Effects of Hypohydration and Menstrual Phase on Pain

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ABSTRACT

Chronic pain is a pervasive health problem and is associated with tremendous societal and economic costs. However, current pain treatments are often ineffective because there are multiple factors that contribute to a person's experience of pain. Recent research showed that mild hypohydration increases experimental pain sensitivity in men, but whether this also occurs in women has not been examined. The fluctuations in ovarian hormone (i.e., 17ß-oestradiol and progesterone) concentrations throughout the menstrual cycle may influence a woman's pain sensitivity, as well as hydration levels. Therefore, interactions between hypohydration and the menstrual phase on pain may exist. To test this hypothesis, this thesis investigated the effects of hypohydration (induced by 24 hr of fluid restriction) on ischaemic pain sensitivity in 14 healthy, eumenorrheic women during the early follicular and mid-luteal phases of their menstrual cycle. In addition, the potential efficacy of acute water ingestion as a countermeasure to the negative impact of hypohydration on pain was also examined. Blood and urinary markers of hydration status indicated that 24 hr of fluid restriction successfully induced mild hypohydration. The major finding is that mild hypohydration reduced ischaemic pain tolerance (by 34 ± 46 s; P = .02, η_p^2 = .37) and increased subjective ratings of both pain intensity (by 0.7 ± 0.7 cm; *P* = .004; η_p^2 = .55) and pain unpleasantness (0.7 ± 0.9 cm; *P* = .02; η_p^2 = .40), irrespective of menstrual phase. Menstrual phase had no apparent effect on pain sensitivity or on hydration status. Acute water ingestion decreased thirst sensation (by 2.3 ± 0.9 cm; *P* < .001, η_p^2 = .88) but did not reverse the hyperalgesic effects of hypohydration. The effects of hypohydration on pain sensitivity were not explained by differences in state anxiety levels or mood state. In conclusion, the findings from this thesis extend to women, previous data in men that showed increases in pain sensitivity with mild hypohydration. This thesis also provides strong evidence that the menstrual phase does not

influence pain sensitivity, after hydration status was controlled, the influence of potential confounders was minimised, and when menstrual phases were accurately verified. Lastly, these findings underscore the importance of ingesting fluids regularly throughout the day to maintain adequate hydration and avoid dehydration, especially for individuals experiencing pain.

