4-14-2014 12:00 AM

Sex Hormones and Sympathetic Nerve Activity

Charlotte W. Usselman, *The University of Western Ontario*

Supervisor: Dr. J. Kevin Shoemaker, *The University of Western Ontario*
A thesis submitted in partial fulfillment of the requirements for the Doctor of Philosophy degree in Kinesiology
© Charlotte W. Usselman 2014

Sex Hormones and Sympathetic Nerve Activity

(Thesis format: Integrated Article)

by

Charlotte Willemina <u>Usselman</u>

Graduate Program in Kinesiology

A thesis submitted in partial fulfillment
of the requirements for the degree of
Doctor of Philosophy

The School of Graduate and Postdoctoral Studies
The University of Western Ontario
London, Ontario, Canada

Abstract

The purpose of this thesis was to test the hypothesis that changes in circulating sex hormone levels are associated with changes in muscle sympathetic nerve activity. The hypothesis was tested through the comparison of low- (early follicular [EF]) and high-hormone (midluteal [ML]) phases of the menstrual cycle and of hormonal contraceptive use (low hormone [LH] versus high hormone [HH]). The microneurography technique was used to compare both the frequency and size of bursts in muscle sympathetic nerve activity (MSNA) at baseline and during two sympatho-excitatory maneuvers: baroreceptor unloading elicited through lower body negative pressure, and chemoreflex stimulation elicited through a hypoxic-hypercapnic end-inspiratory apnea. Sympathetic responses to chemoreflex stimulation were also compared between women and men. All associations between MSNA and hormone phases occurred similarly between users and non-users of hormonal contraceptives. At baseline, MSNA was relatively elevated during the high hormone phases (ML and HH), at which point baseline sympathetic activity was similar to that observed in men. However, stimulation of the chemoreflex resulted in greater sympathetic activation during the low hormone phases (EF and LH) relative to the high hormone phases. Further, this hormone phase effect was mediated largely by greater increases in burst size, rather than the burst frequency component. This may indicate that central integration sites for MSNA are affected by circulating sex hormone levels. Finally, the sympathetic responses to baroreceptor unloading were graded to reductions in stroke volume, which, in turn, were affected by hormone levels. However, no evidence was observed to suggest a change in the central integration of baroreceptor afferent input occurred across phases of the menstrual cycle or hormonal contraceptive use in terms of baroreflex function. Together, these studies

confirm that sympathetic nerve activity at baseline and sympathetic recruitment during

chemoreflex stimulation are affected by hormone phase, while baroreceptor-mediated

responses are not affected by the transition from low (EF and LH) to high hormone phases

(ML and HH).

Keywords

Co-Authorship Statement

Charlotte W. Usselman was the first author and Dr. J. Kevin Shoemaker was the senior author on all papers included in this thesis. The co-authors on the Chapter 3 paper were Dr. Stan H. M. van Uum, Torri A. Luchyshyn, Chantelle A. Nielson, and Tamara I. Gimon. The papers in Chapters 2 & 4 included the same coauthors with the addition of Nicole S. Coverdale.

Specific contributions to the papers are listed as follows:

Conception and design: Charlotte W. Usselman & Dr. J. Kevin Shoemaker

Data collection: Charlotte W. Usselman, Dr. J. Kevin Shoemaker, Torri A. Luchyshyn, Chantelle A. Nielson, Tamara I. Gimon, & Nicole S. Coverdale

Data analysis and interpretation: Charlotte W. Usselman , Dr. J. Kevin Shoemaker & Dr. Stan H. M. van Uum

Writing and revisions: Charlotte W. Usselman with revisions and feedback from all co-authors.

Epigraph

"The physiology of today is the medicine of tomorrow."

- Ernest Henry Starling

Dedication

The past 5 years have taught me that no academic endeavour is successful without two things: genuine interest in the subject matter and perseverance through adversity. I would like to dedicate this thesis to my parents, who have instilled both of these in me. Dad, you have a scientific curiosity that is not only contagious, but also extremely accessible. You've taught me to see the world through the miraculous lens of science, and for that I will be forever grateful. Mom, you are endlessly brave with a ferocious tenacity, yet somehow you combine those traits with kindness and empathy. From you I have learned the importance of balance in all of these qualities.

Mom and Dad, thank you for helping me to become the person and researcher that I am today. I love you!

Acknowledgements

The following individuals provided technical assistance with this project:

Arlene Fleischhauer trained the phlebotomists (T. I. Gimon, T. A. Luchyshyn, C. A. Nielson, & N. S. Coverdale) and assisted with blood draws as needed.

 Dr. Craig Steinback assisted in the design of the chemoreflex protocol.

Table of Contents

List of Tables

List of Figures

List of Abbreviations

ANOVA – Analysis of Variance

APN-P1 – Initial Phase of Apnea

APN-P2 – Latter Phase of Apnea

BMI – Body Mass Index

BSL – Baseline

DBP – Diastolic Blood Pressure

E2 – 17β-Estradiol

EE – Ethinyl Estradiol

EF – Early Follicular

HC – Hormonal Contraceptives

HH – High Hormone (phase of hormonal contraceptive use)

HR – Heart Rate

HUT – Head-Up Tilt

LBNP – Lower Body Negative Pressure

LH – Low Hormone (phase of hormonal contraceptive use)

MAP – Mean Arterial Pressure

ML – Midluteal

MSNA – Muscle Sympathetic Nerve Activity

P4 – Progesterone

PCO_2 – Partial Pressure of Carbon Dioxide

PO_2 – Partial Pressure of Oxygen

PP – Pulse Pressure

Q – Cardiac Output

Q_i – Cardiac Index

REBR – Rebreathing Period

REC – Recovery Period

SBP – Systolic Blood Pressure

SV – Stroke Volume

SV_i – Stroke Volume Index

T – Testosterone

TPR – Total Peripheral Resistance

Chapter 1

1   Introduction

1.1   Overview

Cardiovascular disease risk is reduced in premenopausal women relative to age-matched men (Rosenthal & Oparil, 2000;Young *et al.*, 1993;Eaker *et al.*, 1989) (Figure 1.1, for example). Following menopause, this relative cardioprotection is lost such that cardiovascular disease risk is increased significantly in post-menopausal women (Rosano *et al.*, 2007). The sympathetic nervous system has been implicated as a contributor to the loss of premenopausal cardioprotection based on its importance in the control of cardiovascular function (Wallin & Charkoudian, 2007) and the positive associations which exist between heightened sympathetic nerve activity and incidence of cardiovascular disease (Leimbach, Jr. *et al.*, 1986;Yamada *et al.*, 1989;Miyajima *et al.*, 1991;Carlson *et al.*, 1993). Accordingly, examinations of baseline conditions have indicated that sympathetic nerve activity is elevated in men compared to pre-menopausal women (Ng *et al.*, 1993;Matsukawa *et al.*, 1998;Narkiewicz *et al.*, 2005). Moreover, sympathetic nerve activity increases following menopause, matching or exceeding levels observed in similarly aged men (Matsukawa *et al.*, 1998;Narkiewicz *et al.*, 2005) (Figure 1.2). Together, the similar patterns of sex- and age-related changes in sympathetic nerve activity and cardiovascular disease support a possible role for the sympathetic nervous system in the reduced incidence of cardiovascular morbidity in premenopausal women. However, the source of the relative sympatho-inhibition in premenopausal women is less clear.
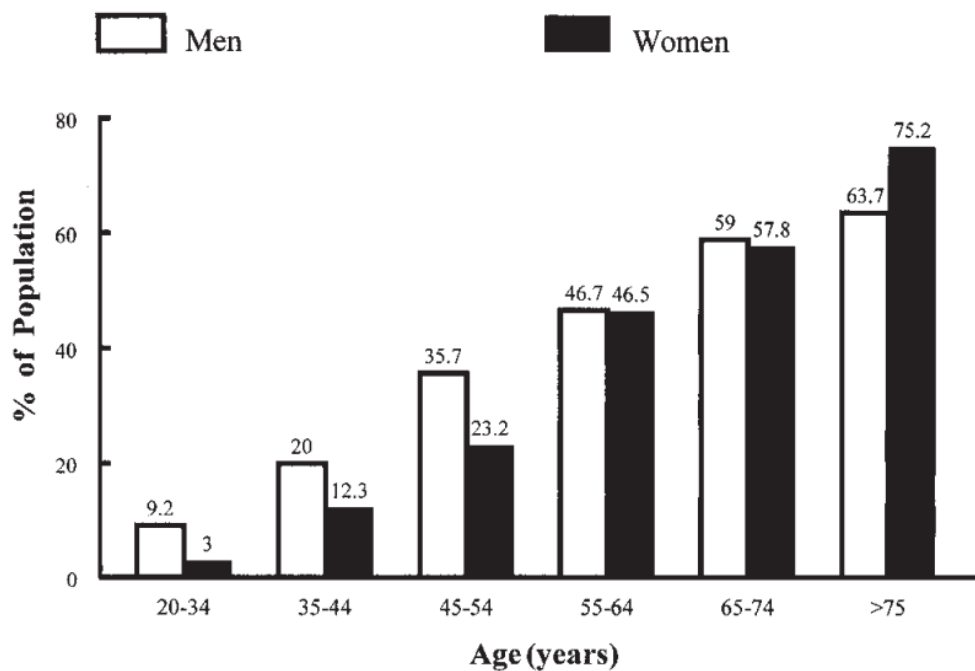
Figure 1.1: Prevalence of hypertension in men and women.

Hypertension is more prevalent in young men than young women, but this sex difference

disappears following menopause. Reproduced with permission from Nature Publishing

Group: Journal of Human Hypertension (Rosenthal & Oparil, 2000).
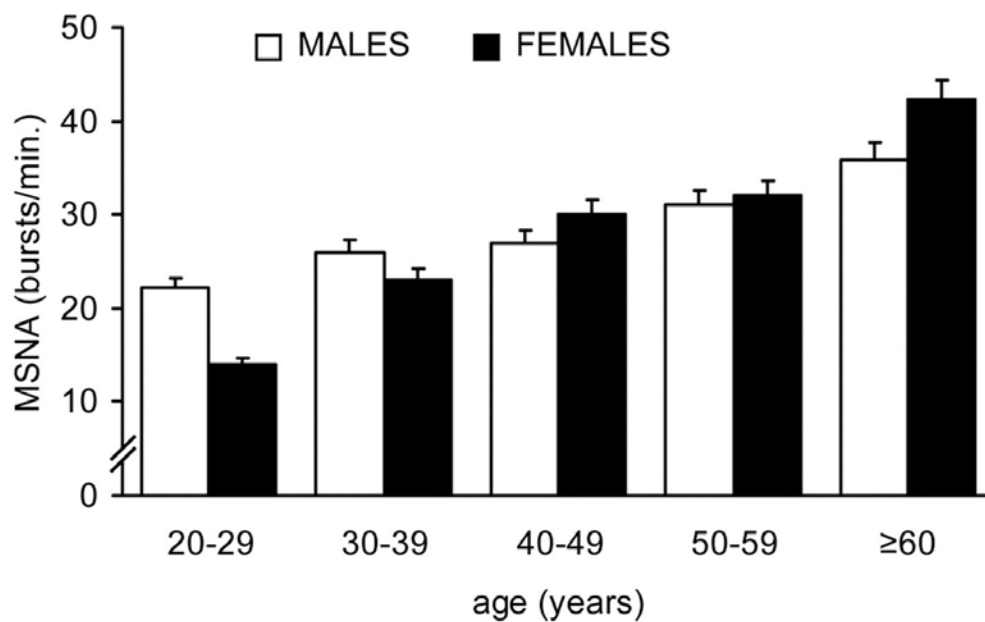
Figure 1.2: Baseline sympathetic nerve activity in men and women

MSNA is lower in young women than in age-matched men. In middle aged and older

adults, MSNA is similar or greater in women than in men. Adapted with permission from

Lippincott Williams and Wilkins/Wolters Kluwer Health: Hypertension (Narkiewicz *et al.*, 2005).

Circulating concentrations of estrogen and progesterone are elevated in women relative to men and are dramatically reduced in women following menopause (Conte & Grumbach, 2007). Together with the data summarized above, this suggests that sex hormones may affect sympathetic regulation in women with important cardiovascular consequences. Further support for this hypothesis has come from women with polycystic ovary syndrome. In these women, levels of circulating testosterone and baseline sympathetic nerve activity are elevated relative to control subjects (Sverrisdottir *et al.*, 2008). However, both the polycystic ovary syndrome and post-menopausal conditions represent alterations to the normal hormonal milieu which is observed in healthy young premenopausal women. The concept that sex hormones exert an influence over levels of sympathetic nerve activity and potentially contribute to the high degree of interindividual variability in baseline sympathetic nervous system activity which is observed in young healthy subjects (Joyner *et al.*, 2010;Wallin, 2006) has only been examined relatively recently.

The sympathetic effects of "normal" fluctuations in hormone levels have been examined through the study of the menstrual cycle, which is associated with large endogenous fluctuations in circulating estrogen and progesterone. Several such studies have observed relative increases in baseline sympathetic nerve activity during the menstrual cycle phase associated with heightened levels of both estrogen and progesterone, the midluteal phase, in comparison to the early follicular phase which is associated with low levels of estrogen and progesterone (Minson *et al.*, 2000a;Park & Middlekauff, 2009;Middlekauff *et al.*, 2012;Carter *et al.*, 2013). While these data support a possible link between sex hormones and sympathetic regulation in young healthy

women, it is important to consider that an almost equal number of studies have failed to observe the same effect (Carter *et al.*, 2009b;Fu *et al.*, 2009;Carter & Lawrence, 2007). The lack of ubiquity in these results might imply that the association between sympathetic nerve activity and menstrual cycle phase is a mild one. It is also possible that this association is strengthened during sympatho-excitation: studies of sympathetic responses to the sympatho-excitatory stress of baroreceptor unloading have consistently observed greater sympathetic nerve activity in the midluteal phase of the menstrual cycle than during the early follicular phase (Fu *et al.*, 2009;Carter *et al.*, 2009b). Also, the differences in sympathetic activity between menstrual cycle phase appear to be most pronounced during the most severe phases of baroreceptor unloading (Fu *et al.*, 2009;Carter *et al.*, 2009b). Therefore, one purpose of these studies was to assess whether menstrual cycle-driven fluctuations in hormone levels affect sympathetic outflow during states of high reflex stress. To achieve this objective, sympathetic regulation across the menstrual cycle was measured during baroreceptor unloading evoked using high levels of lower body negative pressure and during chemoreflex activation which is known to be associated with large increases in sympathetic nerve activity (Morgan *et al.*, 1995;Saito *et al.*, 1988).

A notable addendum to this field of research is the consideration of women whose endogenous fluctuations of sex hormones are impeded and supplemented by synthetic, exogenous hormones. This is an important issue given that at least 20% of women of child-bearing age currently use hormonal contraceptives (Mosher & Jones, 2010) which alter the types and profile of monthly variations in hormones levels. The influence of hormonal contraceptive use on sympathetic regulation has become the focus of a few

recent studies which have targeted similar hormone phases as those studied across the menstrual cycle (specifically, the low-hormone, inactive phase of contraceptive use *versus* the high-hormone, active phase) (Minson *et al.*, 2000b;Carter *et al.*, 2009a;Middlekauff *et al.*, 2012). However, the effects of hormonal contraceptive use on sympathetic regulation have been under-studied relative to menstrual cycle studies, and to date only one study has directly compared the two (Middlekauff *et al.*, 2012). Therefore, another purpose of these studies was to establish the effects of hormonal contraceptive use on sympathetic regulation patterns.

The overall objective of this research is to understand the effects of sex hormones on sympathetic nerve activity by comparing the responses to sympatho-excitatory maneuvers during the low and high hormone phases of the menstrual cycle and of hormonal contraceptive use. The **working hypothesis** of these studies is that hormone phase affects sympathetic nerve activity particularly during reflex activation. This, in turn, affects the observation of male-female differences in sympathetic regulation.

Study 1. Hormone phase influences neurovascular responses to high levels of lower body negative pressure.

> *Purpose:* To compare MSNA responses to moderate to high levels of lower body negative pressure between low- and high-hormone phases of both the regular menstrual cycle and hormonal contraceptive use.

> *Hypothesis:* Hormone phase (regardless of group) would affect sympathetic responses such that in both groups of women the higher hormone phases would be associated with greater sympathetic responses than the lower hormone phases.

Study 2. Hormone phase dependency of neural responses to chemoreflex-driven sympatho-excitation in young women using hormonal contraceptives.

Purpose: To compare muscle sympathetic nerve activation patterns between low and high hormone phases of exogenous contraceptive hormone use across a range of chemoreflexive stimuli.

Hypothesis: Baseline and reflex sympathetic nerve activation would be greater in the high hormone phase relative to the low hormone phase of hormonal contraceptive use.

Study 3. Sex and menstrual cycle effects on sympathetic responses to chemoreflex stimulation.

Purposes: (1) To compare sympathetic responses between men and women during a severe chemoreflex stress, and (2) to determine whether the menstrual cycle is associated with changes in chemoreflex-driven sympatho-excitation.

Hypotheses: (1) Acute hypercapnia-hypoxia would be associated with greater increases in sympathetic activity in young healthy women relative to men; (2) the low hormone phase of the menstrual cycle would be associated with greater increases in sympathetic activity than the high hormone phase.

1.2   Muscle Sympathetic Nerve Activity

The study of the activity of the sympathetic nervous system was greatly advanced by the development of the microneurographic technique by Hagbarth and Vallbo in the late 1960s (Hagbarth & Vallbo, 1968). In this technique, a recording electrode is inserted into a nerve and advanced in close proximity to efferent sympathetic nerve fibres which innervate the vasculature of peripheral muscles. Microneurography allows for the direct, real-time recording of muscle sympathetic nerve activity (MSNA) which has paved the way for decades of studies detailing sympathetic regulation mechanisms.

The microneurography technique involves the use of two tungsten electrodes, one of which is inserted percutaneously 1-3 cm from the active recording site and serves as reference electrode. The other is the recording electrode and is inserted transcutanously, commonly into the peroneal nerve. This nerve is ideal for the study of MSNA as it is easily accessible, relatively large, and contains a large number of efferent sympathetic neurons (Tompkins *et al.*, 2013). As described by Delius and colleagues, adequate MSNA recording sites are determined through the observation of several criteria (Delius *et al.*, 1972). The MSNA signal produces pulse synchronous bursts of activity which increase in frequency during apnea and are unaffected by arousal to a loud noise or light brushing of the skin (rather, these last two criteria are characteristic of skin sympathetic neurons). Signal processing methods are then used to amplify (e.g., 75 000x), filter (bandpass: 700-2000 Hz), rectify, and integrate (0.1s time constant) the raw sympathetic signal, producing the characteristic bursts of activity which are initiated during diastole (Figure 1.3).
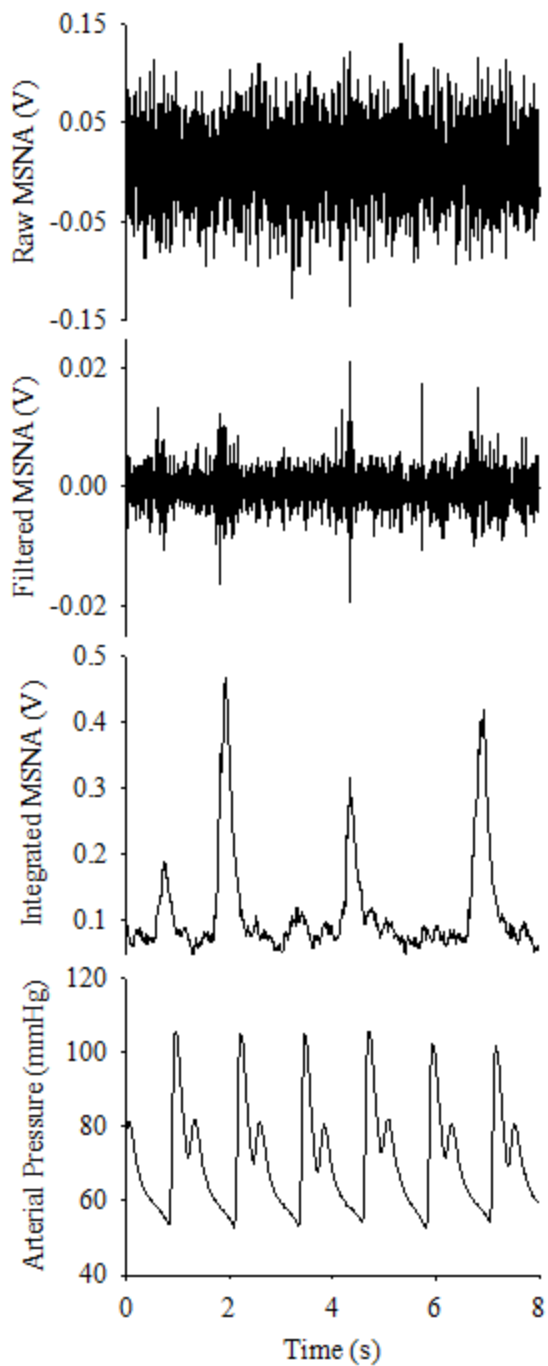
Figure 1.3: Analysis of the muscle sympathetic nerve activity signal.

Bursts of activity which are initiated during diastole are clearly visible following signal

processing.

A total quantification of the integrated MSNA signal consists of two components which appear to be regulated differently: burst frequency and burst size, often referred to as burst amplitude. The firing frequency is expressed as either burst frequency or burst incidence. Burst frequency is expressed in bursts per minute, while burst incidence involves a normalization of burst frequency to heart rate and is expressed as bursts per hundred heart beats. In this way, burst incidence reflects baroreceptor processing of efferent MSNA.

While the methods used to quantify the frequency component of the integrated MSNA signal are widely accepted and reproducible over time (Sundlof & Wallin, 1977;Kimmerly *et al.*, 2004), the quantification of the amplitude component is more complicated. The complication lies in the meaning of the absolute amplitude signal and its implications for interindividual comparisons. Absolute burst amplitude, expressed as a voltage, is an indication of the number (Ninomiya *et al.*, 1993) and size (Steinback *et al.*, 2010b) of neurons within the recording range of the electrode and is affected by electrode position. In the absence of a change in recording site, changes in burst amplitude can be interpreted to indicate alterations in the central regulation of MSNA burst amplitude. On the other hand, the extent to which the regulation of burst amplitude differs between subjects cannot be derived from measures of absolute burst voltages. Sverrisdottir and colleagues have used a method of normalization which allows for interindividual burst amplitude comparisons which isolates the burst amplitude component of MSNA from the burst frequency component (Sverrisdottir *et al.*, 2000). This method of burst amplitude analysis involves the normalization of all baseline bursts to a given burst (for example, the largest burst in the baseline period). The normalized burst amplitudes are then divided

into bins, and the frequency with which each bin occurs is graphed. The statistical characteristics of these distribution curves can then be compared between subjects in terms of mean, median, and/or modes. Similar to burst frequency measures, this method of determining burst amplitude has been shown to be reproducible between test dates (Kimmerly *et al.*, 2004).

Twenty years ago, Malpas and colleagues put forward the hypothesis that the frequency of sympathetic bursts is controlled independently from the number of active sympathetic fibres within a given burst of activity (Malpas, 1995). Several experiments have provided support for this hypothesis (Malpas & Ninomiya, 1992b;Malpas & Ninomiya, 1992a;Sverrisdottir *et al.*, 2000), including observations of altered sympathetic burst amplitude in the presence of unchanged burst frequency during mental stress (Hjemdahl *et al.*, 1989), hypoxic stimulation (Malpas *et al.*, 1996), local heating (DiBona & Jones, 1998), and baroreceptor stimulation (DiBona *et al.*, 1997). A hypothetical model by which sympathetic burst amplitude and frequency are regulated independently stipulates that a variety of afferent inputs converge on unknown central sites of sympathetic integration to cause graded activation of sympathetic efferent pathways (i.e. the control of burst amplitude). Baroreceptor afferent feedback is then thought to act as a gating mechanism to either allow or disallow a burst of efferent sympathetic activity (i.e. the control of burst frequency) (Kienbaum *et al.*, 2001) (Figure 1.4). Within the context of this model, it is conceivable that an increase in burst frequency could occur in conjunction with a reduction in burst amplitude (Kienbaum *et al.*, 2001). Such a possibility warrants the reporting of both burst frequency and burst amplitude measures when examining sympathetic responsiveness.
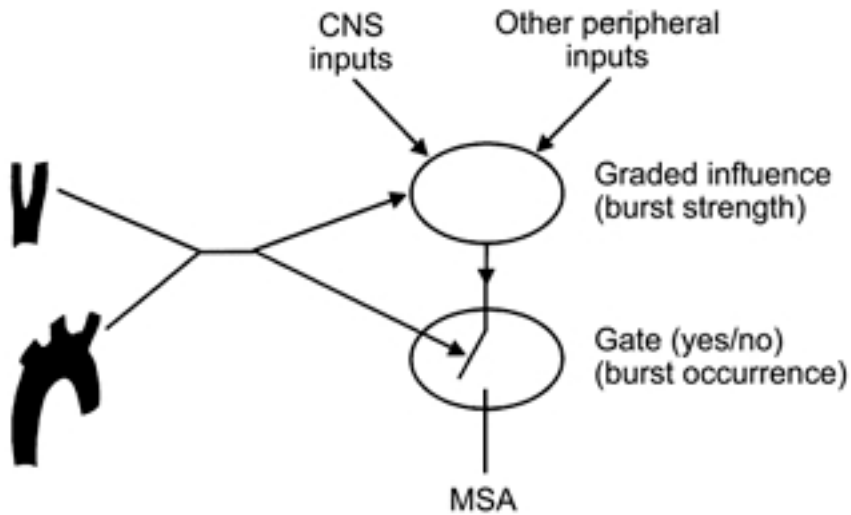
Figure 1.4: Hypothetical model for arterial baroreceptor influence on sympathetic nerve activity.

Baroreceptor afferent activity (left) is incorporated with other inputs to create a graded influence which is reflected by burst amplitude. The gating of the resultant burst strength is then regulated largely by the baroreceptors, affecting the frequency with which efferent sympathetic bursts occur. Reproduced with permission from John Wiley and Sons: Journal of Physiology (Kienbaum *et al.*, 2001).

1.3 Sympathetic Activity during Baroreceptor Unloading

The orthostatic stress which arises during upright posture due to the effect of gravity is associated with the peripheral pooling of blood. This, in turn, can limit venous return and reduce cardiac output, thereby threatening the maintenance of an arterial pressure necessary to adequately perfuse the body with blood. Such a reduction in cardiac output is detected by the mechano-sensitive baroreceptors which are unloaded by the associated reduction in local blood pressure. The subsequent reduction in the firing rates of baroreceptor afferent projections to central sympathetic sites are integrated to produce sympatho-excitation which is graded to the level of baroreceptor unloading and evokes an increase in peripheral resistance in an effort to maintain mean arterial pressure (Victor & Leimbach, Jr., 1987;Rowell, 1993;Johnson *et al.*, 1974) .

In a laboratory setting, microneurographic recordings of MSNA are difficult to measure during the transition from supine to standing as the movements involved can dislodge the recording electrode and/or necessitate the activation of postural muscles which confound the MSNA signal. Methods which simulate orthostasis such as head-up tilt and lower body negative pressure (LBNP) have been developed to allow subjects to maintain relaxation in the leg in which MSNA is being recorded, thus avoiding these issues. Lower body negative pressure also excludes the sympathetic effects of vestibular otolith stimulation which occur during head-up tilt (Ray, 2000;Kaufmann *et al.*, 2002). During LBNP, suction is created around the lower body below the level of the iliac crests which draws blood into the vascular beds contained therein. This method allows for careful titrations of the sympathetic response as suction can be controlled with great precision. Applications of LBNP as low as -5 mmHg have been associated with small but

significant increases in MSNA with no change in mean arterial blood pressure (Victor & Leimbach, Jr., 1987). Progressive increases in suction are associated with graded increases in MSNA (Sundlof & Wallin, 1978;Victor & Leimbach, Jr., 1987) which occur in response to the LBNP-induced reductions in stroke volume (Figure 1.5) (Ryan *et al.*, 2011). Recently, Ichinose and colleagues (Ichinose *et al.*, 2004;Ichinose *et al.*, 2006) demonstrated that increases in the amplitudes and firing frequencies of sympathetic bursts may depend on the severity of LBNP. Their data indicate that at levels of LBNP up to approximately -30 mmHg, increases in total MSNA are achieved through increases in both firing frequencies and burst amplitudes, while at higher levels of suction increases in total MSNA are achieved primarily through elevations in burst amplitude (Ichinose *et al.*, 2006;Ichinose *et al.*, 2004).
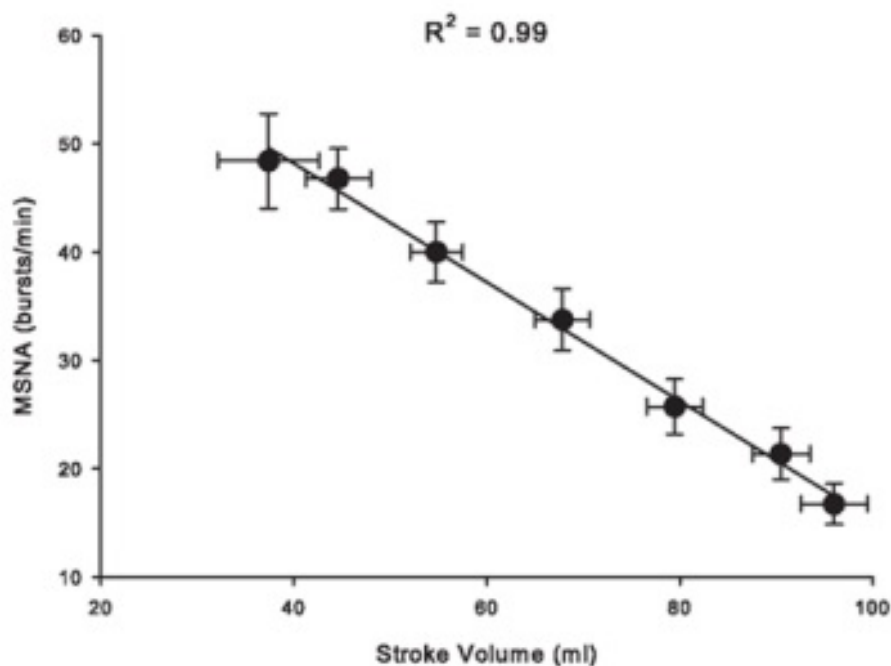
Figure 1.5: Relationship between sympathetic nerve activity and stroke volume during baroreceptor unloading.

Muscle sympathetic nerve activity (MSNA) is negatively related with stroke volume during a progressive LBNP protocol to presyncope. Reproduced from an open access article under the terms of the Creative Commons Attribution Non Commercial License (Ryan *et al.*, 2012).

1.4 Sympathetic Activity during Chemoreflex Stimulation

The activation of the chemoreflex is associated with a large sympatho-excitatory response (Morgan *et al.*, 1995;Saito *et al.*, 1988). Chemoreceptors are located both peripherally and centrally, with different sensitivities to the nature of the chemoreflex stress – central chemoreceptors are sensitive to circulating carbon dioxide, while peripheral chemoreceptors are sensitive to both oxygen and carbon dioxide (Kara *et al.*, 2003). Similar to the effects of baroreceptor unloading, chemoreceptor stimulation produces increases in MSNA which are graded to the intensity and duration of the chemoreflex stimulus (Smith & Muenter, 2000). Importantly, the effects of hypoxia and hypercapnia are additive, such that combined hypoxia-hypercapnia produces a greater increase in MSNA than either hypoxia or hypercapnia alone (Somers *et al.*, 1989). Chemoreflex stress has also been shown to elevate both components of the sympathetic signal, that is, the amplitude and the firing frequency of integrated bursts (Steinback *et al.*, 2009;Malpas *et al.*, 1996).

Independent of chemoreflex stress, there also exists a respiratory modulation of sympathetic nerve activity. Muscle sympathetic nerve activity is increased during expiration and inhibited during inspiration (Eckberg *et al.*, 1985;Hagbarth & Vallbo, 1968), an effect mediated by pulmonary stretch receptors which are activated during inspiration (Seals *et al.*, 1993). As a result of the sympatho-inhibitory influence of the lung stretch receptors, the sympathetic responses to chemoreflex stress are amplified during the absence of active respiration, such as during an apnea maneuver (Steinback *et al.*, 2010a;Narkiewicz *et al.*, 1999) (Figure 1.6). Therefore, a combination of hypoxic-

hypercapnic and apneic stress evokes very large increases in MSNA (Steinback *et al.*,

2010a).

	BASELINE	**THIRD MINUTE OF HYPOXIA**	**APNEA**

ECG
HR — 65 beats/min — 70 beats/min

Neurogram

O_2 Sat — 99% — 81%
V_E — 7.9 L/min — 10.4 L/min
MAP — 87 mm Hg — 89 mm Hg
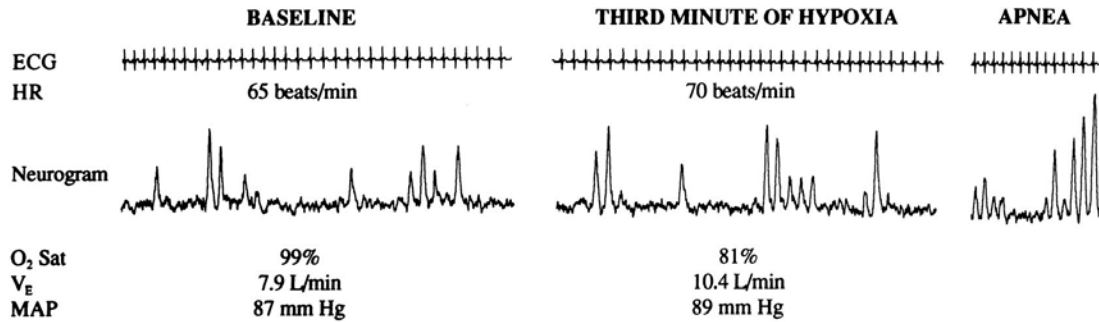
Figure 1.6: Sympathetic nerve activity during hypoxia with and without apnea.
Hypoxia was generated through breathing of a 10% oxygen gas mixture which was
titrated to maintain isocapnia. A 10-second end-expiratory apnea immediately followed
the 3-minute period of hypoxic breathing. The mean sympathetic response to apnea was a
50% increase over that observed during hypoxic breathing alone. ECG,
electrocardiogram; HR, heart rate; O_2 Sat, oxygen saturation; V_E, ventilation rate; MAP,
mean arterial pressure. Adapted with permission from Lippincott Williams and
Wilkins/Wolters Kluwer Health: Circulation (Narkiewicz *et al.*, 1999).

1.5   Hormone Fluctuations across the Menstrual Cycle

The primary endogenous sex hormones which have been hypothesized to affect sympathetic regulation are 17-β-estradiol (E2) and progesterone (Carter *et al.*, 2013). Circulating concentrations of these hormones change dramatically over the menstrual cycle and thus provide opportunities to study the effects of nadirs and peaks of E2 and progesterone. In a normal 28 day menstrual cycle, the first 14 days constitute the follicular phase which involves the ovarian maturation of a primary oocyte, while the latter 14 days are referred to as the luteal phase and are associated with the preparation of the uterus for the possible implantation of a fertilized oocyte (Conte & Grumbach, 2007). The progression of these events is regulated by circulating sex hormones.

