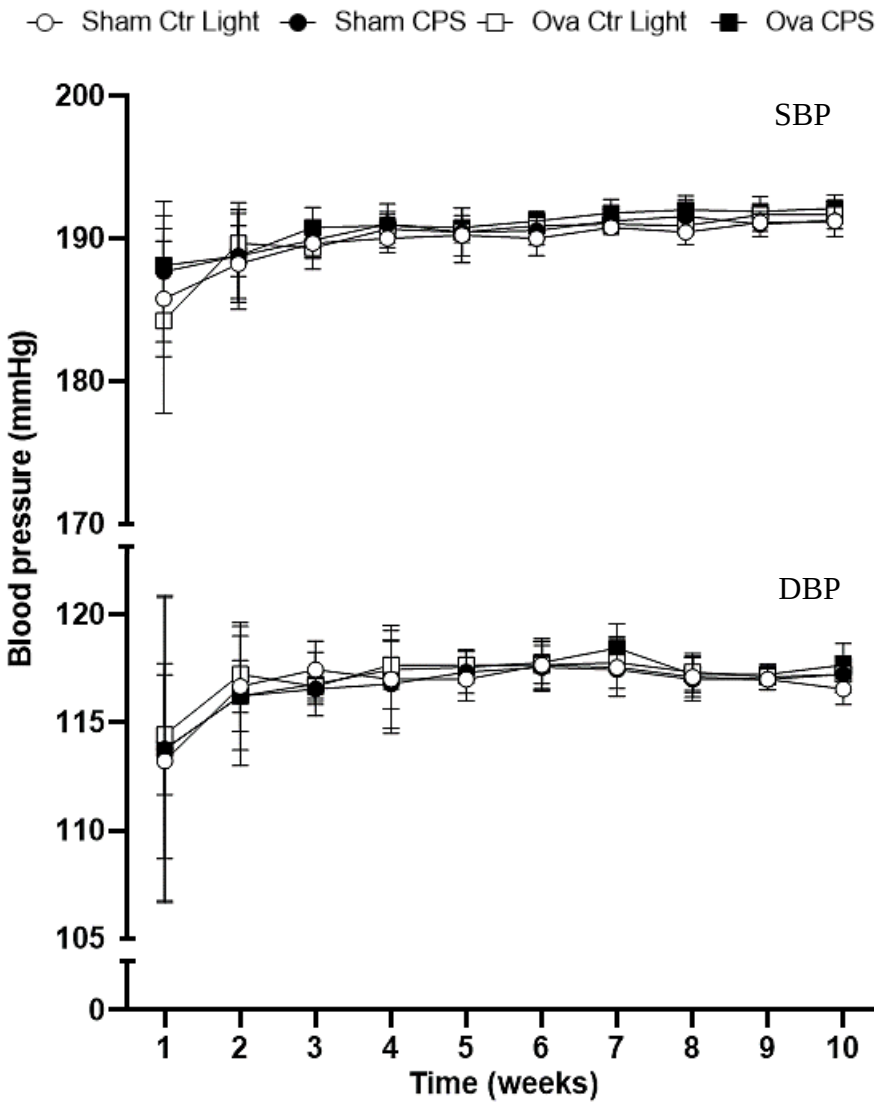

Figure 3.6: Changes in weekly blood pressure (BP) in female SHR during the 10-week CPS intervention. The BP was measured non-invasively using a NIBP250 monitor system. The SBP and DBP remained similar across all groups, with no significant effect of CPS or estrogen deficiency. Data expressed as mean ± SD, n=9 per group. Ctr Light, control lighting; CPS, chronic phase shift; Ova, ovariectomy. SBP, systolic blood pressure; DBP, diastolic blood pressure.

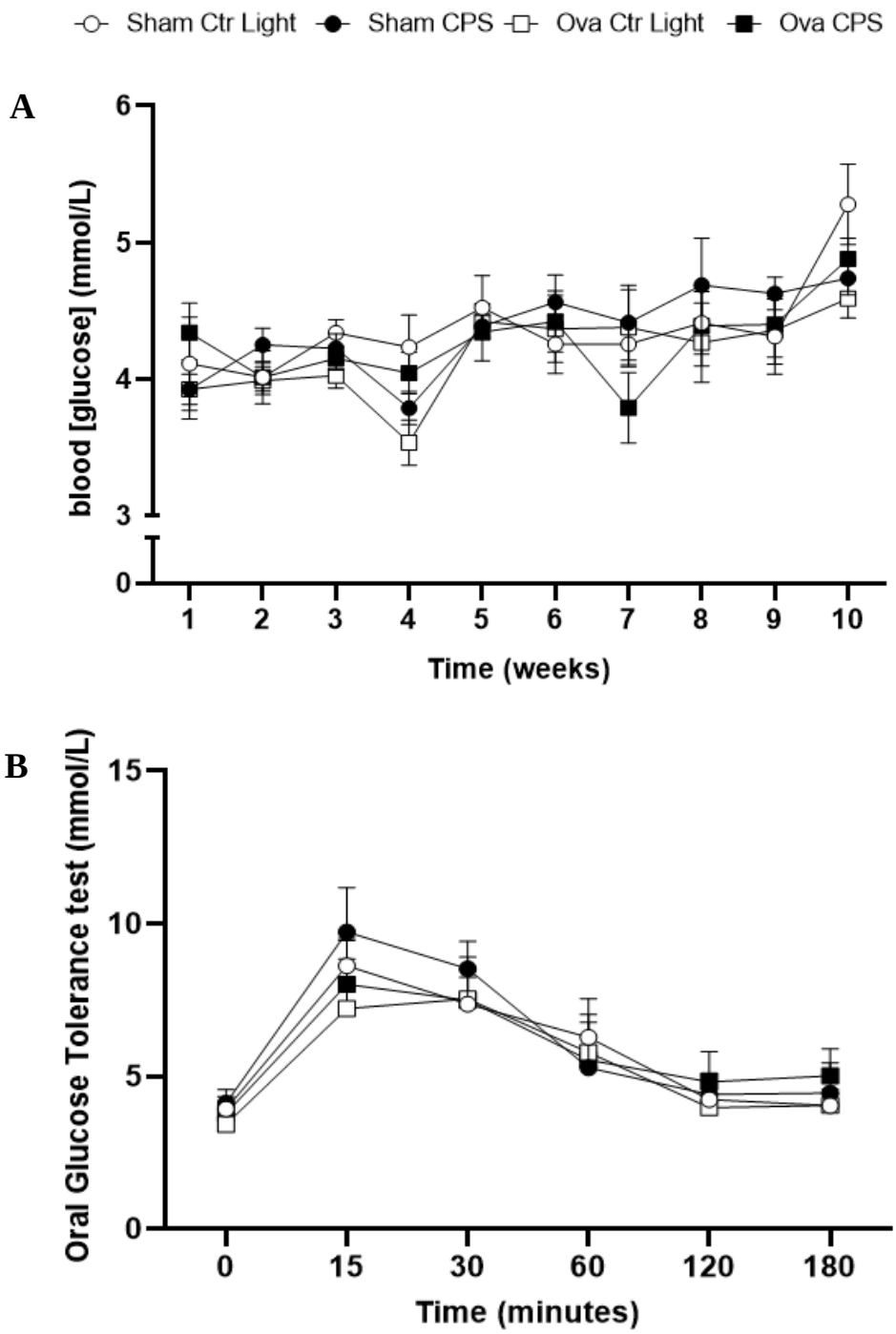

Figure 3.7: Changes in weekly fasting blood glucose concentrations (A) and 3-hour oral glucose tolerance test (OGTT) results (B) in female SHR. The fasting blood glucose concentrations were measure weekly during the 10-week CPS intervention. The 3-hour OGTT was performed 3 days before the end of the 10-week CPS intervention. No significant differences were observed in fasting blood glucose or glucose tolerance between all the groups. Data expressed as mean ± SD, n=9 per group. Ctr Light, control lighting; CPS, chronic phase shift; Ova, ovariectomy; SHR, spontaneously hypertensive rats.