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TABLE OF CONTENTS

ABSTRACT I

ACKNOWLEDGMENTS III

TABLE OF CONTENTS IV

LIST OF ABBREVIATIONS X

LIST OF TABLES XII

LIST OF FIGURES XIII

CHAPTER ONE 1

1.0. Introduction 1

CHAPTER TWO 5

2.0. Review of Literature 5

2.1. Human Pain 6

2.1.1. Why Study Pain? 6

2.1.2. How to Study Pain and on Whom? 7

2.2. Pain in Women 10

2.2.1. Overview of Menstrual Cycle Physiology 10

2.2.2. Acute Experimental Pain and the Menstrual Phase 11

2.2.3. Experimental Pain Modulation and the Menstrual Phase 13

2.2.4. Chronic Pain Severity and the Menstrual Phase 14

2.2.5. Summary of Pain in Women 15

2.3. Hydration, Menstrual Phase and Pain 17

2.3.1. Hypohydration and Pain 17

2.3.2. Hydration and the Menstrual Phase 18

2.3.3. Implications of Hydration for Pain in Women 20

2.4. Other Female Populations 22

2.4.1. Oral Contraceptive Pill Users 22

2.4.2. Postmenopausal Women 24

2.5. Conclusions 26

CHAPTER THREE 27

3.0. Research Aims and Hypotheses 27

3.2. Aims 28

3.2. Hypotheses 29

CHAPTER FOUR 30

4.0. General Methodology 30

4.1. Experimental Pain Assessment 31

4.1.1. Experimental Pain Stimulus 31

4.1.2. Ischaemic Pain Test 32

4.1.3. Ischaemic Pain Measures 35

4.2. Hydration Status 40

4.2.1. Dehydration Method 40

4.2.2. Measures of Hydration Status 42

4.3. Hormonal Status 59

4.3.1. Selection of Menstrual Phases 59

4.3.2. Menstrual Phase Verification 59

4.4. Psychological Variables 64

4.4.1. Depressive Symptomatology 64

4.4.2. State Anxiety 67

4.4.3. Mood State 69

4.5. Ethical Approval 72

4.6. Participant Characteristics 72

4.7. Experimental Overview 75

4.8. Familiarisation 77

4.8.1. Maximal Handgrip Strength Assessment 77

4.8.2. Test-retest Reliability of Ischaemic Pain Measures 78

4.9. Pre-Experimental Controls....80
4.10. Experimental Protocol....83
4.10.1. Ischaemic Pain Test Protocol....84
4.11. Measurements....86
4.11.1. Anthropometric....86
4.11.2. Blood Analyses....86
4.12. Statistical Analyses....88
CHAPTER FIVE....89
5.0. Results....89
5.1. Self-Reported Sleep Duration and Depressive Symptomatology....91
5.2. Menstrual Phase Identification and Verification....92
5.2.1. Ovarian Hormone Concentrations....92
5.3. Hydration Status....94
5.3.1. Hydration Markers....94
5.3.2. Thirst Sensation Ratings....96
5.4. Ischaemic Pain Measures....97
5.4.1. Pain Tolerance....97
5.4.2. Pain Intensity and Pain Unpleasantness Ratings....98

5.5. Psychological Variables 101

5.5.1. State Anxiety 101

5.5.2. Mood State 102

5.6. Correlations 104

5.6.1. Hydration Variables and Ischaemic Pain Measures 104

5.6.2. Ovarian Hormones and Ischaemic Pain Measures 105

CHAPTER SIX 108

6.0. Discussion 108

6.1. Hypohydration and Pain in Women 109

6.2. Menstrual Phase and Pain Sensitivity 113

6.3. Psychological Variables 117

6.4. Hydration Status 119

6.5. Effects of Acute Water Ingestion 121

6.6. Considerations and Limitations 125

6.7. Perspectives 131

6.8. Conclusions and Future Directions 133

6.9. Reflections 135

REFERENCES 139

APPENDIX I 188

Statement of Contribution 188

APPENDIX II 189

Explanation of COVID-19 Impacts 189

LIST OF ABBREVIATIONS

A

ANOVA	Analysis of variance
AVP	Arginine vasopressin

B

BMI	Body mass index
BRUMS	Brunel Mood Scale

C

CES-D	Centre for Epidemiologic Studies Depression scale
CPM	Conditioned pain modulation

E

EF	Early follicular phase
EUH	Euhydrated trial
ELISA	Enzyme-linked immune assay

H

HRT	Hormone replacement therapy
HYPO	Hypohydrated trial

L

LH	Luteinising hormone

M

ML	Mid-luteal phase
N	
NRS	Numeric rating scale
O	
OCP	Oral contraceptive pill
P	
η_p^2	Partial-eta squared
P_{osm}	Plasma osmolality
POMS	Profile of Mood States
P_4:E_2	Ratio of progesterone to 17ß-oestradiol
S	
S_{osm}	Serum osmolality
STAI-S	State scale of the State-Trait Anxiety Inventory
T	
TMD	Total mood disturbance
U	
USG	Urine specific gravity
V	
VRS	Verbal rating scale
VAS	Visual analogue scale

LIST OF TABLES

Table 1. Characteristics of each participant.....74

Table 2. Mean ovarian hormone concentrations in each hydration condition and menstrual phase. 93

Table 3. Hydration markers at baseline (i.e., before water ingestion) in each hydration condition and menstrual phase.....95

Table 4. Mean scores on each subscale of the BRUMS (all n = 14) and the TMD scores (n = 14) at baseline (i.e., before water ingestion) and after water ingestion in each hydration condition and menstrual phase.....103

Table 5. Pearson correlation coefficients, r, for linear relationships between hydration markers, ovarian hormone concentrations and ischaemic pain measures.....107

LIST OF FIGURES

Figure 1. Fluctuations of oestradiol and progesterone across a conventional 28-day menstrual cycle. Figure from McNulty et al. (2020). 11

Figure 2. Force output form a participant across the 25 handgrip exercises. Black horizontal line represents the target force (i.e., 30% of maximum strength). 33