The follicular phase begins with menstruation, which is triggered by falling blood plasma concentrations of progesterone and E2 (Figure 1.7). Thus, the early follicular phase is associated with a nadir in circulating E2 and progesterone. During the late follicular phase, the ovaries produce testosterone which is immediately and locally converted to E2 by the enzyme aromatase. The resultant E2 circulates through the body, and, at the level of the hypothalamus, initiates a positive feedback loop with the ovaries to dramatically increase further production of E2 (Conte & Grumbach, 2007). Thus, the late follicular phase is associated with high levels of circulating E2 while progesterone levels remain low. The follicular phase ends with ovulation, during which E2 levels are reduced following termination of the positive feedback loop, and the oocyte is released for potential fertilization.
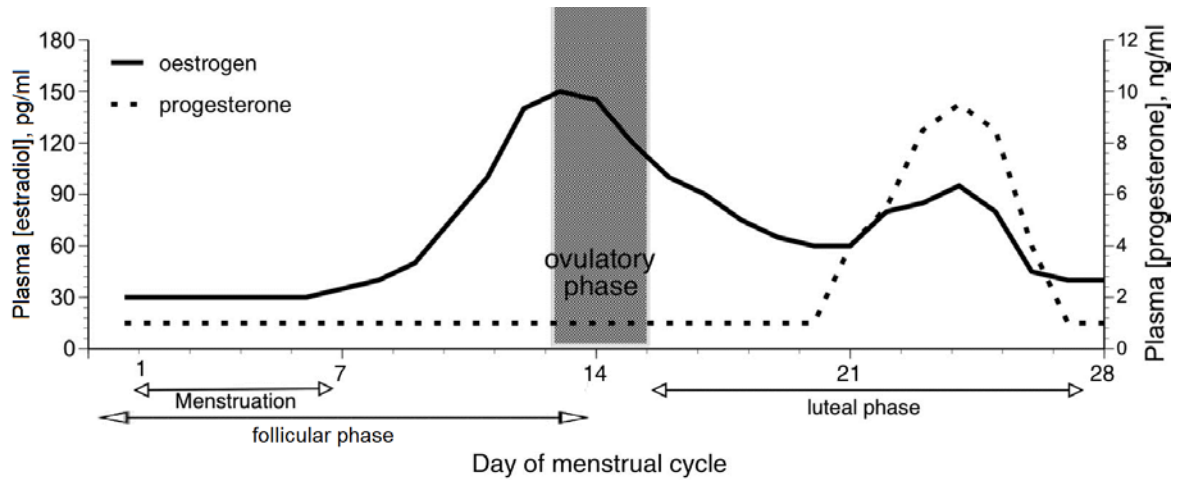
Figure 1.7: Plasma hormone concentrations across a regular menstrual cycle.

Adapted with permission from John Wiley and Sons: Journal of Physiology (Wenner & Stachenfeld, 2012).

The production of sex hormones during the luteal phase is accomplished by the corpus luteum, a temporary endocrine organ which is formed following ovulation (Conte & Grumbach, 2007). The corpus luteum synthesizes and secretes E2 and progesterone which act to prepare the uterine wall for implantation in the event of oocyte fertilization. However, E2 and progesterone also enter the circulation and as such the luteal phase is characterized by gradual increases in E2 and progesterone which plateau mid-way through the luteal phase. In the event that the oocyte is not fertilized, the corpus luteum degenerates, resulting in reductions in E2 and progesterone which in turn trigger menstruation and the beginning of a new menstrual cycle. The menstrual cycle therefore presents naturally occurring opportunities to study different hormonal milieus.

1.6 Hormonal Contraceptives

Many different types of hormonal contraceptives exist which act to inhibit ovulation and therefore prevent pregnancy. Binding to the progesterone receptor is the main requirement for this action, but progesterone itself is expensive and difficult to isolate for the purpose of hormone supplementation (Conte & Grumbach, 2007). Researchers have, therefore, spent the past half century developing synthetic progesterone analogs, called progestins. In the early development of hormonal contraceptive pills, the inclusion of an estrogen component was found to stabilize the endometrium and minimize side effects associated with contraceptive use (Burkman *et al.*, 2011;Conte & Grumbach, 2007). Although progestin-only contraceptives exist, their use is largely restricted to special circumstances, such as women who are breast-feeding or who have contraindications to estrogen (Conte & Grumbach, 2007). More common are combined hormonal contraceptives which contain both an estrogen (ethinyl estradiol) and a progestin component.

Early generations of contraceptives included high doses of ethinyl estradiol which exceeded 50 µg (Conte & Grumbach, 2007). However, the majority of contraceptives used today contain less than 35 µg of ethinyl estradiol (Burkman *et al.*, 2011). The progestin content of hormonal contraceptives has also evolved over time. This evolution has stemmed largely from the finding that most progestins interact not only with the progesterone receptor, but also with the androgen receptor (Sitruk-Ware & Nath, 2013). This "androgenicity" was thought to adversely affect lipid profiles and glucose tolerance in the early generation progestins, which were derived from testosterone (Sitruk-Ware, 2005;Conte & Grumbach, 2007). Newer progestins, which include desogestrel,

gestodene, and norgestimate, were improvements on the previous generation due to having low androgenic activity (Speroff & DeCherney, 1993). However, progesterone itself is known to have antiandrogenic activity which is not mimicked by these progestins (Sitruk-Ware, 2005). Drospirenone is a new progestin which binds to the progesterone receptor with the same affinity as progesterone and has antiandrogenic activity which may surpass endogenous progesterone (Sitruk-Ware, 2005).

The development of hormonal contraceptives continues today. While the prevention of ovulation and therefore pregnancy is well executed by current hormonal contraceptives, the simulation of the physiological effects of endogenous hormones has not yet been achieved with current synthetic hormones (Sitruk-Ware, 2005).

1.7 Associations between Hormone Levels and Sympathetic Nerve Activity

In a relatively new field of research, studies have begun to test women at various phases of the menstrual cycle to examine the effects that changes in hormonal milieus might exert over sympathetic regulation in young healthy women. The majority of these studies have focused on the early follicular (EF) and midluteal (ML) phases of the menstrual cycle to examine the combined effects of low (i.e. EF) or high levels of E2 and progesterone (i.e. ML). Fewer studies have compared low (LH) and high hormone (HH) phases of hormonal contraceptive use.

Baseline studies of MSNA in recumbent subjects have, for the most part, demonstrated elevations in MSNA burst frequency during the ML phase relative to the EF phase (Middlekauff *et al.*, 2012;Park & Middlekauff, 2009;Minson *et al.*, 2000a). Several studies have reported similar baseline MSNA in EF and ML phases (Fu *et al.*, 2009;Carter *et al.*, 2009b;Lawrence *et al.*, 2008), in line with the observations from hormonal contraceptive users, in whom no hormone phase-dependent differences in baseline MSNA have been observed (Minson *et al.*, 2000b;Middlekauff *et al.*, 2012;Carter *et al.*, 2009a). It is worth noting that, of the studies which also reported baseline vascular resistance, none have observed a difference in vascular resistance between phases, regardless of hormonal contraceptive use or whether a difference in baseline MSNA was observed (Minson *et al.*, 2000a;Fu *et al.*, 2009;Minson *et al.*, 2000b). Similarly, the majority of studies have observed similar baseline blood pressures between hormone phases in both users or non-users of hormonal contraceptives (Fu *et al.*,

2009;Carter *et al.*, 2009b;Minson *et al.*, 2000a;Middlekauff *et al.*, 2012;Carter *et al.*, 2009a).

In studies of baroreceptor unloading, both LBNP and head-up tilt (HUT) have been used to simulate orthostasis. These studies support a divergence between users and non-users of hormonal contraceptives. In non-users, greater total MSNA responses to baroreceptor unloading have been observed in the ML phase than the EF phase during both HUT and LBNP (Fu *et al.*, 2009;Carter *et al.*, 2009b). Although HUT activates the vestibulosympathetic reflex (Ray, 2000;Kaufmann *et al.*, 2002) and has the potential to exaggerate the observed sympathetic responses to baroreceptor unloading (Shortt & Ray, 1997), otolith-driven sympatho-excitation appears to be similar between EF and ML phases (Lawrence *et al.*, 2008). Therefore, this hormone phase effect is thought to result from a baroreceptor-driven mechanism alone. However, in the majority of studies, differences in sympathetic baroreflex sensitivity have not been observed between EF and ML menstrual cycle phases (Fu *et al.*, 2009;Carter *et al.*, 2009b;Middlekauff *et al.*, 2012). The lack of a difference in sympathetic baroreflex sensitivity, which expresses the extent to which MSNA increases for a given fall in DBP, implies that venous return and, therefore, cardiac output are reduced to a greater extent in the ML phase, thereby necessitating a larger sympatho-excitatory response (Fu *et al.*, 2009).

In the only study to date which has compared sympathetic responses to simulated orthostasis across hormone phases in hormonal contraceptive users, increases in total MSNA were similar between LH and HH phases (Carter *et al.*, 2009a). In comparisons of sympathetic baroreflex sensitivity, one study observed an elevation in sympathetic baroreflex sensitivity in the LH phase of contraceptive use compared to the HH phase

(Minson *et al.*, 2000b). However, this observation has not been repeated, with subsequent studies reporting similar baroreflex sensitivity between hormone phases in hormonal contraceptive users (Middlekauff *et al.*, 2012;Carter *et al.*, 2009a).

In the study by Carter and colleagues (Carter *et al.*, 2009b), the authors noted that, over the range of LBNP which was applied between -5 and -40 mmHg, the menstrual cycle-based differences in sympathetic activation were most visible during the higher levels of baroreceptor unloading. A similar trend was observed in the study by Fu and colleagues (Fu *et al.*, 2009), with the differences in total MSNA appearing greatest in the latter 20 minutes of the 45-minute HUT protocol. Therefore, it is possible that hormone phase may affect sympathetic responses to a greater extent during more severe reflex stress. Whether this effect is baroreceptor-dependent remains to be determined. These questions provided an impetus for this series of studies.

Notably, two studies which have reported an effect of hormone phase on sympathetic activation during baroreceptor unloading have observed these effects on total MSNA without a concomitant effect on MSNA burst frequency (Fu *et al.*, 2009;Carter *et al.*, 2009b). As the authors note, this strongly implies that elevations in E2 and progesterone are associated with specific sympatho-excitatory effects in the burst amplitude domain of MSNA. However, due to the limitations outlined in Section 1.2, MSNA burst amplitude is seldom reported. Therefore another purpose of these studies was to explore MSNA burst amplitude in greater detail, and led to the inclusion of chemoreflex stimulation in these studies as a means to evoke large increases in burst amplitude (Steinback *et al.*, 2009;Malpas *et al.*, 1996).

While the majority of research has focused on hormone phase effects on baroreceptor-drive regulation of sympathetic activity, other studies have compared high and low hormone phases during other sources of sympatho-excitation. These results have proved mostly equivocal; MSNA responses do not appear to be different between EF and ML menstrual cycle phases during handgrip exercise or post-exercise occlusion (Jarvis *et al.*, 2011), mental stress (Carter & Lawrence, 2007), or the cold pressor test (Middlekauff *et al.*, 2012;Jarvis *et al.*, 2011). The cold pressor test has also been repeated in users of hormonal contraceptives, with similar sympathetic responses observed between LH and HH phases as well (Middlekauff *et al.*, 2012). Taken together with MSNA responses to simulated orthostasis, these data suggest that if hormone phases affect neural regulation during sympatho-excitation, it is in a reflex-dependent manner. Interestingly, there have been no studies to date which have systematically compared the sympathetic responses to chemoreflex stimulation between hormone phases in users or non-users of hormonal contraceptives. The lack of research in this area was another impetus for these studies.

1.8 References

Burkman R, Bell C, & Serfaty D (2011). The evolution of combined oral contraception: improving the risk-to-benefit ratio. *Contraception* 84, 19-34.

Carlson JT, Hedner J, Elam M, Ejnell H, Sellgren J, & Wallin BG (1993). Augmented resting sympathetic activity in awake patients with obstructive sleep apnea. *Chest* 103, 1763-1768.

Carter JR, Fu Q, Minson CT, & Joyner MJ (2013). Ovarian cycle and sympathoexcitation in premenopausal women. *Hypertension* 61, 395-399.

Carter JR & Lawrence JE (2007). Effects of the menstrual cycle on sympathetic neural responses to mental stress in humans. *J Physiol* 585, 635-641.

Carter JR, Klein JC, & Schwartz CE (2009a). Effects of oral contraceptives on sympathetic nerve activity during orthostatic stress in young, healthy women. *Am J Physiol Regul Integr Comp Physiol* 298, R9-R14.

Carter JR, Lawrence JE, & Klein JC (2009b). Menstrual cycle alters sympathetic neural responses to orthostatic stress in young, eumenorrheic women. *Am J Physiol Endocrinol Metab* 297, E85-E91.

Conte FA & Grumbach MM (2007). Female Reproductive Endocrinology & Infertility. In *Greenspan's Basic & Clinical Endocrinology*, eds. Gardner DG & Shoback D, pp. 502-561. The McGraw-Hill Companies.

Delius W, Hagbarth KE, Hongell A, & Wallin BG (1972). Manoeuvres affecting sympathetic outflow in human muscle nerves. *Acta Physiol Scand* 84, 82-94.

DiBona GF & Jones SY (1998). Reflex effects on components of synchronized renal sympathetic nerve activity. *Am J Physiol* 275, F441-F446.

DiBona GF, Jones SY, & Sawin LL (1997). Reflex effects on renal nerve activity characteristics in spontaneously hypertensive rats. *Hypertension* 30, 1089-1096.

Eaker ED, Packard B, & Thom TJ (1989). Epidemiology and risk factors for coronary heart disease in women. *Cardiovasc Clin* 19, 129-145.

Eckberg DL, Nerhed C, & Wallin BG (1985). Respiratory modulation of muscle sympathetic and vagal cardiac outflow in man. *J Physiol* 365, 181-196.

Fu Q, Okazaki K, Shibata S, Shook RP, VanGunday TB, Galbreath MM, Reelick MF, & Levine BD (2009). Menstrual cycle effects on sympathetic neural responses to upright tilt. *The Journal of Physiology* 587, 2019-2031.

Hagbarth KE & Vallbo AB (1968). Pulse and respiratory grouping of sympathetic impulses in human muscle-nerves. *Acta Physiol Scand* 74, 96-108.

Hjemdahl P, Fagius J, Freyschuss U, Wallin BG, Daleskog M, Bohlin G, & Perski A (1989). Muscle sympathetic activity and norepinephrine release during mental challenge in humans. *Am J Physiol* 257, E654-E664.

Ichinose M, Saito M, Fujii N, Kondo N, & Nishiyasu T (2006). Modulation of the control of muscle sympathetic nerve activity during severe orthostatic stress. *J Physiol* 576, 947-958.

Ichinose M, Saito M, Ogawa T, Hayashi K, Kondo N, & Nishiyasu T (2004). Modulation of control of muscle sympathetic nerve activity during orthostatic stress in humans. *Am J Physiol Heart Circ Physiol* 287, H2147-H2153.

Jarvis SS, VanGundy TB, Galbreath MM, Shibata S, Okazaki K, Reelick MF, Levine BD, & Fu Q (2011). Sex differences in the modulation of vasomotor sympathetic outflow during static handgrip exercise in healthy young humans. *Am J Physiol Regul Integr Comp Physiol* 301, R193-R200.

Johnson JM, Rowell LB, Neiderberger MANF, & Eisman MM (1974). Human splanchnic and forearm vasoconstrictor responses to reductions of right atrial and aortic pressures. *Circ Res* 34, 515-524.

Joyner MJ, Charkoudian N, & Wallin BG (2010). Sympathetic nervous system and blood pressure in humans: individualized patterns of regulation and their implications. *Hypertension* 56, 10-16.

Kara T, Narkiewicz K, & Somers VK (2003). Chemoreflexes--physiology and clinical implications. *Acta Physiol Scand* 177, 377-384.

Kaufmann H, Biaggioni I, Voustianiouk A, Diedrich F, Costa F, Clarke R, Gizzi M, Raphan T, & Cohen B (2002). Vestibular control of sympathetic activity: An otolith-sympathetic reflex in humans. *Exp Brain Res* 143, 463-469.

Kienbaum P, Karlssonn T, Sverrisdottir YB, Elam M, & Wallin BG (2001). Two sites for modulation of human sympathetic activity by arterial baroreceptors? *J Physiol* 531, 861-869.

Kimmerly DS, O'Leary DD, & Shoemaker JK (2004). Test-retest repeatability of muscle sympathetic nerve activity: influence of data analysis and head-up tilt. *Auton Neurosci* 114, 61-71.

Lawrence JE, Ray CA, & Carter JR (2008). Vestibulosympathetic reflex during the early follicular and midluteal phases of the menstrual cycle. *Am J Physiol Endocrinol Metab* 294, E1046-E1050.

Leimbach WN, Jr., Wallin BG, Victor RG, Aylward PE, Sundlof G, & Mark AL (1986). Direct evidence from intraneural recordings for increased central sympathetic outflow in patients with heart failure. *Circulation* 73, 913-919.

Malpas SC (1995). A new model for the generation of sympathetic nerve activity. *Clin Exp Pharmacol Physiol* 22, 11-15.

Malpas SC, Bendle RD, Head GA, & Ricketts JH (1996). Frequency and amplitude of sympathetic discharges by baroreflexes during hypoxia in conscious rabbits. *Am J Physiol* 271, H2563-H2574.

Malpas SC & Ninomiya I (1992a). Effect of chemoreceptor stimulation on the periodicity of renal sympathetic nerve activity in anesthetized cats. *J Auton Nerv Syst* 37, 19-28.

Malpas SC & Ninomiya I (1992b). The amplitude and periodicity of synchronized renal sympathetic nerve discharges in anesthetized cats: differential effect of baroreceptor activity. *J Auton Nerv Syst* 40, 189-198.

Matsukawa T, Sugiyama Y, Watanabe T, Kobayashi F, & Mano T (1998). Gender difference in age-related changes in muscle sympathetic nerve activity in healthy subjects. *Am J Physiol Regul Integr Comp Physiol* 275, R1600-R1604.

Middlekauff HR, Park J, & Gornbein JA (2012). Lack of effect of ovarian cycle and oral contraceptives on baroreceptor and nonbaroreceptor control of sympathetic nerve activity in healthy women. *Am J Physiol Heart Circ Physiol* 302, H2560-H2566.

Minson CT, Halliwill JR, Young TM, & Joyner MJ (2000a). Influence of the Menstrual Cycle on Sympathetic Activity, Baroreflex Sensitivity, and Vascular Transduction in Young Women. *Circulation* 101, 862-868.

Minson CT, Halliwill JR, Young TM, & Joyner MJ (2000b). Sympathetic Activity and Baroreflex Sensitivity in Young Women Taking Oral Contraceptives. *Circulation* 102, 1473-1476.

Miyajima E, Yamada Y, Yoshida Y, Matsukawa T, Shionoiri H, Tochikubo O, & Ishii M (1991). Muscle sympathetic nerve activity in renovascular hypertension and primary aldosteronism. *Hypertension* 17, 1057-1062.

Morgan BJ, Crabtree DC, Palta M, & Skatrud JB (1995). Combined hypoxia and hypercapnia evokes long-lasting sympathetic activation in humans. *J Appl Physiol* (1985) 79, 205-213.

Mosher WD & Jones J (2010). Use of contraception in the United States: 1982-2008. *Vital Health Stat* 23 1-44.

Narkiewicz K, van de Borne PJ, Pesek CA, Dyken ME, Montano N, & Somers VK (1999). Selective potentiation of peripheral chemoreflex sensitivity in obstructive sleep apnea. *Circulation* 99, 1183-1189.

Narkiewicz K, Phillips BG, Kato M, Hering D, Bieniaszewski L, & Somers VK (2005). Gender-Selective Interaction Between Aging, Blood Pressure, and Sympathetic Nerve Activity. *Hypertension* 45, 522-525.

Ng AV, Callister R, Johnson DG, & Seals DR (1993). Age and gender influence muscle sympathetic nerve activity at rest in healthy humans. *Hypertension* 21, 498-503.

Ninomiya I, Malpas SC, Matsukawa K, Shindo T, & Akiyama T (1993). The amplitude of synchronized cardiac sympathetic nerve activity reflects the number of activated pre- and postganglionic fibers in anesthetized cats. *J Auton Nerv Syst* 45, 139-147.

Park J & Middlekauff HR (2009). Altered pattern of sympathetic activity with the ovarian cycle in female smokers. *Am J Physiol Heart Circ Physiol* 297, H564-H568.

Ray CA (2000). Interaction of the vestibular system and baroreflexes on sympathetic nerve activity in humans. *Am J Physiol Heart Circ Physiol* 279, H2399-H2404.

Rosano GM, Vitale C, Marazzi G, & Volterrani M (2007). Menopause and cardiovascular disease: the evidence. *Climacteric* 10 Suppl 1, 19-24.

Rosenthal T & Oparil S (2000). Hypertension in women. *J Hum Hypertens* 14, 691-704.

Rowell LB (1993). *Human Cardiovascular Control* Oxford University Press, Inc., New York, N.Y.

Ryan KL, Rickards CA, Hinojosa-Laborde C, Cooke WH, & Convertino VA (2011). Arterial pressure oscillations are not associated with muscle sympathetic nerve activity in individuals exposed to central hypovolaemia. *J Physiol* 589, 5311-5322.

Ryan KL, Rickards CA, Hinojosa-Laborde C, Cooke WH, & Convertino VA (2012). Sympathetic responses to central hypovolemia: new insights from microneurographic recordings. *Front Physiol* 3, 110.

Saito M, Mano T, Iwase S, Koga K, Abe H, & Yamazaki Y (1988). Responses in muscle sympathetic activity to acute hypoxia in humans. *J Appl Physiol* (1985) 65, 1548-1552.

Seals DR, Suwarno NO, Joyner MJ, Iber C, Copeland JG, & Dempsey JA (1993). Respiratory modulation of muscle sympathetic nerve activity in intact and lung denervated humans. *Circ Res* 72, 440-454.

Shortt TL & Ray CA (1997). Sympathetic and vascular responses to head-down neck flexion in humans. *Am J Physiol* 272, H1780-H1784.

Sitruk-Ware R (2005). Pharmacology of different progestogens: the special case of drospirenone. *Climacteric* 8 Suppl 3, 4-12.

Sitruk-Ware R & Nath A (2013). Characteristics and metabolic effects of estrogen and progestins contained in oral contraceptive pills. *Best Pract Res Clin Endocrinol Metab* 27, 13-24.

Smith ML & Muenter NK (2000). Effects of hypoxia on sympathetic neural control in humans. *Respir Physiol* 121, 163-171.

Somers VK, Mark AL, Zavala DC, & Abboud FM (1989). Contrasting effects of hypoxia and hypercapnia on ventilation and sympathetic activity in humans. *J Appl Physiol* (1985) 67, 2101-2106.

Speroff L & DeCherney A (1993). Evaluation of a new generation of oral contraceptives. The Advisory Board for the New Progestins. *Obstet Gynecol* 81, 1034-1047.

Steinback CD, Breskovic T, Frances M, Dujic Z, & Shoemaker JK (2010a). Ventilatory restraint of sympathetic activity during chemoreflex stress. *Am J Physiol Regul Integr Comp Physiol* 299, R1407-R1414.

Steinback CD, Salmanpour A, Breskovic T, Dujic Z, & Shoemaker JK (2010b). Sympathetic neural activation: an ordered affair. *J Physiol* 588, 4825-4836.

Steinback CD, Salzer D, Medeiros PJ, Kowalchuk J, & Shoemaker JK (2009). Hypercapnic vs. hypoxic control of cardiovascular, cardiovagal, and sympathetic function. *Am J Physiol Regul Integr Comp Physiol* 296, R402-R410.

Sundlof AG & Wallin BG (1977). The variability of muscle nerve sympathetic activity in resting recumbent man. *The Journal of Physiology* 272, 383-397.

Sundlof G & Wallin BG (1978). Effect of lower body negative pressure on human muscle nerve sympathetic activity. *J Physiol* 278, 525-532.

Sverrisdottir YB, Rundqvist B, Johannsson G, & Elam M (2000). Sympathetic neural burst amplitude distribution: A more specific indicator of sympathoexcitation in human heart failure. *Circulation* 102, 2076-2081.

Sverrisdottir YB, Mogren T, Kataoka J, Janson PO, & Stener-Victorin E (2008). Is polycystic ovary syndrome associated with high sympathetic nerve activity and size at birth? *Am J Physiol Endocrinol Metab* 294, E576-E581.

Tompkins RP, Melling CW, Wilson TD, Bates BD, & Shoemaker JK (2013). Arrangement of sympathetic fibers within the human common peroneal nerve: implications for microneurography. *J Appl Physiol* (1985) 115, 1553-1561.

Victor RG & Leimbach WN, Jr. (1987). Effects of lower body negative pressure on sympathetic discharge to leg muscles in humans. *J Appl Physiol* 63, 2558-2562.

Wallin BG (2006). Regulation of sympathetic nerve traffic to skeletal muscle in resting humans. *Clin Auton Res* 16, 262-269.

Wallin BG & Charkoudian N (2007). Sympathetic neural control of integrated cardiovascular function: insights from measurement of human sympathetic nerve activity. *Muscle Nerve* 36, 595-614.

Wenner MM & Stachenfeld NS (2012). Blood pressure and water regulation: understanding sex hormone effects within and between men and women. *J Physiol* 590, 5949-5961.

Yamada Y, Miyajima E, Tochikubo O, Matsukawa T, & Ishii M (1989). Age-related changes in muscle sympathetic nerve activity in essential hypertension. *Hypertension* 13, 870-877.

Young T, Palta M, Dempsey J, Skatrud J, Weber S, & Badr S (1993). The occurrence of sleep-disordered breathing among middle-aged adults. *N Engl J Med* 328, 1230-1235.

Chapter 2

2   Hormone phases influence neurovascular responses to high levels of lower body negative pressure

2.1   Introduction

There exists an increased incidence of orthostatic intolerance in young women relative to young men (Montgomery *et al.*, 1977;Christou *et al.*, 2005;Hordinsky *et al.*, 1981). Orthostatic stress tolerance is contingent upon adequate neurovascular responses to compensate for the gravity-induced peripheral pooling of blood. As such, several studies have made use of the microneurography technique to determine whether a reduced muscle sympathetic nerve activity (MSNA) response might contribute to the increase in orthostatic intolerance in women. While some studies have reported that MSNA responses to simulated orthostasis are blunted in women relative to men (Kimmerly *et al.*, 2007;Yang *et al.*, 2012;Shoemaker *et al.*, 2001), some studies have determined that MSNA responses are equivocal between the sexes (Fu *et al.*, 2005;Fu *et al.*, 2009). It is possible that differences in the concentrations of circulating sex hormones in the female subjects may have contributed to this discrepancy in the results, but the impact of changes in sex hormones on the regulation of MSNA have only recently begun to be elucidated.

Recent evidence suggests that concentrations of circulating sex hormones exert an influence over the regulation of baseline MSNA (Carter *et al.*, 2013;Day *et al.*, 2011). Cyclical changes in sex hormones occur across the regular menstrual cycle, and several studies have provided support for a relative sympatho-excitation during the high hormone midluteal phase of the menstrual cycle (ML) relative to the low hormone early follicular

phase (EF) (Minson *et al.*, 2000a;Park & Middlekauff, 2009;Middlekauff *et al.*, 2012;Carter *et al.*, 2013). Several studies have also compared sympathetic responses to simulated orthostasis. These stimuli cause peripheral pooling of blood which reduces venous return and cardiac output and threaten the maintenance of blood pressure. Muscle sympathetic neural responses to orthostasis elicit increases in peripheral resistance, thereby maintaining blood pressure at baseline levels across mild to moderate levels of orthostasis. Sympathetic responses to the simulated orthostasis techniques of head up tilt (Fu *et al.*, 2009) and lower body negative pressure (LBNP) (Carter *et al.*, 2009b) are elevated in the ML phase relative to the EF phase. Notably, the differences in the MSNA responses between EF and ML are greatest during the most severe stages of baroreceptor unloading (Fu *et al.*, 2009;Carter *et al.*, 2009b). These studies have also reported a lack of change in sympathetic baroreflex sensitivity across the menstrual cycle (Fu *et al.*, 2009;Carter *et al.*, 2009b). This observation is in line with a greater vascular stress which arises from the same orthostatic stimulus, such as a greater fall in stroke volume. However, menstrual cycle differences in the orthostasis-induced reduction in stroke volume have not yet been observed (Fu *et al.*, 2009).

In comparison with studies of the menstrual cycle in eumenorrheic women, less is known regarding sympathetic regulation in women taking hormonal contraceptives (HC), despite the fact that at least 20% of women of child-bearing age currently use hormonal contraceptives (HC) in the United States (Mosher & Jones, 2010). Moreover, HC use has been associated with small yet significant increases in blood pressures relative to control subjects (Hickson *et al.*, 2011;Atthobari *et al.*, 2007), suggesting that blood pressure regulation mechanisms such as the baroreflex may be affected by HC use. Indeed, recent

studies have indicated that phases of HC use may not mirror the menstrual cycle in their effects on sympathetic regulation. For instance, differences in baseline MSNA have not been observed between low- (LH) and high-hormone (HH) phases of HC use (Minson *et al.*, 2000b;Carter *et al.*, 2009a;Middlekauff *et al.*, 2012). A study examining sympathetic responses to simulated orthostasis observed that moderate levels of LBNP (0 to -40 mmHg) were associated with similar MSNA responses between LH and HH phases, during which blood pressure responses were also similar between phases (Carter *et al.*, 2009a). However, to the best of our knowledge there have been no studies which have directly compared the effects of hormone phases on sympathetic responses to simulated orthostasis between users and non-users of HC.

Therefore, the purpose of this study was to compare MSNA responses to moderate to high levels of LBNP between low- and high-hormone phases of both the regular menstrual cycle and HC use. We tested the hypothesis that hormone phase (regardless of group) would affect sympathetic and blood pressure responses such that in both groups of women the higher hormone phases (HH and ML) would be associated with greater sympathetic responses than the lower hormone phases (LH and EF).

2.2 Methods
2.2.1   Subjects

Seventeen undergraduate and graduate students enrolled in the School of Kinesiology at The University of Western Ontario in London, Ontario participated in the study. These were recruited in two groups: users and non-users of HC. Subject characteristics are presented in Table 2.1. The baseline characteristics for the HC group,

and their MSNA responses to chemoreflex stress, have been reported previously (Usselman *et al.*, 2013). All subjects were regularly active non-smokers who were free of cardiovascular and respiratory disease, and were not taking any medications, with the exception of HC. Within users of HC, with the exception of 1 subject who was using a patch with norelgestromin and 1 subject using a triphasic pill containing norgestimate, all subjects were using monophasic combination pills with either drospirenone (2 subjects), desogestrel (1 subject), or levonorgestrel (3 subjects). All HCs contained 20-30 µg of ethinyl estradiol. Participants provided signed consent to the study protocols which were approved by the Health Sciences Research Ethics Board at The University of Western Ontario, Canada, and conformed to the standards set by the *Declaration of Helsinki.*

Women in the HC group were tested between days 1 and 4 (day 1 being the first day of menstruation) to represent the LH phase which is associated with no contraceptive use or placebo use. Women taking HC were also tested between days 20 and 24 to represent the HH phase, associated with active hormonal contraceptive treatment. Due to the inhibitory effect of exogenous hormones on endogenous hormone production, there was no significant change in circulating levels of endogenous 17-β-estradiol or progesterone from LH (117 ± 89 pmol/L and 1.0 ± 0.7 nmol/L, respectively) to HH (51 ± 18 pmol/L and 1.1 ± 0.7 nmol/L; $P = 0.1$ and 0.6, respectively). Non-users of HC were tested between days 1 and 4 of the menstrual cycle during the EF phase, and days 20-24 during the ML phase. Significant increases in 17-β-estradiol and progesterone from EF (151 ± 50 pmol/L and 1.2 ± 0.5 nmol/L, respectively) to ML (638 ± 175 pmol/L and 35.8 ± 9.3 nmol/L) confirmed the target menstrual cycle phases.

2.2.2   Experimental Design

Each subject visited the laboratory on 3 occasions. The first was a familiarization visit during which the subjects practiced the LBNP protocol and experienced all non-invasive aspects of data acquisition. Hormone phase testing occurred following familiarization, and the order of hormone phase testing was counterbalanced among the subjects. Time of day was kept constant within each subject. Subjects arrived for test days a minimum of 3 hours postprandial and having abstained from exercise, caffeine, and alcohol for a minimum of 12 hours. Subjects were positioned supine with their legs and hips sealed in a LBNP chamber. The LBNP chamber was connected to a vacuum fed through a variable transformer (Staco Energy Products Co., Dayton, Ohio, USA) which allowed precise control of suction inside the chamber. After subjects had been instrumented for data acquisition including microneurography, 5 minutes of stable baseline were recorded. Subjects were then exposed to LBNP at -30, -60, and -80 mmHg. The order of LBNP testing was quasi-random: -60 mmHg always preceded -80 mmHg to ensure that all subjects could tolerate -60 before advancing to -80 mmHg. Therefore, only the order of -30 mmHg *versus* the severe levels of suction was randomized. An additional five minutes of baseline were recorded prior each LBNP level to account for any possible drift in the sympathetic or hemodynamic signals over the course of the test session. All LBNP levels were maintained for 3 minutes, and were tolerated in all subjects except one woman who reported mild nausea following LBNP -60 mmHg; LBNP -80 mmHg was not conducted in this subject.

2.2.3   Measures

Heart rate (HR) was measured through a 3-lead electrocardiogram. Blood pressure waveforms were obtained through finger photoplethysmography (Finometer; Finapres Medical Systems, Amsterdam, The Netherlands) and calibrated to a resting blood pressure which was the average of three values obtained through manual sphygmomanometry. Cardiac output (Q) was calculated online using the Modelflow algorithm (Finometer). Muscle sympathetic nerve activity was assessed through microneurography at the peroneal nerve (Vallbo *et al.*, 1979). Briefly, a tungsten recording electrode with an uninsulated tip was inserted transcutaneously and an additional electrode was inserted subcutaneously as reference. The recording electrode was maneuvered into the nerve until a recording site was obtained. Adequate recording sites were associated with pulse synchronous bursts of activity which increased in frequency during end-expiratory apnea and were unaffected by arousal to a loud noise (Delius *et al.*, 1972). The MSNA signal was amplified 1000x by a preamplifier and 75x by a variable-gain, isolated amplifier, then band-pass filtered from 700 to 2000 Hz. The signal was then rectified and integrated (0.1 s time constant; model 662C-3; Iowa University Bioengineering, Iowa, USA). The MSNA signal was sampled at 10 000 Hz by an online data acquisition and analysis package (Powerlab /16SP with LabChart 7, ADInstruments, Colorado Springs, Colorado, USA). Hemodynamic measures were sampled at 1000 Hz.

2.2.4   Data Analysis

Mean arterial pressure (MAP), systolic (SBP), diastolic (DBP), and pulse pressures (PP) were calculated from the calibrated brachial blood pressure waveform.

Stroke volume (SV) was calculated as Q divided by HR. Total peripheral resistance (TPR) was calculated as MAP divided by Q. Total MSNA was calculated as burst frequency (bursts/min) multiplied by mean normalized burst amplitude; burst amplitudes were normalized within each LBNP condition by expressing all bursts relative to the largest burst in the preceding baseline period, which was assigned a value of 100.

All data were analysed statistically using mixed repeated measures analyses of variance (ANOVA; Statistical Analysis System V.9.1.3, SAS Institute Inc., Cary, NC, USA). At baseline, a 2-way mixed ANOVA assessed the main effects of group (HC *vs* no HC) and hormone phase (EF/LH *vs* ML/HH) on the sympathetic and hemodynamic outcome measures, as well as possible group x phase interactions. Responses to LBNP were assessed using a 3-way mixed ANOVA, which assessed the main effects of group, phase, and experimental condition (BSL, -30, -60, and -80 mmHg LBNP). These analyses on responses to baroreceptor unloading were performed in a slightly smaller group than baseline comparisons: 7 women taking HC and 7 women not taking HC. The Tukey-Kramer correction was applied in all *post hoc* comparisons. Alpha was set at 0.05.

2.3 Results

Baseline hemodynamics and MSNA characteristics are summarized in Table 2.2. No differences were observed in MSNA or hemodynamics between users and non-users of HC. A main effect of hormone phase was observed for HR which was significantly greater in the high versus low hormone phases. Likewise, MSNA burst frequency and total MSNA were elevated in the high hormone phases relative to the low hormone phases.