Table 3. 1: Characteristics of 5-month-old female ovariectomized and sham operated SHR after10-week chronic phase shift or control light protocol.

Characteristics	**Sham Control light n=9**	**Sham CPS n=9**	**Ova Control light n=9**	**Ova CPS n=9**
Rectal temperature, °C	36.6 ± 0.2	36.3 ± 0.6	36.0 ± 0.4	36.2 ± 0.3
AUC OGTT, min*mmol/L	28.3 ± 4.2	29.6 ± 5.9	28.0 ± 3.7	27.6 ± 4.3
Peak OGTT, mmol/L	7.2 ± 1.3	8.7 ± 2.2	7.1 ± 1.5	7.1 ± 1.9
Final body mass, g	191 ± 10	193 ± 11	251 ± 15***	250 ± 9***
Heart mass, g	0.86 ± 0.06	0.88 ± 0.06	0.95 ± 0.07***	0.95 ± 0.06***
Left ventricle mass, g	0.64 ± 0.04	0.65 ± 0.05	0.72 ± 0.04***	0.71 ± 0.05***
Liver mass, g	7.1 ± 0.5	6.8 ± 0.4†	8.1 ± 0.8***	7.6 ± 0.5***†
Kidney mass, g	0.66 ± 0,07	0.64 ± 0.09	0.71 ± 0.05*	0.69 ± 0.06*
Normalized heart mass, g/100g	0.45 ± 0.03	0.46 ± 0.04	0.38 ± 0.02***	0.38 ± 0.02***
Normalized left ventricle mass, g/100g	0.34 ± 0.02	0.34 ± 0.02	0.29 ± 0.01***	0.29 ± 0.02***
Normalized liver mass, g/100g	3.7 ± 0.3	3.5 ± 0.2†	3.2 ± 0.2***	3.1 ± 0.2***†
Normalized kidney mass, g/100g	0.34 ± 0.03	0.33 ± 0.04	0.29 ± 0.02***	0.28 ± 0.02***
LDL, ug/mL	9.1 ± 6.5	13.9 ± 10.0	9.7 ± 6.6	15.8 ± 12.1

Data expressed as mean ± SD. SHR, spontaneously hypertensive rats; CPS, chronic phase shift; Ova, ovariectomy; AUC, area under graph; OGTT, oral glucose tolerance test; LDL, low-density lipoprotein. *, $p < 0.05$ vs sham-operated groups; **, $p < 0.01$ vs sham-operated groups; ***, $p < 0.001$ vs sham-operated groups; †, $p \leq 0.05$ vs control light groups.

3.3 Echocardiographic parameters of 5-month-old female SHR

Table 3.2 shows the M–mode and M–mode derived echocardiographic parameters of ovariectomized and sham-operated SHR under CPS or control lighting conditions. There were no significant interactions across these parameters. The LV end-diastolic diameter (LVEDd) was significantly greater in the ovariectomized rats compared to the sham-operated rats ($p = 0.03$). The LV end-systolic diameter (LVEDs) was significantly greater in the ovariectomized rats compared to the sham-operated rats ($p = 0.004$). The endocardial fractional shortening (FSend) was significantly lower in the ovariectomized rats compared to the sham-operated rats ($p = 0.007$). The LV ejection fraction (EF) was significantly lower in the ovariectomized rats compared to the sham-operated rats ($p = 0.007$).

There was no significant difference in the intraventricular septal diameter end diastole (IVSTd), the intraventricular septal diameter end-systole (IVSTs), the LV posterior wall thickness in diastole (PWTd), the LV posterior wall thickness in systole (PWTs), the relative wall thickness (RWT), the midwall fractional shortening (FSmid) and stroke volume (SV) between the experimental groups. Table 3.3 shows the tissue and pulse Doppler echocardiography parameters of ovariectomized and sham-operated SHR under CPS or control lighting conditions. There were no significant interactions across these parameters, and there was no significant difference in all these parameters between the experimental groups.

Table 3. 2: M–mode and M–mode derived echocardiographic parameters of 5-month-old female ovariectomized and sham operated SHR after 10-week chronic phase shift or control light protocol.

Parameters	**Sham Control light n=9**	**Sham CPS n=9**	**Ova Control light n=9**	**Ova CPS n=9**
Interventricular (mm)				
IVSTd	2.4 ± 0.3	2.3 ± 0.4	2.2 ± 0.5	2.7 ± 0.4
IVSTs	3.4 ± 0.1	3.3 ± 0.5	3.1 ± 0.7	3.5 ± 0.3
Left ventricular diameters (mm)				
LVEDd	4.5 ± 0.6	4.6 ± 0.7	5.1 ± 0.5*	5.0 ± 0.4*
LVEDs	2.4 ± 0.4	2.4 ± 0.5	3.0 ± 0.7**	3.0 ± 0.4**
Posterior wall thickness (mm)				
LVPWTd	2.1 ± 0.6	2.1 ± 0.6	2.3 ± 0.4	2.0 ± 0.5
LVPWTs	2.8 ± 0.6	2.8 ± 0.5	2.9 ± 0.5	2.6 ± 0.6
Derived				
Relative wall thickness	1.0 ± 0.4	1.0 ± 0.4	0.9 ± 0.2	0.8 ± 0.3
FS_{end}, %	48.2 ± 3.3	49.0 ± 5.2	41.7 ± 10.0**	40.5 ± 6.0**
FS_{mid}, %	23.1 ± 3.3	23.1 ± 3.7	20.4 ± 5.5	19.9 ± 4.7
EF, %	79.6 ± 3.3	80.1 ± 4.8	71.2 ± 12.5**	70.6 ± 7.2**
SV, µL	76.7 ± 23.2	79.9 ± 24.5	87.8 ± 19.2	84.9 ± 16.9

Data expressed as mean ± SD. SHR, spontaneously hypertensive rats; CPS, chronic phase shift; Ova, ovariectomy; IVSTd, intraventricular septal diameter end diastole; IVSTs, intraventricular septal diameter end systole; LVEDd, left ventricular end-diastolic diameter; LVEDs, left ventricular end-diastolic diameter; LVPWTd, left ventricular posterior wall thickness in diastole; LVPWTs, left ventricular posterior wall thickness in systole; FSend, endocardial fractional shortening; FSmid, mid wall fractional shortening; EF, ejection fraction; SV, stroke volume. *, $p \leq 0.05$ vs sham-operated groups; **, $p < 0.01$ vs sham-operated groups

Table 3. 3: Tissue and pulse Doppler echocardiography parameters of 5-month-old female ovariectomized and sham-operated SHR after 10-week chronic phase shift or control light protocol.

Parameters	**Sham Control light n=9**	**Sham CPS n=9**	**Ova Control light n=9**	**Ova CPS n=9**
Tissue doppler				
e', cm/s	5.1 ± 0.8	6.4 ± 2.4	6.1 ± 0.7	5.6 ± 1.2
a', cm/s	3.3 ± 0.5	4.3 ± 0.9	3.7 ± 0.6	4.4 ± 1.9
s', cm/s	4.8 ± 0.7	5.3 ± 0.4	4.6 ± 0.7	4.6 ± 1.1
Pulse doppler				
E, cm/s	84.3 ± 18.8	86.6 ± 26.7	86.3 ± 22.8	89.9 ± 10.4
A, cm/s	52.9 ± 20.6	57.5 ± 20.3	57.6 ± 20.3	55.9 ± 15.3
Derived				
E/A	1.71 ± 0.45	1.57 ± 0.35	1.62 ± 0.47	1.73 ± 0.51
E/e'	17.5 ± 7.1	15.7 ± 9.0	14.4 ± 3.9	17.0 ± 4.8
e'/a'	1.6 ± 0.3	1.6 ± 0.7	1.7 ± 0.3	1.4 ± 0.5

Data expressed as mean ± SD. SHR, spontaneously hypertensive rats; CPS, chronic phase shift; Ova, ovariectomy; e', early peak tissue lengthening velocity at the lateral mitral annulus; a', late peak tissue lengthening velocity at the lateral mitral annulus; s', peak systolic velocity; E, maximum early mitral inflow velocity; A, maximum late mitral inflow velocity; E/e', early diastolic mitral inflow velocity to early diastolic mitral annulus velocity; E/A, early to late diastolic filling velocity ratio; e'/a', early to late peak tissue lengthening velocity ratio.