Figure 3. Schematic diagram of study design, along with the hormonal profile during each phase, for participants who started their trials in the (A) early follicular phase and (B) mid-luteal phase 76

Figure 4. Individual and mean (A) pain tolerance, (B) pain intensity ratings and (C) pain unpleasantness ratings across three separate sessions. 79

Figure 5. Schematic diagram of the experimental protocol. 84

Figure 6. Mean CES-D score (n = 13) in each menstrual phase. 91

Figure 7. Mean ratings of thirst sensation (n = 13) at baseline (i.e., before water ingestion) and after water ingestion in each hydration condition and menstrual phase. 96

Figure 8. Mean pain tolerance (n = 14) at baseline (i.e., before water ingestion) and after water ingestion in each hydration condition and menstrual phase. 97

Figure 9. Mean ratings of (A) pain intensity (n = 12) and (B) pain unpleasantness (n = 12) at 1 min after the handgrip exercises, at baseline (i.e., before water ingestion) and after water ingestion in each hydration condition and menstrual phase. 99

Figure 10. Mean ratings of (A) pain intensity (n = 14) and (B) pain unpleasantness (n = 14) at pain tolerance, at baseline (i.e., before water ingestion) and after water ingestion in each hydration condition and menstrual phase. 100

Figure 11. Mean scores on the STAI-S (n = 14) at baseline (i.e., before water ingestion) and after water ingestion in each hydration condition and menstrual phase. 101

Figure 12. Linear relationship between 17ß-oestradiol concentrations and (A) pain tolerance and (B) pain unpleasantness ratings.....106

CHAPTER ONE

1.0. Introduction

Total body water represents 50 to 70% of body mass, making it the principal constituent of the human body (Altman and Katz, 1961). Humans lose approximately 1 to 3 L of body water per day through insensible (i.e., non-sweating) means, which must be constantly replaced by drinking water, or from the consumption of water in foods and beverages, to maintain body fluid homeostasis Adequate hydration is vital for optimal functioning of almost all physiological systems in the body, including temperature regulation, cardiovascular function and digestion (Sawka et al., 2005; Jéquier and Constant, 2010). The importance of adequate fluid intake cannot be understated; yet, many individuals do not meet the recommended guidelines for daily water intake (Drewnowski et al., 2013; Gibson and Shirreffs, 2013), which may increase their risk of becoming hypohydrated. Furthermore, a considerable proportion of the general population, including children and the elderly, appear to be inadequately hydrated (Stookey, 2005; Perrier et al., 2013b; Stookey, 2019). In a recent nationwide survey, around 70% of adults in the United States did not meet the hydration criteria set by the authors (Stookey, 2019). This is especially important in the context of increasing heat exposure due to a warming climate and more frequent and severe heatwaves (Meehl and Tebaldi, 2004; Luber and McGeehin, 2008).

Hypohydration of ≥ 2% body mass loss is known to negatively impact physical performance, cognitive function, psychological state and health (Popkin et al., 2010; Cheuvront and Kenefick, 2014; Masento et al., 2014). Some of these deleterious consequences may even manifest with milder levels of hypohydration (< 2% body mass loss) that often occur during

activities of daily living (Maughan, 2003; Bardis et al., 2013; Benton et al., 2016). More recently, mild hypohydration was also shown to increase subjective pain ratings in men (Ogino et al., 2014; Moyen et al., 2015; Bear et al., 2016), although research in this area is still in its infancy. This is pertinent as pain has been recognised as a public health problem (Goldberg and McGee, 2011). However, the treatment of pain has proven difficult due to the complex and multi-factorial nature of pain (Turk et al., 2011). Furthermore, whether hypohydration also affects pain in women has not been examined.