Baroreceptor unloading by LBNP was associated with increases in MSNA burst amplitude, burst frequency, and total MSNA (Figure 2.1). A main effect of hormone phase indicated that higher hormone phases were associated with higher levels of total MSNA and burst frequency than the lower hormone phases while there was no significant effect of hormone phase on burst amplitude. These patterns of sympathetic activation were not different between users and non-users of HC.

The higher hormone phases were associated with higher HR and lower SV relative to the lower hormone phases (Figure 2.2). Conversely, the reductions in Q and MAP and increases in TPR were similar between low and high hormone phases. However, significant group x LBNP interactions were observed for MAP and TPR. These indicated that MAP was reduced during LBNP only in women taking HC while TPR was increased only in non-users of HC.

In order to determine whether the exaggerated MSNA responses observed in the high hormone phases were accounted for by the greater reductions in SV, we performed regression analyses to determine the slope of the relationship between the change in SV and the change in MSNA across the 3 levels of LBNP (Figure 2.3). Significant, negative relationships were observed between Δ-SV and Δ-total MSNA during both low hormone ($R = -0.7$) and high hormone phases ($R = -0.6$), the slopes of which were not different between hormone phases.

Table 2.1: Subject characteristics

	HC Users	Non-Users of HC
n	8	9
Age (y)	24 ± 3	24 ± 3
Height (cm)	167 ± 3	166 ± 6
Weight (kg)	60 ± 4	64 ± 9
BMI (kg • m^2)	22 ± 2	23 ± 3

Values are mean ± standard deviation. BMI, body mass index; HC, hormonal contraceptives.

Table 2.2: Baseline hemodynamics and muscle sympathetic nerve activity

	HC Users		Non-Users of HC		Hormone Phase P Value
	LH	HH	EF	ML	
MAP (mmHg)	92 ± 4	96 ± 9	86 ± 6	84 ± 6	0.5
SBP (mmHg)	119 ± 10	123 ± 14	114 ± 13	112 ± 10	0.7
DBP (mmHg)	68 ± 4	70 ± 8	67 ± 9	67 ± 4	0.7
PP (mmHg)	51 ± 7	53 ± 10	48 ± 18	44 ± 10	0.7
HR (beats · min^{-1})	64 ± 8	68 ± 13	61 ± 7	64 ± 7	0.03
Q (L · min^{-1})	5.1 ± 0.7	5.6 ± 1.0	4.9 ± 1.0	5.1 ± 1.0	0.2
SV (mL)	81 ± 13	83 ± 10	80 ± 10	80 ± 14	0.7
TPR (mmHg · L^{-1} · min^{-1})	18 ± 2	18 ± 3	18 ± 3	17 ± 5	0.7
MSNA burst frequency (bursts · min^{-1})	11 ± 7	16 ± 8	10 ± 5	14 ± 6	<0.01
Total MSNA (a.u.)	493 ± 303	740 ± 398	466 ± 203	740 ± 306	<0.01

Values in central columns are mean ± standard deviation in women taking hormonal contraceptives (n=8) during low- (LH) and high-hormone (HH) phases and in women not taking exogenous hormones during early follicular (EF) and midluteal (ML) phases of the menstrual cycle. Rightmost columns are split-plot ANOVA P values for the main effect of hormone phases (EF/LH *versus* ML/HH). No significant main effects of group (HC users *versus* non-users of HC) or phase x group interactions were observed. HC, hormonal contraceptives; MAP, mean arterial pressure; SBP, systolic blood pressure; DBP, diastolic blood pressure; PP, pulse pressure; HR, heart rate; Q, cardiac output; SV, stroke volume; TPR, total peripheral resistance; MSNA, muscle sympathetic nerve activity.
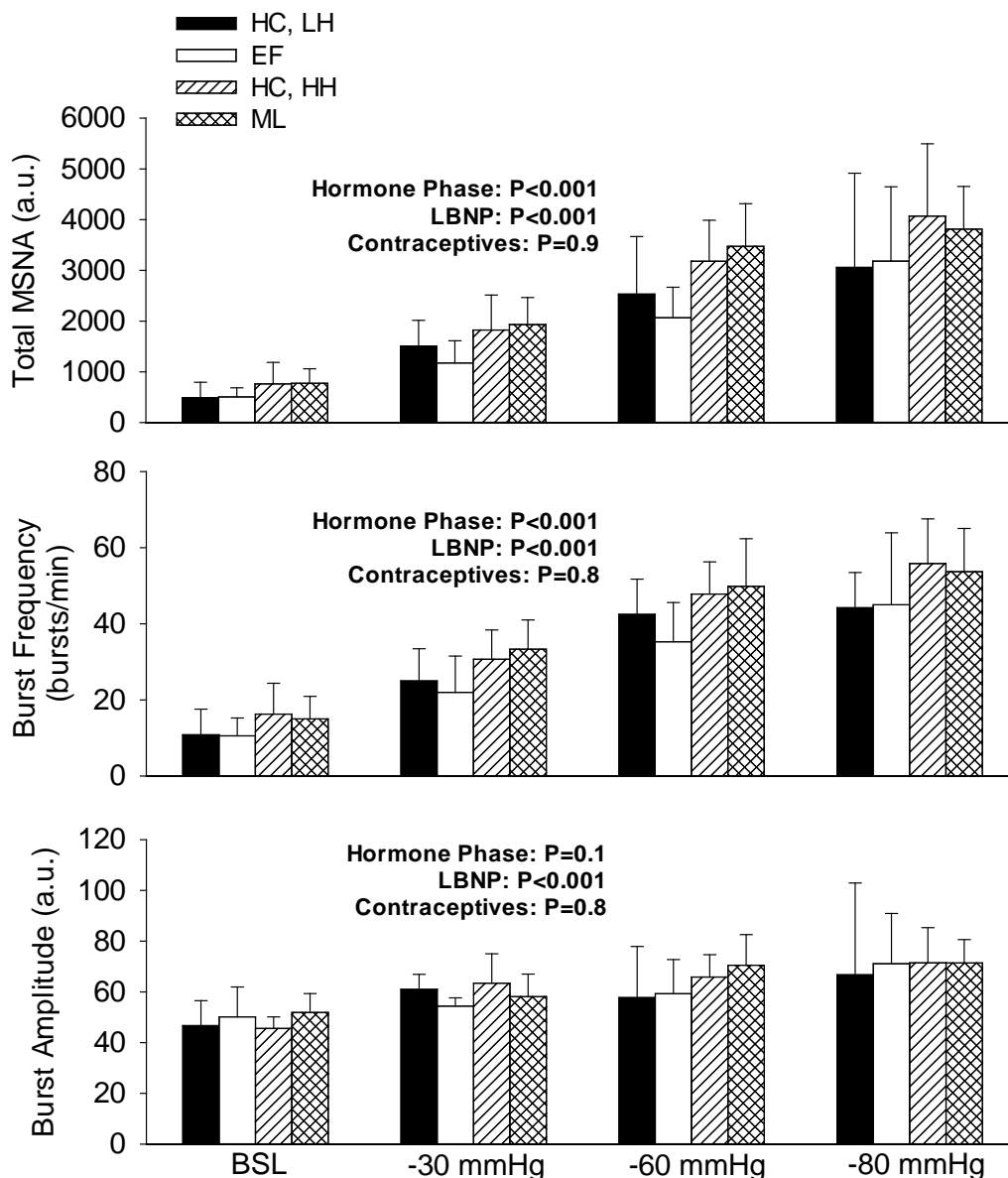
Figure 2.1: Sympathetic nerve activity during lower body negative pressure across hormone phases.

Total muscle sympathetic nerve activity (MSNA) was elevated in the menstrual cycle phases associated with elevated hormones (ML and HH) relative to the low hormone phases (EF and LH) across all levels of baroreceptor unloading, regardless of exogenous hormone use. HC, hormonal contraceptives; EF, early follicular; LH, low hormone; ML, midluteal; HH, high hormone; BSL, baseline. Values are means ± standard deviations.
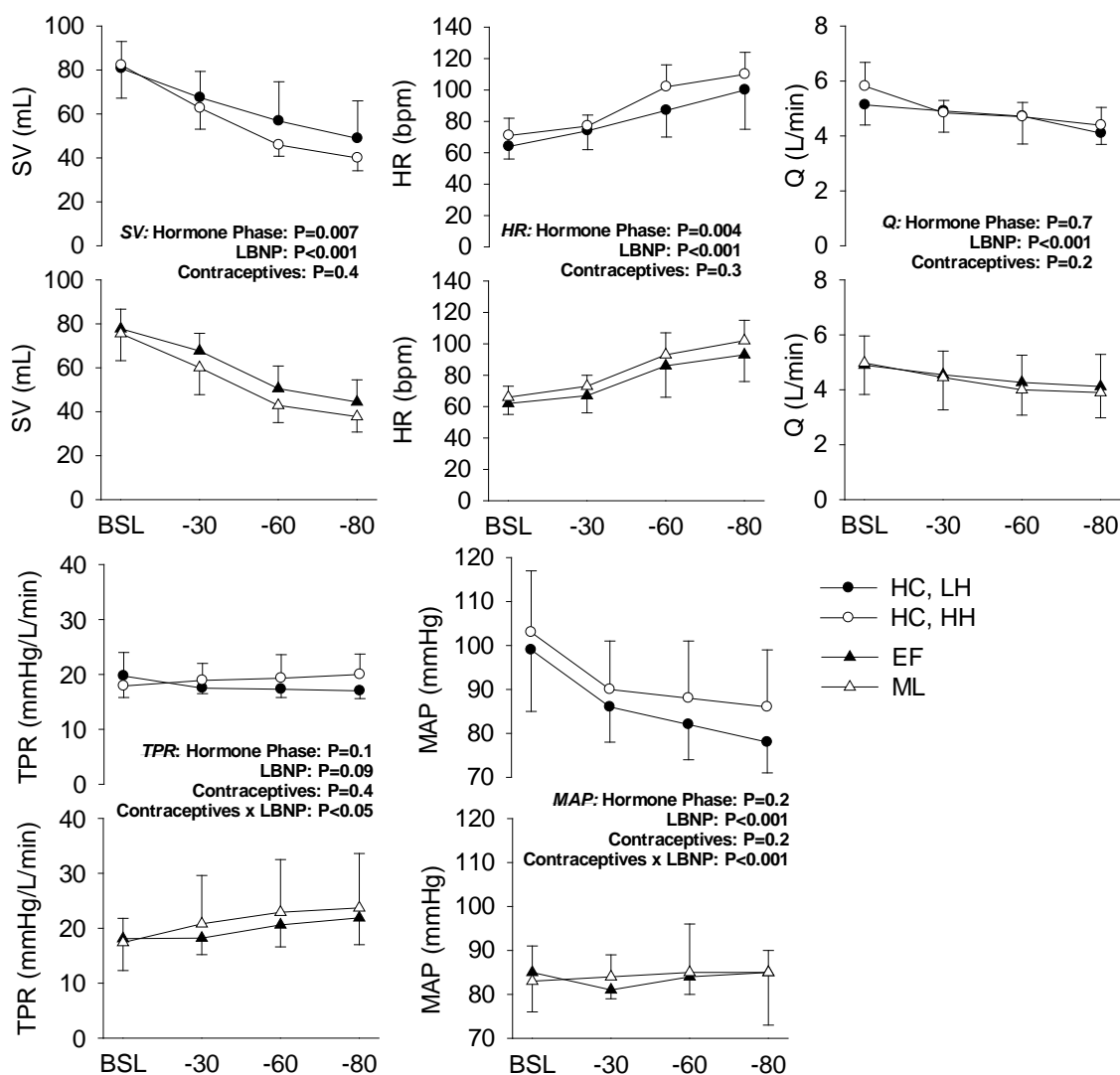
Figure 2.2: Hemodynamic responses to lower body negative pressure across hormone phases

Stroke volume (SV) was lower during the high hormone phases (HH and ML) relative to the low hormone phases (LH and EF), while heart rate (HR) was higher during the high hormone phases, thus cardiac output (Q) was similar between phases. All of SV, HR, and Q were similar between women taking and not taking hormonal contraceptives. LBNP was associated with an increase in total peripheral resistance (TPR) only in women not taking contraceptives. Mean arterial pressure (MAP) was reduced during LBNP only in women taking contraceptives. Values are means ± standard deviations.
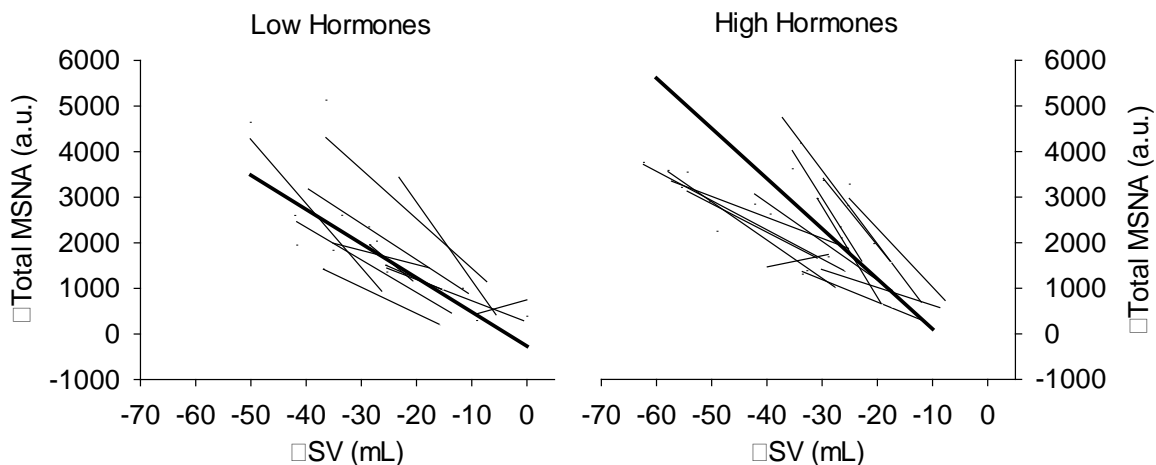
Figure 2.3: Associations between stroke volume and sympathetic nerve activity during lower body negative pressure

The change in stroke volume (SV) during lower body negative pressure (LBNP) was negatively associated with the change in total muscle sympathetic nerve activity (MSNA) in both low hormone and high hormone conditions. The slopes of the SV-MSNA relationships were similar between hormone phases (-75 ± 55 a.u./mL *vs* -110 ± 58 a.u./mL, low hormones *vs* high hormones; P=0.2). Thin lines are individual subject data across LBNP -30, -60, and -80; thick lines represent the mean relationships between Δ-SV and Δ-MSNA.

2.4 Discussion

This study compared sympathetic responses to moderate to severe levels of LBNP during low and high hormone phases of the endogenous menstrual cycle and exogenous hormonal contraceptive use. We observed several main findings. First, baseline MSNA levels were similar between users and non-users of HC. In both groups of women, MSNA burst frequency and total MSNA were reduced during the lower hormone phases (LH and EF) compared to the higher hormone phases (HH and ML). Second, the sympathetic responses to LBNP were greater in the higher hormone phases than the lower hormone phases of both groups. In users and non-users of HC, the exaggerated sympatho-excitation associated with the higher hormone phase occurred in response to greater falls in SV, suggesting a relative increase in the severity of baroreceptor unloading in the higher hormone phases compared with the lower hormone phases. Finally, an LBNP-driven increase in TPR was observed only in women not taking HC. No increase in TPR was observed in women taking HC, and as such, HC users experienced a significant fall in MAP, which was not observed in non-users of HC. Together, these data indicate that higher hormone phases of the menstrual cycle and HC use are associated with greater baroreceptor-mediated increases in sympathetic nerve activity than the lower hormone phases. However, the resultant end-organ responses associated with sympathetic nerve activation may be affected by HC use.

An increase in baseline MSNA from the low hormone to the high hormone phase has been observed several times in non-users of HC, with heightened MSNA observed in the ML phase of the menstrual cycle relative to the EF phase (Minson *et al.*, 2000a;Park & Middlekauff, 2009;Middlekauff *et al.*, 2012;Carter *et al.*, 2013). However, a similar

pattern has not yet been observed across low and high hormone phases of HC use (Minson *et al.*, 2000b;Carter *et al.*, 2009a;Middlekauff *et al.*, 2012). It is possible that differences between endogenous hormones and the exogenous hormones in contraceptives may contribute to this discrepancy. Significant correlations have been observed between the magnitude of the increases in endogenous 17-β-estradiol (E2) and progesterone from EF to ML and the increase in MSNA burst frequency across the menstrual cycle (Carter *et al.*, 2013). Specifically, the largest ML phase-driven increases in MSNA coincided with limited increases in E2 and larger increases in progesterone, in support of a sympatho-inhibitory influence of E2 and a sympatho-excitatory influence of progesterone (Carter *et al.*, 2013). Along similar lines, some progestins commonly used in HC have androgenic properties, and an androgenic hormonal milieu has been associated with elevations in baseline MSNA in a clinical population of women (Sverrisdottir *et al.*, 2008). Therefore, while the increased progestin concentrations associated with the HH phase of HC use could potentially elevate MSNA, all of the progestins contained in the contraceptives used in the present study have low androgenic activity (Speroff & DeCherney, 1993) and/or antiandrogenic activity (Fuhrmann *et al.*, 1996). Thus, it remains unclear why an increase in MSNA was observed in the women taking HC in the present study, in contrast to the previous studies (Minson *et al.*, 2000b;Carter *et al.*, 2009a;Middlekauff *et al.*, 2012).

Previous studies examining the sympathetic responses to baroreceptor unloading across the menstrual cycle in women in non-users of HC have used mild to moderate LBNP (-5 to -40 mmHg) (Carter *et al.*, 2009b) and prolonged 60° head-up tilt (Fu *et al.*, 2009). Both studies observed greater increases in total MSNA in the ML phase than the

EF phase. On the other hand, the same effect has not been observed in women taking HC: graded LBNP up to -40 mmHg resulted in similar MSNA responses between LH and HH phases of HC use (Carter *et al.*, 2009a). However, in each of these previous studies, hemodynamic responses to baroreceptor unloading were similar between hormone phases (Carter *et al.*, 2009a;Carter *et al.*, 2009b;Fu *et al.*, 2009), in contrast with the present study.

In the present study, acute, severe LBNP was used to evoke large sympatho-excitatory responses driven by baroreceptor unloading. Unlike previous studies, the SV stimulus and MSNA response were greater in high hormone phases (ML and HH) than low hormone phases (EF and LH). Total MSNA increases were linearly related to the decrements in SV in both low and high hormone conditions. Moreover, the slope of this relationship was similar between hormone phases, indicating similar baroreceptor-driven MSNA sensitivity across hormone phases. In other words, these results indicate that the greater sympathetic response in high hormone phases was due to greater baroreceptor unloading rather than a centrally driven amplification of the efferent sympathetic response. These observations confirm those of Middlekauff and colleagues (Middlekauff *et al.*, 2012) who showed that sympathetic baroreflex sensitivity was similar between low and high hormone phases of both the menstrual cycle and HC use as assessed by the modified Oxford test. On the other hand, Minson and colleagues have also used the modified Oxford model of baroreflex assessment in comparisons of hormone phases in users and non-users of HC, and observed greater sympathetic baroreflex sensitivity in the ML phase of the endogenous menstrual cycle relative to the EF phase (Minson *et al.*, 2000a) while users of HC experience increased sympathetic baroreflex sensitivity in the

LH phase relative to the HH phase (Minson *et al.*, 2000b). Thus, although evidence exists to indicate that sympathetic responses to a given stimulus of baroreceptor unloading are similar between women taking and not taking HC, further work is required in this area.

In the present study, the only statistical difference between the users and non-users of HC was observed in the hemodynamic outcomes during LBNP. In women not taking exogenous hormones, LBNP was associated with a small but significant increase in TPR, an effect which was not observed in the users of HC. The reduction in cardiac output during LBNP was similar between both groups of women, therefore MAP was maintained in non-users of HC but reduced in users of HC. These data are suggestive of a reduced neurovascular response to an acute orthostatic challenge in women taking HC. Previously, Carter and colleagues repeated a graded LBNP protocol from -5 to -40 mmHg in women across the endogenous menstrual cycle and in women taking HC in both the low and high hormone phases (Carter *et al.*, 2009b;Carter *et al.*, 2009a). In these studies, the authors observed no significant changes in MAP with LBNP in either group of women. However, their levels of LBNP were less severe than those used in the current study. The combined data illustrate the potential deleterious impact of HC on neurovascular and blood pressure control, but an effect that is observed only at high levels of stress.

Limitations

The type of hormonal contraceptive was not controlled in this study, and although all subjects were using combination formulations, the type of progestin varied among the subjects. Given the varying effects that different progestins exert over endothelial

function (Meendering *et al.*, 2010;Meendering *et al.*, 2009;Torgrimson *et al.*, 2007), it is unclear to what extent the hemodynamic measures in this study were affected by the range of HC types. Also, this experimental design compared phases in which progesterone or progestins were elevated at the same time as E2 or ethinyl estradiol. These hormones are thought to exert opposing influences over sympathetic regulation (Carter *et al.*, 2013), and in the present design we could not tease apart these separate and perhaps competing influences.

Perspectives

In this study we did not observe a difference in sympathetic regulation between users and non-users of hormonal contraceptives. In both groups, similar lower body negative pressure stimuli were associated with greater reductions in SV in the high hormone phases, implying more severe venous pooling occurred for a given level of negative pressure relative to the low hormone phases. As a result, the sympathetic responses to lower body negative pressure were greater during the high hormone phases than the low hormone phases. However, the subsequent increase in total peripheral vascular resistance was dependent on whether or not a woman was taking hormonal contraceptives. Women taking hormonal contraceptives lacked the reflex increase in vascular resistance during baroreceptor unloading, and as a result mean arterial pressure was not maintained during LBNP as it was in women not taking exogenous hormones. These results suggest that both hormone phase and use of HC influence the regulation of blood pressure during acute orthostasis in young healthy women.

2.5 References

Atthobari J, Gansevoort RT, Visser ST, de Jong PE, & de Jong-van den Berg LT (2007). The impact of hormonal contraceptives on blood pressure, urinary albumin excretion and glomerular filtration rate. *Br J Clin Pharmacol* 63, 224-231.

Carter JR, Fu Q, Minson CT, & Joyner MJ (2013). Ovarian cycle and sympathoexcitation in premenopausal women. *Hypertension* 61, 395-399.

Carter JR, Klein JC, & Schwartz CE (2009a). Effects of oral contraceptives on sympathetic nerve activity during orthostatic stress in young, healthy women. *Am J Physiol Regul Integr Comp Physiol* 298, R9-R14.

Carter JR, Lawrence JE, & Klein JC (2009b). Menstrual cycle alters sympathetic neural responses to orthostatic stress in young, eumenorrheic women. *Am J Physiol Endocrinol Metab* 297, E85-E91.

Christou DD, Jones PP, Jordan J, Diedrich A, Robertson D, & Seals DR (2005). Women have lower tonic autonomic support of arterial blood pressure and less effective baroreflex buffering than men. *Circulation* 111, 494-498.

Day DS, Gozansky WS, Bell C, & Kohrt WM (2011). Acute sex hormone suppression reduces skeletal muscle sympathetic nerve activity. *Clin Auton Res* 21, 339-345.

Delius W, Hagbarth KE, Hongell A, & Wallin BG (1972). Manoeuvres affecting sympathetic outflow in human muscle nerves. *Acta Physiol Scand* 84, 82-94.

Fu Q, Okazaki K, Shibata S, Shook RP, VanGunday TB, Galbreath MM, Reelick MF, & Levine BD (2009). Menstrual cycle effects on sympathetic neural responses to upright tilt. *The Journal of Physiology* 587, 2019-2031.

Fu Q, Witkowski S, Okazaki K, & Levine BD (2005). Effects of gender and hypovolemia on sympathetic neural responses to orthostatic stress. *Am J Physiol Regul Integr Comp Physiol* 289, R109-R116.

Fuhrmann U, Krattenmacher R, Slater EP, & Fritzemeier KH (1996). The novel progestin drospirenone and its natural counterpart progesterone: biochemical profile and antiandrogenic potential. *Contraception* 54, 243-251.

Hickson SS, Miles KL, McDonnell BJ, Yasmin, Cockcroft JR, Wilkinson IB, & McEniery CM (2011). Use of the oral contraceptive pill is associated with increased large artery stiffness in young women: the ENIGMA study. *J Hypertens* 29, 1155-1159.

Hordinsky JR, Gebhardt U, Wegmann HM, & Schafer G (1981). Cardiovascular and biochemical response to simulated space flight entry. *Aviat Space Environ Med* 52, 16-18.

Kimmerly DS, Wong S, Menon R, & Shoemaker JK (2007). Forebrain neural patterns associated with sex differences in autonomic and cardiovascular function during baroreceptor unloading. *Am J Physiol Regul Integr Comp Physiol* 292, R715-R722.

Meendering JR, Torgrimson BN, Miller NP, Kaplan PF, & Minson CT (2009). Ethinyl estradiol-to-desogestrel ratio impacts endothelial function in young women. *Contraception* 79, 41-49.

Meendering JR, Torgrimson BN, Miller NP, Kaplan PF, & Minson CT (2010). A combined oral contraceptive containing 30 mcg ethinyl estradiol and 3.0 mg drospirenone does not impair endothelium-dependent vasodilation. *Contraception* 82, 366-372.

Middlekauff HR, Park J, & Gornbein JA (2012). Lack of effect of ovarian cycle and oral contraceptives on baroreceptor and nonbaroreceptor control of sympathetic nerve activity in healthy women. *Am J Physiol Heart Circ Physiol* 302, H2560-H2566.

Minson CT, Halliwill JR, Young TM, & Joyner MJ (2000a). Influence of the Menstrual Cycle on Sympathetic Activity, Baroreflex Sensitivity, and Vascular Transduction in Young Women. *Circulation* 101, 862-868.

Minson CT, Halliwill JR, Young TM, & Joyner MJ (2000b). Sympathetic Activity and Baroreflex Sensitivity in Young Women Taking Oral Contraceptives. *Circulation* 102, 1473-1476.

Montgomery LD, Kirk PJ, Payne PA, Gerber RL, Newton SD, & Williams BA (1977). Cardiovascular responses of men and women to lower body negative pressure. *Aviat Space Environ Med* 48, 138-145.

Mosher WD & Jones J (2010). Use of contraception in the United States: 1982-2008. *Vital Health Stat 23* 1-44.

Park J & Middlekauff HR (2009). Altered pattern of sympathetic activity with the ovarian cycle in female smokers. *Am J Physiol Heart Circ Physiol* 297, H564-H568.

Shoemaker JK, Hogeman CS, Khan M, Kimmerly DS, & Sinoway LI (2001). Gender affects sympathetic and hemodynamic response to postural stress. *Am J Physiol Heart Circ Physiol* 281, H2028-H2035.

Speroff L & DeCherney A (1993). Evaluation of a new generation of oral contraceptives. The Advisory Board for the New Progestins. *Obstet Gynecol* 81, 1034-1047.

Sverrisdottir YB, Mogren T, Kataoka J, Janson PO, & Stener-Victorin E (2008). Is polycystic ovary syndrome associated with high sympathetic nerve activity and size at birth? *Am J Physiol Endocrinol Metab* 294, E576-E581.

Torgrimson BN, Meendering JR, Kaplan PF, & Minson CT (2007). Endothelial function across an oral contraceptive cycle in women using levonorgestrel and ethinyl estradiol. *Am J Physiol Heart Circ Physiol* 292, H2874-H2880.

Usselman CW, Luchyshyn TA, Gimon TI, Nielson CA, Van Uum SH, & Shoemaker JK (2013). Hormone phase-dependency of neural responses to chemoreflex-driven sympatho-excitation in young women using hormonal contraceptives. *J Appl Physiol*.

Vallbo AB, Hagbarth KE, Torebjork HE, & Wallin BG (1979). Somatosensory, proprioceptive, and sympathetic activity in human peripheral nerves. *Physiol Rev* 59, 919-957.

Yang H, Cooke WH, Reed KS, & Carter JR (2012). Sex differences in hemodynamic and sympathetic neural firing patterns during orthostatic challenge in humans. *J Appl Physiol (1985)* 112, 1744-1751.

Chapter 3

3 Hormone phase dependency of neural responses to chemoreflex-driven sympatho-excitation in young women using hormonal contraceptives

(Published in J Appl Physiol 115(10): 1415-1422, 2013. Used with permission – see Appendix D)

3.1 Introduction

Endogenous female sex hormones affect autonomic regulation of the heart and vasculature (Saleh *et al.*, 2005;Herbison *et al.*, 2000). Concentrations of circulating sex hormones fluctuate across the regular menstrual cycle, and recent studies (Minson *et al.*, 2000a;Park & Middlekauff, 2009;Middlekauff *et al.*, 2012;Carter *et al.*, 2009b;Jones *et al.*, 1996;Fu *et al.*, 2009;Jarvis *et al.*, 2011;Carter & Lawrence, 2007) have used the measurement of muscle sympathetic nerve activity (MSNA) to examine whether the menstrual cycle affects neural regulation of the peripheral muscle vasculature (Wallin *et al.*, 1974). However, despite the high prevalence of oral contraceptive use (Mosher & Jones, 2010), relatively few studies have investigated the possible influence that fluctuations in exogenous sex hormone levels, brought on through hormonal contraceptive use, exert on MSNA.

Studies that have examined the effect of the menstrual cycle on sympathetic activation in young eumenorrheic women not taking hormonal supplementation most often compare the early follicular phase, associated with the nadir of estrogen and progesterone levels, to the mid-luteal phase, associated with an elevated plateau of these hormones. Some (Minson *et al.*, 2000a;Park & Middlekauff, 2009;Middlekauff *et al.*,

2012;Carter *et al.*, 2013), but not all (Carter *et al.*, 2009b;Jones *et al.*, 1996;Fu *et al.*,

2009;Jarvis *et al.*, 2011;Carter & Lawrence, 2007), studies have observed elevated

baseline MSNA during the mid-luteal phase of the menstrual cycle relative to the early

follicular phase. Hormone phase-dependent effects on MSNA have been observed during

baroreceptor unloading (Carter *et al.*, 2009b;Fu *et al.*, 2009) and following mental stress

(Carter & Lawrence, 2007), with larger MSNA responses and greater baroreflex

sensitivity (Minson *et al.*, 2000a) during the mid-luteal phase. Thus, the high-hormone

mid-luteal phase is associated with heightened MSNA relative to the low-hormone early

follicular phase under conditions of normally-fluctuating endogenous sex hormones.

    While the bulk of recent research has focused on the impact of endogenous sex

hormone fluctuations in eumenorrheic women, fewer studies have examined the effects

of exogenous hormone supplementation on MSNA regulation in young healthy women.

Hormonal contraceptive use has been associated with elevated systolic blood pressures

(Le-Ha *et al.*, 2012;Hickson *et al.*, 2011) through elevations in arterial stiffness (Hickson

et al., 2011), which could be attributable to altered patterns of sympathetic outflow in

these women compared to those previously observed in eumenorrheic women. Thus, in

order to examine patterns of sympathetic outflow during contraceptive use, three previous

studies made observations of MSNA at two phases of hormone levels – the low hormone

phase (LH; brought on through placebo pills or cessation of active pill ingestion) and the

high hormone phase (HH; the monthly plateau of EE and progestin ingestion). To date,

the comparison of LH and HH phases has not revealed differences in absolute levels of

baseline MSNA (Minson *et al.*, 2000b;Carter *et al.*, 2009a;Middlekauff *et al.*, 2012). That

is, women taking oral contraceptives appear to lack the reduction in MSNA which occurs

during the early follicular phase in eumenorrheic women not taking contraceptives. However, one study observed higher baseline mean and diastolic blood pressures during LH unaccompanied by a difference in MSNA from HH (Minson *et al.*, 2000b), suggesting either a change in the transduction of the neural signal into a vascular outcome, or a change in central baroreflex integration from LH to HH. To the latter end, sympathetic baroreflex sensitivity elicited through the modified Oxford method may be greater during the LH phase relative to HH (Minson *et al.*, 2000b) although others have failed to replicate that finding using the same technique (Middlekauff *et al.*, 2012) or spontaneous measures of sensitivity (Carter *et al.*, 2009a). Similarly, mild to moderate levels of lower body negative pressure (Carter *et al.*, 2009a) have elicited similar MSNA responses in each hormone phase. Given the severe vasoactive consequences of the modified Oxford technique which involves pharmacologically induced depressor (nitroprusside) and pressor (phenylephrine) effects (Rudas *et al.*, 1999), it is possible that the influence of hormone phase on MSNA control may be observed only under conditions of severe stress. Another stressor known to elicit large changes in sympathetic discharge patterns is chemoreflex stress (Steinback *et al.*, 2010;Malpas & Ninomiya, 1992a), but this has not yet been examined in women taking hormonal contraceptives. Thus, both the nature and severity of reflex stress may be important in determining sex-hormone specific effects on sympathetic reflex activation. Taken together, previous research suggests that sympathetic regulation may be affected by fluctuations in exogenous hormones, although the low hormone-low MSNA, high hormone-high MSNA pattern observed in the conditions of cycling endogenous hormones has yet to be observed under conditions of contraceptive use.

Therefore, the purpose of this study was to compare muscle sympathetic nerve activation patterns during LH *versus* HH phases of exogenous contraceptive hormone use across a range of chemoreflexive stimuli. We hypothesized that baseline MSNA and sympathetic reflex responses would be greater in the HH phase relative to the LH phase of hormonal contraceptive use.

3.2   Methods

3.2.1   Participants

Ten healthy female participants were enrolled in the study after providing written informed consent; repeated nerve sites in each hormone phase were obtained in seven participants. The seven research subjects were 24 ± 2 years of age, 167 ± 4 cm in height and 60 ± 4 kg in weight (BMI $= 22 \pm 2$ kg/m^2). They were healthy non-smokers who had no history of cardiovascular or respiratory disease. All participants were physically active, performing a combination of aerobic and resistance exercise an average of 4 times each week, 50 minutes per day. Participants were not taking any medications with the exception of hormonal contraceptives. Average age of menarche was 12 ± 1 years. Hormonal contraceptive data are presented in Table 3.1. All protocols were approved by the Health Sciences Research Ethics Board at The University of Western Ontario, Canada, and conformed to the Helsinki Accord.

3.2.2   Experimental Design

Prior to data collection, all participants attended a familiarization session in the same laboratory used for testing at which time they practiced the test protocol and experienced the non-invasive components of data acquisition. On test dates, participants

arrived at the laboratory at least 3 hours postprandial and having abstained from alcohol, caffeine, and other stimulants for 12 hours. Participants were studied twice: during the low hormone phase (LH; day 1-4; day 1 = the first day of menses) and during the high hormone phase (HH; day 20-24). Phases were designated based on the daily doses of the contraceptives. Test sessions were conducted at the same time of day for each subject, and the order of hormone phase data collection was counterbalanced among participants.

3.2.3   Rebreathing and End-Inspiratory Apnea Protocol

Participants were supine and breathed through a mouthpiece (series 9060, Hans Rudolph, Inc., Kansas City, MO) attached to a three-way valve which allowed the subject to breathe either room air, or through a Y-connector (VacuMed, Ventura, CA) leading to two 3-litre breathing bags. Participants were instrumented with a pulse oximetry ear clip (Dura-Y D-YSE, Covidien-Nellcor, Boulder, CO) connected to a pulse oximeter (OxiMax N-560, Covidien-Nellcor) to monitor blood oxygen saturation throughout the protocol. Gases were analyzed using an infrared carbon dioxide sensor and optical oxygen detector fed from a damped micro vacuum sampling pump (ML206 Gas Analyzer, ADInstruments, Colorado Springs, CO). These values were calibrated using ambient air pressure values and converted to online measurements of oxygen (PO_2) and carbon dioxide (PCO_2) partial pressures. Prior to beginning baseline, subjects filled the breathing bags with expired air in preparation for the rebreathing period which followed the five minutes of baseline recording. After baseline collection, the 3-way valve was turned to initiate rebreathing, a procedure to induce progressive hypoxia and hypercapnia and thereby maximize the sympatho-excitatory stress evoked during the subsequent apnea. Once PO_2 reached 70 Torr, subjects were instructed to perform the maximal end-

inspiratory apnea on their next inspiration. Upon exhalation at voluntary cessation of the apnea, subjects breathed twice in and out of the bags to allow measurement of the end-apnea PO_2 and PCO_2.