Chapter 4: Discussion

The present study determined the effects of a circadian misalignment protocol on cardiometabolic parameters in estrogen-deficient female spontaneously hypertensive rats (SHR). The main findings of the present study are that circadian misalignment resulting from a 10-week chronic phase shift (CPS) intervention did not induce changes in the cardiometabolic parameters measured. Circadian misalignment only induced an increase in water intake and a decrease in liver mass. On the other hand, estrogen deficiency resulting from ovariectomy induced an increase in body mass, food intake and organ masses. However, estrogen deficiency induced a decrease in food intake and organ masses once normalized to body mass. Importantly, estrogen deficiency induced increased left ventricle (LV) chamber dimensions, and decreased LV contraction, measured by M-mode echocardiography. Finally, the present study showed no interaction between circadian misalignment and estrogen deficiency. The present dissertation hypothesises that the absence of effect of circadian misalignment on cardiometabolic parameters as well as the lack of interaction with estrogen deficiency may be due to the abnormal circadian rhythms reported in SHR and the age at which ovariectomy was performed.

4.1 Effects of estrogen deficiency

One aim of the present study was to investigate the effects of estrogen deficiency on cardiometabolic parameters in female SHR. The present study utilized an ovariectomized rat model to induce an abrupt and sudden decline in ovarian hormones, particularly estrogen. Thus, for this discussion "estrogen deficiency" will be referring to ovariectomized rodents to distinguish from the natural age-related withdrawal of estrogen, like that observed in menopausal women.

First, a decline in estrogen levels have been linked to alterations in body mass (Barrea *et al*., 2021; Roa-Díaz *et al*., 2021). Indeed, previous research demonstrates that postmenopausal women experience increased body mass attributed to estrogen withdrawal (Davis *et al*., 2012; Fenton, 2021). Similar to postmenopausal women, numerous studies have shown that estrogen deficiency leads to a rapid increase in body mass in normotensive rats (Tarttelin and Gorski, 1971; Blaustein and Wade, 1976; McElroy and Wade, 1987; Geary and Asarian, 1999) and SHR (Santos *et al*., 2004; Lin *et al*., 2016; da Silva *et al*., 2017). In agreement with previous studies, estrogen deficiency led to an increased body mass in female SHR in the present study. The increased body mass may then serve to confirm that the concentration of estrogen in the blood has decreased following ovariectomy in the present study.

Studies have proposed that the decline in estrogen levels contribute to changes in body mass through alterations in appetite regulation (Clegg *et al*., 2007; Butera *et al*., 2014). Lower levels of estrogen can lead to an increased appetite (Gao *et al*., 2007; Hirschberg, 2012). Indeed, postmenopausal women have been shown to have an increased appetite and food intake, which resulted in body mass gain (Duval *et al*., 2014; Karim *et al*., 2015; Ranasinghe *et al*., 2017). Similar results have been shown in estrogen-deficient normotensive rats (Wade, 1975; Blaustein and Wade, 1976; Asarian and Geary, 2002) and SHR (Wallen *et al*., 2002). In the present study, the increased body mass with estrogen deficiency paralleled an increase in food intake before and after the commencement of the intervention.

Note that, in the present study, the body mass gain rate and the food intake were increased in the first 2 weeks following the surgery with estrogen deficiency. The body mass gain rate stabilized, and the food intake decreased in all the groups once the rats reached 3-month-old. Rats are considered adult at 3-month-old (Quinn, 2005; Klein and Romeo, 2013; Smith *et al*., 2019; Sudakov *et al*., 2021). During adulthood, the calorie and energy requirements start to decrease (Wakimoto and Block, 2001; Roberts and Dallal, 2005; Amarya *et al*., 2015). The present results suggest that the observed body mass increase with estrogen deficiency throughout the study period may have been influenced by the rapid body mass gain during the first 2 weeks following ovariectomy. In addition, despite an increased food intake with estrogen deficiency throughout the study period, the estrogen-deficient female SHR consumed less food relative to their body size in the present study. Previous research proposed that estrogen-deficient rats may have lower calorie requirements than sham-operated rats due to a decreased physical activity and energy expenditure (Lovejoy *et al*., 2008; Rogers *et al*., 2009). The present study then hypothesises that the lower normalized food intake with estrogen deficiency may be indicative of decreased physical activity and altered metabolic processes.

As the body grows, organs need to adapt and grow to support the increased body size (Lui and Baron, 2011; Lui *et al*., 2015). Human studies demonstrate a correlation between body mass increase and the mass of certain organs - including the heart, the LV, the liver and kidneys (Brumback et al., 2010; Mandal *et al*., 2012; Molina and DiMaio, 2015a; Mubbunu *et al*., 2018). Research in rat models also demonstrate that organ masses tend to correlate with body mass (Bailey *et al*., 2004; Lazic *et al*., 2020). Additionally, an increased body mass with estrogen deficiency has been associated with an increase in size of certain organs, especially the heart and LV mass of rat models (Xu *et al*., 2003; Tivesten *et al*., 2006; Phungphong *et al*.,

2020), including SHR (Santos *et al*., 2004; Lin *et al*., 2016; da Silva *et al*., 2017). In the present study, increased organ masses were observed with estrogen deficiency. However, once normalized to body mass, the organ masses were decreased with estrogen deficiency. These results suggest that, because the increase in organ masses is proportionally lower than the overall body mass gain with estrogen deficiency, the body and organ growth was lower with estrogen deficiency. The body mass gain with estrogen deficiency may have been due to a change in the body composition towards accumulation of fat. Indeed, alterations in body mass following estrogen deficiency may be associated with changes in body composition (Siemińska *et al*., 2006; Sowers *et al*., 2007). Moreover, changes in body composition play a significant role in metabolic processes that affect body mass gain (Sowers *et al*., 2007).

The intriguing observation that the increase in organ masses did not proportionally match overall body mass gain with estrogen deficiency in the present study prompts further investigation into potential metabolic alterations beyond changes in body composition. Specifically, alterations of metabolic processes crucial to energy homeostasis, such as glucose and lipid metabolism. Studies consistently demonstrate a strong correlation between a decline in estrogen levels and metabolic dysfunction in postmenopausal women and rodents (Misso *et al*., 2003; Salpeter *et al*., 2006; Ainy *et al*., 2007; Jeong and Park, 2022). Postmenopausal women often present with insulin resistance (Sathya Bhama *et al*., 2012) and increased fasting blood glucose concentrations (Kanaya *et al*., 2003; Bermingham *et al*., 2022). In estrogen-deficient normotensive rats, some studies have reported no alterations in fasting blood glucose concentrations (Swislocki *et al*., 2002; Sivasinprasasn *et al*., 2015; Min *et al*., 2018; Maluleke *et al*., 2022), while others report increased fasting blood glucose concentrations (Sanker *et al*., 2015; Fahmy *et al*., 2018; Minta *et al*., 2018). Notably, studies indicating no alteration in fasting blood glucose concentrations induced estrogen deficiency in young female rats (6 to 12-week-old), while those reporting increased concentrations induced estrogen deficiency at 3 to 4-month-old and subjected the rats to a high-fat diet. The age at which estrogen deficiency is induced and the diet may then influence these alterations.