The ovarian hormones influence many of the pain processing and modulation pathways (Aloisi and Bonifazi, 2006; Amandusson and Blomqvist, 2013).The fluctuations in these hormones throughout the menstrual cycle have also been associated with changes in a woman's perception of pain (Riley et al., 1999; Martin, 2009). Although it is quite clear that the severity of pain symptoms in several chronic pain conditions varies across the menstrual cycle (Martin, 2009; Hassan et al., 2014), whether the menstrual phase affects experimental pain sensitivity in healthy, pain-free women is still up for debate (Sherman and LeResche, 2006; Iacovides et al., 2015a). This is mostly because many studies on the topic did not accurately verify menstrual phases or account for various potential confounders [e.g., dysmenorrhoea (menstrual pain), dietary and lifestyle factors]. Although some studies have attempted to address these issues (e.g., Kowalczyk et al., 2006a; Klatzkin et al., 2010), there is a dearth of such studies. Therefore, the true effect of menstrual phase on experimental pain sensitivity remains to be determined. Moreover, none of the previous studies measured hydration status, which could vary across the menstrual cycle (Giersch et al., 2019).

In addition to their effects on pain, the ovarian hormones also affect the mechanisms involved in body fluid regulation (Stachenfeld, 2008), with implications for hydration status and the

responses to a given hydration challenge (e.g., dehydration) (Giersch et al., 2019). Thus, there may be potential interactions between hypohydration and the menstrual phase on pain, but this hypothesis has yet to be tested.

Another unanswered question is whether the potential mechanisms by which hypohydration increases pain sensitivity are related to physiological changes, or the subjective feelings of thirst. Previous studies suggest a role for thirst (Farrell et al., 2006; Ogino et al., 2014; Geuter et al., 2016), but the independent effect of thirst *per se* on pain sensitivity cannot be determined from those studies.

These questions surrounding the separate and combined effects of hypohydration and the menstrual phase on pain sensitivity in women may have important implications for the treatment of pain in women, interpretation of previous research, design of future pain studies and accuracy of fluid intake guidelines for women. For example, women may need to be more cognisant of their fluid intake during certain phases of their menstrual cycle, especially if they are dealing with pain. Therefore, rather than conducting a series of studies, this thesis consists of one large study that aims to answer the following questions:

1. Does hypohydration affect pain sensitivity in healthy, eumenorrheic women?

2. Do the effects of hypohydration on pain sensitivity vary as a function of menstrual phase?

3. Does menstrual phase have an independent effect on pain sensitivity, when menstrual phases are properly verified, when hydration status is controlled and when potential confounders are minimised (e.g., dietary and lifestyle factors, dysmenorrhea)?

4. Can the negative impacts of hypohydration on pain sensitivity be remedied immediately by having a drink of water to quench thirst?

To answer these questions, the relevant literature surrounding the effects of menstrual phase on both pain and hydration, as well as the effects of hypohydration on pain, will first be reviewed (**Chapter Two**). From this focussed review of the literature, the aims and hypotheses of this thesis are subsequently formulated and described (**Chapter Three**). In **Chapter Four**, the strengths and limitations of the methodology, experimental measures and procedures used in this thesis will first be reviewed, along with justification for selecting them. This will be followed by a description of the study design and experimental protocol. Next, the findings from this thesis will be described (**Chapter Five**). **Chapter Six** will start with an in-depth discussion of the relevant findings from this thesis, followed by a characterisation of the considerations and limitations of the study. The findings of this thesis will then be summarised with recommendations for future research. Lastly, the chapter will conclude with a personal reflection on this doctoral journey.

CHAPTER TWO

2.0. Review of Literature

Publication based on this chapter:

Tan, B., Philipp, M., Hill, S., Muhamed, A.M.C., Mündel, T. (2020). Pain across the menstrual cycle: Considerations of hydration. *Front Physiol* 11, 585667. doi: 10.3389/fphys.2020.585667.

This chapter contains a focussed review of the relevant literature concerning the effects of the menstrual phase on both pain and hydration, as well as the effects of hypohydration on pain. The purposes of this review are to: (i) summarise the existing literature in these areas, and (ii) explore how hypohydration and the menstrual phase may interact to influence pain. Based upon this literature review, the primary focus of this thesis will be stated, followed by a description of the aims and hypotheses in the subsequent chapter (**Chapter Three**).