3.2.4   Measurements

Heart rate (HR) was measured through a standard three-lead electrocardiogram. A blood pressure waveform was obtained through finger photoplethysmography while online calculations produced continuous brachial artery pressure waveforms and cardiac output (Q) measures (Finometer; Finapres Medical Systems, Amsterdam, The Netherlands).

Multiunit recordings of postganglionic sympathetic nerve activity were obtained from the peroneal nerve of the right leg by microneurography. When test dates were separated by fewer than 4 weeks, microneurography was performed on the left leg on the second visit. A tungsten microelectrode (35 mm long, 200 µm diameter, tapered to a 1-5 µm tip) was inserted transcutaneously into the nerve posterior to the fibular head. A reference electrode was positioned subcutaneously 1-3 cm from the recording site. A suitable sympathetic nerve site produced a characteristic pulse-synchronous burst pattern which increased firing frequency in response to a voluntary apnea but was not associated with skin paresthesias or skin afferent activity and was not affected by arousal to a loud noise. Neuronal recordings were amplified 1000 times by a preamplifier and 75 times by a variable-gain, isolated amplifier before being band-pass filtered at 700 – 2000 Hz and then rectified and integrated to obtain a mean voltage neurogram (0.1 s time constant) (model 662C-3; Iowa University Bioengineering). Raw, filtered, and integrated MSNA

data were sampled at 10 000 Hz (Powerlab Software, ADInstruments). All data were stored for offline analysis.

3.2.5   Data Analysis

Rebreathing data were selected based on PO_2: all data between 85 and 70 Torr PO_2 (i.e. prior to the beginning of the apnea) were averaged to generate mean responses to rebreathing. Apnea data were divided into two halves corresponding to an initial phase of relative neural suppression to be followed by a larger sympathetic response. Therefore, apnea data for each subject were averaged in two phases hereafter referred to as the initial phase (APN-P1) and the latter phase (APN-P2). All relative changes were calculated against the 5-minute baseline period.

After calibrating the reconstructed brachial blood pressure signal to a manual sphygmomanometer blood pressure reading obtained at rest, the brachial arterial blood pressure waveform was used to calculate systolic blood pressure (SBP), diastolic blood pressure (DBP), pulse pressure (PP), and mean arterial pressure (MAP). Total peripheral resistance (TPR) was calculated as MAP/Q.

Sympathetic activity was quantified as burst frequency (bursts/min) and burst incidence (bursts/100 heartbeats). Burst amplitudes were normalized to the largest burst recorded during the baseline period, which was given a value of 100. To examine burst amplitude at LH and HH during baseline conditions, burst amplitude distribution curves were generated and the medians of the distributions were compared (Sverrisdottir *et al.*, 2000). Total MSNA in each condition was calculated as the mean normalized burst amplitude x burst frequency.

3.2.6   Statistical Analysis

Baseline data were compared between LH and HH using 1-tailed paired t-tests. A two-way repeated measures ANOVA was used to examine the main effects of hormone phase (LH *vs* HH) and experimental condition (baseline, rebreathing, APN-P1, and APN-P2) on the outcome measures. Point-wise comparisons for chemoreflex conditions were conducted using two-tailed paired t-tests. *Post hoc* comparisons were corrected using the Bonferroni correction. Alpha was set at 0.05.

3.3 Results

Chemoreflex characteristics are presented in Table 3.2; respiration rate, PO_2, and PCO_2 were similar between LH and HH during baseline, rebreathing, and following cessation of the apnea.

Baseline levels of MAP, SBP, DBP, PP, and TPR were similar between LH and HH phases (Table 3.3). Small differences in HR and Q between phases did not reach statistical significance. Baseline MSNA burst incidence and burst frequency were greater in the HH phase relative to the LH phase, a pattern observed in the majority of subjects (Figure 3.1). Median MSNA burst amplitude was not different between LH and HH (Figure 3.2).

A sample tracing of a representative subject completing the chemoreflex protocol in the LH phase is presented in Figure 3.3.

The hemodynamic consequences of rebreathing were similar between hormone phases (Figure 3.4). Specifically, rebreathing during LH and HH elicited similar levels of MAP (P=0.72), Q (P=0.23), and HR (P=0.26). TPR was unchanged during rebreathing and was similar between hormone phases.

Compared with baseline, rebreathing was associated with significant elevations in MSNA burst frequency and amplitude (P<0.05) in both hormone phases (Figure 3.4). The HH phase produced higher absolute levels of MSNA burst frequency and burst incidence. However, when MSNA firing patterns were expressed relative to baseline levels, no differences between LH and HH were observed in rebreathe MSNA levels (Figure 3.5).

Apnea. Apneas were maintained for similar durations in each hormone phase (22 ± 11 *vs* 22 ± 7 s, LH *vs* HH, P=0.90). Relative to APN-P1, APN-P2 produced larger increases in MAP and TPR and was associated with a reduction in Q (P<0.05; Figure 3.4). Changes in these hemodynamics were not statistically different between LH and HH during either APN-P1 or APN-P2.

Both apnea segments elicited significant increases in all MSNA variables from baseline (P<0.01; Figure 3.4). Burst frequency rose to similar absolute levels in each hormone phase during APN-P1 (P=0.15) and APN-P2 (P=0.36). As a result of the baseline hormone phase-based differences in MSNA firing frequency, the relative increases in burst frequency (P=0.03) and incidence (P=0.02) were greater in the LH phase than the HH phase in APN-P1 but not APN-P2 (frequency: P=0.11; incidence: P=0.23; Figure 3.5). Conversely, burst amplitude increased similarly between hormone phases in APN-P1 (P=0.63) while APN-P2 elicited a greater increase in burst amplitude in the LH compared to the HH phase (P=0.04; Figure 3.4). Together, these changes in burst characteristics contributed to a similar total MSNA response in APN-P1 between LH and HH (P=0.49) but a greater total MSNA response in LH than HH in APN-P2 (P=0.02).

Table 3.1: Hormonal contraceptive data

Contraceptive	Type	n	Daily EE dose (µg)	Daily progestin dose(s) (mg)
Alesse	monophasic pill	1	20	0.1
Aviane	monophasic pill	1	20	0.1
Evra	monophasic transdermal patch	1	35*	0.2*
Minovral	monophasic pill	1	30	0.15
Tri-Cyclen Lo	triphasic pill	1	25	0.18, 0.215, 0.25
Yasmin	monophasic pill	1	30	3
Yaz	monophasic pill	1	20	3
Mean ± S.D.:			**26 ± 6**	**0.8 ± 1.2**

Data obtained from pharmaceutical websites. Triphasic pill data presented as week 1, week 2, week 3. *=estimated daily dose. EE, ethinyl estradiol.

Table 3.2: Baseline and chemoreflex respiration characteristics

	Respiration Rate (breaths/min)		PO$_2$ (torr)		PCO$_2$ (torr)	
	LH	HH	LH	HH	LH	HH
Baseline	13 ± 3	13 ± 4	126 ± 5	126 ± 6	18 ± 4	18 ± 4
Rebreathe	13 ± 4	12 ± 4	79 ± 1	79 ± 1	45 ± 4	44 ± 4
End-Apnea	-	-	62 ± 3	62 ± 3	49 ± 4	47 ± 4

Data presented as mean ± standard deviation. PO$_2$ = partial pressure of oxygen; PCO$_2$ = partial pressure of carbon dioxide; LH = low hormone phase; HH = high hormone phase.

Table 3.3: Baseline hemodynamic and muscle sympathetic nerve activity

Variable	Low Hormone Phase	High Hormone Phase	*P* Value
MAP (mmHg)	99 ± 14	103 ± 14	0.22
HR (beats · min^{-1})	64 ± 8	71 ± 11	0.06
SBP (mmHg)	128 ± 16	129 ± 16	0.43
DBP (mmHg)	70 ± 5	73 ± 12	0.15
PP (mmHg)	57 ± 13	55 ± 9	0.36
Q (L · min^{-1})	5.1 ± 0.7	5.8 ± 0.9	0.08
TPR (mmHg · L^{-1} · min^{-1})	20 ± 4	18 ± 2	0.14
MSNA burst incidence (bursts · 100 heartbeats^{-1})	16 ± 8	23 ± 9	**0.03**
MSNA burst frequency (bursts · min^{-1})	11 ± 6	16 ± 8	**0.02**
Total MSNA (a.u.)	522 ± 270	744 ± 350	**0.04**

Data presented as mean ± standard deviation. MAP = mean arterial pressure; HR = heart rate; SBP = systolic blood pressure; DBP = diastolic blood pressure; PP = pulse pressure; Q = cardiac output; TPR = total peripheral resistance; MSNA = muscle sympathetic nerve activity.
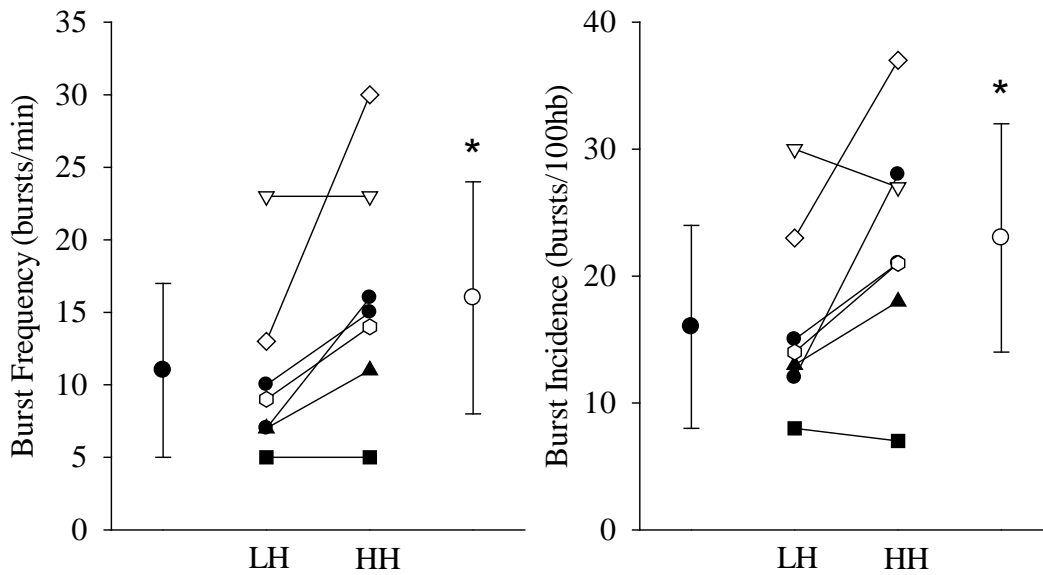
Figure 3.1: Individual patterns of sympathetic burst frequency and incidence.

The majority of subjects increased muscle sympathetic nerve activity (MSNA) firing

rates from low (LH) to high hormone phases (HH). Closed circles with error bars

represent LH mean ± standard deviation; open circles with error bars represent HH mean

± standard deviation. *P<0.05 *vs* LH.
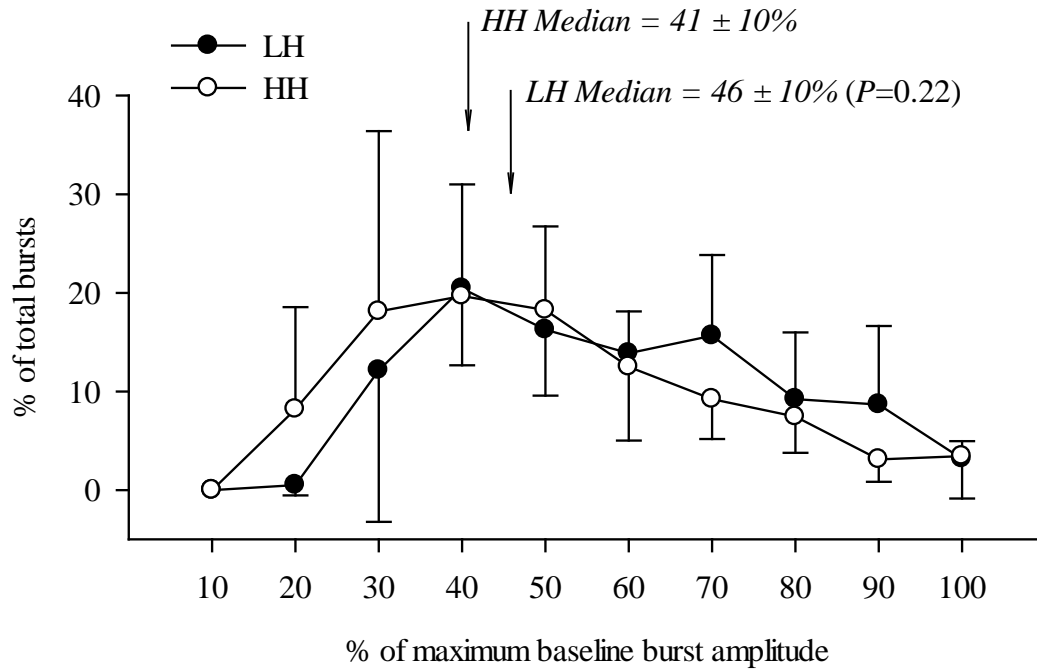
Figure 3.2: Sympathetic burst amplitude distributions at baseline.

Medians of the distributions were not different between LH and HH phases (P=0.22).

Data are presented as mean ± standard deviation.
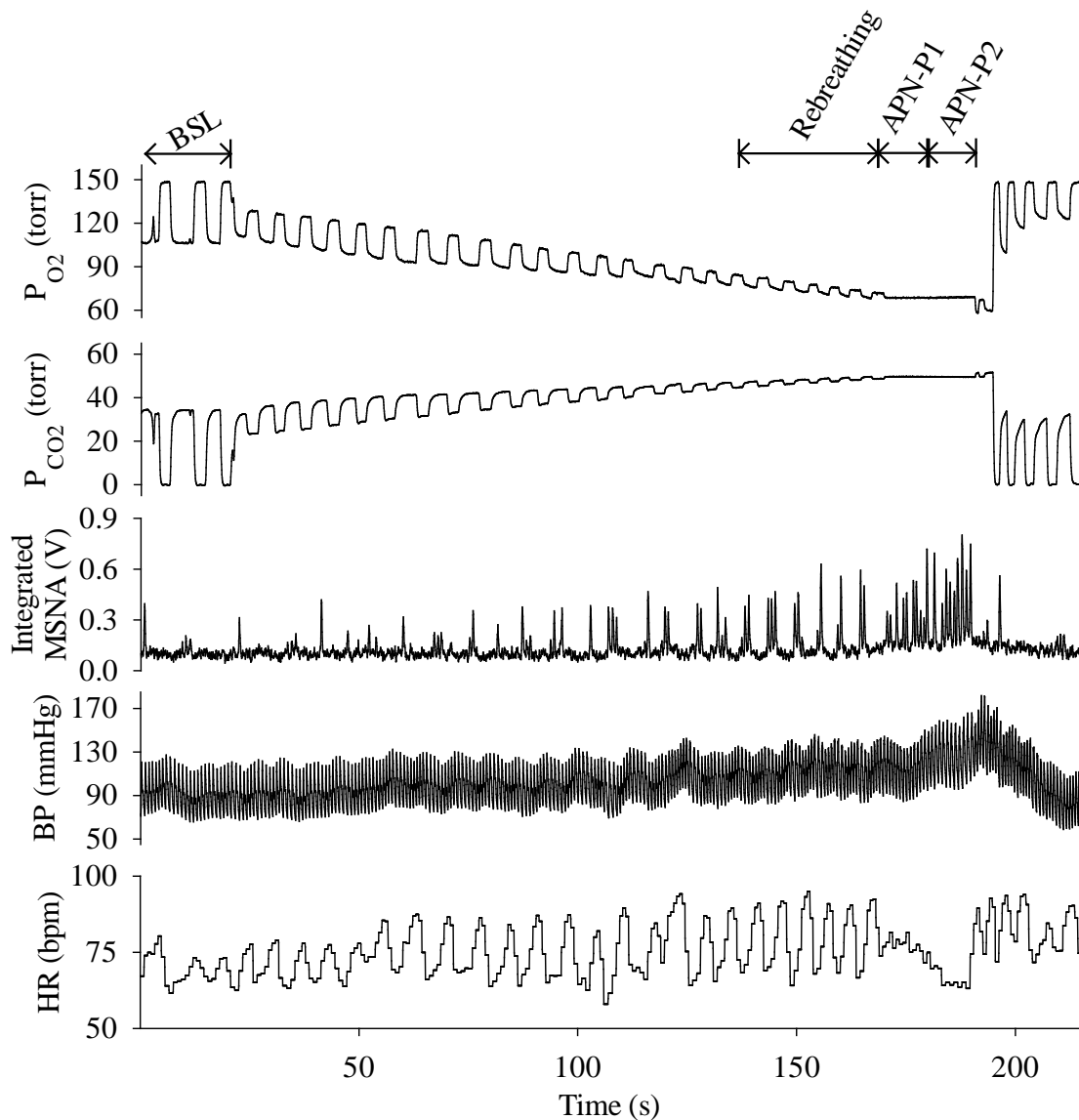
Figure 3.3: Sample tracing from a subject performing the rebreathing and apnea protocol.

Data are from low hormone phase. Lines at top represent data selected for analysis. BSL = baseline; APN-P1 = initial half of apnea; APN-P2 = latter half of apnea; PO_2 = partial pressure of oxygen; PCO_2 = partial pressure of carbon dioxide; MSNA = muscle sympathetic nerve activity; BP = blood pressure; HR = heart rate.
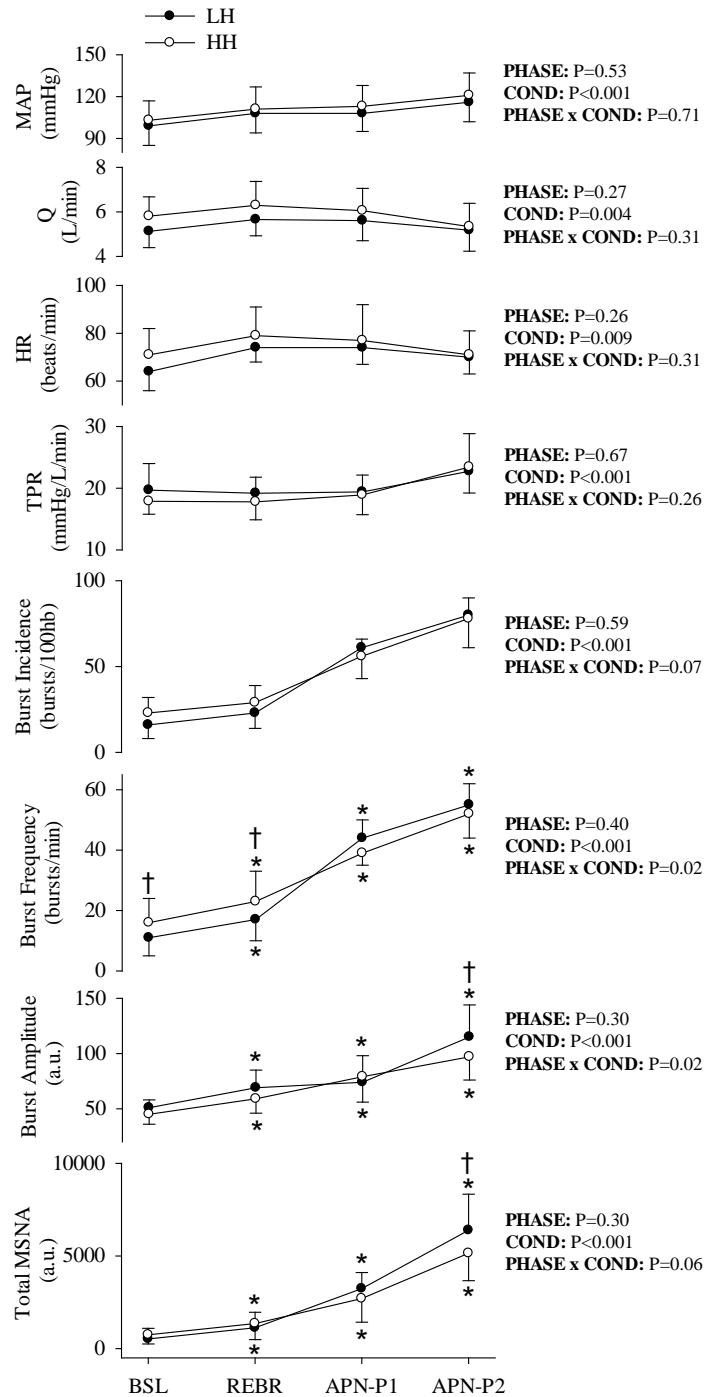
Figure 3.4: Hemodynamics and sympathetic activity responses.

A main effect of chemoreflex was observed across all measures. Significant hormone phase x chemoreflex condition interactions were observed in burst frequency and burst amplitude. *P<0.05 *vs* BSL; †P<0.05 LH *vs* HH. Data are presented as mean ± standard deviation. COND = chemoreflex condition; LH = low hormone phase; HH = high hormone phase.
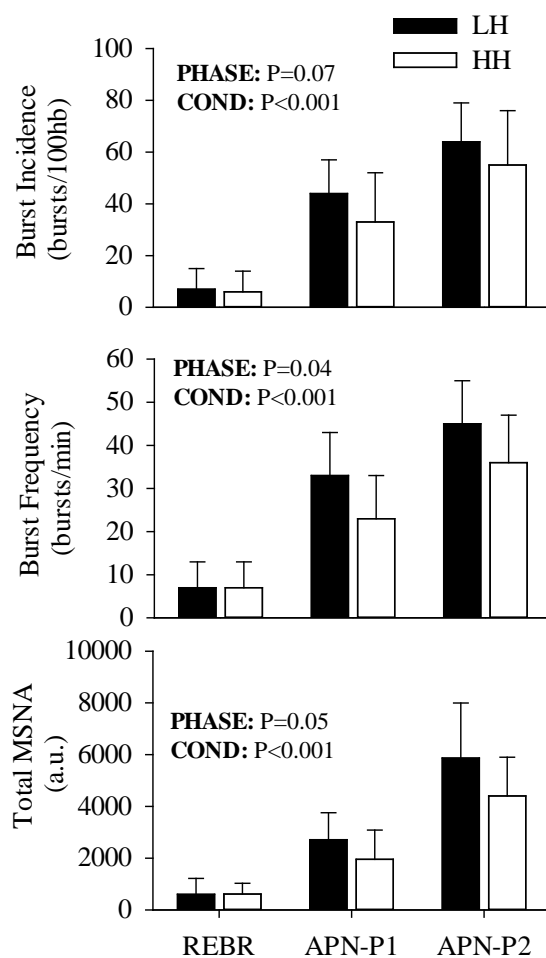
Figure 3.5: Relative changes from baseline in sympathetic characteristics.

Greater increases in muscle sympathetic nerve activity (MSNA) in response to

chemoreflex stimulation were observed in the low hormone (LH) phase compared to the

high hormone (HH) phase of hormonal contraceptive use. No significant Phase x

Condition interactions were observed. Data are presented as mean ± standard deviation.

COND = chemoreflex condition.

3.4   Discussion

This study was the first to observe differences in baseline levels of MSNA between hormone phases in women taking hormonal contraceptives. Specifically, we observed elevations in baseline MSNA burst frequency and burst incidence with no change in baseline burst amplitude distribution from the low- to the high-hormone phase of hormonal contraceptive use. In addition to baseline effects, hormonal contraceptives also affected the reflex increase in MSNA, but in a manner that was different than baseline effects. Specifically, severe chemoreflex stimulation elicited greater increases from baseline in sympathetic firing frequency and burst amplitude in the LH phase relative to the HH phase. Importantly, the greater chemoreflex response in the LH phase included a shift in burst amplitude distribution suggesting greater neuronal recruitment. Therefore, these data suggest that endogenous hormone phase affects the regulation of muscle sympathetic nerve activity both at baseline and during chemoreflex stress.

In the current study, elevated baseline MSNA in the HH phase relative to the LH phase was observed without concurrent change to blood pressure, Q, or TPR. While previous studies have not observed baseline differences in MSNA between LH and HH (Minson *et al.*, 2000b;Carter *et al.*, 2009a;Middlekauff *et al.*, 2012), Minson and colleagues (Minson *et al.*, 2000b) observed reduced baseline MAP and diastolic blood pressure, with elevated calf blood flow and a trend toward reduced calf vascular resistance (P=0.06) during the HH phase relative to LH. In the presence of similar MSNA, this points to a reduction in the transduction of the neural signal into a vascular outcome in the HH phase of hormonal contraceptive use, which is consistent with the data from the present study. It is possible that changes to the hormonal milieu induced

through contraceptive use may contribute to the change in baseline sympathetic regulation, as research conducted in eumenorrheic women without hormonal supplementation suggests that the concentrations of circulating hormones affect baseline MSNA. A recent study by Carter and colleagues (Carter *et al.*, 2013) retrospectively correlated circulating concentrations of estradiol and progesterone to resting MSNA in 30 young premenopausal women who were not taking any exogenous hormones. In their analysis comparing the magnitude of the change in hormone levels from the early follicular to the mid-luteal phase to the magnitude of the increase in MSNA across the menstrual cycle, the authors observed that smallest changes in the ratio of estradiol to progesterone were associated with the greatest increases in baseline MSNA (Carter *et al.*, 2013), suggesting that increasing levels of progesterone promote sympathoexcitation, while elevated levels of estradiol are associated with sympathoinhibition. Furthermore, sex hormones are well known to be vasoactive; the influence estrogens exert over the vasculature has been the subject of thorough review (Miller & Duckles, 2008). Estradiol administration enhances basal nitric oxide release in resistance arteries (Sudhir *et al.*, 1996), which would counteract MSNA-induced vasoconstriction. Conversely, progesterone antagonizes the vasodilatory properties of estradiol observed during FMD (Miner *et al.*, 2011). Together, these data support a hormonal basis for the menstrual cycle-based changes in MSNA observed in eumenorrheic women since the mid-luteal phase, associated with high progesterone and estradiol, is associated with heightened MSNA (Minson *et al.*, 2000a;Park & Middlekauff, 2009;Middlekauff *et al.*, 2012;Carter *et al.*, 2013). Similar patterns observed in the present study suggest that it is the hormone levels which are driving phase-based changes in MSNA in women taking hormonal

contraceptives. However, it is important to note that endogenous and exogenous hormones differ in their physiological effects. Ethinyl estradiol is not oxidized the same way as endogenous estradiol, thus its potency is much greater than the endogenous counterpart (Kuhl, 2005). Endogenous progesterone and the synthetic progestins vary in their progestogenic, antiestrogenic, and androgenic effects, dependent on the presence of molecular subgroups which determine receptor binding (Kuhl, 2005). Even within the realm of synthetic hormones, the vascular effects of various progestins are dependent on the precise progestin studied (Meendering *et al.*, 2010;Meendering *et al.*, 2009). Thus, caution must be taken when applying conclusions based on endogenous hormones to exogenous hormonal supplementation. This issue is further confounded due to the complexities involved in quantifying the hormonal milieu in women supplementing endogenous hormone production with exogenous hormones. Bioavailability of exogenous hormones varies among individuals (Goldzieher & Stanczyk, 2008), metabolites of hormones may remain in tissue for days following cessation of contraceptive use (Wenner & Stachenfeld, 2012), and endogenous production of hormones can increase during hormonal contraceptive withdrawal (van Heusden & Fauser, 1999). A complete picture of circulating hormone levels in women taking hormonal contraceptives would therefore require the measurement of both exogenous and endogenous levels of sex hormones. However, conventional ELISA techniques are plagued by cross-reactivity between ethinyl estradiol and 17β-estradiol. This issue requires further attention with precise quantification of hormone levels, such as those achieved through mass spectrometry, in order to determine the extent to which circulating hormones exert an influence over baseline MSNA.

The present study was the first to compare chemoreflex-induced increases in MSNA across the phases of hormonal contraceptive use. The application of progressive chemoreflex stress (i.e. rebreathing then apnea) revealed hormone phase-specific effects based on severity of chemoreflex stimulation. For instance, mild chemoreflex stimulation during rebreathing did not change overall patterns of MSNA from those observed at baseline. However, despite the lower baseline levels of MSNA, the LH phase was associated with greater relative changes in MSNA burst frequency and incidence during the initial phase of apnea, as well as greater increases in burst amplitude in the latter phase of the apnea, such that total MSNA in the LH phase exceeded that observed in the HH phase during the most severe chemoreflex condition examined in this study. These observations suggest that elevated baseline activity in HH may encroach upon the ceiling of maximal sympathetic outflow. The current observations are supported by previous observations insofar that mild to moderate baroreceptor unloading (Carter *et al.*, 2009a) did not elicit hormone phase-dependent differences in MSNA regulation while application of the modified Oxford method, which induces large, supraphysiological fluctuations in MAP, revealed greater sympathetic baroreflex sensitivity in subjects in the LH phase (Minson *et al.*, 2000b). Therefore, it appears to be important to examine a range of severities in sympatho-excitatory stimuli when examining the effects of hormonal contraceptive use on sympathetic regulation.

The most commonly reported characteristics of MSNA are burst frequency, a general indicator of efferent sympathetic outflow (what the end organ "sees"), and burst incidence, which takes into account the central integration of beat-to-beat afferent feedback originating from the baroreceptors. Burst amplitude, however, is reported less

often, yet is correlated to the magnitude of vascular responses (Fairfax *et al.*, 2013) and is

controlled in a manner different from burst frequency (Sverrisdottir *et al.*,

2000;Kienbaum *et al.*, 2001;Malpas & Ninomiya, 1992b). This supports the presence of

two discrete mechanisms by which vasoconstrictor signals might be altered: strength and

occurrence of sympathetic bursts (Kienbaum *et al.*, 2001). The quantification of burst

amplitude presents a difficulty, as raw amplitude values are indicative of recording

electrode placement within the nerve (Vallbo *et al.*, 1979) and/or axonal recruitment

(Ninomiya *et al.*, 1993). However, when raw burst amplitude voltages are normalized

and expressed as burst distribution plots, changes in the distribution of burst amplitude

have been shown to discriminate between conditions where similar levels of burst

frequency were observed (Sverrisdottir *et al.*, 2000;Shoemaker *et al.*, 2001). In the

present study, the distribution of burst amplitude was similar between LH and HH during

conditions in which burst frequency and incidence were different between hormone

phases. In the latter half of the apnea condition, however, in which firing frequencies had

approached maximal levels and no longer differed between LH and HH, burst amplitude

increased in the LH phase, resulting in overall greater total MSNA in the LH phase

relative to the HH phase during severe chemoreflex stress. These data emphasize the

importance of burst amplitude as a mechanism by which alterations to peripheral nerve

activity may be accomplished, particularly when burst frequencies are at or near maximal

levels.

Limitations

The end-inspiratory apnea protocol was designed to maximize sympathetic

activation. Although the technique would affect primarily a change in chemoreflex stress,

the increases in intrathoracic pressures which accompany such apneas will also affect cardiac function and baroreflex contributions to the response. Also, the chemoreflex protocol induced concurrent hypercapnia and hypoxia. Therefore, whether hormone phase has an effect on the singular stimulus of hypoxia or hypercapnia on MSNA remains unknown.

Importantly, the participants in the current study were taking different hormonal contraceptives, with one subject taking a transdermal patch and one subject taking a triphasic pill formulation. While all contraceptives contained relatively similar amounts of EE, the type and amount of progestin varied, and these factors are known to affect vascular function (Meendering *et al.*, 2010;Meendering *et al.*, 2009). Therefore, it is unclear how the specific types of hormonal contraceptives contributed to our findings.

Conclusions

The data from the present study support the conclusion that exogenous sex hormones exert an effect on baseline sympathetic outflow, in a manner which similar to that observed in studies examining regularly cycling women not taking hormonal contraceptives (Minson *et al.*, 2000a;Park & Middlekauff, 2009;Middlekauff *et al.*, 2012;Carter *et al.*, 2013). Specifically, baseline MSNA was higher in the high hormone phase. Moreover, chemoreflex-driven sympathetic responses (both burst frequency and amplitude) were smaller in the HH versus the LH phase. Thus, the higher baseline MSNA levels in HH did not translate into high maximal levels of sympathetic outflow. Observations of similar hemodynamic outcomes in the face of altered MSNA imply a

contraceptive phase-dependent change in the transduction of neural inputs into vascular

outcomes.

3.5 References

Carter JR, Fu Q, Minson CT, & Joyner MJ (2013). Ovarian cycle and sympathoexcitation in premenopausal women. *Hypertension* **61**, 395-399.

Carter JR & Lawrence JE (2007). Effects of the menstrual cycle on sympathetic neural responses to mental stress in humans. *J Physiol* **585**, 635-641.

Carter JR, Klein JC, & Schwartz CE (2009a). Effects of oral contraceptives on sympathetic nerve activity during orthostatic stress in young, healthy women. *Am J Physiol Regul Integr Comp Physiol* **298**, R9-R14.

Carter JR, Lawrence JE, & Klein JC (2009b). Menstrual cycle alters sympathetic neural responses to orthostatic stress in young, eumenorrheic women. *Am J Physiol Endocrinol Metab* **297**, E85-E91.

Fairfax ST, Padilla J, Vianna LC, Davis MJ, & Fadel PJ (2013). Spontaneous bursts of muscle sympathetic nerve activity decrease leg vascular conductance in resting humans. *Am J Physiol Heart Circ Physiol* **304**, H759-H766.

Fu Q, Okazaki K, Shibata S, Shook RP, VanGunday TB, Galbreath MM, Reelick MF, & Levine BD (2009). Menstrual cycle effects on sympathetic neural responses to upright tilt. *The Journal of Physiology* **587**, 2019-2031.

Goldzieher JW & Stanczyk FZ (2008). Oral contraceptives and individual variability of circulating levels of ethinyl estradiol and progestins. *Contraception* **78**, 4-9.

Herbison AE, Simonian SX, Thanky NR, & Bicknell RJ (2000). Oestrogen modulation of noradrenaline neurotransmission. *Novartis Found Symp* **230**, 74-85.

Hickson SS, Miles KL, McDonnell BJ, Yasmin, Cockcroft JR, Wilkinson IB, & McEniery CM (2011). Use of the oral contraceptive pill is associated with increased large artery stiffness in young women: the ENIGMA study. *J Hypertens* **29**, 1155-1159.

Jarvis SS, VanGundy TB, Galbreath MM, Shibata S, Okazaki K, Reelick MF, Levine BD, & Fu Q (2011). Sex differences in the modulation of vasomotor sympathetic outflow during static handgrip exercise in healthy young humans. *Am J Physiol Regul Integr Comp Physiol* **301**, R193-R200.

Jones PP, Spraul M, Matt KS, Seals DR, Skinner JS, & Ravussin E (1996). Gender does not influence sympathetic neural reactivity to stress in healthy humans. *Am J Physiol Heart Circ Physiol* **270**, H350-H357.

Kienbaum P, Karlssonn T, Sverrisdottir YB, Elam M, & Wallin BG (2001). Two sites for modulation of human sympathetic activity by arterial baroreceptors? *J Physiol* **531**, 861-869.

Kuhl H (2005). Pharmacology of estrogens and progestogens: influence of different routes of administration. *Climacteric* **8 Suppl 1**, 3-63.

Le-Ha C, Beilin LJ, Burrows S, Huang RC, Oddy WH, Hands B, & Mori TA (2012). Oral contraceptive use in girls and alcohol consumption in boys are associated with increased blood pressure in late adolescence. *Eur J Prev Cardiol*.

Malpas SC & Ninomiya I (1992a). Effect of chemoreceptor stimulation on the periodicity of renal sympathetic nerve activity in anesthetized cats. *J Auton Nerv Syst* **37**, 19-28.

Malpas SC & Ninomiya I (1992b). The amplitude and periodicity of synchronized renal sympathetic nerve discharges in anesthetized cats: differential effect of baroreceptor activity. *J Auton Nerv Syst* **40**, 189-198.