In SHR, unovariectomized adult and ageing female SHR are shown to exhibit increased blood glucose concentrations and impaired glucose tolerance from 3-month-old (Iwase *et al*., 1994; Kinney LaPier *et al*., 2001). Estrogen-deficient female SHR are also reported to present with increased fasting blood glucose concentrations and decreased insulin sensitivity at 5-month-old (Sanches *et al*., 2012; Conti e*t al*., 2015; Brito-Monzani *et al*., 2017). In these studies,

estrogen deficiency was induced at 3-month-old after 10 weeks of receiving fructose in drinking water and the glucose levels were determined using the constant rate for blood glucose disappearance (KITT). Otherwise, studies in female SHR report unchanged fasting blood glucose concentrations (Swislocki *et al*., 2002; das Graças Abeles *et al*., 2012) and glucose tolerance (Iwase *et al*., 1996) with estrogen deficiency induced at 3 to 12-week-old. Similarly, estrogen deficiency was induced at 7-week-old in the present study and no changes in fasting blood glucose concentrations and glucose tolerance were observed. These findings suggest that age-related estrogen withdrawal and diet may lead to increased glucose levels in female SHR. Implying that estrogen deficiency alone may not influence fasting blood glucose levels in young female SHR. As proposed by Iwase et al. (1996), gonadectomy (removal of testes or ovaries) may have protective effects against the onset of increased blood glucose levels in male SHR, while showing no effect on blood glucose levels in young female SHR.

A decline in estrogen levels and the metabolic changes induced may also lead to a shift in the lipid profile, including LDL cholesterol (Awa *et al*., 2012; Yonezawa *et al*., 2012; Wietlisbach *et al*., 2013). Contrasting results were reported in postmenopausal women. Some studies reported that postmenopausal women present with increased levels of LDL cholesterol (Mathews *et al*., 1994; Anagnostis *et al*., 2015; Inaraja *et al*., 2020). In contrast, other studies reported unchanged LDL cholesterol levels in postmenopausal women (Kanaya *et al*., 2003; McNeill *et al*., 2005). In normotensive rats, estrogen deficiency induced at 4 to 13-week-old increased LDL cholesterol concentrations (Van Lenten *et al*., 1983; Packer *et al*., 2002; Babaei *et al*., 2010; Yoon *et al*., 2015). However, when estrogen deficiency was induced in older normotensive female rats (6-month-old), there were no changes in LDL cholesterol concentrations (Dost *et al*., 2014). These findings suggest that the age of when estrogen deficiency was induced plays a role in altering LDL cholesterol.

In SHR, the effects of estrogen deficiency on LDL cholesterol are contradictory. Packer et al. (2002) and Kim et al. (2011) showed increased LDL cholesterol concentrations in estrogen-deficient female SHR. The room temperature was maintained at 22 °C, and the female SHR sacrificed at 4-month-old. In contract, Delgado et al. (2017) found no changes in LDL cholesterol concentrations. The room temperature was maintained at 24 °C, and the female SHR sacrificed at 3-month-old. Although contradictory, these studies induced estrogen deficiency between 4 and 9-week-old, and the LDL cholesterol was calculated using an equation adopted from Friedewald et al. (1972). In the present study, the LDL cholesterol

concentrations remained unchanged with estrogen deficiency induced at 7-week-old. The room temperature and relative humidity were maintained at 26 °C and 28%, respectively. The female SHR were sacrificed at 5.5-month-old in the present study. Therefore, unlike normotensive female rats, the age at which estrogen deficiency was induced may not play a role in altering LDL cholesterol concentrations in female SHR. The discrepancies may have resulted from the different environmental conditions housing the female SHR.

According to the National Institutes of Health (NIH) Guide, it is important to maintain rats within a room temperature range of 26-30 °C and a relative humidity range of 30-70% (National Research Council, 2011). Extremes in temperature and humidity can impact the cardiometabolic functions (Baldwin *et al*., 2022; Chen and Zhang, 2023). Notably, housing rats in room temperature of 20-26 °C is reported to cause cold stress (Hankenson *et al*., 2018). A room temperature of 22 °C is cooler than the recommended 26-30 °C, which may be perceived as mildly stressful for rats. Cold stress may trigger the hypothalamus-pituitary-adrenal (HPA) axis, resulting in the release of cortisol (Umishio *et al*., 2022). Cortisol is a steroid hormone synthesized from cholesterol for mediating the stress response (Hu *et al*., 2010; Lee *et al*., 2015). Prolonged cold exposure may result in excess cortisol, leading to high cholesterol levels (Umishio *et al*., 2022). Indeed, studies demonstrated that LDL cholesterol concentrations and triglycerides increased with decreasing room temperature (Hong *et al*., 2012; Shiue, 2016; Sartini *et al*., 2017). Therefore, the present study suggests that estrogen deficiency alone does not alter LDL cholesterol in young female SHR, as environmental factors may also play a role.

Similar to the metabolic profiles, findings on the effect of reduced estrogen levels on blood pressure (BP) are contradictory. Evidence indicates that estrogen withdrawal is a contributing factor to the increased BP (hypertension) observed in postmenopausal women when compared to their premenopausal counterparts (Zanchetti *et al*., 2005; Lima *et a*l., 2012; Tasić *et al*., 2022). In contrast, some studies have found no significant BP difference in postmenopausal women when compared to their premenopausal counterparts (Wu *et al*., 1990; Armellini *et al*., 1990; Zamboni *et al*., 1992; Casiglia *et al*., 1996). Finally, a lower BP with menopause has also been reported (Lindquist and Bengtsson, 1980; van Beresteijn *et al*., 1992). These contrary findings in the postmenopausal women suggest that estrogen withdrawal alone may not be the main contributing factor to changes in BP. Some studies argue that changes in BP in postmenopausal women may be because of an increase in body mass index (BMI) and age

concurrently than the hormonal status alone (Casiglia *et al*., 1996; Portaluppi *et al*., 1997; Khoo *et al*., 1998).

Similar to findings in postmenopausal women, results in estrogen-deficient rats are contradictory. In normotensive rats, estrogen deficiency was associated with increases in BP (Hernández *et al*., 2000; Clark *et al*., 2004; Xu *et al*., 2008; Zhang *et al*., 2019). In contrast, other studies reported no significant changes in BP with estrogen deficiency (Wallen *et al*., 2000; Tamaya-Mori *et al*., 2002; El-Mas and Abdel-Rahman, 2009; Maluleke *et al*., 2022). Notably, in studies that demonstrated an increased BP, estrogen deficiency was induced in adult rats (2 to 10-month-old), while those reporting no changes induced estrogen deficiency in young rats (3 to 10-week-old). Indeed, studies comparing BP changes in young vs. old rats show no changes in BP when estrogen deficiency was induced at a younger age, whereas significant increases in BP were observed when estrogen deficiency was induced at an older age (Fortepiani *et al*., 2003; Clark *et al*., 2004). The age at which estrogen deficiency is induced appears to play a crucial role in its effect on BP.

This phenomenon is supported by human findings, which proposes that the differences in BP changes are influenced by the age at which menopause occurs (Portaluppi *et al*., 1997; Zanchetti *et al*., 2005; Izumi *et al*., 2007; Son *et al*., 2015). The BP does not increase immediately when the ovaries stop producing estrogen, but rather, an average of five to 20 years after menopause (Fortepiani *et al*., 2003; Lin *et al*., 2016). This may suggest that a gradual decline of estrogen levels over several years may be more detrimental than the abrupt change observed in ovariectomy. Therefore, gradual withdrawal of the cardioprotective effects of estrogen may pose a greater risk for developing hypertension.

Interestingly, the findings in female SHR are contradicting and not supportive of this phenomenon. Some studies reported that estrogen deficiency further increases BP in female SHR (Dantas *et al*., 1999; Peng *et al*., 2003; Lin *et al*., 2016; Shimojo *et al*., 2018), while others reported that estrogen deficiency does not exacerbate BP levels in female SHR (Giménez *et al*., 2006; Jazbutyte *et al*., 2008; Martin *et al*., 2008; Tatchum-Talom *et al*., 2011; Costa *et al*., 2018; Lin *et al*., 2020; Chen *et al*., 2021). In these studies, estrogen deficiency was induced at different ages (both young adolescence and adult female SHR). Moreover, the increase in BP is not of a constant value and varies largely between studies. In the present study, estrogen deficiency was induced at 7-week-old, and the hypertensive state of the young female SHR remained unchanged. The discrepancies could be due to most studies using an arterial catheter

for BP recording and the inconsistent number of subjects. The present study speculates that the hypertensive state and BP regulation in response to estrogen deficiency in female SHR may be influenced differently. Nevertheless, the present study indicates that BP was not affected by estrogen deficiency in female SHR.