2.1. Human Pain

2.1.1. Why Study Pain?

Chronic pain – typically defined as pain that persists for more than 3 months (Treede et al., 2015) – is a major health issue with a high and increasing prevalence both locally and globally. In New Zealand, almost 780,000 adults (or 21%) reported experiencing chronic pain in the year 2016. Compared to the year 2007, this represented a 37% increase in the total number adults affected by pain (Ministry of Health, 2016). A survey conducted in the United States estimated that 17% of the adult population (39 million adults) suffer from chronic pain (Nahin, 2015), while a recent meta-analysis reported a 43% (28 million adults) prevalence of chronic pain in the United Kingdom (Fayaz et al., 2016). The prevalence of chronic pain is expected to continue to rise, especially with an ageing population. Apart from its debilitating impact on numerous aspects of the lives of the affected individuals and their families (Turk et al., 2011), chronic pain is also associated with tremendous direct and indirect economic costs. A report estimated that the total cost of arthritis in New Zealand in the year 2010 was $3.2 billion (1.7% of gross domestic product), with almost half of this amount being attributed to productivity losses alone (Access Economics, 2010). The annual cost of pain in the United States in 2008 was reported to be between $560 and $635 billion dollars, which was more than that for heart disease, cancer and diabetes (Gaskin and Richard, 2012).

Chronic pain can be caused by numerous reasons, such as an existing physical injury, infection and various diseases (e.g., cancer, sickle cell disease, diabetes) (Institute of Medicine, 2011; Schubiner et al., 2017). However, in many cases, chronic pain is not attributable to any detectable pathology or structural issue (Jacobs, 2013; Schubiner et al., 2017). For example, among patients with low back pain, around 85% of them do not have a

specific and diagnosable physical cause for their pain (Deyo et al., 1992). This is partly because pain is a highly subjective and individual experience, with an abundance of physical, biological and psychosocial factors that independently and interactively influence an individual's pain experience (Gatchel et al., 2007; Fillingim, 2017b). This complex nature of pain makes treating chronic pain very difficult. In fact, many current pain treatments are often only minimally effective in eliminating pain in improving quality of life (Martin et al., 2008; Turk et al., 2011). One review found that the average success rate of pain treatments was only "roughly 30% in about half of treated patients" (Turk et al., 2011, pp. 2232). In a survey performed across 15 European countries and Israel, 40% of chronic pain sufferers reported feeling dissatisfied with the effectiveness of their pain treatment(s) (Breivik et al., 2006). Therefore, further knowledge and understanding of the mechanisms and factors that contribute to pain is fundamental for the development of more effective pain treatments and management strategies.

2.1.2. How to Study Pain and on Whom?

Research on pain is usually conducted by inducing acute pain using various experimental modalities in healthy, pain-free individuals. Although recruiting patients with chronic pain as participants would enhance ecological validity of these studies, and is often the target population of interventions, this is often difficult to do due to the associated logistical and ethical considerations. Furthermore, chronic pain sufferers often have several comorbidities (e.g., depression, anxiety disorders, insomnia) that can directly affect pain perception (Bruce et al., 2006; Asmundson and Katz, 2009; Ostovar-Kermani et al., 2020).

Unlike chronic pain, acute pain is characterised as being temporary and is caused by a specific stimulus (e.g., injury, surgery, dental work). It serves as a warning sign of actual or

impending tissue damage and usually goes away once the damage has healed (Grichnik and Ferrante, 1991; Świeboda et al., 2013). A range of pain stimuli have been used in studies on acute experimental pain. The more commonly used pain modalities are the cold pressor task, muscle ischaemia, mechanical pressure, noxious thermal pain (heat or cold) and electrical stimulation. The type of pain evoked by each stimulus differ in a number of characteristics, such as the sensations they produce, pain mechanisms they activate, and more important, their resemblance to clinical pain (Rainville et al., 1992; Fillingim and Ness, 2000). For example, muscle ischaemia is thought to be the most clinically relevant pain stimulus, because the deep and aching pain produced by this stimulus may better replicate the type of pain experienced by chronic pain patients (Moore et al., 1979; Rainville et al., 1992; Iacovides et al., 2015a). The responses to these pain stimuli are commonly assessed using the following outcome measures: pain threshold (least amount of a stimulus that elicits feelings of pain), pain tolerance (maximum amount of a painful stimulus that an individual can, or is willing to, endure), subjective ratings of pain intensity and/or pain unpleasantness (Edens and Gil, 1995; Hastie et al., 2005; IASP, 2017). For clarity, throughout this thesis, *pain sensitivity* will refer to the outcome of one, or a combination, of these measures. For example, an increase in pain sensitivity would indicate a decrease in pain threshold, decrease in pain tolerance and/or increase in subjective pain ratings.