Meendering JR, Torgrimson BN, Miller NP, Kaplan PF, & Minson CT (2009). Ethinyl estradiol-to-desogestrel ratio impacts endothelial function in young women. *Contraception* **79**, 41-49.

Meendering JR, Torgrimson BN, Miller NP, Kaplan PF, & Minson CT (2010). A combined oral contraceptive containing 30 mcg ethinyl estradiol and 3.0 mg drospirenone does not impair endothelium-dependent vasodilation. *Contraception* **82**, 366-372.

Middlekauff HR, Park J, & Gornbein JA (2012). Lack of effect of ovarian cycle and oral contraceptives on baroreceptor and nonbaroreceptor control of sympathetic nerve activity in healthy women. *Am J Physiol Heart Circ Physiol* **302**, H2560-H2566.

Miller VM & Duckles SP (2008). Vascular actions of estrogens: functional implications. *Pharmacol Rev* **60**, 210-241.

Miner JA, Martini ER, Smith MM, Brunt VE, Kaplan PF, Halliwill JR, & Minson CT (2011). Short-term oral progesterone administration antagonizes the effect of transdermal

estradiol on endothelium-dependent vasodilation in young healthy women. *Am J Physiol Heart Circ Physiol* **301**, H1716-H1722.

Minson CT, Halliwill JR, Young TM, & Joyner MJ (2000a). Influence of the Menstrual Cycle on Sympathetic Activity, Baroreflex Sensitivity, and Vascular Transduction in Young Women. *Circulation* **101**, 862-868.

Minson CT, Halliwill JR, Young TM, & Joyner MJ (2000b). Sympathetic Activity and Baroreflex Sensitivity in Young Women Taking Oral Contraceptives. *Circulation* **102**, 1473-1476.

Mosher WD & Jones J (2010). Use of contraception in the United States: 1982-2008. *Vital Health Stat 23* 1-44.

Ninomiya I, Malpas SC, Matsukawa K, Shindo T, & Akiyama T (1993). The amplitude of synchronized cardiac sympathetic nerve activity reflects the number of activated pre- and postganglionic fibers in anesthetized cats. *J Auton Nerv Syst* **45**, 139-147.

Park J & Middlekauff HR (2009). Altered pattern of sympathetic activity with the ovarian cycle in female smokers. *Am J Physiol Heart Circ Physiol* **297**, H564-H568.

Rudas L, Crossman AA, Morillo CA, Halliwill JR, Tahvanainen KU, Kuusela TA, & Eckberg DL (1999). Human sympathetic and vagal baroreflex responses to sequential nitroprusside and phenylephrine. *Am J Physiol* **276**, H1691-H1698.

Saleh TM, Connell BJ, & Cribb AE (2005). Sympathoexcitatory effects of estrogen in the insular cortex are mediated by GABA. *Brain Res* **1037**, 114-122.

Shoemaker JK, Hogeman CS, Khan M, Kimmerly DS, & Sinoway LI (2001). Gender affects sympathetic and hemodynamic response to postural stress. *Am J Physiol Heart Circ Physiol* **281**, H2028-H2035.

Steinback CD, Salmanpour A, Breskovic T, Dujic Z, & Shoemaker JK (2010). Sympathetic neural activation: an ordered affair. *J Physiol* **588**, 4825-4836.

Sudhir K, Jennings GL, Funder JW, & Komesaroff PA (1996). Estrogen enhances basal nitric oxide release in the forearm vasculature in perimenopausal women. *Hypertension* **28**, 330-334.

Sverrisdottir YB, Rundqvist B, Johannsson G, & Elam M (2000). Sympathetic neural burst amplitude distribution: A more specific indicator of sympathoexcitation in human heart failure. *Circulation* **102**, 2076-2081.

Vallbo AB, Hagbarth KE, Torebjork HE, & Wallin BG (1979). Somatosensory, proprioceptive, and sympathetic activity in human peripheral nerves. *Physiol Rev* **59**, 919-957.

van Heusden AM & Fauser BC (1999). Activity of the pituitary-ovarian axis in the pill-free interval during use of low-dose combined oral contraceptives. *Contraception* **59**, 237-243.

Wallin BG, Delius W, & Sundlof G (1974). Human muscle nerve sympathetic activity in cardiac arrhythmias. *Scand J Clin Lab Invest* **34**, 293-300.

Wenner MM & Stachenfeld NS (2012). Blood pressure and water regulation: understanding sex hormone effects within and between men and women. *J Physiol* **590**, 5949-5961.

Chapter 4

4 Sex and menstrual cycle effects on sympathetic responses to chemoreflex activation

4.1 Introduction

Rates of cardiovascular morbidity and mortality tend to be lower in premenopausal women than in age-matched men (Rosenthal & Oparil, 2000;Eaker *et al.*, 1989;Criqui *et al.*, 1985). Included in the category of morbidity are the sleep apnea syndromes, which are more prevalent in men than in women (Young *et al.*, 1993). Sleep-disordered breathing is associated with elevated MSNA (Hedner *et al.*, 1988;Carlson *et al.*, 1993) and is an independent risk factor for hypertension (Hla *et al.*, 1994), an association which may be strengthened in men relative to women (Nieto *et al.*, 2000). However, the factors contributing to the elevated risk in men are not yet well established (Jordan & McEvoy, 2003).

Given that individuals with sleep apnea experience repeated bouts of hypoxia and hypercapnia over the course of a night, the relative female protection from sleep apnea may stem from an improved ability to respond to chemoreflex stress. In a study of the sympathetic responses to acute hypoxic stress in humans, muscle sympathetic nerve activity increased with a shorter latency in women relative to men, and following cessation of hypoxia returned back to baseline levels faster than men (Jones *et al.*, 1999). Together with data indicating that females are better able to survive prolonged exposure to hypoxia than males (Britton & Kline, 1945), these data support a sex effect on sympathetic regulation in response to hypoxic stress. However, the apneic events which accompany sleep apnea are associated with a combination of hypoxia and hypercapnia,

and it is not yet known whether the sympathetic response to this combined stimulus differs between men and women.

In the consideration of sympathetic regulation mechanisms in women, several recent studies suggest that menstrual cycle should be taken into account. For instance, several studies have observed that muscle sympathetic nerve activity (MSNA) peaks during the midluteal (ML) phase of the menstrual cycle, associated with elevations in 17β-oestradiol (E2) and progesterone (P4), and declines during the early follicular (EF) phase, associated with the nadir of E2 and P4 (Minson *et al.*, 2000;Park & Middlekauff, 2009;Middlekauff *et al.*, 2012;Carter *et al.*, 2013). This same pattern has been observed during baroreceptor unloading, which elicits reflex increases in MSNA that are larger in the ML phase than in the EF phase (Fu *et al.*, 2009;Carter *et al.*, 2009). Furthermore, the differences in MSNA responses between menstrual cycle phases are exaggerated as the magnitude of baroreceptor unloading is increased (Fu *et al.*, 2009;Carter *et al.*, 2009), suggesting that the observation of menstrual cycle-based effects on sympathetic regulation may depend on the intensity of the stimulus. This hypothesis may be an important point to consider when examining responses to the chemoreflex, as the stimulation of the chemoreflex is known to elicit large increases in sympathetic nerve activity (Morgan *et al.*, 1995;Saito *et al.*, 1988) which are graded to the intensity and duration of the stimulus (Smith & Muenter, 2000).

To the best of our knowledge, no studies have systematically compared MSNA responses to chemoreflex stimulation between EF and ML. However, our laboratory recently examined chemoreflex regulation in women who were regular users of hormonal contraceptives (Usselman *et al.*, 2013). In those women, the low hormone phase of

hormonal contraceptive use was associated with a greater sympatho-excitatory response to chemoreflex activation than the high hormone phase (Usselman *et al.*, 2013). If these results are generalized to pertain to endogenous hormone phases, this may indicate that lower levels of circulating hormones are associated with greater chemoreflex-induced increases in MSNA than higher hormone levels.

Therefore, the purpose of the present study was to compare sympathetic responses between men and women during acute, severe chemoreflex stress. We tested the hypothesis that hypercapnic-hypoxic rebreathing and apnea would be associated with greater increases in MSNA in young healthy women relative to men. To determine whether the menstrual cycle is associated with changes in chemoreflex-driven sympatho-excitation, we compared sympathetic responses in women during the early follicular (EF) and midluteal (ML) phases of the menstrual cycle. We tested the hypothesis that the EF phase would be associated with greater increases in MSNA than the ML phase.

4.2 Methods

4.2.1 Subjects

Subjects were eligible to participate if they were healthy, non-smoking, free of cardiovascular and respiratory disease, and not taking any medications. All women reported regular menstrual cycles of approximately 28 days' duration. Data were collected from eighteen undergraduate and graduate students enrolled at The University of Western Ontario in London, Ontario: nine females (24 ± 3 y, 166 ± 6 cm, 64 ± 9 kg; mean ± standard deviation) and nine males (26 ± 2 y, 179 ± 4 cm, 82 ± 11 kg). All participants provided written, informed consent. The protocols were approved by the Health Sciences

Research Ethics Board at The University of Western Ontario, Canada and conformed to

the standards set by the latest revision of the *Declaration of Helsinki*. All subjects were

physically active, engaging in both endurance and resistance exercise on a regular basis

(women: 4 ± 2 bouts/week, 81 ± 43 min/bout; men: 5 ± 2 bouts/week, 66 ± 25 min/bout).

4.2.2   Experimental Design

Women were tested during EF (days 1-4 after the onset of menstruation) and ML

(days 20-24) phases of the menstrual cycle. The order of menstrual cycle phase testing

was counter-balanced. Menstrual cycle phases were confirmed through analysis of the

hormonal milieu, including measures for E2 (Enhanced Estradiol eE2 assay; Siemens

ADVIA Centaur Immunoassay System; Siemens Healthcare, Erlangen, Germany), P4

(Progesterone [PRGE] assay; Siemens ADVIA Centaur Immunoassay System), and

testosterone (T; Elecsys Testosterone II assay; Roche Cobas e411 Analyzer; Roche

Diagnostics, Basel, Switzerland) (Table 4.1). Time of day was held constant within each

subject across test days.

Table 4.1: Serum sex hormone levels in women and men

	Women		Men
	EF	ML	
E2 (pmol · L^{-1})	$151 \pm 50^{\dagger}$	638 ± 175	$161 \pm 64^{\dagger}$
P4 (nmol · L^{-1})	$1.2 \pm 0.5^{\dagger}$	35.8 ± 9.3	$1.5 \pm 0.3^{\dagger}$
T (nmol · L^{-1})	1.0 ± 0.2	1.0 ± 0.4	$19.6 \pm 6.5^{*\dagger}$

Data presented as mean ± standard deviation. Assay reference ranges, E2: men, ≤ 146 pmol/L; EF phase, 72-529 pmol/L; ML phase, 205-786 pmol/L; P4: EF phase, 0.5-4.5 nmol/L; ML phase, 14.0-89.0 nmol/L; T: men, 8.6-29.0 nmol/L; women, 0.3-1.7 nmol/L. *$P<0.05$ *versus* EF, †$P<0.05$ *versus* ML.

Before testing, all subjects attended a familiarization session during which they became accustomed to the experimental protocols and the non-invasive aspects of data acquisition. On test dates, subjects arrived at the laboratory having fasted for 3 hours, and having abstained from caffeine, alcohol, and exercise for 12 hours. Subjects were positioned supine and were instrumented following an intravenous blood draw from the antecubital vein for the assessment of hormone levels. Sympathetic nerve sites were located using microneurography.

4.2.3　End-Inspiratory Apnea Protocol

Participants breathed through a mouthpiece (series 9060, Hans Rudolph, Inc., Kansas City, MO) attached to a three-way valve which opened to either room air or a Y-connector (VacuMed, Ventura, CA) leading to two 3-litre rebreathing bags. A nose clip prevented nasal breathing (series 9015, Hans Rudolph) and a pulse oximetry ear clip (Dura-Y D-YSE, Covidien-Nellcor, Boulder, CO) connected to a pulse oximeter (OxiMax N-560, Covidien-Nellcor) was used to monitor blood oxygen saturation. Gases were analyzed online using an infrared carbon dioxide sensor and an optical oxygen detector fed from a damped micro vacuum sampling pump (ML206 Gas Analyzer, ADInstruments, Colorado Springs, CO). In order to maximize the sympatho-excitatory stress, a period of rebreathing was conducted immediately preceding the end-inspiratory apnea. The order of testing was conducted as follows: (1) subjects filled the rebreathing bags with expired air; (2) five minutes of baseline during room air breathing; (3) rebreathing to induce progressive hypoxia-hypercapnia; (4) at 70 Torr PO_2, maximal end-inspiratory apnea; (5) upon termination of apnea, subjects breathed twice in and out of

the bags for quantification of end-apnea hypoxia-hypercapnia; (6) three minutes of recovery data.

4.2.4   Measurements

Sympathetic nerve activity was assessed using the microneurographic technique (Vallbo *et al.*, 1979). A tungsten recording electrode (diameter: 200 µm, length: 35 mm) with an uninsulated 1-5 µm tip was inserted transcutaneously into the peroneal nerve and a reference electrode was inserted subcutaneously 1-3 cm from the recording site. Adequate MSNA recording sites produced pulse-synchronous bursts of activity which increased in frequency during apnea maneuvers and were unaffected by arousal to a loud noise (Delius *et al.*, 1972). The MSNA signal was amplified 1000 times by a preamplifier and 75 times by a variable-gain, isolated amplifier and then band-pass filtered (700-2000 Hz). The signal was then rectified and integrated (time constant 0.1 s) (model 662C-3; Iowa University Bioengineering, Iowa, USA).

Baseline blood pressures were assessed using manual sphygmomanometry; the average of three blood pressure values was used to calibrate beat-to-beat blood pressures obtained through photoplethysmographic methods (Finometer; Finapres Medical Systems, Amsterdam, The Netherlands). Cardiac output (Q) was calculated using the Modelflow algorithm in the Finometer. Heart rate (HR) was measured using a standard 3-lead electrocardiogram. All signals were sampled at 1000 Hz with an online data acquisition and analysis package (PowerLab /16SP with LabChart 7, ADInstruments, Colorado Springs, Colorado, USA) except MSNA, which was sampled at 10 000 Hz.

4.2.5   Data Analysis

Data from the final section of rebreathing (PO_2 between 80 and 70 Torr) were

averaged to reflect hypercapnia-hypoxia while breathing. Apnea data were divided into

two halves, corresponding to an initial phase of relative neural suppression (APN-P1)

followed by the largest sympathetic response occurring during the latter half of the apnea

(APN-P2). Recovery data were averaged over the final two minutes of recovery, after

values had returned to baseline levels.

The brachial blood pressure waveform was analyzed to determine mean (MAP),

systolic (SBP), and diastolic (DBP) blood pressures. Body surface area was estimated

using the Mosteller formula (Mosteller, 1987;Lam & Leung, 1988) and was used to

normalize Q and Q-derived measures to body size in men and women. Q and stroke

volume were divided by body surface area to determine cardiac index (Q_i) and stroke

volume index (SV_i), respectively. Total peripheral resistance (TPR) was calculated as

MAP/Q.

Sympathetic firing frequency was quantified as burst frequency (bursts/min). The

amplitude component was considered because of its importance in distinguishing

between conditions which have similar burst frequencies (Sverrisdottir *et al.*, 2000) and

because burst amplitude is regulated in a manner distinct from burst frequency

(Kienbaum *et al.*, 2001;Malpas & Ninomiya, 1992) which may reflect axonal recruitment

(Steinback *et al.*, 2010b;Salmanpour *et al.*, 2011). The amplitude component was

considered in three ways. Baseline amplitudes were compared between groups and

conditions through the comparison of frequency probability distribution curves. In this

baseline analysis, all bursts were scaled to the largest burst in each recording which was

assigned a value of 100. The median value of each curve was then used to compare normalized burst amplitudes between groups (Sverrisdottir *et al.*, 2000;Kimmerly *et al.*, 2004). Next, to evaluate changes in burst amplitude during chemoreflex stimulation, all burst amplitude voltages during apnea were normalized to the largest amplitude achieved during the previous baseline period. The mean normalized burst amplitude during each condition was then used to compare between groups. Finally, raw voltages were also compared between baseline and recovery periods to validate burst amplitude measures by ensuring that sympathetic nerve sites had not shifted during the chemoreflex protocol.

A representation of the total MSNA signal was calculated by multiplying mean normalized burst amplitude by burst frequency. In women, baseline total MSNA was regressed against concentrations of circulating sex hormones in the EF phase and in the ML phase to determine whether baseline MSNA was graded to the level of circulating E2, P4, or T in either phase. Also, to determine whether the magnitude of the increase in baseline MSNA from the EF to the ML phase was associated with the magnitude of the change in circulating sex hormone concentrations, EF total MSNA levels and sex hormone concentrations were subtracted from ML values in each subject. The resulting delta values were then regressed against each other.

4.2.6   Statistical Analyses

Comparisons of hormones and baseline measures were conducted using paired (EF *vs* ML) or unpaired (men *vs* EF and ML) T-tests; the alpha value for these and all *post hoc* comparisons were corrected for multiple comparisons using the Bonferroni method. Hemodynamic and sympathetic responses to chemoreflex stimulation were

analyzed using three separate mixed ANOVAs, comparing (1) EF to ML, (2) men to EF, and (3) men to ML.

4.3 Results

Baseline subject characteristics are presented in Table 4.2. Sympathetic burst frequency and total activity were greater in the ML phase of the menstrual cycle than the EF phase. As a result, total MSNA and burst frequency were greater in men than women in the EF phase but similar between men and women in the ML phase. Baseline MSNA burst amplitude distribution median values were similar between EF (46 ± 13), ML (51 ± 9), or men (44 ± 7 a.u.). In women, no significant correlations between baseline total MSNA and concentrations of circulating E2, P4, or T were observed in either the EF phase or the ML phase when the menstrual cycle phases were examined independently (Figure 4.1). A significant, positive relationship was observed between the magnitude of the ML-phase induced increase in total MSNA and the menstrual cycle-induced increase in P4, although this relationship was not observed between total MSNA and E2 or T.

Apnea characteristics are presented in Table 4.3. Apneas were maintained for similar durations and post-apnea PO_2 reached similar levels in all groups. Post-apnea PCO_2 was similar between EF and ML phases, but higher in men relative to the ML phase (P=0.01).

Chemoreflex stimulation was associated with progressive increases in total MSNA during rebreathing, APN-P1, and APN-P2 conditions due to elevations in MSNA burst frequency and amplitude (Figure 4.2). The increases in total MSNA, burst frequency, and burst amplitude were greater in the EF than the ML phase. A statistical

interaction between menstrual cycle phase and chemoreflex condition indicated that the difference in total MSNA and burst amplitude between EF and ML phases was most pronounced during APN-P2. The increases in total MSNA and in burst frequency were greater in women in the EF phase than in men, while burst amplitude was not significantly different between women and men. All changes in MSNA were similar between men and women in the ML phase.

Individual patterns of changes in MSNA from EF to ML menstrual cycle phases are presented in Figure 4.3; from EF to ML menstrual cycle phases, the majority of women experienced an increase in baseline total MSNA, and a reduction in peak total MSNA as evoked by chemoreflex activation.

Hemodynamic responses to chemoreflex stimulation were similar between men and women and across menstrual cycle phases with the exception of HR, which was greater in women than men (Figure 4.4).

Table 4.2: Baseline hemodynamics and sympathetic nerve activity in women and men

	Women		Men
	EF	ML	
MAP (mmHg)	86 ± 6	84 ± 6	$92 \pm 8^{\dagger}$
SBP (mmHg)	114 ± 13	112 ± 11	$127 \pm 9*^{\dagger}$
DBP (mmHg)	67 ± 9	67 ± 4	73 ± 7
HR (beats $\cdot$ min^{-1})	61 ± 7	64 ± 7	$54 \pm 7*^{\dagger}$
SV$_i$ (mL $\cdot$ m^2)	47 ± 8	46 ± 7	51 ± 7
Q$_i$ (L $\cdot$ min$^{-1} \cdot$ m^2)	2.9 ± 0.8	3.0 ± 0.6	2.7 ± 0.5
TPR (mmHg $\cdot$ L$^{-1} \cdot$ min^{-1})	18 ± 3	17 ± 5	17 ± 3
MSNA burst frequency (bursts $\cdot$ min^{-1})	10 ± 5	$14 \pm 7*$	$18 \pm 5*$
Total MSNA (a.u.)	466 ± 203	$714 \pm 317*$	$854 \pm 239*$

Data are mean $\pm$ standard deviation. EF, early follicular phase; ML, midluteal phase; MAP, mean arterial pressure; SBP, systolic blood pressure; DBP, diastolic blood pressure; HR, heart rate; SV$_i$, stroke volume index; Q$_i$, cardiac index; TPR, total peripheral resistance; MSNA, muscle sympathetic nerve activity. * P<0.05 *vs* EF; † P<0.05 *vs* ML

Table 4.3: Apnea characteristics in men and women

	Women		Men
	EF	ML	
Apnea Duration (s)	21 ± 7	22 ± 10	26 ± 8
End-Apnea PO_2 (Torr)	61 ± 4	61 ± 6	59 ± 4
End-Apnea PCO_2 (Torr)	54 ± 4	51 ± 3	56 ± 4

Data presented as mean $\pm$ standard deviation. PO_2 = partial pressure of oxygen; PCO_2 = partial pressure of carbon dioxide; EF = early follicular; ML = midluteal.
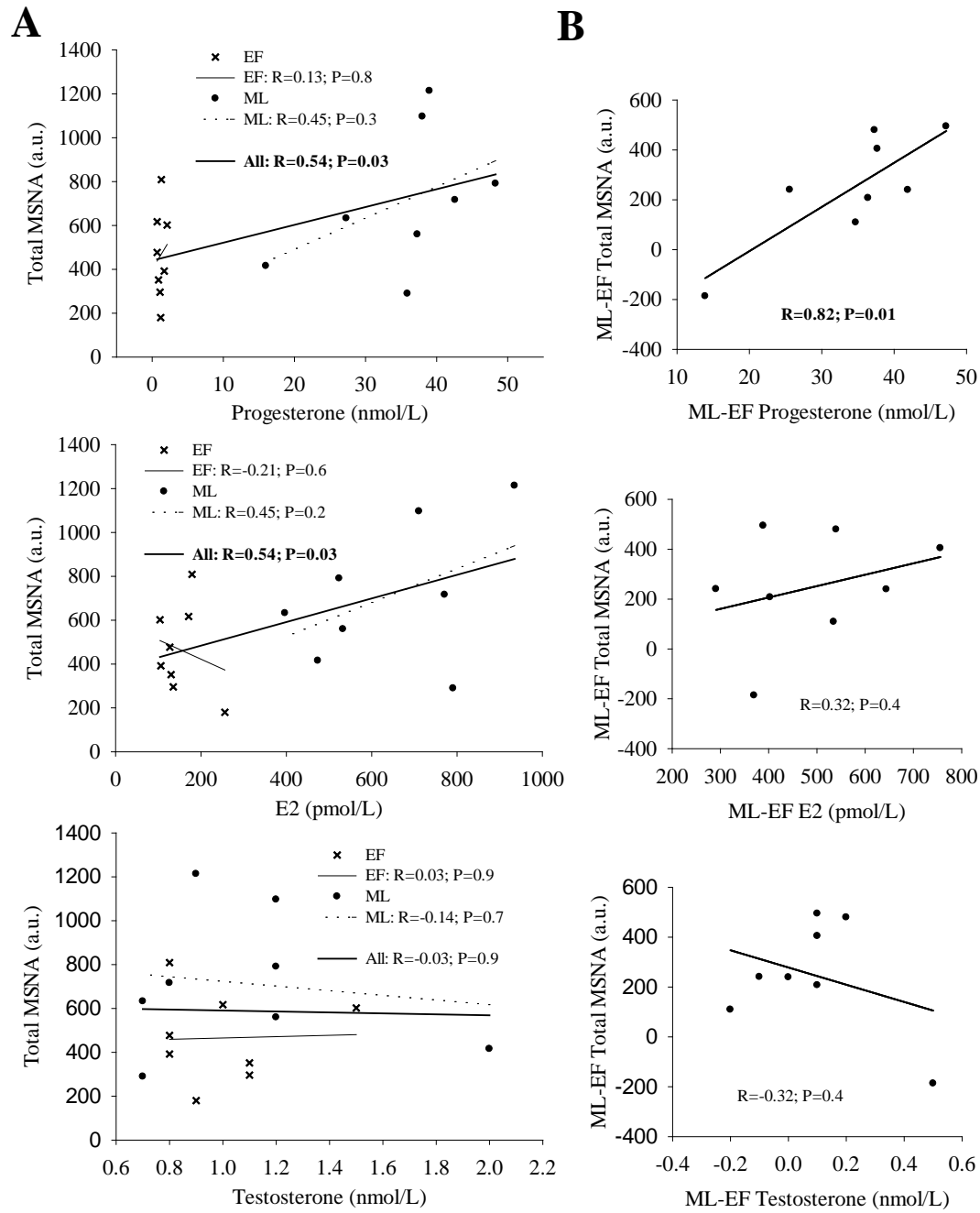
Figure 4.1: Associations between sympathetic nerve activity and circulating sex hormone concentrations at baseline.
No significant associations between sex hormone concentrations and total muscle sympathetic nerve activity (MSNA) were observed in either menstrual cycle phase alone (panel A). However, the magnitude of the change in total MSNA from EF to ML was positively related to the change in circulating progesterone across the menstrual cycle (panel B). EF, early follicular; ML, midluteal.
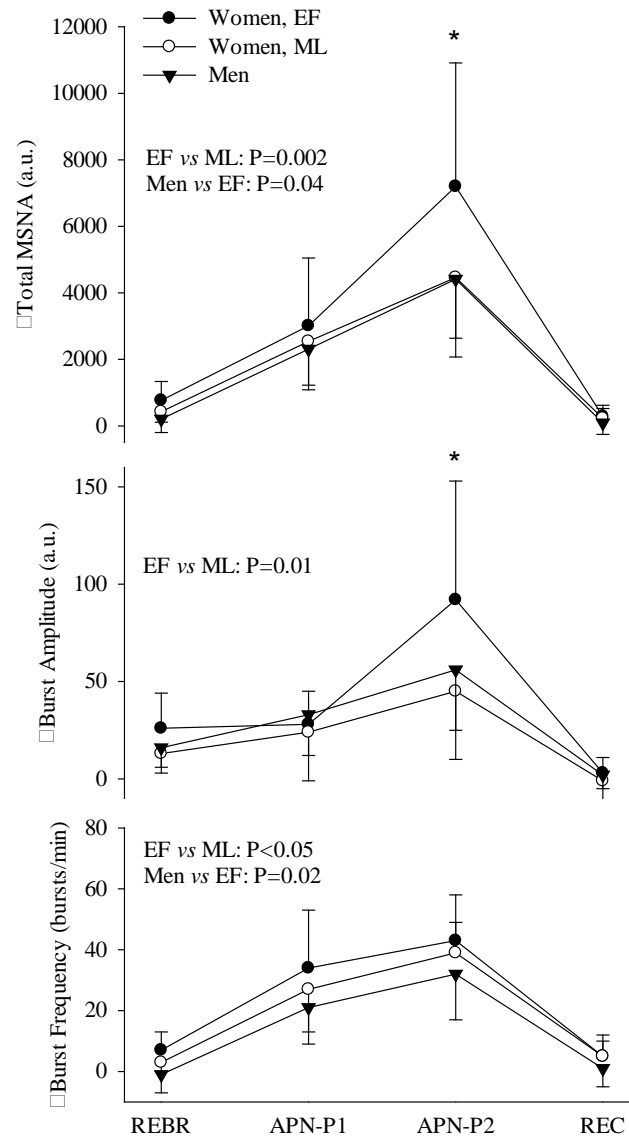
Figure 4.2: Sympathetic responses to chemoreflex stimulation.

Chemoreflex stimulation was associated with progressive increases in Δ-total MSNA, Δ-burst amplitude, and Δ-burst frequency in men and women in early follicular (EF) and midluteal (ML) menstrual cycle phases (all $P<0.001$). Δ-Total MSNA and Δ-burst frequency were greater in the EF phase relative to both the ML phase and to men. Δ-Burst amplitude was greater in the EF phase than ML, but not significantly different from men. Men had similar MSNA responses as women in the ML phase in all comparisons. Significant phase x chemoreflex interactions were observed in Δ-total MSNA ($P=0.02$) and Δ-burst amplitude ($P=0.02$).Data are mean ± standard deviation. * denotes $P<0.05$, EF vs ML; MSNA, muscle sympathetic nerve activity; BSL, baseline; REBR, rebreathing; APN-P1, initial half of apnea; APN-P2, latter half of apnea; REC, recovery.
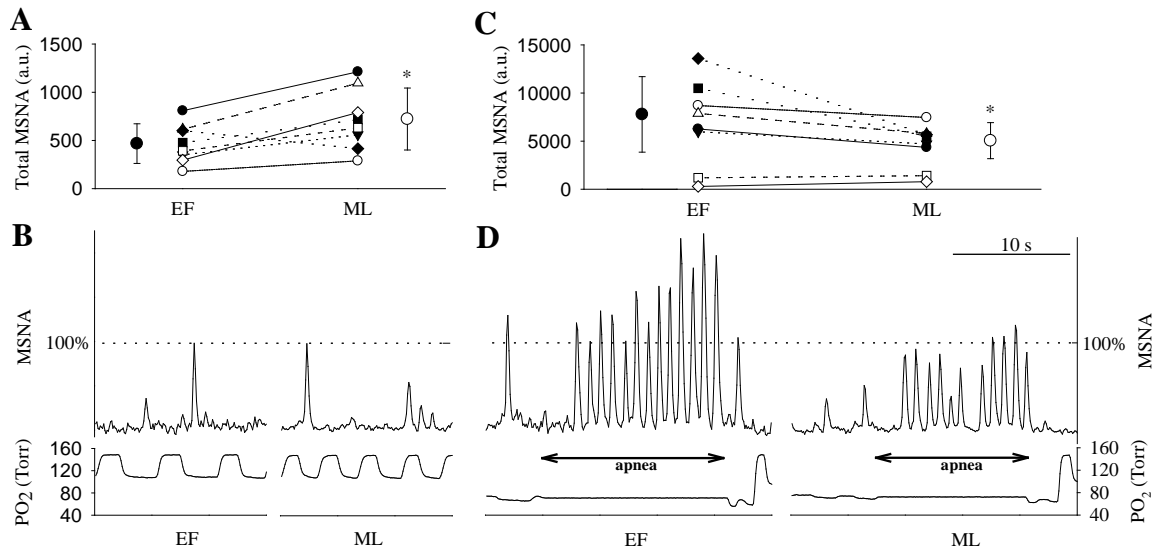
Figure 4.3: Individual patterns of total muscle sympathetic nerve activity across the menstrual cycle.

Panel A contains individual data from all women at baseline; circles and error bars are means and standard deviations of EF and ML phases; * denotes $P< 0.05$ *versus* EF. Panel B shows tracings of 15s of baseline recordings from a representative subject during EF and ML phases. Panel C contains individual data from all women during the latter half of the apnea (APN-P2). Panel D shows tracings of the end-inspiratory apnea performed during EF and ML phases.
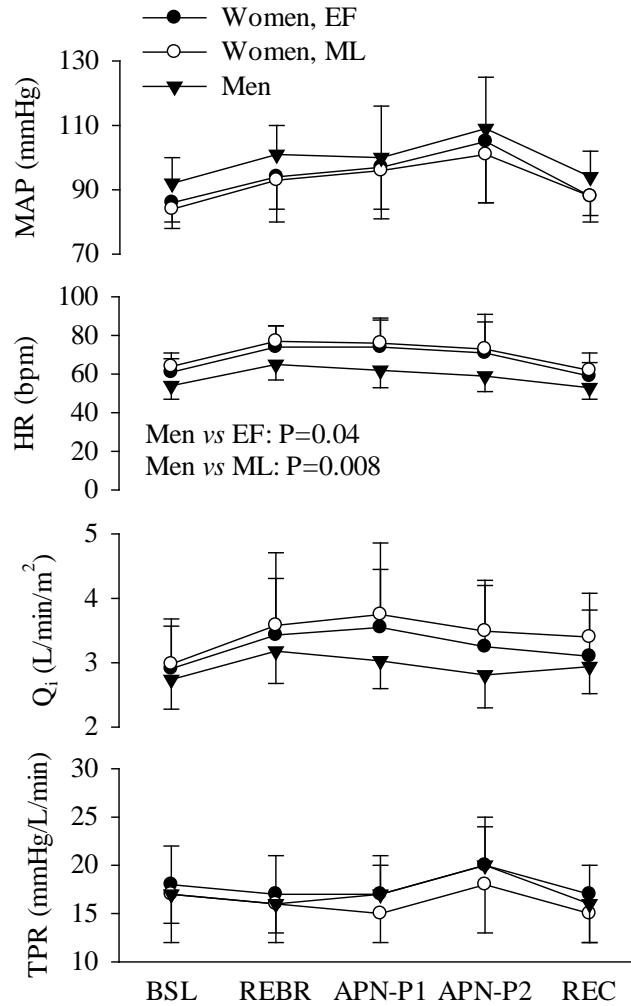
Figure 4.4: Hemodynamic responses to chemoreflex stimulation.

Chemoreceptor stimulation was associated with significant changes in mean arterial pressure (MAP), heart rate (HR), cardiac index (Q_i), and total peripheral resistance (TPR) (all $P<0.01$). Heart rate was higher in women than men. Data are mean ± standard deviation. BSL, baseline; REBR, rebreathing; APN-P1, initial apnea half; APN-P2, latter apnea half; REC, recovery. [2]

4.4   Discussion

In this study, we examined menstrual cycle- and sex-specific regulation of sympathetic nerve activity during severe chemoreflex stimulation. We observed that severe chemoreflex stimulation elicits reflex increases in total MSNA which are larger in women in the EF phase of the menstrual cycle in comparison with men and also with women in the ML phase. This occurred as a result of elevations in the burst frequency component of MSNA which exceeded those observed in men and in women in the ML phase. On the other hand, the chemoreflex response in the burst amplitude component of MSNA was affected by menstrual cycle phase, but was not different between men and women. We also observed that, across all conditions, patterns of MSNA were similar between men and women in the ML phase. Overall, these data indicate that baseline MSNA and the sympatho-excitatory responses to severe chemoreflex stress differ between men and women, but only when women are measured during the EF phase of the menstrual cycle.

Many previous studies have compared baseline MSNA between young healthy women and men. The first direct comparison of MSNA between men and women, made by Ng and colleagues, demonstrated greater resting MSNA burst frequency in men relative to women (Ng *et al.*, 1993). Since then, this finding has been reproduced (Jones *et al.*, 1996b;Ng *et al.*, 1993;Shoemaker *et al.*, 2001;Matsukawa *et al.*, 1998;Yang *et al.*, 2012;Jones *et al.*, 1996a;Hart *et al.*, 2009), albeit not consistently (Kimmerly *et al.*, 2007;Fu *et al.*, 2009;Fu *et al.*, 2005;Jones *et al.*, 1999;Narkiewicz *et al.*, 2005). Of these studies, only a few have accounted for menstrual cycle phase in the female subjects (Kimmerly *et al.*, 2007;Yang *et al.*, 2012;Hart *et al.*, 2009;Fu *et al.*, 2009). It is likely that

this has contributed to the lack of consistency in the observation of sex differences in these studies, as elevations in midluteal baseline MSNA have been observed relative to the early follicular phase (Minson *et al.*, 2000;Park & Middlekauff, 2009;Middlekauff *et al.*, 2012;Carter *et al.*, 2013). Although this observation has not been universal (Carter *et al.*, 2009;Jones *et al.*, 1996b;Fu *et al.*, 2009;Jarvis *et al.*, 2011;Carter & Lawrence, 2007), recent evidence suggests that changes concentrations of circulating sex hormones, which are altered across the menstrual cycle, are associated with changes in baseline MSNA (Carter *et al.*, 2013;Day *et al.*, 2011). These studies indicate that acute increases in circulating P4 mediate a sympatho-excitatory effect, while increases in E2 promote sympatho-inhibition (Carter *et al.*, 2013;Day *et al.*, 2011).However, one study quantified the extent to which a ratio of E2-to-P4 was associated with baseline MSNA, and determined that only approximately 27% of the variance in MSNA was explained by alterations in these sex hormones (Carter *et al.*, 2013). The authors suggested that increases in testosterone might occur across the menstrual cycle and contribute to the ML phase sympatho-excitation. In the present study we observed no change in testosterone across the menstrual cycle, and no associations between testosterone concentrations and baseline MSNA. On the other hand, the present data support a positive association between the increase in P4 from EF to ML menstrual cycle phases and the magnitude of the elevation in resting MSNA.