Despite no significant difference in the hypertensive state, sustained pressure overload can still lead to clinical manifestations of cardiac remodelling. Untreated hypertension in postmenopausal women has been reported to be a key driving force in the development of cardiac remodelling (Pośnik-Urbańska and Kawecka-Jaszcz, 2006, Yanes and Reckelhoff, 2011). Indeed, hypertensive postmenopausal women are reported to present with concentric LVH (Pines *et al*., 1993; Schillaci *et al*., 1998; Hinderliter *et al*., 2002; Düzenli *et al*., 2007; Sung *et al*., 2022). Similarly, LVH has been observed in estrogen-deficient pressure-overload rats (Bhuiyan *et al*., 2007; Dai *et al*., 2008) and in estrogen-deficient hypertensive mRen2.Lewis and Dahl salt-sensitive rats (Wang *et al*., 2012; Ludvigswn *et al*., 2018). Additionally, estrogen-deficient pressure-overload female mice presented with a greater LVH than estrogen-deficient female mice receiving ERT (Van Eickels *et al*., 2001).

Furthermore, LVH is a predominant characteristic of the SHR (Yamori *et al*., 1979; Lundin *et al*., 1982; Kokubo *et al*., 2005). Studies that report increased LVH in estrogen-deficient female SHR determined the hypertrophy either by the LV: body mass ratio or LV: tibial length ratio (Wallen *et al*., 2000; Santos *et al*., 2004; Dalpiaz *et al*., 2014; Wittnich *et al*., 2015; Lin *et al*., 2016). This is in contrast with studies using M-mode echocardiography reporting that estrogen deficiency does not exacerbate the LVH in female SHR (da Silva *et al*., 2017; Magubane *et al*., 2017; Chen *et al*., 2021). Similarly, the present study observed only increased chamber dimensions, while the wall thickness and LV mass remained unchanged with estrogen deficiency. Interestingly, in the study by Chen et al. (2021), estrogen-deficient female SHR receiving ERT presented with improved LVH, whereas estrogen-deficient female SHR receiving estrogen and testosterone supplementation presented with greater LVH. Moreover, gonadectomy in male SHR has been documented to improve LVH (Dalpiaz *et al*., 2014). These findings demonstrate the importance of testosterone's influence on LV remodelling in SHR (Dalpiaz *et al*., 2014; Chen *et al*., 2021). Therefore, the present study speculates that estrogen alone may not be beneficial against the development of LVH in female SHR. It may be the absence of the detrimental effect of testosterone as observed in male SHR protecting estrogen-deficient female SHR from increased LVH.

It is interesting to observe increased chamber dimensions in estrogen-deficient female SHR without exacerbated LVH. This is potentially indicative of worsening systolic or diastolic dysfunction. In previous studies, postmenopausal women who present with LVH frequently had impaired diastolic function (Kangro *et al*., 1995; Düzenli *et al*., 2007; Hirose *et al*., 2017; Sung *et al*., 2022). Similar to postmenopausal women, impaired diastolic function has been observed in hypertensive estrogen-deficient rats with LVH (Groban *et al*., 2008; Mori *et al*., 2011; Murase *et al*., 2012; Wang *et al*., 2012; Ludvigsen *et al*., 2018; Bustamante *et al*., 2019). Female SHR are documented to develop diastolic dysfunction around 12 months of age (Albrecht, 1974; Wexler *et al*., 1981). In support of this, a comparison study between female SHR and WKY showed no differences in diastolic function at 10 months of age (Renna *et al*., 2007). However, a study comparing strain and estrogen deficiency effects revealed that at 5 to 6 months of age, female SHR exhibited greater diastolic impairment compared to female WKY rats (da Silva *et al*., 2017). Within the SHR group, there were no significant differences in diastolic function observed between estrogen-deficient and sham-operated female SHR (da Silva *et al*., 2017). In agreement, Chen et al. (2021) also demonstrated the absence of diastolic impairment in 5 to 6-month-old estrogen-deficient female SHR compared to sham-operated female SHR. Similarly, in the present study all indices measured for LV diastolic function in estrogen-deficient female SHR were unchanged. Although diastolic dysfunction may develop at an advanced age in female SHR (Masuyama *et al*., 2000), estrogen deficiency may not have a direct influence on its development. Nevertheless, estrogen deficiency did not worsen the diastolic dysfunction of female SHR in the present study. Therefore, the increased chamber dimensions are not indicative of worsened diastolic dysfunction.

Changes in systolic and diastolic function may occur simultaneously, however, some degree of systolic abnormalities could also be detected in the absence of diastolic dysfunction and vice versa (Sagie *et al*., 1993; Soma *et al*., 1996; Zabalgoitia *et al*., 1998; de Simone *et al*., 2000). Indeed, postmenopausal women present with impaired systolic function independent of diastolic dysfunction (Prelevic *et al*., 1994; Schillazi *et al*., 1998; Zhao *et al*., 2018; Alexanderson-Rosas *et al*., 2021; Aziz *et al*., 2023). In female rats, estrogen deficiency does not consistently lead to an impaired systolic function. Some studies report no significant effect of estrogen deficiency on systolic function of female rats (Mori *et al*., 2011; Sivasinprasasn *et al*., 2015; Machi *et al*., 2016; Jiang *et al*., 2017), including the hypertensive mRen2.Lewis rats (Jessup *et al*., 2013). In contrast, other studies report a decreased systolic function with estrogen in female rats (Felix *et al*., 2015; Wang *et al*., 2022), including the heart failure-prone

SHHF/Mcc-fa cp rats (Sharkey *et al*., 1998). Notably, the discrepancies in these studies may be due to strain and age differences of the rats. Furthermore, literature states that female SHR develop systolic dysfunction also at an advanced age (around 24 months old) (Pfeffer *et al*., 1982). Indeed, the absence of systolic dysfunction was confirmed with a comparison of 10 months old female SHR and WKY (Renna *et al*., 2007). Additionally, studies have documented lack of systolic dysfunction in estrogen-deficient female SHR (Magubane *et al*., 2007; da Silva *et al*., 2017; Chen *et al*., 2021). Notably, estrogen deficiency was induced while ovaries were still functioning at 3 to 9-month-old in these studies.

In the present study, a decreased systolic function, indexed by lower endocardial fractional shortening (FSend) and ejection fraction, was observed with estrogen deficiency. However, the midwall fractional shortening (FSmid) and stroke volume remained unchanged with estrogen deficiency. The observation of unchanged FSmid in the present study suggests that the SNS-mediated contractility remains unaltered in female SHR. Whereas, decreased FSend in estrogen-deficient female SHR indicates impaired contraction. Impaired contraction potentially leads to reduced blood ejection, limiting systolic emptying (Norman *et al*., 2011; Schwinger, 2021). Decreasing ejection fraction may result in blood retention in the LV following contraction, reducing systolic efficiency (Schwinger, 2021). Ultimately, this may affect the length-tension relationship, contributing to increased chamber dimensions (Schwinger, 2021). Despite the decreased FSend, compensatory mechanisms may maintain the stroke volume (Lekven *et al*., 1973; Kroeker *et al*., 2003; MacIver, 2009). Therefore, the present study suggests that the abrupt and sudden decline of estrogen may pose more detrimental effects on the systolic function of female SHR than gradual withdrawal of the cardioprotective effects of estrogen. Indeed, estrogen is suggested to enhance cardiac contractility, thus estrogen deficiency may result in a reduction of systolic function (Pelzer *et al*., 2005).

4.2 Effects of circadian misalignment

The present study also investigated the effects of circadian misalignment on cardiometabolic parameters in female SHR. Circadian misalignment was induced in female SHR using a 10-week CPS protocol, whereby the light exposure was shifted weekly by 12-hour cycles (12/12 h light/dark) to mimic the light conditions experienced by shift workers. The weekly reversal of the 12-hour light cycle is comparable to the constant and frequent adjustments to the daily routines of shift/night workers (Varcoe *et al*., 2011). The light intensity in the present study

was maintained at approximately 200 lux to mimic daytime (light) and 0 lux to mimic night-time (dark). Similarly, other studies in albino rats used the light intensity of 150-300 lux (Gaston *et al*., 2013; Stenvers *et al*., 2016; Liu *et al*., 2019; Jing *et al*., 2020). The NIH Guide recommends using high light intensity (130 to 325 lux) at cage level in the room (National Research Council, 2011). Despite this recommendation, the findings on the appropriate light intensity for albino rats are inconsistent. Some studies show that albino rats avoid cage areas with low light intensity of less than 25 lux (Castelhano-Carlos and Baumans, 2009; De Vera Mudry *et al*., 2013). However, other studies showed that albino rats are better adapted to low intensity lighting conditions of 1 to 40 lux (Clough, 1982; Vandershuren *et al*., 1995). Similarly, Blom et al. (1995) performed a light intensity preference test demonstrating that albino rats prefer cages with a low light intensity (<100 lux) over cages with high light intensity (100–380 lux).

Albino rats lack the ocular melanin that normally protects the retina against high light intensities (Wasowicz *et al*., 2002). Albino rats exposed to light intensities of 130–270 lux above the recommended threshold may be at risk of retinal damage (Semple-Rowland and Dawson, 1987; Jin *et al*., 1998). A high prevalence of light-dependent retinopathy was observed between 210 and 490 lux in albino rats (De Vera Mudry *et al*., 2013). Other studies even reported risk of photic retinopathy with light intensity lower than the 200 lux and retinal damage in albino rats exposed to light intensities as low as 60 lux (Semple-Rowland and Dawson, 1987; Penn *et al*., 1985; Organisciak *et al.,* 1998). The onset of retinal damage is believed to occur following continuous light exposure, either through short exposure to high light intensities or prolonged exposure to lower light intensities (Wasowicz *et al*., 2002; Egan Benova *et al*., 2021). Indeed, Williams et al. (1985) showed that, in three days, albino rats exposed to 133 lux had lost 50% of their retinal rod nuclei as compared to the control group maintained at 3 lux. Finally, while the present study adhered to the recent NIH-recommended light intensity, the historical variability in appropriate light intensity for albino rats raises concerns about potential retinal damage. Therefore, the female SHR may have been at risk of retinal damage in the present study. The present study cannot provide conclusive evidence on the possible retinal damage as the eyes of the female SHR were not assessed.

Light exposure is a crucial environmental factor that can influence cardiometabolic health. Notably, light exposure is important in regulating circadian rhythms (Duffy and Czeisler, 2009; Blume *et al*., 2019). Evidence has revealed the complex interactions between the circadian

rhythms and CV systems (Durgan and Young, 2010) or metabolic systems (Maury *et al*., 2010). In human studies, circadian misalignment has been associated with increased body mass (Nakamura *et al*., 1997; Karlsson *et al*., 2001; Ha and Park, 2005; Morikawa *et al*., 2007), as well as higher blood glucose levels (Tochikubo *et al*., 1996; Spiegel *et al*., 1999; Nagaya *et al*., 2002; Scheer *et al*., 2009; Buxton *et al*., 2012; Yuan *et al*., 2021). Concerning LDL cholesterol concentrations, the findings in shift workers are contradictory. Some studies in humans report increased LDL cholesterol (Lennernas *et al*., 1994; Ghiasvand *et al*., 2006; Haupt *et al*., 2008; Weigl *et al*., 2019), whereas others report no changes in LDL cholesterol (Sookoian *et al*., 2007; Esquirol *et al*., 2009; Puttonen *et al*., 2009; Loaf *et al*., 2019). The discrepancies in these studies may be due to different types of shift work considered, as well as different demographic parameters (age and sex of the participants). Finally, shift workers present with higher BP or rates of hypertension (Sakata *et al*., 2003; Ha and Park, 2005; Scheer *et al*., 2009; Gamboa Madeira *et al*., 2021).

Hypertension may influence LV contraction and relaxation as discussed in the previous section. Interestingly, female shift workers in their menopausal years have been reported to experience sleep disturbances and poor sleep quality compared to men of the same age (Jehan *et al*., 2015; Pines, 2016; Zhang *et al*., 2022). Overall, women also have a different alignment of their sleep/wake cycle to the circadian cycle (sleeping and waking later in their biological time). This different alignment has been suggested as a possible explanation for the increased complaints of poor sleep quality and sleep disturbances in women. Hence, this baseline misalignment in women could be enhanced with shift work (Cain *et al*., 2010). Therefore, it may be challenging to determine whether the effects of shift work result solely from circadian misalignment or from a combination of circadian misalignment, estrogen withdrawal and existing sleep abnormalities in females.

Similar to shift workers, several studies reported increased body mass with circadian misalignment in rats (Tsai *et al*., 2005; Tsai *et al*., 2007; Salgado-Delgado *et al*., 2010) and mice (Oishi and Itoh, 2013). However, other studies found reduced body mass in rats (Murphy *et al*., 2003; Salgado-Delgado *et al*., 2008) and mice (Sherman *et al*., 2012; Yoon *et al*., 2012; Oyama *et al*., 2014). Notably, studies reporting increased body mass involved rotating light exposure manipulations more than once weekly. Whereas those reporting reduced body mass utilized forced activity or restrictive feeding to stimulate circadian misalignment. Moreover, studies also report no changes in body mass in rats (Gale *et al*., 2011; Leenaars *et al*., 2012;

McDonald *et al*., 2013; Deibel *et al*., 2014; Zeman *et al*., 2016). Notably, these studies involved constant light exposure with either advanced or delayed timing, or weekly rotations. Therefore, the method of stimulating circadian misalignment and the frequency of light exposure manipulations may trigger different metabolic responses compared to constant irregular light/dark regimen. Alterations in body mass can ultimately influence energy metabolism as discussed in the previous section. Indeed, studies documented increased blood glucose levels, triglycerides, and total cholesterol with circadian misalignment in rodents with altered body mass (Salgado-Delgado *et al*., 2008; Sherman *et al*., 2012; Yoon *et al*., 2012).

Similarly, studies investigating the circadian misalignment effect on BP are contradictory. Recent studies report increased BP in rats (Zhang *et al*., 2000; Wang *et al*., 2022) and mice (Doi *et al*., 2009). Notably, these studies were conducted in male rodents exposed to more than a 16-hour dark period or constant darkness. Similarly, in hypertensive and cardiomyopathic rodent models, circadian misalignment increased BP and the expression of CV risk factors (Mosendane *et al*., 2008; Puttonen *et al*., 2010; Oishi and Ohkura; 2013). Interestingly, in an experimental sex comparison study by Zeman et al. (2016), circadian misalignment was associated with decreased BP in young male Wistar rats (6-week-old) and no changes in BP in aged female Wistar rats (12-month-old). Although the same light/dark regimen was employed, the discrepancies may have been due to different techniques used to assess BP. The male-based experiment used a tail-cuff plethysmography, whereas the female-based experiment used implanted telemetry transmitters. It may therefore be challenging to compare findings on the effect of circadian misalignment on BP as previous studies use different types of circadian misalignment and BP techniques. Finally, recent findings also reported increased LV mass, impaired LV contraction, and unchanged diastolic function with circadian misalignment in male normotensive rats (Jing *et al*., 2020; Wang *et al*., 2022). However, there is not enough findings to make comparisons, especially in female rodents.