In the present study we also compared sex and menstrual cycle effects on MSNA regulation during a strong chemoreflex stimulus. In coupling the sympatho-excitatory effect of combined hypoxia-hypercapnia (Morgan *et al.*, 1995) with the elimination of the sympatho-inhibitory effect of the thoracic afferent nerves through the use of apnea

(Somers *et al.*, 1989;Steinback *et al.*, 2010a), the stimulus was designed to elicit a maximal or near-maximal elevation in MSNA. During chemoreflex activation, increases in total MSNA and MSNA burst frequency were greatest in women in the EF phase of the menstrual cycle and exceeded the MSNA responses observed in men and in the ML phase of the menstrual cycle. These data are in partial support of the previous work which reported hypoxia-driven increases in total MSNA which were evoked with a shorter latency in women than in men (Jones *et al.*, 1999). However, this previous study did not systematically study the effect of menstrual cycle on sympathetic responses to chemoreflex stimulation (Jones *et al.*, 1999). Our data, which indicate that chemoreflex-driven increases in MSNA were similar between men and women in the ML phase, suggest that chemoreflex responses are not regulated equally across the menstrual cycle. To the best of our knowledge, this is the first study to systematically study sympathetic responses to chemoreflex stimulation across the menstrual cycle. However, our laboratory recently conducted a similar study in regular users of hormonal contraceptives (Usselman *et al.*, 2013). In that previous study, during chemoreflex activation the low-hormone phase of hormonal contraceptive use was associated with a greater total MSNA response than the high-hormone phase (Usselman *et al.*, 2013). Together, these data indicate that the patterns of sympathetic responses to severe chemoreflex stress appear to be similar between users and non-users of hormonal contraceptives.

An interesting observation in this study was the difference between menstrual cycle phases in the regulation of the burst amplitude component of MSNA. During the EF phase, burst amplitude increased during the most severe phase of chemoreflex stimulation, an increase which was not observed during the ML phase. A similar finding

was observed in our previous work with regular users of hormonal contraceptives: an increase in burst amplitude was observed in the low hormone phase of contraceptive use which exceeded that observed in the high hormone phase (Usselman *et al.*, 2013). Also, in both the users of hormonal contraceptives and the women examined in the present study, the increases in burst amplitude contributed to an overall augmentation of the total MSNA response in the low hormone phases relative to the high hormone phases. While an elevated burst frequency response was also observed in the EF phase relative to the ML, these data might suggest that severe chemoreflex activation preferentially excites the burst amplitude, or strength, component of the MSNA signal, an effect which is amplified when circulating sex hormone levels are lower. Whether this is due to the removal of an inhibitory influence of sex hormones over the amplitude component requires further study.

Limitations

Menstrual cycle phases were selected based on the desire to test the nadir of hormone secretions (i.e. EF) against a prolonged increase in E2 and P4 production (ML). However, we did not study the late follicular phase. In light of our findings, the study of this phase would present an interesting opportunity to observe elevated E2 without a concurrent change in P4, and perhaps further elucidate the relationship between E2 and MSNA.

The chemoreflex stress used in this study was designed to elicit large sympatho-excitatory responses from the subjects. However, the protocol was not designed to

differentiate between the specific hemodynamic and sympathetic effects of hypoxia and hypercapnia (Steinback *et al.*, 2009).

In summary, baseline MSNA burst frequency and total MSNA were affected by both sex and the menstrual cycle, and the observation of baseline sex differences were menstrual cycle-dependent. Similarly, chemoreflex stimulation produced menstrual cycle-dependent sex effects, but to the opposite effect: increases in MSNA burst frequency and total MSNA were greater in women than in men, and greater in the EF phase than the ML phase of the menstrual cycle. The menstrual cycle also affected MSNA burst amplitude, with larger increases in amplitude in the EF phase relative to the ML phase. The results suggest that, when possible, future studies examining sex differences in sympathetic activation should include consideration for menstrual cycle phase. Future studies may also benefit from examining MSNA in women in more than one menstrual cycle phase to generate a more complete picture of factors affecting sympathetic regulation in women.

4.5   References

Britton SW & Kline RF (1945). Age, sex, carbohydrate, adrenal cortex and other factors in anoxia. *Am J Physiol* 145, 190-202.

Carlson JT, Hedner J, Elam M, Ejnell H, Sellgren J, & Wallin BG (1993). Augmented resting sympathetic activity in awake patients with obstructive sleep apnea. *Chest* 103, 1763-1768.

Carter JR, Fu Q, Minson CT, & Joyner MJ (2013). Ovarian cycle and sympathoexcitation in premenopausal women. *Hypertension* 61, 395-399.

Carter JR & Lawrence JE (2007). Effects of the menstrual cycle on sympathetic neural responses to mental stress in humans. *J Physiol* 585, 635-641.

Carter JR, Lawrence JE, & Klein JC (2009). Menstrual cycle alters sympathetic neural responses to orthostatic stress in young, eumenorrheic women. *Am J Physiol Endocrinol Metab* 297, E85-E91.

Criqui MH, Fronek A, Barrett-Connor E, Klauber MR, Gabriel S, & Goodman D (1985). The prevalence of peripheral arterial disease in a defined population. *Circulation* 71, 510-515.

Day DS, Gozansky WS, Bell C, & Kohrt WM (2011). Acute sex hormone suppression reduces skeletal muscle sympathetic nerve activity. *Clin Auton Res* 21, 339-345.

Delius W, Hagbarth KE, Hongell A, & Wallin BG (1972). Manoeuvres affecting sympathetic outflow in human muscle nerves. *Acta Physiol Scand* 84, 82-94.

Eaker ED, Packard B, & Thom TJ (1989). Epidemiology and risk factors for coronary heart disease in women. *Cardiovasc Clin* 19, 129-145.

Fu Q, Okazaki K, Shibata S, Shook RP, VanGunday TB, Galbreath MM, Reelick MF, & Levine BD (2009). Menstrual cycle effects on sympathetic neural responses to upright tilt. *The Journal of Physiology* 587, 2019-2031.

Fu Q, Witkowski S, Okazaki K, & Levine BD (2005). Effects of gender and hypovolemia on sympathetic neural responses to orthostatic stress. *Am J Physiol Regul Integr Comp Physiol* 289, R109-R116.

Hart EC, Charkoudian N, Wallin BG, Curry TB, Eisenach JH, & Joyner MJ (2009). Sex differences in sympathetic neural-hemodynamic balance: Implications for human blood pressure regulation. *Hypertension* 53, 571-576.

Hedner J, Ejnell H, Sellgren J, Hedner T, & Wallin G (1988). Is high and fluctuating muscle nerve sympathetic activity in the sleep apnoea syndrome of pathogenetic importance for the development of hypertension? *J Hypertens Suppl* 6, S529-S531.

Hla KM, Young TB, Bidwell T, Palta M, Skatrud JB, & Dempsey J (1994). Sleep apnea and hypertension. A population-based study. *Ann Intern Med* 120, 382-388.

Jarvis SS, VanGundy TB, Galbreath MM, Shibata S, Okazaki K, Reelick MF, Levine BD, & Fu Q (2011). Sex differences in the modulation of vasomotor sympathetic outflow during static handgrip exercise in healthy young humans. *Am J Physiol Regul Integr Comp Physiol* 301, R193-R200.

Jones PP, Davy KP, & Seals DR (1999). Influence of gender on the sympathetic neural adjustments to alterations in systemic oxygen levels in humans. *Clin Physiol* 19, 153-160.

Jones PP, Snitker S, Skinner JS, & Ravussin E (1996a). Gender differences in muscle sympathetic nerve activity: effect of body fat distribution. *Am J Physiol Endocrinol Metab* 270, E363-E366.

Jones PP, Spraul M, Matt KS, Seals DR, Skinner JS, & Ravussin E (1996b). Gender does not influence sympathetic neural reactivity to stress in healthy humans. *Am J Physiol Heart Circ Physiol* 270, H350-H357.

Jordan AS & McEvoy RD (2003). Gender differences in sleep apnea: epidemiology, clinical presentation and pathogenic mechanisms. *Sleep Med Rev* 7, 377-389.

Kienbaum P, Karlssonn T, Sverrisdottir YB, Elam M, & Wallin BG (2001). Two sites for modulation of human sympathetic activity by arterial baroreceptors? *J Physiol* 531, 861-869.

Kimmerly DS, O'Leary DD, & Shoemaker JK (2004). Test-retest repeatability of muscle sympathetic nerve activity: influence of data analysis and head-up tilt. *Auton Neurosci* 114, 61-71.

Kimmerly DS, Wong S, Menon R, & Shoemaker JK (2007). Forebrain neural patterns associated with sex differences in autonomic and cardiovascular function during baroreceptor unloading. *Am J Physiol Regul Integr Comp Physiol* 292, R715-R722.

Lam TK & Leung DT (1988). More on simplified calculation of body-surface area. *N Engl J Med* 318, 1130.

Malpas SC & Ninomiya I (1992). The amplitude and periodicity of synchronized renal sympathetic nerve discharges in anesthetized cats: differential effect of baroreceptor activity. *J Auton Nerv Syst* 40, 189-198.

Matsukawa T, Sugiyama Y, Watanabe T, Kobayashi F, & Mano T (1998). Gender difference in age-related changes in muscle sympathetic nerve activity in healthy subjects. *Am J Physiol Regul Integr Comp Physiol* 275, R1600-R1604.

Middlekauff HR, Park J, & Gornbein JA (2012). Lack of effect of ovarian cycle and oral contraceptives on baroreceptor and nonbaroreceptor control of sympathetic nerve activity in healthy women. *Am J Physiol Heart Circ Physiol* 302, H2560-H2566.

Minson CT, Halliwill JR, Young TM, & Joyner MJ (2000). Influence of the Menstrual Cycle on Sympathetic Activity, Baroreflex Sensitivity, and Vascular Transduction in Young Women. *Circulation* 101, 862-868.

Morgan BJ, Crabtree DC, Palta M, & Skatrud JB (1995). Combined hypoxia and hypercapnia evokes long-lasting sympathetic activation in humans. *J Appl Physiol (1985)* 79, 205-213.

Mosteller RD (1987). Simplified calculation of body-surface area. *N Engl J Med* 317, 1098.

Narkiewicz K, Phillips BG, Kato M, Hering D, Bieniaszewski L, & Somers VK (2005). Gender-Selective Interaction Between Aging, Blood Pressure, and Sympathetic Nerve Activity. *Hypertension* 45, 522-525.

Ng AV, Callister R, Johnson DG, & Seals DR (1993). Age and gender influence muscle sympathetic nerve activity at rest in healthy humans. *Hypertension* 21, 498-503.

Nieto FJ, Young TB, Lind BK, Shahar E, Samet JM, Redline S, D'Agostino RB, Newman AB, Lebowitz MD, & Pickering TG (2000). Association of sleep-disordered breathing, sleep apnea, and hypertension in a large community-based study. Sleep Heart Health Study. *JAMA* 283, 1829-1836.

Park J & Middlekauff HR (2009). Altered pattern of sympathetic activity with the ovarian cycle in female smokers. *Am J Physiol Heart Circ Physiol* 297, H564-H568.

Rosenthal T & Oparil S (2000). Hypertension in women. *J Hum Hypertens* 14, 691-704.

Saito M, Mano T, Iwase S, Koga K, Abe H, & Yamazaki Y (1988). Responses in muscle sympathetic activity to acute hypoxia in humans. *J Appl Physiol (1985)* 65, 1548-1552.

Salmanpour A, Frances MF, Goswami R, & Shoemaker JK (2011). Sympathetic neural recruitment patterns during the Valsalva maneuver. *Conf Proc IEEE Eng Med Biol Soc* 2011, 6951-6954.

Shoemaker JK, Hogeman CS, Khan M, Kimmerly DS, & Sinoway LI (2001). Gender affects sympathetic and hemodynamic response to postural stress. *Am J Physiol Heart Circ Physiol* 281, H2028-H2035.

Smith ML & Muenter NK (2000). Effects of hypoxia on sympathetic neural control in humans. *Respir Physiol* 121, 163-171.

Somers VK, Mark AL, Zavala DC, & Abboud FM (1989). Contrasting effects of hypoxia and hypercapnia on ventilation and sympathetic activity in humans. *J Appl Physiol (1985)* 67, 2101-2106.

Steinback CD, Breskovic T, Frances M, Dujic Z, & Shoemaker JK (2010a). Ventilatory restraint of sympathetic activity during chemoreflex stress. *Am J Physiol Regul Integr Comp Physiol* 299, R1407-R1414.

Steinback CD, Salmanpour A, Breskovic T, Dujic Z, & Shoemaker JK (2010b). Sympathetic neural activation: an ordered affair. *J Physiol* 588, 4825-4836.

Steinback CD, Salzer D, Medeiros PJ, Kowalchuk J, & Shoemaker JK (2009). Hypercapnic vs. hypoxic control of cardiovascular, cardiovagal, and sympathetic function. *Am J Physiol Regul Integr Comp Physiol* 296, R402-R410.

Sverrisdottir YB, Rundqvist B, Johannsson G, & Elam M (2000). Sympathetic neural burst amplitude distribution: A more specific indicator of sympathoexcitation in human heart failure. *Circulation* 102, 2076-2081.

Usselman CW, Luchyshyn TA, Gimon TI, Nielson CA, Van Uum SH, & Shoemaker JK (2013). Hormone phase-dependency of neural responses to chemoreflex-driven sympatho-excitation in young women using hormonal contraceptives. *J Appl Physiol*.

Vallbo AB, Hagbarth KE, Torebjork HE, & Wallin BG (1979). Somatosensory, proprioceptive, and sympathetic activity in human peripheral nerves. *Physiol Rev* 59, 919-957.

Yang H, Cooke WH, Reed KS, & Carter JR (2012). Sex differences in hemodynamic and sympathetic neural firing patterns during orthostatic challenge in humans. *J Appl Physiol (1985)* 112, 1744-1751.

Young T, Palta M, Dempsey J, Skatrud J, Weber S, & Badr S (1993). The occurrence of sleep-disordered breathing among middle-aged adults. *N Engl J Med* 328, 1230-1235.

Chapter 5

5 General Discussion

5.1 Perspectives

A relatively high level of sympathetic nerve activity is observed in young men in comparison with young women (Ng *et al.*, 1993;Matsukawa *et al.*, 1998;Narkiewicz *et al.*, 2005). This is thought to be linked to men's higher risk of cardiovascular disease, from which women appear to be relatively protected until menopause (Rosenthal & Oparil, 2000;Young *et al.*, 1993;Eaker *et al.*, 1989). It is not known, however, why women lose this cardioprotection following menopause (Rosano *et al.*, 2007). Similar to post-menopausal women, an elevated risk of cardiovascular disease is also present in women with polycystic ovary syndrome (Meyer *et al.*, 2012;Ehrmann *et al.*, 2006;Schlaich *et al.*, 2011). Like post-menopausal women, polycystic ovary syndrome is also associated with a change in the sex hormone milieu and an increase in baseline sympathetic nerve activity (Abbott *et al.*, 2002;Sverrisdottir *et al.*, 2008). In both cases, the roles that sex hormones might play in the alterations to sympathetic and/or cardiovascular control are not well described. Also, although synthetic hormone supplementation is prescribed as a matter of course in both conditions, the effects of synthetic hormones on sympathetic regulation are not well known. Even in young, healthy women, knowledge regarding interactions between changes in sex hormones and sympathetic regulation has only begun to emerge in the past 15 years. Therefore, in these studies we sought to generate a more in-depth understanding of how sympathetic regulation patterns relate to changes in hormone levels. We examined acute changes in sex hormone levels in young, healthy women free of cardiovascular disease and

hormonal abnormalities with the hope that a greater understanding of normal interactions between sex hormones and MSNA might help to shed light on the mechanisms which are disturbed in clinical states.

5.2 Major Findings

In this series of studies we observed several novel findings regarding interactions between sex hormones and MSNA which occur across the menstrual cycle and across phases of hormonal contraceptive use. A major finding of these studies was that associations between sympathetic nerve activity and hormone phase were not dependent on the source of the alteration to the hormonal milieu (i.e. across the menstrual cycle or as a result of the use of hormonal contraceptives). In contrast with previous studies (Middlekauff *et al.*, 2012;Minson *et al.*, 2000b;Minson *et al.*, 2000a;Carter *et al.*, 2009a;Carter *et al.*, 2009b), we observed similar muscle sympathetic nerve activation patterns between the early follicular phase of the menstrual cycle and the low hormone phase of contraceptive use, as well as between the midluteal phase of the menstrual cycle and the high hormone phase of contraceptive use. Furthermore, these similarities were observed under all conditions, including at baseline and during baroreceptor unloading and chemoreflex stimulation. These results imply that the changes in endogenous hormones which occur across the menstrual cycle exert a similar influence over the regulation of MSNA as the changes in the exogenous hormones contained within the hormonal contraceptives used by our subjects. However, it is important to note that while all contraceptives used in this study were combination formulations with low doses of estradiol and low-androgenic activity progestins, we did not control for the specific type of hormonal contraceptive in these studies. Therefore, further research is required to

determine why certain hormonal contraceptives may mimic the sympathetic effects of endogenous hormones while others may not.

Another major finding of these studies was that high hormone phases were associated with relative elevations in baseline sympathetic nerve activity in comparison with the low hormone phases, a trend which was reversed during large, chemoreflex-induced sympatho-excitatory stress. The hypoxic-hypercapnic end-inspiratory apnea produced larger reflex increases in MSNA in the lower hormone phases than those observed during the higher hormone phases (see Table 5.1 for a summary of findings). Although this observation was initially unanticipated (see Chapter 3), known sex differences in sympathetic regulation may provide support for these results. For instance, large-scale studies have reported that baseline MSNA is higher in young men than young women (Ng *et al.*, 1993;Matsukawa *et al.*, 1998;Narkiewicz *et al.*, 2005). On other hand, it has been reported that chemoreflex-driven increases in MSNA are greater in women than in men (Jones *et al.*, 1999). These results could be interpreted to indicate that the factors which drive relative increases in MSNA at baseline are not effective during chemoreflex-driven sympatho-excitation, during which other mechanisms contribute to increases in MSNA.

A main finding of Study 1 (Chapter 2) stemmed from the observation of greater reductions in stroke volume for a given lower body negative pressure stimulus during the high hormone phases when compared to the low hormone phases. This had not been observed by previous studies of simulated orthostasis (Fu *et al.*, 2009;Carter *et al.*, 2009b;Carter *et al.*, 2009a), although it had been hypothesized to occur (Fu *et al.*, 2009). The greater drop in stroke volume implied that a greater unloading of the baroreceptors

occurred in the high hormone phases for an equivalent orthostatic stress. Following the subsequent normalization of the sympathetic responses relative to baroreceptor stimuli, we observed no difference between hormone phases in the sympathetic responses to baroreceptor unloading. These data imply that the integration of afferent baroreceptor information is unaffected by hormone phase, as had been reported previously (Fu *et al.*, 2009;Carter *et al.*, 2009b;Middlekauff *et al.*, 2012).

Table 5.1: Summary of muscle sympathetic nerve activity results

	Strength of Stimulus	Total MSNA Response	MSNA Burst Frequency Response	MSNA Burst Amplitude Response
Baseline	-	HI > LO	HI > LO	LO = HI
Baroreceptor Unloading (LBNP)	HI > LO [†]	HI > LO [††]	HI > LO [††]	LO = HI
Chemoreflex Stimulation (Hypoxic-Hypercapnic Apnea)	LO = HI [‡]	LO > HI	LO > HI	LO > HI

Low (LO) hormone phases are EF phase of menstrual cycle and LH phase of hormonal contraceptive use; high (HI) hormone phases are ML phase of menstrual cycle and HH phase of hormonal contraceptive use.

† Stroke volume was lower in ML and HH than in EF and LH, indicating greater baroreceptor unloading in these phases.

†† When normalized to the fall in stroke volume, these responses were no longer different between high and low hormone phases.

‡ Both indicators of chemoreflex stress (partial pressures of oxygen and carbon dioxide) and the duration of the apnea were similar between low and high hormone phases, indicating a similar sympatho-excitatory stimulus.

In this series of studies, separate consideration was given to the regulation of the individual components of total MSNA: burst frequency and burst amplitude. This was done within the context of the hypothesis that baroreceptor feedback gates efferent sympathetic activity, thereby affecting MSNA burst frequency, while other peripheral inputs are integrated to regulate MSNA burst amplitude (Malpas, 1995;Kienbaum *et al.*, 2001). Baseline MSNA recordings indicated a relative increase of MSNA burst frequency in the high hormone phases with no change in the regulation of burst amplitude. However, chemoreflex activation was associated with a reversal of this trend as greater levels of burst frequency were observed in the low hormone phases. Severe chemoreflex stimulation was also associated with a large increase in MSNA burst amplitude in the low hormone phases which was not observed in the high hormone phases. The baseline data suggest that a relatively elevated sex hormone milieu appears to promote the augmentation of the burst frequency component of MSNA, which is thought to be regulated centrally through a baroreceptor-related mechanism (Kienbaum *et al.*, 2001). However, the chemoreflex data suggest that an elevated sex hormone milieu may exert a sympatho-inhibitory effect over the burst amplitude component of MSNA. Therefore, large reflexive increases in burst amplitude are achieved only in low hormone phases.

An interesting caveat to the data presented here is that the changes in sympathetic responses observed across hormone phases were not coupled with similar changes in peripheral resistance. A lack of congruity between MSNA and peripheral resistance has been reported previously in young women (Hart *et al.*, 2009). Several explanations have been put forth to account for this, including the buffering of the sympathetic signal by activation of vasodilatory beta-adrenoreceptors (Hart *et al.*, 2011). Alternatively, the

vascular production of nitric oxide is increased by elevations in circulating estradiol (Sudhir *et al.*, 1996). An increase in circulating nitric oxide during the midluteal phase of the menstrual cycle would exert a dilatory influence over the vasculature, counteracting the vasoconstrictor influence of MSNA. Although we did not measure these vascular factors in the present study, future research targeting these mechanisms and addressing whether they are affected by changes in circulating sex hormones would provide further insight into the present findings.

In the context of sex hormone effects on sympathetic regulation, future research might also address the separate consideration of sex hormones in their possible effects on sympathetic nerve activity. The late follicular phase of the menstrual presents an opportunity to study sympathetic regulation during an increase in estradiol which is unaccompanied by a change in progesterone (Ettinger *et al.*, 1998;Miner *et al.*, 2011). Alternatively, short-term changes in circulating hormone concentrations can be elicited experimentally through the use of gonadotropin releasing hormone antagonist therapy, which dramatically reduces ovarian production of estradiol and progesterone. Estradiol and progesterone can then be added back to the subjects independently to examine their unique effects. This technique has been coupled with microneurography once to date (Day *et al.*, 2011), and while the study was conducted in a small group of women, the data indicate a significant sympatho-inhibitory effect of estrogen, and a trend towards a sympatho-excitatory effect of progesterone. Further work in this area would shed more light on the roles of sex hormones in the regulation of MSNA, and could have implications for the development of hormonal treatments of sympathetic irregularities within the clinical realm.

5.3   Conclusions

In this series of studies, we have demonstrated for the first time that sympathetic responses to chemoreflex stimulation are affected by hormone phases associated with the menstrual cycle and hormonal contraceptive use. We have also provided the first evidence that the baseline and reflex sympathetic influences of the endogenous hormones of the menstrual cycle may not differ from those exerted by synthetic hormones contained within contraceptives. Collectively, these studies confirm that MSNA recruitment is affected by hormone phase, but in a reflex-specific manner.

5.4 References

Abbott DH, Dumesic DA, & Franks S (2002). Developmental origin of polycystic ovary syndrome - a hypothesis. *J Endocrinol* **174**, 1-5.

Carter JR, Klein JC, & Schwartz CE (2009a). Effects of oral contraceptives on sympathetic nerve activity during orthostatic stress in young, healthy women. *Am J Physiol Regul Integr Comp Physiol* **298**, R9-R14.

Carter JR, Lawrence JE, & Klein JC (2009b). Menstrual cycle alters sympathetic neural responses to orthostatic stress in young, eumenorrheic women. *Am J Physiol Endocrinol Metab* **297**, E85-E91.

Day DS, Gozansky WS, Bell C, & Kohrt WM (2011). Acute sex hormone suppression reduces skeletal muscle sympathetic nerve activity. *Clin Auton Res* **21**, 339-345.

Eaker ED, Packard B, & Thom TJ (1989). Epidemiology and risk factors for coronary heart disease in women. *Cardiovasc Clin* **19**, 129-145.

Ehrmann DA, Liljenquist DR, Kasza K, Azziz R, Legro RS, & Ghazzi MN (2006). Prevalence and predictors of the metabolic syndrome in women with polycystic ovary syndrome. *J Clin Endocrinol Metab* **91**, 48-53.

Ettinger SM, Silber DH, Gray KS, Smith MB, Yang QX, Kunselman AR, & Sinoway LI (1998). Effects of the ovarian cycle on sympathetic neural outflow during static exercise. *J Appl Physiol* **85**, 2075-2081.

Fu Q, Okazaki K, Shibata S, Shook RP, VanGunday TB, Galbreath MM, Reelick MF, & Levine BD (2009). Menstrual cycle effects on sympathetic neural responses to upright tilt. *The Journal of Physiology* **587**, 2019-2031.

Hart EC, Charkoudian N, Wallin BG, Curry TB, Eisenach J, & Joyner MJ (2011). Sex and ageing differences in resting arterial pressure regulation: the role of the beta-adrenergic receptors. *J Physiol* **589**, 5285-5297.

Hart EC, Charkoudian N, Wallin BG, Curry TB, Eisenach JH, & Joyner MJ (2009). Sex differences in sympathetic neural-hemodynamic balance: Implications for human blood pressure regulation. *Hypertension* **53**, 571-576.

Jones PP, Davy KP, & Seals DR (1999). Influence of gender on the sympathetic neural adjustments to alterations in systemic oxygen levels in humans. *Clin Physiol* **19**, 153-160.

Kienbaum P, Karlssonn T, Sverrisdottir YB, Elam M, & Wallin BG (2001). Two sites for modulation of human sympathetic activity by arterial baroreceptors? *J Physiol* **531**, 861-869.

Malpas SC (1995). A new model for the generation of sympathetic nerve activity. *Clin Exp Pharmacol Physiol* **22**, 11-15.

Matsukawa T, Sugiyama Y, Watanabe T, Kobayashi F, & Mano T (1998). Gender difference in age-related changes in muscle sympathetic nerve activity in healthy subjects. *Am J Physiol Regul Integr Comp Physiol* **275**, R1600-R1604.

Meyer ML, Malek AM, Wild RA, Korytkowski MT, & Talbott EO (2012). Carotid artery intima-media thickness in polycystic ovary syndrome: a systematic review and meta-analysis. *Hum Reprod Update* **18**, 112-126.

Middlekauff HR, Park J, & Gornbein JA (2012). Lack of effect of ovarian cycle and oral contraceptives on baroreceptor and nonbaroreceptor control of sympathetic nerve activity in healthy women. *Am J Physiol Heart Circ Physiol* **302**, H2560-H2566.

Miner JA, Martini ER, Smith MM, Brunt VE, Kaplan PF, Halliwill JR, & Minson CT (2011). Short-term oral progesterone administration antagonizes the effect of transdermal estradiol on endothelium-dependent vasodilation in young healthy women. *Am J Physiol Heart Circ Physiol* **301**, H1716-H1722.

Minson CT, Halliwill JR, Young TM, & Joyner MJ (2000a). Influence of the Menstrual Cycle on Sympathetic Activity, Baroreflex Sensitivity, and Vascular Transduction in Young Women. *Circulation* **101**, 862-868.

Minson CT, Halliwill JR, Young TM, & Joyner MJ (2000b). Sympathetic Activity and Baroreflex Sensitivity in Young Women Taking Oral Contraceptives. *Circulation* **102**, 1473-1476.

Narkiewicz K, Phillips BG, Kato M, Hering D, Bieniaszewski L, & Somers VK (2005). Gender-Selective Interaction Between Aging, Blood Pressure, and Sympathetic Nerve Activity. *Hypertension* **45**, 522-525.

Ng AV, Callister R, Johnson DG, & Seals DR (1993). Age and gender influence muscle sympathetic nerve activity at rest in healthy humans. *Hypertension* **21**, 498-503.

Rosano GM, Vitale C, Marazzi G, & Volterrani M (2007). Menopause and cardiovascular disease: the evidence. *Climacteric* **10 Suppl 1**, 19-24.

Rosenthal T & Oparil S (2000). Hypertension in women. *J Hum Hypertens* **14**, 691-704.

Schlaich MP, Straznicky N, Grima M, Ika-Sari C, Dawood T, Mahfoud F, Lambert E, Chopra R, Socratous F, Hennebry S, Eikelis N, Bohm M, Krum H, Lambert G, Esler MD, & Sobotka PA (2011). Renal denervation: a potential new treatment modality for polycystic ovary syndrome? *J Hypertens* **29**, 991-996.

Sudhir K, Jennings GL, Funder JW, & Komesaroff PA (1996). Estrogen enhances basal nitric oxide release in the forearm vasculature in perimenopausal women. *Hypertension* **28**, 330-334.

Sverrisdottir YB, Mogren T, Kataoka J, Janson PO, & Stener-Victorin E (2008). Is polycystic ovary syndrome associated with high sympathetic nerve activity and size at birth? *Am J Physiol Endocrinol Metab* **294**, E576-E581.

Young T, Palta M, Dempsey J, Skatrud J, Weber S, & Badr S (1993). The occurrence of sleep-disordered breathing among middle-aged adults. *N Engl J Med* **328**, 1230-1235.

Appendix A: Ethics Approval

Office of Research Ethics

The University of Western Ontario
Room 4180 Support Services Building, London, ON, Canada N6A 5C1
Telephone: (519) 661-3036 Fax: (519) 850-2466 Email: ethics@uwo.ca
Website: www.uwo.ca/research/ethics

Western

Use of Human Subjects - Ethics Approval Notice

Principal Investigator: Dr. K. Shoemaker	
Review Number: 16912	**Review Level:** Full Board
Review Date: February 23, 2010	**Approved Local # of Participants:** 30
Protocol Title: Sex-specific hormone levels and reflex sympathoexcitation	
Department and Institution: Kinesiology, University of Western Ontario	
Sponsor: NSERC # 217916-2008	
Ethics Approval Date: March 12, 2010	**Expiry Date:** December 31, 2012
Documents Reviewed and Approved: UWO Protocol, Letter of information & consent form & Advertisement	
Documents Received for Information:	

This is to notify you that The University of Western Ontario Research Ethics Board for Health Sciences Research Involving Human Subjects (HSREB) which is organized and operates according to the Tri-Council Policy Statement: Ethical Conduct of Research Involving Humans and the Health Canada/ICH Good Clinical Practice Practices: Consolidated Guidelines; and the applicable laws and regulations of Ontario has reviewed and granted approval to the above referenced study on the approval date noted above. The membership of this REB also complies with the membership requirements for REB's as defined in Division 5 of the Food and Drug Regulations.

The ethics approval for this study shall remain valid until the expiry date noted above assuming timely and acceptable responses to the HSREB's periodic requests for surveillance and monitoring information. If you require an updated approval notice prior to that time you must request it using the UWO Updated Approval Request Form.
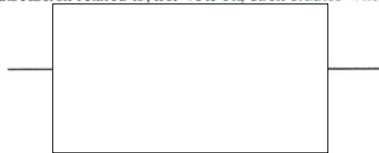
During the course of the research, no deviations from, or changes to, the protocol or consent form may be initiated without prior written approval from the HSREB except when necessary to eliminate immediate hazards to the subject or when the change(s) involve only logistical or administrative aspects of the study (e.g. change of monitor, telephone number). Expedited review of minor change(s) in ongoing studies will be considered. Subjects must receive a copy of the signed information/consent documentation.

Investigators must promptly also report to the HSREB:

- a) changes increasing the risk to the participant(s) and/or affecting significantly the conduct of the study;
- b) all adverse and unexpected experiences or events that are both serious and unexpected;
- c) new information that may adversely affect the safety of the subjects or the conduct of the study.

If these changes/adverse events require a change to the information/consent documentation, and/or recruitment advertisement, the newly revised information/consent documentation, and/or advertisement, must be submitted to this office for approval.

Members of the HSREB who are named as investigators in research studies, or declare a conflict of interest, do not participate in discussion related to, nor vote on, such studies when they are presented to the HSREB.

Chair of HSREB: Dr. Joseph Gilbert
FDA Ref. #: IRB 00000940

Ethics Officer to Contact for Further Information			
☑ Janice Sutherland	☐ Elizabeth Wambolt	☐ Grace Kelly	☐ Denise Grafton

This is an official document. Please retain the original in your files. cc: ORE File

UWO HSREB Ethics Approval - Initial
V.2008-07-01 (rptApprovalNoticeHSREB_Initial)   16912   Page 1 of 1

Office of Research Ethics

The University of Western Ontario
Room 4180 Support Services Building, London, ON, Canada N6A 5C1
Telephone: (519) 661-3036 Fax: (519) 850-2466 Email: ethics@uwo.ca
Website: www.uwo.ca/research/ethics

Western

Use of Human Subjects - Ethics Approval Notice

Principal Investigator: Dr. K. Shoemaker	
Review Number: 16914	**Review Level:** Full Board
Review Date: February 23, 2010	**Approved Local # of Participants:** 50
Protocol Title: Sex-specific hormone levels and reflex sympathoinhibition	
Department and Institution: Kinesiology, University of Western Ontario	
Sponsor: NSERC #217916-2008	
Ethics Approval Date: March 12, 2010	**Expiry Date:** December 31, 2012
Documents Reviewed and Approved: UWO Protocol, Letter of information & consent form & Advertisement	
Documents Received for Information:	

This is to notify you that The University of Western Ontario Research Ethics Board for Health Sciences Research Involving Human Subjects (HSREB) which is organized and operates according to the Tri-Council Policy Statement: Ethical Conduct of Research Involving Humans and the Health Canada/ICH Good Clinical Practice Practices: Consolidated Guidelines; and the applicable laws and regulations of Ontario has reviewed and granted approval to the above referenced study on the approval date noted above. The membership of this REB also complies with the membership requirements for REB's as defined in Division 5 of the Food and Drug Regulations.

The ethics approval for this study shall remain valid until the expiry date noted above assuming timely and acceptable responses to the HSREB's periodic requests for surveillance and monitoring information. If you require an updated approval notice prior to that time you must request it using the UWO Updated Approval Request Form.

During the course of the research, no deviations from, or changes to, the protocol or consent form may be initiated without prior written approval from the HSREB except when necessary to eliminate immediate hazards to the subject or when the change(s) involve only logistical or administrative aspects of the study (e.g. change of monitor, telephone number). Expedited review of minor change(s) in ongoing studies will be considered. Subjects must receive a copy of the signed information/consent documentation.

Investigators must promptly also report to the HSREB:
a) changes increasing the risk to the participant(s) and/or affecting significantly the conduct of the study;
b) all adverse and unexpected experiences or events that are both serious and unexpected;
c) new information that may adversely affect the safety of the subjects or the conduct of the study.

If these changes/adverse events require a change to the information/consent documentation, and/or recruitment advertisement, the newly revised information/consent documentation, and/or advertisement, must be submitted to this office for approval.