Furthermore, there is not enough research investigating circadian misalignment effect on cardiometabolic health of SHR. It is noteworthy that there are significant differences between the circadian timing system of SHR and their normotensive controls (Cui *et al*., 2011; Sládek *et al*., 2012; Polidarová *et al*., 2013; Olejníková *et al*., 2015). Research indicates that an occurrence of disruption of the circadian timing system starts before hypertension and insulin resistance emerge and may be related to the genetic predispositions of the SHR (Miyazaki *et al*., 2011). Indeed, SHR exhibit abnormal circadian rhythms in the SCN and several peripheral

tissues and organs (Cui *et al*., 2011; Sládek *et al*., 2012; Tanaka *et al*., 2018). Evidence suggests that the temporal control of physiological processes in SHR may be influenced by weakened day/night rhythms in BP (Lemmer *et al*., 1993) and the abnormal sleeping patterns (Peters *et al*., 1994; Carley *et al*., 1996). It is also suggested that in SHR the SCN clock may be differently synchronized with the light/dark cycle, and the clock may struggle to generate robust circadian signals needed to regulate output rhythms (Sládek *et al*., 2012). Malfunctions in the SCN clock compromises the internal synchronization within the circadian timing system (Sládek *et al*., 2012). Additionally, SHR exhibit disrupted circadian rhythms in metabolic gene expression in the CV and metabolic tissues (Cui *et al*., 2011; Hou *et al*., 2021). The demonstrated hypertensive retinopathy in SHR likely affects the transmission of light to the SCN, thus limiting the normal entrainment of the SCN by light of mammals (Li *et al*., 2020).

In the present study, circadian misalignment did not induce changes in the body mass, food intake, metabolic function (blood glucose levels, glucose tolerance, LDL cholesterol) and the LV structure and function (BP, systolic and diastolic markers). The already misaligned circadian timing system reported in SHR, may explain to some extent why the CPS protocol, designed to induce circadian misalignment and its resulting cardiometabolic effects, had no visible impact on cardiometabolic parameters in the present study. Interestingly, Rumanova et al. (2019) exposed male SHR (4.5-month-old) to 12-hour of 150 lux light and 12-hour of dim light (2 lux) throughout the dark period for 2 to 5 weeks. In the study, the body mass, daily food intake, blood glucose levels, and LDL cholesterol remained unchanged, similar to the present study. However, there was a gradual increase in BP and loss of light/dark variability for BP with circadian misalignment (Rumanova *et al*., 2019). The lack of complete darkness may have produced the different circadian misalignment effects on BP observed in the study by Rumanova et al. (2019). Light exposure at night, even at low intensity, can potentially influence BP regulation (Verceles *et al*., 2012; Molcan *et al*., 2019).

Finally, in the present study there was a decrease in liver mass and an increased water intake with circadian misalignment, independent of the ovariectomized or sham-operated status. According to a study comparing the gene expression profiles of SHR in the liver and heart, the heart had a more severe impairment in clock gene expression than the liver (Cui *et al*., 2011). The liver clock is more independent and less sensitive to SCN signalling compared to the clocks in other organs like the heart (Sládek *et al*., 2012). This may imply that the hepatic clock, driven by its own set of clock genes, may still experience some circadian disruption independent of

the SCN. Disrupted hepatic circadian rhythms can negatively affect the liver mass through several mechanisms. Under normal physiological conditions, the liver undergoes regular cycles of cell proliferation and programmed cell death (apoptosis) (Alison *et al*., 1994; Gentric and Desdouets, 2014; Passman *et al*., 2023). Thus, the circadian gene targets involved in cell proliferation and apoptosis represent a potential means by which circadian misalignment can influence liver mass. Indeed, the disruption of the hepatic clock may contribute to abnormal cell proliferation and uncontrolled apoptosis (Zhou *et al*., 2022). Therefore, the present study hypothesises that hepatic clock disruption may result in fewer hepatic cells entering cell proliferation cycle and increased cell death, ultimately leading to shrinkage of the liver.

Furthermore, circadian misalignment can affect the thirst mechanisms and lead to increased water intake in several ways. It has been suggested that circadian regulation affects fluid balance hormones, such as vasopressin or antidiuretic hormone (ADH), produced in the posterior hypothalamus and aldosterone (secreted by the adrenal cortex) (George *et al*., 1975; Hiller-Sturmhöfel and Bartke, 1998; Kamperis *et al*., 2004; Scheuermaier *et al*., 2023). ADH stimulates water retention by the kidneys, which reduces urine output, whereby aldosterone indirectly regulates water reabsorption in the kidneys by regulating sodium reabsorption (Hiller-Sturmhöfel and Bartke, 1998; Wang *et al*., 2020). The secretion of these hormones exhibits circadian rhythms, such that their levels fluctuate across a 24-hour day (Duffy *et al*., 2016; Scheuermaier *et al*., 2023). Evidence suggests that circadian misalignment may lead to lower aldosterone production (Thosar *et al*., 2019; McMullan *et al*., 2022). Aldosterone stimulates the production of ANG II (a hormone that indirectly increases ADH release from the posterior hypothalamus) (Takahashi *et al*., 2011; Szczepcanska-Sadowska *et al*., 2018), thus reduced aldosterone may lead to reduced ANG II and lower ADH release (Zheng *et al*., 2020).

ADH stimulates aquaporins to be inserted into the collecting duct's cell membrane, increasing the amount of aquaporins and thus promoting water reabsorption (Hasler *et al*., 2002; Oh *et al*., 2008). Reduced ADH may result in decreased water reabsorption, and the body will attempt to maintain fluid intake through an increased urine output (McKinley and Johnson, 2004; Mutter *et al*., 2021). Ultimately, this may trigger compensatory thirst and an increased water intake (Perrier *et al*., 2020; Mutter *et al*., 2021). It is possible that the CPS protocol in the present study induced a circadian disruption that eventually affected the secretion of ADH and/or aldosterone. The present study speculates that circadian misalignment may indirectly

affect water intake through the fluid balance hormones. Moreover, the present study is the first to show alterations in water intake and liver mass with circadian misalignment. Therefore, the present study speculates that while the SHR may not be sensitive to the CPS protocol, circadian misalignment may still have some effect on other physiological pathways affected by the light exposure.

To confirm whether the circadian timing system of the female SHR were responsive to shifts in the light/dark regimen, the present study assessed the rectal temperature. Rectal temperature measurement reflects the core body temperature (Brown *et al*., 1992; Dilsaver *et al*., 1992; Leduc *et al*., 2000; El-Radhini, 2014), which is regulated by circadian rhythms (Cevoli *et al*., 2002; Okamoto-Mizuno and Mizuno, 2012). Notably, there were no alterations in the rectal temperatures in the present study, however it was assessed only once a day in the last 2 weeks of the CPS protocol and not continuously throughout the 24-hour day. Core temperature fluctuates and varies during the 24-hour period (Mumm *et al*., 1989; Alföldi *et al*., 1990; Hankenson *et al*., 2014), usually peaking in the late afternoon or early evening and dropping to its lowest by early morning hours (Refinetti, 2020). Therefore, the present study cannot make a conclusion on whether the circadian rhythms were misaligned by the CPS protocol.

In summary, the present study suggests that estrogen deficiency increases body mass due to changes in body composition rather than increased food intake. The increase in organ masses was influenced by body mass gain differences rather than a true increase in organ size. Estrogen deficiency may not have direct effects on the glucose and lipid metabolism. Perhaps age and environmental conditions (i.e. diet and temperature) may concurrently have a greater effect on the metabolic profile of young female SHR than estrogen deficiency alone. Additionally, estrogen deficiency also did not further the hypertensive state, LVH development and diastolic dysfunction of the female SHR. However, estrogen deficiency impaired systolic function, affecting the LV contraction and chamber dimensions. Furthermore, the present study suggests that the CPS protocol did not influence the cardiometabolic parameters. The already abnormal circadian timing system in SHR may explain the absence of effect of the CPS protocol in the present study. However, the CPS protocol may still have some effect on other physiological pathways as reflected by alterations in water intake and liver mass.