Members of the HSREB who are named as investigators in research studies, or declare a conflict of interest, do not participate in discussion related to, nor vote on, such studies when they are presented to the HSREB.

Chair of HSREB: Dr. Joseph Gilbert
FDA Ref. #: IRB 00000940

Ethics Officer to Contact for Further Information			
☑ Janice Sutherland	☐ Elizabeth Wambolt	☐ Grace Kelly	☐ Denise Grafton

This is an official document. Please retain the original in your files.   cc: ORE File

Appendix B: Letters of Information

LETTER OF INFORMATION

Sex-Specific Hormone Levels and Reflex Sympathoexcitation

Principal Investigator: Dr. J. Kevin Shoemaker

Research Coordinator: Charlotte W. Usselman, M.Sc.

Sponsor: Natural Sciences and Engineering Research Council of Canada

You are being invited to participate in a research study that will examine how male and female hormone levels influence the nervous system. Before agreeing to participate, please read this Letter of Information. If you would like more details regarding something mentioned in this letter, or information not included here, please ask. Take time to read this carefully and to understand the following information. You will receive a copy of this letter to keep as your own. A total of 30 people will participate in this study.

Introduction

The nervous system plays an important role in controlling your heart rate and blood pressure. Interestingly, it appears that the nervous system is influenced by the sex hormones which circulate in your body (for example, estrogen and testosterone). To better understand how hormones influence the nervous system, this study has two purposes. The first purpose is to compare the nervous systems of men and women. The second purpose is to compare the nervous systems of women at two different phases of the menstrual cycle.

The study will take place on three separate days. The first day will be a familiarization session, lasting approximately 45 minutes. On this day the procedures will be explained and practiced so that you are comfortable with all aspects of the study. The remaining two sessions will each last approximately 3 hours. If you agree to participate, on each day you will be required to come to the laboratory approximately three hours following a light meal, and after having avoided exercise, alcohol, smoking, nicorette gum, recreational drugs, coffee, tea, soft drinks and chocolate for at least 12 hours. On arrival at the laboratory you will be asked questions about the medical history of you and your family.

Participant Inclusion/Exclusion Criteria

You will not be included in the study if you are under 18 or over 35 years of age. You will not be included in the study if you are, or think you could be, pregnant. In addition, you will not be included in the study if you are a smoker. Also, you will not be included in the study if you have any of the following: a resting blood pressure above 139/85 or below 100/55, respiratory disorders (e.g. asthma) or illnesses (e.g. bronchitis), cardiovascular disorders (e.g. Raynaud's disease), metabolic diseases (e.g. diabetes), or a history of fainting.

Measurements

If you agree to participate in this study, testing will be conducted at the Neurovascular Research Laboratory, Room 3110 Thames Hall at The University of Western Ontario. The testing procedure will involve the measurement of several cardiovascular variables during the 3-hour testing period:

1. Small adhesive electrodes will be placed on your chest to record the electrical heart rate tracing (electrocardiogram; ECG).

2. A small cuff will be placed around one finger and a blood pressure cuff will be placed around the upper portion of the same arm. These cuffs are used to measure your blood pressure. When activated, the finger cuff will inflate with air and you should feel a pulsating sensation on your finger.

3. An elastic strap will be placed around your chest to monitor changes in breathing rate and depth.

4. Small probes will be clipped on to one earlobe and one of your toes. These probes emit a light that passes easily through the earlobe and toe and lets us measure the amount of oxygen in your blood.

5. To examine your blood vessels, ultrasound probes will be placed on top of the skin at your elbow and at the base of your neck in order to measure the size of your vessels and the amount of blood flowing through them. These probes are similar to those used on pregnant women for imaging of an unborn child.

6. You are being asked to undergo a procedure called "microneurography". This procedure has two phases. First, the position of a nerve that runs very close to your skin just on the outside of the knee will be located by touch. Second, a thin tungsten electrode (similar to an acupuncture needle, about the size of a large human hair) will be inserted through the intact skin and positioned just under the skin about 2-3 cm from the nerve site. This will be followed by the placement of a

second tungsten electrode through the intact skin into this nerve (called the peroneal nerve). This second electrode will be manipulated gently until the appropriate recording site is found. The microelectrodes are sterilized before use and the area of skin around the knee is cleansed with alcohol before and after the procedure.

7. You will lie down on a table, with your legs and hips sealed inside a box that is connected to a vacuum source. This box is designed to produce suction (negative pressure) around your lower body. This mimics the effects of standing in terms of cardiovascular and nervous system responses. You will undergo 1-3 levels of lower body suction, starting at -60 mmHg (this level is comparable to standing upright) up to a maximum of -80 mmHg, for no longer than 2 minutes at a time.

8. During a portion of the experiment you will be given a mouthpiece to place in your mouth, which allows you to breathe normally. This mouthpiece will be attached to a valve that opens to either room air or a bag. At rest you will breathe room air. During one of the protocols the valve will be turned so that you will be breathing in and out of the bag. By breathing in and out of the bag we will be altering the amounts of oxygen and carbon dioxide in your blood. This will cause your rate and depth of breathing to increase, and will last for approximately 5 minutes. Immediately following this "rebreathing", we will also ask you to hold your breath for "as long as possible"; in most individuals this will not be longer than 30 seconds.

Procedures

Each day of testing will last approximately two hours, and will be carried out according to the order and timing illustrated in the following diagram. The procedures and measures used on each day will be identical.

1. We will begin by setting up the devices listed above in the "Measurements" section. This will take approximately 20 minutes. During this period you will simply be required to lie down and rest on a padded table.

2. Next, a small plastic tube will be inserted into a vein in your elbow by a registered nurse. This tube will be connected to a device that measures blood pressure in that vein. Also, blood will be taken from this tube at one time during the test. The total amount of blood withdrawn will not exceed 10 mL (2 teaspoons). The blood samples will be measured for sex hormones as well as hormones that regulate

blood pressure and blood vessels (e.g. estrogen, testosterone, progesterone, c-reactive protein).

3. Following the blood draw, we will begin the process of locating a recording site in your peroneal nerve through microneurography. While 1 hour has been allotted for the process, this is a maximum. It is possible that a suitable recording site could be found within the 1 hour time frame.

4. You will be asked to lay quietly and rest for a 10 minute "baseline" period.

5. *Breathing Task:*

   1.   This task will be performed with the mouthpiece in your mouth (see "Measurements", item 8). To begin, the valve will be turned from room air to the bag, and you will be asked to breathe in and out of the bag. You will continue to breathe in and out of the bag until the gases in the bag change a desired amount. At this point you will be asked to take in one more breath, and then hold your breath for as long as possible. When you cannot hold your breath any longer, you will exhale, then take 2 final breaths in and out of the bag. The valve will then be turned back to room air.

6. You will be asked to lay quietly and rest for at least 5 minutes.

7. *Lower Body Suction Task:*

   While lying quietly on your back, lower body suction of -60 mmHg will be applied for a duration of 2 minutes, after which you will be allowed at least 2 minutes to recover. You will be asked to brace yourself with one leg (the opposite leg used for microneurography) during suction. Lower body suction will then be increased in 10 mmHg increments up to -80 mmHg, with at least 2 minutes of rest between each level.

   As indicated above, the entire procedure will take approximately 3 hours to complete on each test day. Upon completion of the experiment you will rise slowly from the table into a seated position. After approximately 30-60 seconds of sitting you will be permitted to stand beside the bed and move about the lab when you feel comfortable to do so.

Risks

ECG
The adhesive on the electrodes used to measure your heart rate may cause a small rash to develop under the electrode. However, this rash should disappear in a day or two.

Blood Pressure Cuff

There are no known risks of using the finger cuff methods (Finometer) of examining arterial blood pressure. With the finger cuff the finger tip may turn a little blue and feel numb during the prolonged test sessions but this resolves immediately when the cuff is removed. Standard arm cuff blood pressure measures of arterial pressure will also be obtained periodically, a method that has no known risks.

Ultrasound

There are no known harmful effects with standard diagnostic ultrasound techniques.

Blood Oxygen Saturation

There are no known risks associated with the use of this device.

Blood Draw

There is a small risk of bruising or infection when collecting blood from your vein. Some participants may experience mild pain and discomfort and some may feel nauseated or dizzy when blood is taken.

Microneurography

Insertion of the microneurography electrodes within the nerve may cause some "pins and needles" or muscle cramping sensations. These sensations disappear immediately by changing electrode position. There will be no sensations when the needle is in the correct position. There is small chance of these sensations occurring around the area of needle insertion immediately following the study and a less than 1% chance of these sensations persisting longer than one day. Other extremely rare reactions include infection and/or bleeding when the electrode is removed. Once in place there is no discomfort from the electrodes. You will be advised to partake only in normal day-to-day activities and abstain from aggressive physical effort or exercise for 24 hours after the study, such as lifting weights, running or competitive sports.

Lower Body Suction

During lower body suction you may feel faint or dizzy. These symptoms may lead to actual fainting. To ensure this does not happen, we will ask you to inform us if you feel any of the following symptoms: nausea, light-headedness, tunnel vision, blurry vision, excessive heat and/or sweating. These symptoms are alleviated quickly by turning off the lower body suction. You may stop the test at any time. Also, we will be monitoring you throughout the experiment to ensure that you are okay and we will stop the test if we feel that it is necessary.

Breathing Protocol

The rebreathing protocol may last for a duration of up to 5 minutes. Subsequent breath-holding may last for a duration of up to 30 seconds. The decreasing amount of oxygen and increasing amount of carbon dioxide may give you a sensation of breathlessness. This sensation will go away immediately when you are switched to breathe room air. In the case that you become uncomfortable due to the breathless sensation, you will be returned immediately to air breathing which will alleviate the sensation. The increase in carbon dioxide in the air you breathe during rebreathing may cause a slight headache to occur, which will be reversed immediately upon returning to breathing room air. Decreases in oxygen much greater than used here carry the risk of dizziness or fainting. To date, the reduction in blood oxygen as used in this study has been conducted on 83 individuals. In one of these participants, the study was stopped early because the individual became dizzy. Given the limited number of people that this procedure has been conducted on, the exact level of risk has not yet been determined. To ensure your safety during these studies your blood oxygen content will be monitored continuously and we will return you to breathing room air if the amount of oxygen in your blood decreases below 80%. Also, your heart rate and blood pressure will be monitored continuously. The study will be stopped if your heart rate or blood pressure fall to levels below normal for more than 10 heart beats. You may stop the test at any time.

Staying Still
During the experiment, you will have to remain still in a lying down position for 2 hours. You may develop a sore back in the middle of the experiment. These sensations will diminish very quickly when you sit up from the bed after the experiment.

In the event that you suffer injury as a result of participating in this research no compensation will be provided for you by The University of Western Ontario, or the Researchers. You still have all your legal rights. Nothing said here about treatment or compensation in any way alters your right to recover damages.

Alternatives to Participating

You may choose not to participate in this study.

Benefits to You if You Take Part in the Study

There are no direct benefits to you as a result of participating in this study.

Voluntary Participation

You are encouraged to ask questions regarding the purpose of this study and the outcome of your testing. Participation in this study is voluntary. You may refuse to participate, refuse to answer any questions, or withdraw from the study at any time with no effect on your academic or employment status. We ask that you do not get involved with any other study while you are involved in this study. However, participation in this study will not stop you from being involved in future studies. You do not waive any legal rights by signing the consent form.

Confidentiality

All information that you provide will be kept strictly confidential. No information that could reveal your identity will be released to anyone unless disclosure is required legally. All of the information collected for this study will be stored in a locked filing cabinet or a password-protected computer that will only be accessible to the research team. To further protect your confidentiality, your name will be replaced with a subject ID number on all documents.

Representatives of The University of Western Ontario Health Sciences Research Ethics Board may contact you or require access to your study-related records or may follow up with you to monitor the conduct of the research.

Compensation

You will be reimbursed for travel and/or parking expenses (not exceeding $20 per visit).

Publication of Results

Published results from this study will not identify you by name. New findings from this study may be forwarded to each interested participant upon request. You may keep a copy of this letter of information.

Contact Persons

If you have any questions regarding this study, please feel free to contact:

Dr. Kevin Shoemaker

If you have any questions about your rights as a participant or about the conduct of the study you may contact The University of Western Ontario Office of Research Ethics,

LETTER OF INFORMED CONSENT

Sex-Specific Hormone Levels and Reflex Sympathoexcitation

Principal Investigator: Dr. J. Kevin Shoemaker

Research Coordinator: Charlotte W. Usselman, M.Sc.

I have read the Letter of Information, have had the nature of the study explained to me, and I agree to participate. All questions have been answered to my satisfaction.

_____ _____
Name of participant (Please print) Name of person obtaining consent

_____ _____
Signature of participant Signature of person obtaining consent

_____   _____
Date   Date

LETTER OF INFORMATION

Sex-Specific Hormone Levels and Reflex Sympathoinhibition

Principal Investigator: Dr. J. Kevin Shoemaker

Research Coordinator: Charlotte W. Usselman, M.Sc.

Sponsor: Natural Sciences and Engineering Research Council of Canada

      You are being invited to participate in a research study that will examine how male and female hormone levels influence the nervous system. Before agreeing to participate, please read this Letter of Information. If you would like more details regarding something mentioned in this letter, or information not included here, please ask. Take time to read this carefully and to understand the following information. You will receive a copy of this letter to keep as your own. A total of 50 people will participate in this study.

Introduction

      The nervous system plays an important role in controlling your heart rate and blood pressure. Interestingly, it appears that the nervous system is influenced by the sex hormones which circulate in your body (for example, estrogen and testosterone). To better understand how hormones influence the nervous system, this study has two purposes. The first purpose is to compare the nervous systems of men and women. The second purpose is to compare the nervous systems of women at two different phases of the menstrual cycle.

      The study will take place on three separate days. The first day will be a familiarization session, lasting approximately 45 minutes. On this day the procedures will be explained and practiced so that you are comfortable with all aspects of the study. The remaining two sessions will each last approximately 3 hours. If you agree to participate, on each day you will be required to come to the laboratory approximately three hours following a light meal, and after having avoided exercise, alcohol, smoking, nicorette gum, recreational drugs, coffee, tea, soft drinks and chocolate for at least 12 hours. On arrival at the laboratory you will be asked questions about the medical history of you and your family.

Participant Inclusion/Exclusion Criteria

You will not be included in the study if you are under 18 or over 35 years of age. You will not be included in the study if you are, or think you could be, pregnant. In addition, you will not be included in the study if you are a smoker. Also, you will not be included in the study if you have any of the following: a resting blood pressure above 139/85 or below 100/55, respiratory disorders (e.g. asthma) or illnesses (e.g. bronchitis), cardiovascular disorders (e.g. Raynaud's disease), metabolic diseases (e.g. diabetes), or a history of fainting.

Measurements

If you agree to participate in this study, testing will be conducted at the Neurovascular Research Laboratory, Room 3110 Thames Hall at The University of Western Ontario. The testing procedure will involve the measurement of several cardiovascular variables:

1. Small adhesive electrodes will be placed on your chest to record the electrical heart rate tracing (electrocardiogram; ECG).

2. A small cuff will be placed around one finger and a blood pressure cuff will be placed around the upper portion of the same arm. These cuffs are used to measure your blood pressure. When activated, the finger cuff will inflate with air and you should feel a pulsating sensation on your finger.

3. An elastic strap will be placed around your chest to monitor changes in breathing rate and depth.

4. A small probe will be clipped on one of your toes. This probe emits a light that passes through the toe and lets us measure the amount of oxygen in your blood.

5. To examine your blood vessels, ultrasound probes will be placed on top of the skin at your elbow and at the base of your neck in order to measure the size of your vessels and the amount of blood flowing through them. These probes are similar to those used on pregnant women for imaging of an unborn child.

6. You are being asked to undergo a procedure called "microneurography". This procedure has two phases. First, the position of a nerve that runs very close to your skin just on the outside of the knee will be located by touch. Second, a thin tungsten electrode (similar to an acupuncture needle, about the size of a large human hair) will be inserted through the intact skin and positioned just under the skin about 2-3 cm from the nerve site. This will be followed by the placement of a second tungsten electrode through the intact skin into this nerve (called the

peroneal nerve). This second electrode will be manipulated gently until the appropriate recording site is found. The microelectrodes are sterilized before use and the area of skin around the knee is cleansed with alcohol before and after the procedure.

7. You will lie down on a table which can be tilted automatically. You will be tilted to a slight head-down position (6° from the horizontal plane) for 1 minute periods. This will cause a small increase in the amount of blood in your chest, and a small decrease in the amount of blood contained in your legs.

8. You will be asked to perform a very mild handgrip exercise task. During this task you will squeeze a small air-filled bag at a level that requires 5% of your maximal voluntary strength (i.e. the hardest you can possibly squeeze the bag) for 1 minute periods.

9. You will be asked to breathe at a rate of 15 breaths per second. A metronome set to the same rate will assist you in maintaining this pattern.

10. During (6) and (7), you will be lying down with your legs and hips sealed inside a box that is connected to a vacuum source. This box is designed to produce suction (negative pressure) around your lower body. This mimics the effects of standing in terms of cardiovascular and nervous system responses. During two of the tilt tests, and two of the handgrips, the vacuum source will be turned on to a lower body suction level of -30 mmHg. This is a mild to moderate level of suction, less stressful than standing up.

Procedures

Each day of testing will last approximately two hours, and will be carried out according to the order and timing illustrated in the following diagram. The three experimental protocols (head-down tilt, handgrip, and paced breathing) are illustrated as "A", "B", and "C" because you will determine the order in which they are carried out by rolling a dice. The order of protocols determined on the first day of testing will be repeated on the second day of testing.

Instrumentation	Blood Draw	Microneurography Setup	Rest	Protocol "A"	Rest	Protocol "B"	Rest	Protocol "C"	Rest

Time (min) 0   20   30   90   100   125 130   145 150   165 170

1. We will begin by setting up the devices listed above in the "Measurements" section. This will take approximately 20 minutes. During this period you will simply be required to lie down and rest on a padded table.

2. Next, a small plastic tube will be inserted into a vein in your elbow by a registered nurse. This tube will be connected to a device that measures blood pressure in that vein. Also, blood will be taken from this tube at one time during the test. The total amount of blood withdrawn will not exceed 10 mL (2 teaspoons). The blood samples will be measured for sex hormones as well as hormones that regulate blood pressure and blood vessels (e.g. estrogen, testosterone, progesterone, c-reactive protein).

3. Following the blood draw, we will begin the process of locating a recording site in your peroneal nerve through microneurography. While 1 hour has been allotted for the process, this is a maximum. It is possible that a suitable recording site could be found within the 1 hour time frame.

4. You will be asked to lay quietly and rest for a 10 minute "baseline" period.

5. *Head-Down Tilt Task:*

   To begin, you will lie horizontally (zero degrees of tilt) on the table with your lower body sealed inside a box (see "Measurements", item 8). In total, you will be tilted to a head-down position (6 degrees of tilt) 4 times. Two of the tilts will be completed without lower body suction, and will last 2 minutes each. In the remainder of the trials, we will first turn on lower body suction to -30 mmHg for 2 minutes. At this, you will be tilted head-down. That is, you will be tilted while suction is applied to your lower body. You will then be returned to the horizontal position for a final 2 minutes of lower body suction. This will be followed by 2 minutes of rest.

6. You will be asked to lay quietly and rest for at least 5 minutes.

7. *Handgrip Task:*

   Before beginning the handgrip task, you will be asked to squeeze a small air-filled bag as hard as you can. This represents handgrip exercise at 100%. For the rest of the task, you will squeeze the bag at 5% of your maximal strength for 1 minute at a time. You will be able to see a screen that tells you how hard you are squeezing. You will complete 4 trials in total. Similar to the tilt task, you will perform 2 trials consisting of handgrip alone, and the other two trials will consist of a combination of lower body suction and handgrip. Each handgrip trial will be followed by 1 minute of quiet rest.

8. *Paced Breathing Task:*

During this task, you will be asked to breathe in time with a metronome, which will be set to 15 breaths per minute. That is, you will breathe in and out every 4 seconds for a total of 5 minutes.

As indicated above, the entire procedure will take approximately 3 hours to complete on each test day. Upon completion of the experiment you will rise slowly from the table into a seated position. After approximately 30-60 seconds of sitting you will be permitted to stand beside the bed and move about the lab when you feel comfortable to do so.

Risks

ECG
The adhesive on the electrodes used to measure your heart rate may cause a small rash to develop under the electrode. However, this rash should disappear in a day or two.

Blood Pressure Cuff
There are no known risks of using the finger cuff methods (Finometer) of examining arterial blood pressure. With the finger cuff the finger tip may turn a little blue and feel numb during the prolonged test sessions but this resolves immediately when the cuff is removed. Standard arm cuff blood pressure measures of arterial pressure will also be obtained periodically, a method that has no known risks.

Ultrasound
There are no known harmful effects with standard diagnostic ultrasound techniques.

Blood Oxygen Saturation
There are no known risks associated with the use of this device.

Blood Draw
There is a small risk of bruising or infection when collecting blood from your vein. Some participants may experience mild pain and discomfort and some may feel nauseated or dizzy when blood is taken.

Head-Down Tilt
There are no known risks associated with the mild level of head-down tilt used in this protocol. Head-down tilt is commonly used in physiotherapy.

Handgrip
There are no known risks associated with the low intensity of handgrip exercise used in this protocol.

Microneurography
Insertion of the microneurography electrodes within the nerve may cause some "pins and needles" or muscle cramping sensations. These sensations disappear immediately by changing electrode position. There will be no sensations when the needle is in the correct position. There is small chance of these sensations occurring around the area of needle insertion immediately following the study and a less than 1% chance of these sensations persisting longer than one day. Other extremely rare reactions include infection and/or bleeding when the electrode is removed. Once in place there is no discomfort from the electrodes. You will be advised to partake only in normal day-to-day activities and abstain from aggressive physical effort or exercise for 24 hours after the study, such as lifting weights, running or competitive sports.

Lower Body Suction
During lower body suction you may feel faint or dizzy. These symptoms may lead to actual fainting. To ensure this does not happen, we will ask you to inform us if you feel any of the following symptoms: nausea, light-headedness, tunnel vision, blurry vision, excessive heat and/or sweating. These symptoms are alleviated quickly by turning off the lower body suction. You may stop the test at any time. Also, we will be monitoring you throughout the experiment to ensure that you are okay and we will stop the test if we feel that it is necessary.

Paced Breathing
There are no known risks associated with paced breathing. This technique is commonly used during meditation.

Staying Still
During the experiment, you will have to remain still in a lying down position for 2 hours. You may develop a sore back in the middle of the experiment. These sensations will diminish very quickly when you sit up from the bed after the experiment.

In the event that you suffer injury as a result of participating in this research no compensation will be provided for you by The University of Western Ontario, or the Researchers. You still have all your legal rights. Nothing said here about treatment or compensation in any way alters your right to recover damages.

Alternatives to Participating

You may choose not to participate in this study.

Benefits to You if You Take Part in the Study

There are no direct benefits to you as a result of participating in this study.

Voluntary Participation

You are encouraged to ask questions regarding the purpose of this study and the outcome of your testing. Participation in this study is voluntary. You may refuse to participate, refuse to answer any questions, or withdraw from the study at any time with no effect on your academic or employment status. We ask that you do not get involved with any other study while you are involved in this study. However, participation in this study will not stop you from being involved in future studies. You do not waive any legal rights by signing the consent form.

Confidentiality

All information that you provide will be kept strictly confidential. No information that could reveal your identity will be released to anyone unless disclosure is required legally. All of the information collected for this study will be stored in a locked filing cabinet or a password-protected computer that will only be accessible to the research team. To further protect your confidentiality, your name will be replaced with a subject ID number on all documents.

Representatives of The University of Western Ontario Health Sciences Research Ethics Board may contact you or require access to your study-related records or may follow up with you to monitor the conduct of the research.

Compensation

You will be reimbursed for travel and/or parking expenses (not exceeding $20 per visit).

Publication of Results

Published results from this study will not identify you by name. New findings from this study may be forwarded to each interested participant upon request. You may keep a copy of this letter of information.

Contact Persons

If you have any questions regarding this study, please feel free to contact:

Dr. Kevin Shoemaker

If you have any questions about your rights as a participant or about the conduct of the study you may contact The University of Western Ontario Office of Research Ethics,

LETTER OF INFORMED CONSENT

Sex-Specific Hormone Levels and Reflex Sympathoinhibition

Principal Investigator: Dr. J. Kevin Shoemaker

Research Coordinator: Charlotte W. Usselman, M.Sc.

I have read the Letter of Information, have had the nature of the study explained to me, and I agree to participate. All questions have been answered to my satisfaction.

_____   _____
Name of participant (Please print)   Name of person obtaining consent

_____   _____
Signature of participant   Signature of person obtaining consent

_____   _____
Date   Date

Appendix C: Copyright Licenses

Figure 1.1:

<div align="center">

NATURE PUBLISHING GROUP LICENSE
TERMS AND CONDITIONS

</div>

Mar 06, 2014

This is a License Agreement between Charlotte W Usselman ("You") and Nature Publishing Group ("Nature Publishing Group") provided by Copyright Clearance Center ("CCC"). The license consists of your order details, the terms and conditions provided by Nature Publishing Group, and the payment terms and conditions.

All payments must be made in full to CCC. For payment instructions, please see information listed at the bottom of this form.

License Number 3343191277755
License date Mar 06, 2014
Licensed content publisher Nature Publishing Group
Licensed content
publication
Journal of Human Hypertension
Licensed content title Hypertension in women
Licensed content author T Rosenthal, S Oparil
Licensed content date Oct 16, 2000
Volume number 14
Issue number 0
Type of Use reuse in a dissertation / thesis
Requestor type academic/educational
Format print and electronic
Portion figures/tables/illustrations
Number of
figures/tables/illustrations
1
Figures Figure 1
Author of this NPG article no
Your reference number
Title of your thesis /
dissertation
Sex hormones and muscle sympathetic nerve activity
Expected completion date Apr 2014
Estimated size (number of
pages)
200
Total 0.00 USD
Terms and Conditions

Terms and Conditions for Permissions

Nature Publishing Group hereby grants you a non-exclusive license to reproduce this material for

this purpose, and for no other use,subject to the conditions below:

1. NPG warrants that it has, to the best of its knowledge, the rights to license reuse of this material. However, you should ensure that the material you are requesting is original to Nature Publishing Group and does not carry the copyright of another entity (as credited in the published version). If the credit line on any part of the material you have requested indicates that it was reprinted or adapted by NPG with permission from another source, then you should also seek permission from that source to reuse the material.

2. Permission granted free of charge for material in print is also usually granted for any electronic version of that work, provided that the material is incidental to the work as a whole and that the electronic version is essentially equivalent to, or substitutes for, the print version.Where print permission has been granted for a fee, separate permission must be obtained for any additional, electronic re-use (unless, as in the case of a full paper, this has already been accounted for during your initial request in the calculation of a print run).NB: In all cases, web-based use of full-text articles must be authorized separately through the 'Use on a Web Site' option when requesting permission.

3. Permission granted for a first edition does not apply to second and subsequent editions and for editions in other languages (except for signatories to the STM Permissions Guidelines, or where the first edition permission was granted for free).

4. Nature Publishing Group's permission must be acknowledged next to the figure, table or

abstract in print. In electronic form, this acknowledgement must be visible at the same time as the figure/table/abstract, and must be hyperlinked to the journal's homepage.

5. The credit line should read:

Reprinted by permission from Macmillan Publishers Ltd: [JOURNAL NAME] (reference citation), copyright (year of publication)

For AOP papers, the credit line should read:

Reprinted by permission from Macmillan Publishers Ltd: [JOURNAL NAME], advance online publication, day month year (doi: 10.1038/sj.[JOURNAL ACRONYM].XXXXX)

Note: For republication from the *British Journal of Cancer,* the following credit lines apply.

Reprinted by permission from Macmillan Publishers Ltd on behalf of Cancer Research UK: [JOURNAL NAME] (reference citation), copyright (year of publication)For AOP papers, the credit line should read:

Reprinted by permission from Macmillan Publishers Ltd on behalf of Cancer Research UK: [JOURNAL NAME], advance online publication, day month year (doi: 10.1038/sj.[JOURNAL ACRONYM].XXXXX)

6. Adaptations of single figures do not require NPG approval. However, the adaptation should be credited as follows:

Adapted by permission from Macmillan Publishers Ltd: [JOURNAL NAME] (reference citation), copyright (year of publication)

Note: For adaptation from the *British Journal of Cancer,* the following credit line applies.

Adapted by permission from Macmillan Publishers Ltd on behalf of Cancer Research UK: [JOURNAL NAME] (reference citation), copyright (year of publication)

7. Translations of 401 words up to a whole article require NPG approval. Please visit http://www.macmillanmedicalcommunications.com for more information.Translations of up to a 400 words do not require NPG approval. The translation should be credited as follows:

Translated by permission from Macmillan Publishers Ltd: [JOURNAL NAME] (reference

citation), copyright (year of publication).
Note: For translation from the *British Journal of Cancer,* the following credit line applies.
Translated by permission from Macmillan Publishers Ltd on behalf of Cancer Research UK: [JOURNAL NAME] (reference citation), copyright (year of publication)
We are certain that all parties will benefit from this agreement and wish you the best in the use of
this material. Thank you.
Special Terms:
v1.1

Figure 1.2:

WOLTERS KLUWER HEALTH LICENSE
TERMS AND CONDITIONS

Feb 14, 2014

This is a License Agreement between Charlotte W Usselman ("You") and Wolters Kluwer Health ("Wolters Kluwer Health") provided by Copyright Clearance Center ("CCC"). The license consists of your order details, the terms and conditions provided by Wolters Kluwer Health, and the payment terms and conditions.

All payments must be made in full to CCC. For payment instructions, please see information listed at the bottom of this form.

License Number 3327690015454
License date Feb 14, 2014
Licensed content publisher Wolters Kluwer Health
Licensed content
publication
Hypertension
Licensed content title Gender-Selective Interaction Between Aging, Blood Pressure, and Sympathetic Nerve Activity
Licensed content author Krzysztof Narkiewicz, Bradley G. Phillips, Masahiko Kato, Dagmara
Hering, Leszek Bieniaszewski, Virend K. Somers
Licensed content date Apr 1, 2005
Volume Number 45
Issue Number 4
Type of Use Dissertation/Thesis
Requestor type Individual
Portion Figures/table/illustration
Number of
figures/tables/illustrations
1
Figures/tables/illustrations
used
Figure 1
Author of this Wolters
Kluwer article
No
Title of your thesis /
dissertation
Sex hormones and muscle sympathetic nerve activity
Expected completion date Apr 2014
Estimated size(pages) 200
Billing Type Invoice
Billing address School of Kinesiology

Canada
Total 0.00 USD
Terms and Conditions

Terms and Conditions

1. A credit line will be prominently placed and include: for books - the author(s), title of book, editor, copyright holder, year of publication; For journals - the author(s), title of article, title of journal, volume number, issue number and inclusive pages.
2. The requestor warrants that the material shall not be used in any manner which may be considered derogatory to the title, content, or authors of the material, or to Wolters Kluwer.
3. Permission is granted for a one time use only within 12 months from the date of this invoice. Rights herein do not apply to future reproductions, editions, revisions, or other derivative works. Once the 12-month term has expired, permission to renew must be submitted in writing.
4. Permission granted is non-exclusive, and is valid throughout the world in the English language and the languages specified in your original request.
5. Wolters Kluwer cannot supply the requestor with the original artwork or a "clean copy."
6. The requestor agrees to secure written permission from the author (for book material only).
7. Permission is valid if the borrowed material is original to a Wolters Kluwer imprint (Lippincott-Raven Publishers, Williams & Wilkins, Lea & Febiger, Harwal, Igaku-Shoin, Rapid Science, Little Brown & Company, Harper & Row Medical, American Journal of Nursing Co, and Urban & Schwarzenberg - English Language).
8. If you opt not to use the material requested above, please notify Rightslink within 90 days of the original invoice date.
9. Please note that articles in the ahead-of-print stage of publication can be cited and the content may be re-used by including the date of access and the unique DOI number. Any final changes in manuscripts will be made at the time of print publication and will be reflected in the final electronic version of the issue.?Disclaimer: Articles appearing in the Published Ahead-of-Print section have been peer-reviewed and accepted for publication in the relevant journal and posted online before print publication. Articles appearing as publish ahead-of-print may contain statements, opinions, and information that have errors in facts, figures, or interpretation. Accordingly, Lippincott Williams & Wilkins, the editors and authors and their respective employees are not responsible or liable for the use of any such inaccurate or misleading data, opinion or information contained in the articles in this section.
10. 1This permission does not apply to images that are credited to publications other than Wolters Kluwer journals. For images credited to non-Wolters Kluwer journal publications, you will need to obtain permission from the journal referenced in the figure or table legend or credit line before making any use of the image(s) or table(s).
11. In case of Disease Colon Rectum, Plastic Reconstructive Surgery, The Green Journal, Critical Care Medicine, Pediatric Critical Care Medicine, the American Heart Publications, the American Academy of Neurology the following guideline applies: no drug brand/trade name or logo can be included in the same page as the material reused
12. When requesting a permission to translate a full text article, Wolters Kluwer/Lippincott Williams & Wilkins requests to receive the pdf of the translated document
13. "Adaptations of single figures do not require Wolters Kluwer further approval if the permission has been granted previously. However, the adaptation should be credited as follows:?Adapted with permission from Lippincott Williams and Wilkins/Wolters Kluwer Health: [JOURNAL NAME] (reference citation), copyright (year of publication)" Please note that modification of text within figures or full-text articles is strictly forbidden.

14. The following statement needs to be added when reprinting the material in Open Access journals only: 'promotional and commercial use of the material in print, digital or mobile device format is prohibited without the permission from the publisher Lippincott Williams & Wilkins. Please contact journalpermissions@lww.com for further information".
15. Other Terms and Conditions:

v1.8

Figure 1.4:

<div align="center">

JOHN WILEY AND SONS LICENSE
TERMS AND CONDITIONS

</div>

<div align="right">

Mar 03, 2014

</div>

This is a License Agreement between Charlotte W Usselman ("You") and John Wiley and Sons ("John Wiley and Sons") provided by Copyright Clearance Center ("CCC"). The license consists of your order details, the terms and conditions provided by John Wiley and Sons, and the payment terms and conditions.

All payments must be made in full to CCC. For payment instructions, please see information listed at the bottom of this form.

License Number 3341440776254
License date Mar 03, 2014
Licensed content publisher John Wiley and Sons
Licensed content
publication
Journal of Physiology
Licensed content title Two sites for modulation of human sympathetic activity by arterial baroreceptors?
Licensed copyright line Copyright © 2004, John Wiley and Sons
Licensed content author Peter Kienbaum,Tomas Karlsson,Yrsa B. Sverrisdottir,Mikael Elam,B. Gunnar Wallin
Licensed content date Aug 5, 2004
Start page 861
End page 869
Type of use Dissertation/Thesis
Requestor type University/Academic
Format Print and electronic
Portion Figure/table
Number of figures/tables 1
Original Wiley figure/table
number(s)
Figure 4
Will you be translating? No
Title of your thesis /
dissertation
Sex hormones and muscle sympathetic nerve activity
Expected completion date Apr 2014
Expected size (number of
pages)
200
Total 0.00 USD
Terms and Conditions

<div align="center">

TERMS AND CONDITIONS

</div>

This copyrighted material is owned by or exclusively licensed to John Wiley & Sons, Inc. or one of its group companies (each a "Wiley Company") or a society

for whom a Wiley Company has exclusive publishing rights in relation to a particular journal (collectively "WILEY"). By clicking "accept" in connection with completing this licensing transaction, you agree that the following terms and conditions apply to this transaction (along with the billing and payment terms and conditions established by the Copyright Clearance Center Inc., ("CCC's Billing and Payment terms and conditions"), at the time that you opened your RightsLink account (these are available at any time at http://myaccount.copyright.com).

Terms and Conditions

1. The materials you have requested permission to reproduce (the "Materials") are protected by copyright.

2.You are hereby granted a personal, non-exclusive, non-sublicensable, non-transferable, worldwide, limited license to reproduce the Materials for the purpose specified in the licensing process. This license is for a one-time use only with a maximum distribution equal to the number that you identified in the licensing process. Any form of republication granted by this license must be completed within two years of the date of the grant of this license (although copies prepared before may be distributed thereafter). The Materials shall not be used in any other manner or for any other purpose. Permission is granted subject to an appropriate acknowledgement given to the author, title of the material/book/journal and the publisher. You shall also duplicate the copyright notice that appears in the Wiley publication in your use of the Material. Permission is also granted on the understanding that nowhere in the text is a previously published source acknowledged for all or part of this Material. Any third party material is expressly excluded from this permission.

3. With respect to the Materials, all rights are reserved. Except as expressly granted by the terms of the license, no part of the Materials may be copied, modified, adapted (except for minor reformatting required by the new Publication), translated, reproduced, transferred or distributed, in any form or by any means, and no derivative works may be made based on the Materials without the prior permission of the respective copyright owner. You may not alter, remove or suppress in any manner any copyright, trademark or other notices displayed by the Materials. You may not license, rent, sell, loan, lease, pledge, offer as security, transfer or assign the Materials, or any of the rights granted to you hereunder to any other person.

4. The Materials and all of the intellectual property rights therein shall at all times remain the exclusive property of John Wiley & Sons Inc or one of its related companies (WILEY) or their respective licensors, and your interest therein is only that of having possession of and the right to reproduce the Materials pursuant to Section 2 herein during the continuance of this Agreement. You agree that you own no right, title or interest in or to the Materials or any of the intellectual property rights therein. You shall have no rights hereunder other than the license as provided for above in Section 2. No right, license or interest to any trademark,

trade name, service mark or other branding ("Marks") of WILEY or its licensors is granted hereunder, and you agree that you shall not assert any such right, license or interest with respect thereto.

5. NEITHER WILEY NOR ITS LICENSORS MAKES ANY WARRANTY OR REPRESENTATION OF ANY KIND TO YOU OR ANY THIRD PARTY, EXPRESS, IMPLIED OR STATUTORY, WITH RESPECT TO THE MATERIALS OR THE ACCURACY OF ANY INFORMATION CONTAINED IN THE MATERIALS, INCLUDING, WITHOUT LIMITATION, ANY IMPLIED WARRANTY OF MERCHANTABILITY, ACCURACY, SATISFACTORY QUALITY, FITNESS FOR A PARTICULAR PURPOSE, USABILITY, INTEGRATION OR NON-INFRINGEMENT AND ALL SUCH WARRANTIES ARE HEREBY EXCLUDED BY WILEY AND ITS LICENSORS AND WAIVED BY YOU.

6. WILEY shall have the right to terminate this Agreement immediately upon breach of this Agreement by you.

7. You shall indemnify, defend and hold harmless WILEY, its Licensors and their respective directors, officers, agents and employees, from and against any actual or threatened claims, demands, causes of action or proceedings arising from any breach of this Agreement by you.

8. IN NO EVENT SHALL WILEY OR ITS LICENSORS BE LIABLE TO YOU OR ANY OTHER PARTY OR ANY OTHER PERSON OR ENTITY FOR ANY SPECIAL, CONSEQUENTIAL, INCIDENTAL, INDIRECT, EXEMPLARY OR PUNITIVE DAMAGES, HOWEVER CAUSED, ARISING OUT OF OR IN CONNECTION WITH THE DOWNLOADING, PROVISIONING, VIEWING OR USE OF THE MATERIALS REGARDLESS OF THE FORM OF ACTION, WHETHER FOR BREACH OF CONTRACT, BREACH OF WARRANTY, TORT, NEGLIGENCE, INFRINGEMENT OR OTHERWISE (INCLUDING, WITHOUT LIMITATION, DAMAGES BASED ON LOSS OF PROFITS, DATA, FILES, USE, BUSINESS OPPORTUNITY OR CLAIMS OF THIRD PARTIES), AND WHETHER OR NOT THE PARTY HAS BEEN ADVISED OF THE POSSIBILITY OF SUCH DAMAGES. THIS LIMITATION SHALL APPLY NOTWITHSTANDING ANY FAILURE OF ESSENTIAL PURPOSE OF ANY LIMITED REMEDY PROVIDED HEREIN.

9. Should any provision of this Agreement be held by a court of competent jurisdiction to be illegal, invalid, or unenforceable, that provision shall be deemed amended to achieve as nearly as possible the same economic effect as the original provision, and the legality, validity and enforceability of the remaining provisions of this Agreement shall not be affected or impaired thereby.

10. The failure of either party to enforce any term or condition of this Agreement shall not constitute a waiver of either party's right to enforce each and every term and condition of this Agreement. No breach under this agreement shall be deemed waived or excused by either party unless such waiver or consent is in writing signed by the party granting such waiver or consent. The waiver by or consent of a

party to a breach of any provision of this Agreement shall not operate or be construed as a waiver of or consent to any other or subsequent breach by such other party.

11. This Agreement may not be assigned (including by operation of law or otherwise) by you without WILEY's prior written consent.

12. Any fee required for this permission shall be non-refundable after thirty (30) days from receipt

13. These terms and conditions together with CCC's Billing and Payment terms and conditions (which are incorporated herein) form the entire agreement between you and WILEY concerning this licensing transaction and (in the absence of fraud) supersedes all prior agreements and representations of the parties, oral or written. This Agreement may not be amended except in writing signed by both parties. This Agreement shall be binding upon and inure to the benefit of the parties' successors, legal representatives, and authorized assigns.

14. In the event of any conflict between your obligations established by these terms and conditions and those established by CCC's Billing and Payment terms and conditions, these terms and conditions shall prevail.

15. WILEY expressly reserves all rights not specifically granted in the combination of (i) the license details provided by you and accepted in the course of this licensing transaction, (ii) these terms and conditions and (iii) CCC's Billing and Payment terms and conditions.

16. This Agreement will be void if the Type of Use, Format, Circulation, or Requestor Type was misrepresented during the licensing process.

17. This Agreement shall be governed by and construed in accordance with the laws of the State of New York, USA, without regards to such state's conflict of law rules. Any legal action, suit or proceeding arising out of or relating to these Terms and Conditions or the breach thereof shall be instituted in a court of competent jurisdiction in New York County in the State of New York in the United States of America and each party hereby consents and submits to the personal jurisdiction of such court, waives any objection to venue in such court and consents to service of process by registered or certified mail, return receipt requested, at the last known address of such party.

Wiley Open Access Terms and Conditions

Wiley publishes Open Access articles in both its Wiley Open Access Journals program [http://www.wileyopenaccess.com/view/index.html] and as Online Open articles in its subscription journals. The majority of Wiley Open Access Journals have adopted the Creative Commons Attribution License (CC BY) which permits the unrestricted use, distribution, reproduction, adaptation and commercial exploitation of the article in any medium. No permission is required to use the article in this way provided that the article is properly cited and other license terms are observed. A small number of Wiley Open Access journals have retained the

For non-commercial and non-promotional purposes individual non-commercial users may access, download, copy, display and redistribute to colleagues Wiley Open Access articles.
In addition, articles adopting the CC BY-NC may be adapted, translated, and text- and data-mined subject to the conditions above.

Use by commercial "for-profit" organizations
Use of non-commercial Wiley Open Access articles for commercial, promotional, or marketing purposes requires further explicit permission from Wiley and will be subject to a fee. Commercial purposes include:
Copying or downloading of articles, or linking to such articles for further redistribution, sale or licensing;
Copying, downloading or posting by a site or service that incorporates advertising with such content;
The inclusion or incorporation of article content in other works or services (other than normal quotations with an appropriate citation) that is then available for sale or licensing, for a fee (for example, a compilation produced for marketing purposes, inclusion in a sales pack)
Use of article content (other than normal quotations with appropriate citation) by forprofit organizations for promotional purposes
Linking to article content in e-mails redistributed for promotional, marketing or educational purposes;
Use for the purposes of monetary reward by means of sale, resale, license, loan, transfer or other form of commercial exploitation such as marketing products
Print reprints of Wiley Open Access articles can be purchased from: corporatesales@wiley.com
The modification or adaptation for any purpose of an article referencing the CC BYNC-ND License requires consent which can be requested from RightsLink@wiley.com .

Other Terms and Conditions:
BY CLICKING ON THE "I AGREE..." BOX, YOU ACKNOWLEDGE THAT YOU HAVE READ AND FULLY UNDERSTAND EACH OF THE SECTIONS OF AND PROVISIONS SET FORTH IN THIS AGREEMENT AND THAT YOU ARE IN AGREEMENT WITH AND ARE WILLING TO ACCEPT ALL OF YOUR OBLIGATIONS AS SET FORTH IN THIS AGREEMENT.
v1.8

Figure 1.6:

WOLTERS KLUWER HEALTH LICENSE
TERMS AND CONDITIONS

Feb 21, 2014

This is a License Agreement between Charlotte W Usselman ("You") and Wolters Kluwer Health ("Wolters Kluwer Health") provided by Copyright Clearance Center ("CCC"). The license consists of your order details, the terms and conditions provided by Wolters Kluwer Health, and the payment terms and conditions.

All payments must be made in full to CCC. For payment instructions, please see information listed at the bottom of this form.

License Number 3333720049493
License date Feb 21, 2014
Licensed content publisher Wolters Kluwer Health
Licensed content
publication
Circulation
Licensed content title Selective Potentiation of Peripheral Chemoreflex Sensitivity in Obstructive Sleep Apnea
Licensed content author Krzysztof Narkiewicz, Philippe J. H. van de Borne, Catherine A. Pesek, Mark E. Dyken, Nicola Montano, Virend K. Somers
Licensed content date Mar 9, 1999
Volume Number 99
Issue Number 9
Type of Use Dissertation/Thesis
Requestor type Individual
Portion Figures/table/illustration
Number of
figures/tables/illustrations
1
Figures/tables/illustrations
used
Figure 1
Author of this Wolters
Kluwer article
No
Title of your thesis /
dissertation
Sex hormones and muscle sympathetic nerve activity
Expected completion date Apr 2014
Estimated size(pages) 200
Billing Type Invoice

Total 0.00 USD

Terms and Conditions

1. A credit line will be prominently placed and include: for books - the author(s), title of book, editor, copyright holder, year of publication; For journals - the author(s), title of article, title of journal, volume number, issue number and inclusive pages.

2. The requestor warrants that the material shall not be used in any manner which may be considered derogatory to the title, content, or authors of the material, or to Wolters Kluwer.

3. Permission is granted for a one time use only within 12 months from the date of this invoice. Rights herein do not apply to future reproductions, editions, revisions, or other derivative works. Once the 12-month term has expired, permission to renew must be submitted in writing.

4. Permission granted is non-exclusive, and is valid throughout the world in the English language and the languages specified in your original request.

5. Wolters Kluwer cannot supply the requestor with the original artwork or a "clean copy."

6. The requestor agrees to secure written permission from the author (for book material only).

7. Permission is valid if the borrowed material is original to a Wolters Kluwer imprint (Lippincott-Raven Publishers, Williams & Wilkins, Lea & Febiger, Harwal, Igaku-Shoin, Rapid Science, Little Brown & Company, Harper & Row Medical, American Journal of Nursing Co, and Urban & Schwarzenberg - English Language).

8. If you opt not to use the material requested above, please notify Rightslink within 90 days of the original invoice date.

9. Please note that articles in the ahead-of-print stage of publication can be cited and the content may be re-used by including the date of access and the unique DOI number. Any final changes in manuscripts will be made at the time of print publication and will be reflected in the final electronic version of the issue.?Disclaimer: Articles appearing in the Published Ahead-of-Print section have been peer-reviewed and accepted for publication in the relevant journal and posted online before print publication. Articles appearing as publish ahead-of-print may contain statements, opinions, and information that have errors in facts, figures, or interpretation. Accordingly, Lippincott Williams & Wilkins, the editors and authors and their respective employees are not responsible or liable for the use of any such inaccurate or misleading data, opinion or information contained in the articles in this section.

10. 1This permission does not apply to images that are credited to publications other than Wolters Kluwer journals. For images credited to non-Wolters Kluwer journal publications, you will need to obtain permission from the journal referenced in the figure or table legend or credit line before making any use of the image(s) or table(s).

11. In case of Disease Colon Rectum, Plastic Reconstructive Surgery, The Green Journal, Critical Care Medicine, Pediatric Critical Care Medicine, the American Heart Publications, the American Academy of Neurology the following guideline applies: no drug brand/trade name or logo can be included in the same page as the material reused

12. When requesting a permission to translate a full text article, Wolters Kluwer/Lippincott Williams & Wilkins requests to receive the pdf of the translated document

13. "Adaptations of single figures do not require Wolters Kluwer further approval if the permission has been granted previously. However, the adaptation should be credited as follows:?Adapted with permission from Lippincott Williams and Wilkins/Wolters Kluwer Health: [JOURNAL NAME] (reference citation), copyright (year of publication)" Please note that modification of text within figures or full-text articles is strictly forbidden.

14. The following statement needs to be added when reprinting the material in Open Access journals only: 'promotional and commercial use of the material in print, digital or

mobile device format is prohibited without the permission from the publisher Lippincott Williams & Wilkins. Please contact journalpermissions@lww.com for further information".
15. Other Terms and Conditions:

v1.8

Figure 1.7:

TERMS AND CONDITIONS

You agree that you own no right, title or interest in or to the Materials or any of the intellectual property rights therein. You shall have no rights hereunder other than the license as provided for above in Section 2. No right, license or interest to any trademark, trade name, service mark or other branding ("Marks") of WILEY or its licensors is granted hereunder, and you agree that you shall not assert any such right, license or interest with respect thereto.

5. NEITHER WILEY NOR ITS LICENSORS MAKES ANY WARRANTY OR REPRESENTATION OF ANY KIND TO YOU OR ANY THIRD PARTY, EXPRESS, IMPLIED OR STATUTORY, WITH RESPECT TO THE MATERIALS OR THE ACCURACY OF ANY INFORMATION CONTAINED IN THE MATERIALS, INCLUDING, WITHOUT LIMITATION, ANY IMPLIED WARRANTY OF MERCHANTABILITY, ACCURACY, SATISFACTORY QUALITY, FITNESS FOR A PARTICULAR PURPOSE, USABILITY, INTEGRATION OR NON-INFRINGEMENT AND ALL SUCH WARRANTIES ARE HEREBY EXCLUDED BY WILEY AND ITS LICENSORS AND WAIVED BY YOU.

6. WILEY shall have the right to terminate this Agreement immediately upon breach of this Agreement by you.

7. You shall indemnify, defend and hold harmless WILEY, its Licensors and their respective directors, officers, agents and employees, from and against any actual or threatened claims, demands, causes of action or proceedings arising from any breach of this Agreement by you.

8. IN NO EVENT SHALL WILEY OR ITS LICENSORS BE LIABLE TO YOU OR ANY OTHER PARTY OR ANY OTHER PERSON OR ENTITY FOR ANY SPECIAL, CONSEQUENTIAL, INCIDENTAL, INDIRECT, EXEMPLARY OR PUNITIVE DAMAGES, HOWEVER CAUSED, ARISING OUT OF OR IN CONNECTION WITH THE DOWNLOADING, PROVISIONING, VIEWING OR USE OF THE MATERIALS REGARDLESS OF THE FORM OF ACTION, WHETHER FOR BREACH OF CONTRACT, BREACH OF WARRANTY, TORT, NEGLIGENCE, INFRINGEMENT OR OTHERWISE (INCLUDING, WITHOUT LIMITATION, DAMAGES BASED ON LOSS OF PROFITS, DATA, FILES, USE, BUSINESS OPPORTUNITY OR CLAIMS OF THIRD PARTIES), AND WHETHER OR NOT THE PARTY HAS BEEN ADVISED OF THE POSSIBILITY OF SUCH DAMAGES. THIS LIMITATION SHALL APPLY NOTWITHSTANDING ANY FAILURE OF ESSENTIAL PURPOSE OF ANY LIMITED REMEDY PROVIDED HEREIN.

9. Should any provision of this Agreement be held by a court of competent jurisdiction to be illegal, invalid, or unenforceable, that provision shall be deemed amended to achieve as nearly as possible the same economic effect as the original provision, and the legality, validity and enforceability of the remaining provisions of this Agreement shall not be affected or impaired thereby.

10. The failure of either party to enforce any term or condition of this Agreement shall not constitute a waiver of either party's right to enforce each and every term

and condition of this Agreement. No breach under this agreement shall be deemed waived or excused by either party unless such waiver or consent is in writing signed by the party granting such waiver or consent. The waiver by or consent of a party to a breach of any provision of this Agreement shall not operate or be construed as a waiver of or consent to any other or subsequent breach by such other party.

11. This Agreement may not be assigned (including by operation of law or otherwise) by you without WILEY's prior written consent.

12. Any fee required for this permission shall be non-refundable after thirty (30) days from receipt

13. These terms and conditions together with CCC's Billing and Payment terms and conditions (which are incorporated herein) form the entire agreement between you and WILEY concerning this licensing transaction and (in the absence of fraud) supersedes all prior agreements and representations of the parties, oral or written. This Agreement may not be amended except in writing signed by both parties. This Agreement shall be binding upon and inure to the benefit of the parties' successors, legal representatives, and authorized assigns.

14. In the event of any conflict between your obligations established by these terms and conditions and those established by CCC's Billing and Payment terms and conditions, these terms and conditions shall prevail.

15. WILEY expressly reserves all rights not specifically granted in the combination of (i) the license details provided by you and accepted in the course of this licensing transaction, (ii) these terms and conditions and (iii) CCC's Billing and Payment terms and conditions.

16. This Agreement will be void if the Type of Use, Format, Circulation, or Requestor Type was misrepresented during the licensing process.

17. This Agreement shall be governed by and construed in accordance with the laws of the State of New York, USA, without regards to such state's conflict of law rules. Any legal action, suit or proceeding arising out of or relating to these Terms and Conditions or the breach thereof shall be instituted in a court of competent jurisdiction in New York County in the State of New York in the United States of America and each party hereby consents and submits to the personal jurisdiction of such court, waives any objection to venue in such court and consents to service of process by registered or certified mail, return receipt requested, at the last known address of such party.

Wiley Open Access Terms and Conditions
Wiley publishes Open Access articles in both its Wiley Open Access Journals program [http://www.wileyopenaccess.com/view/index.html] and as Online Open articles in its subscription journals. The majority of Wiley Open Access Journals have adopted the Creative Commons Attribution License (CC BY) which permits the unrestricted use, distribution, reproduction, adaptation and commercial exploitation of the article in any medium. No permission is required to use the

article in this way provided that the article is properly cited and other license terms are observed. A small number of Wiley Open Access journals have retained the Creative Commons Attribution Non Commercial License (CC BY-NC), which permits use, distribution and reproduction in any medium, provided the original work is properly cited and is not used for commercial purposes.

Online Open articles - Authors selecting Online Open are, unless particular exceptions apply, offered a choice of Creative Commons licenses. They may therefore select from the CC BY, the CC BY-NC and the Attribution-NoDerivatives (CC BY-NC-ND). The CC BY-NC-ND is more restrictive than the CC BY-NC as it does not permit adaptations or modifications without rights holder consent. Wiley Open Access articles are protected by copyright and are posted to repositories and websites in accordance with the terms of the applicable Creative Commons license referenced on the article. At the time of deposit, Wiley Open Access articles include all changes made during peer review, copyediting, and publishing. Repositories and websites that host the article are responsible for incorporating any publisher-supplied amendments or retractions issued subsequently. Wiley Open Access articles are also available without charge on Wiley's publishing platform, Wiley Online Library or any successor sites.

Conditions applicable to all Wiley Open Access articles:
The authors' moral rights must not be compromised. These rights include the right of "paternity" (also known as "attribution" - the right for the author to be identified as such) and "integrity" (the right for the author not to have the work altered in such a way that the author's reputation or integrity may be damaged).
Where content in the article is identified as belonging to a third party, it is the obligation of the user to ensure that any reuse complies with the copyright policies of the owner of that content.
If article content is copied, downloaded or otherwise reused for research and other purposes as permitted, a link to the appropriate bibliographic citation (authors, journal, article title, volume, issue, page numbers, DOI and the link to the definitive published version on Wiley Online Library) should be maintained.
Copyright notices and disclaimers must not be deleted.
Creative Commons licenses are copyright licenses and do not confer any other rights, including but not limited to trademark or patent rights.
Any translations, for which a prior translation agreement with Wiley has not been agreed, must prominently display the statement: "This is an unofficial translation of an article that appeared in a Wiley publication. The publisher has not endorsed this translation."

Conditions applicable to non-commercial licenses (CC BY-NC and CC BY-NC-ND)

For non-commercial and non-promotional purposes individual non-commercial users may access, download, copy, display and redistribute to colleagues Wiley Open Access articles.
In addition, articles adopting the CC BY-NC may be adapted, translated, and text- and data-mined subject to the conditions above.

Use by commercial "for-profit" organizations
Use of non-commercial Wiley Open Access articles for commercial, promotional, or marketing purposes requires further explicit permission from Wiley and will be subject to a fee. Commercial purposes include:
Copying or downloading of articles, or linking to such articles for further redistribution, sale or licensing;
Copying, downloading or posting by a site or service that incorporates advertising with such content;
The inclusion or incorporation of article content in other works or services (other than normal quotations with an appropriate citation) that is then available for sale or licensing, for a fee (for example, a compilation produced for marketing purposes, inclusion in a sales pack)
Use of article content (other than normal quotations with appropriate citation) by forprofit organizations for promotional purposes
Linking to article content in e-mails redistributed for promotional, marketing or educational purposes;
Use for the purposes of monetary reward by means of sale, resale, license, loan, transfer or other form of commercial exploitation such as marketing products
Print reprints of Wiley Open Access articles can be purchased from: corporatesales@wiley.com
The modification or adaptation for any purpose of an article referencing the CC BYNC-ND License requires consent which can be requested from RightsLink@wiley.com .

Other Terms and Conditions:
BY CLICKING ON THE "I AGREE..." BOX, YOU ACKNOWLEDGE THAT YOU HAVE READ AND FULLY UNDERSTAND EACH OF THE SECTIONS OF AND PROVISIONS SET FORTH IN THIS AGREEMENT AND THAT YOU ARE IN AGREEMENT WITH AND ARE WILLING TO ACCEPT ALL OF YOUR OBLIGATIONS AS SET FORTH IN THIS AGREEMENT.
v1.8

Appendix D: Rights of Authors of APS Articles

Copyright

The APS Journals are copyrighted for the protection of authors and the Society. The Mandatory Submission Form serves as the Society's official copyright transfer form.

Rights of Authors of APS Articles

For educational purposes only, authors may make copies of their own articles or republish parts of these articles (e.g., figures, tables), without charge and without requesting permission, provided that full acknowledgement of the source is given in the new work. Authors may not post a PDF of their published article on any website; instead, links may be posted to the article on the APS journal website.

Posting of articles or parts of articles is restricted and subject to the conditions below:

- Theses and dissertations. APS permits whole published articles to be reproduced without charge in dissertations and posted to thesis repositories. Full citation is required.

- Open courseware. Articles, or parts of articles, may be posted to a public access courseware website. Permission must be requested from the APS. A copyright fee will apply during the first 12 months of the article's publication by the APS. Full citation is required.

- Institutional websites. The author's published article (in whole or in part) may not be posted to an institutional website, neither at the institutional nor departmental level. This exclusion includes, but is not limited to, library websites and national government websites. Instead, a link to the article on the APS journal website should be used. (See also the APS Policy on Depositing Articles in PMC.)

- Institutional repositories (non-theses). The author's published article (in whole or in part) may not be posted to any institutional repository. This exclusion includes, but is not limited to, library repositories and national government repositories. Instead, a link to the APS journal website should be used. (See also the APS Policy on Depositing Articles in PMC.)

- Author's article in presentations. Authors may use their articles (in whole or in part) for presentations (e.g., at meetings and conferences). These presentations may be reproduced (e.g., in

monographs) on any type of media including, but not limited to, CDs, DVDs, and flash drives, for educational use only in materials arising from the meeting or conference such as the proceedings of a meeting or conference. A copyright fee will apply if there is a charge to the user or if the materials arising are directly or indirectly commercially supported.

- Reuse in another journal before final publication is prohibited. Permission for reuse of an article (whether in whole or in part) in another publication is restricted to the final-published version of the article. If an article is currently published on the APS "publish ahead of print" website (Articles in PresS), then the author must wait to request permission to reuse the article, or any part of the article, until such time when the article appears in final-published form on the APS journal website.

Authors who do not have access to a subscription and/or who are not APS members may:
> - purchase the article through the pay-per-view option, or
> - purchase a Toll-Free Link from APS, which will allow them to post a link to the APS journals website (directly to the article) enabling unlimited free downloads for any user accessing the article via this Toll-Free Link.

Curriculum Vitae

CHARLOTTE WILLEMINA USSELMAN

EDUCATION:

2009 – 2014	**Doctor of Philosophy in Integrative Physiology** School of Kinesiology, Western University, London, ON *Thesis:* Sex and sex hormones: Associations with muscle sympathetic nerve activity *Supervisor:* Dr. J. Kevin Shoemaker
2007 – 2009	**Master's of Science** School of Kinesiology, Western University, London, ON *Thesis:* Lower body negative pressure in rats: Effect of plane of anaesthesia *Supervisor:* Dr. J. Kevin Shoemaker
2003 – 2007	**Honours Bachelor's of Science** Department of Physical Education and Kinesiology, Brock University, St. Catharines, ON *Thesis:* Child-adult differences in force kinetics and neuro-motor function during muscle contraction *Supervisor:* Dr. Bareket Falk

ACADEMIC AWARDS:

		Value
2011-2013	CIHR Frederick Banting and Charles Best Canada Graduate Scholarship (Doctorate)	$81 667
2010-2011	Ontario Graduate Scholarship in Science and Technology (Doctorate)	$10 000
2009-2010	Ontario Graduate Scholarship (Doctorate)	$15 000
2009-2013	Western Graduate Research Scholarship	$8000/yr
2007-2009	NSERC Alexander Graham Bell Canada Graduate Scholarship (Master's)	$17 500
2007	Ontario Graduate Scholarship (declined)	$15 000
2007-2009	Western Graduate Research Scholarship	$2000/yr
2006	Brock Undergraduate Student Research Award (BUSRA)	$3250
2003-2006	Brock Scholars Award	$2000/yr
2003-2006	UWO Faculty Dependents Scholarship	$2100/yr
2004-2006	Brock University Dean's Honour List	n/a

TEACHING EXPERIENCE:

09/2010 – 04/2013   **Tutor** – PHYSIOL 1020, 1021, and 2021: Human Physiology

• Introductory courses in human physiology, emphasizing the principles of human/mammalian physiology and the general properties of the living cell and the internal environment

09/2010 – 04/2013   **Teaching Assistant/Seminar Coordinator** – KIN 9401 and KIN 9501: Bioscience Seminar
Supervised by Dr. Earl Noble (2010-2011, 2012-2013) and Dr. J. Kevin Shoemaker (2011-2012), School of Kinesiology, Western University, London, ON

• Graduate seminar series for the Bioscience stream of Kinesiology; duties included recruiting professors both within UWO and from other universities in Ontario to give guest lectures, coordinating visiting speaker visits, scheduling graduate student lectures

01/2012 – 04/2012   **Tutor** – KIN 2230: Introductory Exercise Physiology

• Kinesiology course outlining the physiological basis of muscular exercise and training, examining metabolic, cardiorespiratory and muscular adaptations to acute and chronic exercise

09/2009 – 12/2009   **Teaching Assistant** – KIN 4432 and PHYSIOL 4442a: Physiology of Exercise
Supervised by Dr. Mark Babcock, School of Kinesiology, Western University, London, ON

• Study of the integrated regulation of blood flow and blood pressure during exercise in health and disease; duties included proctoring and marking midterm and final exams

RESEARCH EXPERIENCE:

05/2007 – 12/2008   **Research Assistant** – Supervised by Dr. J. Kevin Shoemaker, School of Kinesiology, Western University, London, ON

Topic: Cortical associations with cardiovascular responses to isometric hand grip and lower body negative pressure
• fMRI data analysis (Matlab; Alice; SPM2)
• fMRI experimental set-up and data collection

05/2007 - 08/2008	**Research Assistant** – Supervised by Dr. J. Kevin Shoemaker, School of Kinesiology, Western University, London, ON

Topic: Internet-based Strategic Transdisciplinary Approach to Risk Reduction and Treatment
• Obtaining measurements using doppler ultrasound, tonometry

01/2007 - 04/2007	**Research Assistant** – Supervised by Dr. Brian Roy, Department of Physical Education and Kinesiology, Brock University, St. Catharines, ON

Topic: Osmotic stress and glucose uptake in skeletal muscle
• Muscle biochemical analysis
• Immunohistochemistry

05/2006 - 04/2007	**Research Assistant** – Supervised by Dr. Bareket Falk, Dr. Panagiota Klentrou, and Dr. David Gabriel, Department of Physical Education and Kinesiology, Brock University, St. Catharines, ON

Topic: Muscle force development in children and adults
• Use of force dynamometer, electromyography
• Data analysis using MatLab
• Anthropometry (adults and children)

04/2006 - 12/2006	**Research Assistant** – Supervised by Dr. Bareket Falk, Department of Physical Education and Kinesiology, Brock University, St. Catharines, ON

Topic: Bone age and strength in young adult male athletes
• Facilitating physical activity questionnaires for children
• Anthropometry (children)

11/2005 - 04/2006	**Research Assistant** – Supervised by Dr. Bareket Falk, Department of Physical Education and Kinesiology, Brock University, St. Catharines, ON

Topic: Neuro-motor function in child and adult males
• Electromyography, data analysis using MatLab
• Anthropometry

PUBLICATIONS:

Papers Published in Refereed Journals:

1. **Usselman, C.W.,** Luchyshyn, T. A., Gimon, T.I., Nielson, C.A., van Uum, S.H.M., & Shoemaker, J.K. Hormone phase-dependency of neural responses to chemoreflex-driven sympatho-excitation in young women using hormonal contraceptives. *J Appl Physiol* 115(10): 1415-1422, 2013.

2. Shoemaker, J.K., **Usselman, C.W.,** Rothwell, A., & Wong, S.W. Altered cortical activation patterns associated with baroreflex unloading following 24 h of physical deconditioning. *Exp Physiol* 97(12): 1249-1262, 2012.

3. **Usselman, C.W.,** Mattar, L., Twynstra, J., Welch, I., & Shoemaker, J.K. Rodent cardiovascular responses to baroreceptor unloading: Effect of plane of anaesthesia. *Appl Physiol Nutr Metab* 36(3): 376-381, 2011.

4. Salmanpour, A., Brown, L.J., Steinback, C.D., **Usselman, C.W.,** Goswami, R., & Shoemaker, J.K. Relationship between size and latency of action potentials in human muscle sympathetic nerve activity. *J Neurophysiol* 105(6): 2830-2842, 2011.

5. Falk, B., Brunton, L., Dotan, R., **Usselman, C.,** Klentrou, P., & Gabriel, D. Muscle strength and contractile kinetics of isometric elbow flexion in girls and women. *Pediatr Exerc Sci* 21(3): 354-364, 2009.

6. Falk, B., **Usselman, C.,** Dotan, R., Brunton, L., Klentrou, P., Shaw, J. & Gabriel, D. Child-adult differences in muscle strength and activation pattern during isometric elbow flexion and extension. *Appl Physiol Nutr Metab* 34(4): 609-615, 2009.

7. Zamir, M., Norton, K., Fleischhauer, A., Frances, M.F., Goswami, R., **Usselman, C.W.,** Nolan, R.P. & Shoemaker, J.K. Dynamic responsiveness of the vascular bed as a regulatory mechanism in vasomotor control. *J Gen Physiol* 134(1): 69-75, 2009.

Published Contributions to a Collective Work:

8. **Usselman, C.,** Gabriel, D., Dotan, R., Brunton, L., Klentrou, P., Shaw, J. & Falk, B. Muscle force and activation pattern of boys vs. men in isometric elbow flexion and extension. In: T. Jurimae, N. Armstrong, J. Jurimae (Eds.), Children and Exercise XXIV. The proceedings of the 24th Pediatric Work Physiology Meeting. London, Routledge, Taylor & Francis Group, 2009, pp. 264-269.

Published Abstracts:

1. **Usselman, C.W.,** Nielson, C.A., Coverdale, N.S., Luchyshyn, T.A., Gimon, T.I., van Uum, S.H.M., & Shoemaker, J.K. Menstrual cycle-induced variations in estradiol and progesterone associated with baseline and baroreflex-induced muscle sympathetic nerve activation patterns. *Autonomic Neuroscience* 177(1): 51, 2013.

2. **Usselman, C.W.,** Luchyshyn, T.A., Gimon, T.I., Nielson, C.A., Van Uum, S.H.M. & Shoemaker, J.K. Hormone-phase dependence of muscle sympathetic nerve activity in young healthy women using hormonal contraceptives. *FASEB J.* 27: 1118.19, 2013.

3. Coverdale, N., **Usselman, C.W.,** Gimon, T.I., Nielson, C.A., Luchyshyn, T.A. & Shoemaker, J.K. Sympathoinhibition in men during paced breathing at 0.25 Hz. *FASEB J.* 27: 1118.14, 2013.

4. **Usselman, C.W.,** Nielson, C.A., Luchyshyn, T.A., Gimon, T.I. & Shoemaker, J.K. Sex-specific sympathetic responses to chemoreflex stress in healthy young men and women. *FASEB J.* 26: 1091.10, 2012.

5. **Usselman, C.W.,** Luchyshyn, T.A., Gimon, T.I., Nielson, C.A. & Shoemaker, J.K. Sympathetic responses to chemoreflex stress across the menstrual cycle in young eumenorrheic women. *Appl Physiol Nutr Metab* 36(S2): S355, 2011.

6. **Usselman, C.W.**, Welch, I., Mattar, L. & Shoemaker, J.K. Very deep plane of urethane/α-chloralose anaesthesia induces unexpected bradycardia during lower body negative pressure in male Sprague-Dawley rats. *Autonomic Neuroscience* 149(1-2): 155, 2009.

7. **Usselman, C.W.**, Welch, I., Mattar, L. & Shoemaker, J.K. Effect of depth of anaesthesia and postganglionic sympathetic blockade on reflex heart rate control in rodents during lower body negative pressure. *FASEB J.* 23: 609.11, 2009.

8. Norton, K.N., Zamir, J., Frances, M., **Usselman, C.W.**, Fleischhauer, A. & Shoemaker, J.K. Modeling forearm vascular mechanics in hypertension. *FASEB J.* 23: 1017.41, 2009.

9. Brunton, L., Gabriel, D., Dotan, R., **Usselman, C.**, Klentrou, P. & Falk, B. Muscle force and muscle activation in girls and women during isometric elbow flexion and extension. *Ped Exerc Sci* abs http://www.humankinetics.com/PES/NASPEM_abstracts_2008.pdf

10. Frances, M.F., Zamir, M., **Usselman, C.W.**, Goswami, R., Massé, N., Fleischhauer, A., Nolan, R. & Shoemaker, J.K. Vasomotor changes in controlled and uncontrolled hypertension. *FASEB J.* 22: 737, 2008.

11. **Usselman, C.W.**, Wong, S.W., Kimmerly, D.S., Rothwell, A. & Shoemaker, J.K. Hypovolemia affects cortical activity patterns associated with the cardiovascular response to moderate lower body negative pressure (LBNP). *FASEB J.* 22: 740, 2008.

12. **Usselman, C.**, Gabriel, D., Dotan, R., Brunton, L., Klentrou, P., Shaw, J. & Falk, B. Muscle force and activation pattern of boys vs. men in isometric elbow flexion. *Acta Kinesiologiae Universitatis Tartuensis* 12(S): 195, 2007.

13. MacIntosh, S., **Usselman, C.**, Dotan, R., Falk, B., Klentrou, N. & Gabriel, D. Child-adult differences in force kinetics and neuro-motor function during isometric elbow flexion. *Ped Exerc Sci* abs http://www.humankinetics.com/pes/Content/pdfs/NASPEM_Abstracts_For_PES_2006.pdf