Chapter 5: Conclusions

5.1 Conclusions

The present study investigated the effects of a 10-week CPS protocol on cardiometabolic parameters in 3 months old estrogen-deficient female SHRs. The present study shows that 10 weeks of CPS did not worsen the cardiometabolic parameters measured in female SHR. The SHR may show limited effects of circadian misalignment during shift work paradigm study due to their abnormal circadian rhythms, possibly making SHR not a good model for circadian misalignment research. However, the altered liver mass and water intake observed warrants further investigations as it highlights the complexity of circadian misalignment and its potential effects on different physiological systems. On the other hand, estrogen deficiency did not worsen the metabolic profile of female SHR in the present study. The age at which estrogen deficiency was induced may not be detrimental to the metabolic function of young female SHR. However, estrogen deficiency did influence the body mass gain in female SHR. This warrants further investigations, considering possible compensatory mechanisms for metabolism regulation in young female SHR. Furthermore, estrogen deficiency significantly compromised the systolic function in female SHR, evident through decreased LV contraction and enlarged chamber dimensions. The extent of this systolic impairment suggests an early vulnerability to heart failure influenced by estrogen deficiency, highlighting the critical role of estrogen in maintaining proper cardiac function in female SHR.

5.2 Limitations and future studies

The following are possible limitations of this study: i) the 10-week CPS protocol did not result in significant cardiometabolic changes possibly due to the already dysregulated circadian system in SHR. Future studies may employ a different rat strain or model to determine whether CPS may directly or indirectly cause cardiac and metabolic changes in estrogen-deficient subjects; ii) the findings of the present study are specific to hypertensive estrogen-deficient rats. Without normotensive control groups, it may be difficult to determine whether the observed changes may have resulted from the combined effects of hypertension, circadian misalignment and estrogen deficiency. Therefore, future studies may incorporate normotensive control groups to establish a more comprehensive understanding of how circadian misalignment interacts with different BP levels and estrogen deficiency in affecting cardiometabolic health or to strengthen the study design; iii) core body temperature was measured once a day using rectal thermometer. Although this serves as a valuable tool, it is time-consuming, requires fixation of the animal, it is invasive and stressful (Schmid *et al*., 2021), which may affect its accuracy. The temperature of pulmonary arterial blood is the gold

standard for determining core temperature (Parikh and Rosenberg, 2013). More accurate methods of tracking daily core body temperatures in rodents require the surgical implantation of temperature-sensitive sensors or data loggers (Refinetti, 2020). Moreover, core body temperature alone may not be enough to confirm circadian misalignment. Rest activity cycles are usually used to investigate circadian rhythms in rodents, which we were not able to monitor in the present study. Therefore, future studies may add other assessments such as monitoring of rest activity cycles by light detection loggers (equipped with light sensors that continuously record movement and location in a cage), or wheel running activity monitors (establishing the timing and duration of the active and rest phases) to provide with more information on circadian synchronization. Additionally, future studies may measure melatonin levels (closely related to the sleep/wake cycle) (Brown, 1994) at specific time points during the light/dark cycles to provide valuable insight into potential circadian misalignment; iv) relying on single-point measurements for LDL, fasting blood glucose, food intake, and water intake may not fully capture the diurnal fluctuations associated with these parameters. Future studies could benefit from incorporating more frequent measurements throughout the day or employing telemetry techniques to obtain a more comprehensive picture of these patterns under manipulated light exposure conditions; v) the inclusion of uterine mass measurements, vaginal smear cytology and measures of estrogen concentrations may have strengthened the interpretation of the findings of the present study; vi) The non-invasive tail-cuff technique was used to measure BP in the present study, where measurements are obtained in restrained rats. This has been reported to possibly induce stress to animals and affect BP readings. Prior to the start of data collection, the rats were habituated to the BP monitor and the restrainers for two weeks to minimize stress in the present study. However, with the non-invasive tail-cuff technique, small changes in BP may not be detected. Future research should employ the more reliable telemetry technique, which involves the implantation of a radio transmitter in the carotid artery of a rat at the start of the study or intervention. The telemetry technique is also utilized to assess the accuracy of non-invasive BP readings; vii) the inclusion of the tibia length measurements and body composition may have given a better indication of growth and body mass gain in the present study; viii) conventional echocardiography is a clinically useful tool, however, it has limitations in terms of reproducibility, angle dependency and accuracy (Hanrath and Schlüter, 1983). Subtle alterations in cardiac function parameters may require more specialized testing, such as strain imaging (speckle tracking echocardiography, STE) (Nesbitt *et al*., 2008; Hensel

et al., 2014). Therefore, the present study suggests further analysis using STE to assess possible subclinical cardiac impairments in female SHR.

5.3 Possible clinical implications and recommendations

A decline in estrogen levels contributing to early vulnerability of heart failure in humans aligns with clinical observations linking menopause and increased risk for heart failure. Therefore, it remains of most importance to strengthen the research on hormonal therapy as a strategy for treatment or prevention of menopause-related risks and conditions. Indeed, early interventions to maintain or supplement estrogen concentrations in menopausal years may be beneficial for preserving the cardiac function, however, further human studies are needed with careful considerations of individual risk factors and potential side effects. Furthermore, androgens play an important role in cardiac health, partially explaining the absence of improved CV risks after estrogen supplementation (Chen *et al*., 2021). Therefore, it is important to strengthen the research on combined androgen and estrogen interventions. On the other hand, the absence of significant effects of circadian misalignment on cardiometabolic parameters in the present study may not directly translate to humans. However, it highlights the complexities of studying circadian misalignment and its individual variability. Therefore, further investigation is required to clarify the complex consequences of circadian misalignment in humans, considering potential influencing factors.

Chapter 6: References

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Appendices

STRICTLY CONFIDENTIAL

ANIMAL ETHICS SCREENING COMMITTEE (AESC)

CLEARANCE CERTIFICATE NO. 2017/011/76/C

APPLICANT: Dr F Michel

SCHOOL: Physiology
DEPARTMENT:
LOCATION:

PROJECT TITLE: The effect of circadian misalignment on cardiometabolic parameters in ovariectomised SHR

Number and Species

72X 5 week old female SHR rats

Approval was given for the use of animals for the project described above at an AESC meeting held on 2017/11/28. This approval remains valid until 2019/11/27. ,
Unreported changes to the application may invalidate the clearance given by the AESC
An annual progress report must be provided

The use of these animals is subject to AESC guidelines for the use and care of animals, is limited to the procedures described in the application form and is subject to any additional conditions listed below:

Signed: _____ Date: 15 December 2017
(Chairperson, AESC)

I am satisfied that the persons listed in this application are competent to perform the procedures therein, in terms of Section 23 (1) (c) of the Veterinary and Para-Veterinary Professions Act (19 of 1982)

Signed: _____ Date: 15 December 2017
(Registered Veterinarian)

cc: Supervisor: N/A
Director: CAS

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AESC 2012 M&E

Please note that only type written applications will be accepted.

UNIVERSITY OF THE WITWATERSRAND

ANIMAL ETHICS SCREENING COMMITTEE
MODIFICATIONS AND EXTENSIONS TO EXPERIMENTS

a. Name: Frederic Michel

b. Department: School of Physiology

c. Experiment to be modified / extended **AESC NO**

Original AESC number	**2017**	**11**	**76C**
Other M&Es			9

d. Project Title: the effect of circadian misalignment on cardiometabolic parameters in ovariectomised SHR

		No.	**Species**
e.	Number and species of animals originally approved:	72	SHR
f.	Number of additional animals previously allocated on M&Es:	11	SHR
g.	Total number of animals allocated to the experiment to date:	83	SHR
h.	Number of animals used to date:	46	SHR

i. Specific modification / extension requested:

- Addition of coworkers: Siluleko Mkhize (PhD student, #843928) and Amandus Nthlane (MSc student #1448621)

j. Motivation for modification / extension:

Amandus Nthlane is a new MSc student at the School of Physiology under my supervision. Her MSc project will be about the effect of circadian misalignment on cardiovascular parameters in ovariectomised SHR. She will be responsible with all aspects of the study. Siluleko is also a new PhD student at the School of Physiology under my supervision and will be responsible of echocardiography at termination.

Date:22/02/2022 Signature: 

RECOMMENDATIONS

Approval for the addition of co-workers S. Mkhize and A. Nthlane to the project.

Date:25 February 2022 Signature: 

Deputy Chair, AESC