To date, most of the studies on acute experimental pain have been performed exclusively in male participants, with only a handful performed in women. For instance, among non-human animal studies on pain, 79% of studies that were published from the year 1996 to 2005 were performed solely in male animals, compared to a mere 8% in female animals (Mogil and Chanda, 2005). In the human literature, a similar bias towards males has been observed across many disciplines, including behavioural research (Beery and Zucker, 2011). Yet, there

are clear sex differences in both chronic pain and acute experimental pain (Unruh, 1996; Berkley, 1997; Riley et al., 1998; Fillingim et al., 2009). In women specifically, the ovarian hormones and their fluctuations across the menstrual cycle can influence pain. Furthermore, these hormones may also affect hydration status – a variable that has recently been implicated as a contributing factor to pain in men (Ogino et al., 2014; Bear et al., 2016), although this has not been investigated in women. Given that women make up half of the global population, it is important to focus specific research on pain in women. Increased understanding of pain in women could have important implications for the treatment and management of pain in this population, which could ultimately help alleviate the detrimental economic and social consequences of pain.

Several reviews on the menstrual phase and pain have been published (Riley et al., 1999; Sherman and LeResche, 2006; Martin, 2009; Hassan et al., 2014; Iacovides et al., 2015a). However, none of these reviews have addressed the topic with a consideration of hydration status. Therefore, the purposes of this literature review are to: (i) summarise the existing literature on pain in women, specifically as it relates to the menstrual phase and(potentially) hydration status, and (ii) make recommendations for future research, thereby providing a platform upon which this experimental thesis could operate. A search of the published literature was performed through July 2020 using the PubMed database and Google Scholar search engine, whilst second- and third-order reference lists were checked manually for relevant articles.

2.2. Pain in Women

Sex differences in pain have been extensively studied and research in this area has dominated the pain literature for years (Fillingim et al., 2009). Several comprehensive reviews and meta-analyses on this topic have since been published, which readers are referred to (e.g., Riley et al., 1998; Fillingim et al., 2009; Mogil, 2012; Hashmi and Davis, 2014; Fillingim, 2017a). Overall, there appears to be an agreement that several chronic pain conditions are more prevalent among women than in men (Fillingim et al., 2009; Mogil, 2012). In laboratory settings, women also tend to display greater sensitivity to various experimental pain stimuli compared to men (Fillingim and Maixner, 1995; Riley et al., 1998). Sex differences in endogenous pain modulation pathways, such as opioid analgesia and conditioned pain modulation, have also been reported (Fillingim and Ness, 2000; Paller et al., 2009; Niesters et al., 2010; Popescu et al., 2010; Pisanu et al., 2019). As such, these findings underscore the importance of studying pain specifically in women, as it can have implications for the treatment of their pain.

2.2.1. Overview of Menstrual Cycle Physiology

Blood concentrations of the primary ovarian hormones – 17ß-oestradiol and progesterone – change cyclically during the menstrual cycle. The menstrual cycle, which lasts for an average of 28 days, can be broadly divided into two phases: follicular and luteal phase. The follicular phase begins with the first day of menses (i.e., menstrual bleeding) and lasts until ovulation (~ day 14). During this phase, concentrations of 17ß-oestradiol initially remain low and stable while menses occurs, increase steadily after cessation of menses, then rise sharply and peak during the last few days of the follicular phase. Progesterone concentrations, on the other hand, remain low throughout the follicular phase. Following the spike in 17ß-oestradiol, a

mid-cycle surge in luteinising hormone (LH) occurs, resulting in ovulation and the start of the luteal phase. The luteal phase is generally characterised by a rise in progesterone levels to its highest point and a concomitant rise in 17ß-oestradiol to moderate levels. Towards the end of the luteal phase, concentrations of both progesterone and 17ß-oestradiol fall rapidly, resulting in the onset of menses and the start of a new cycle (Figure 1).

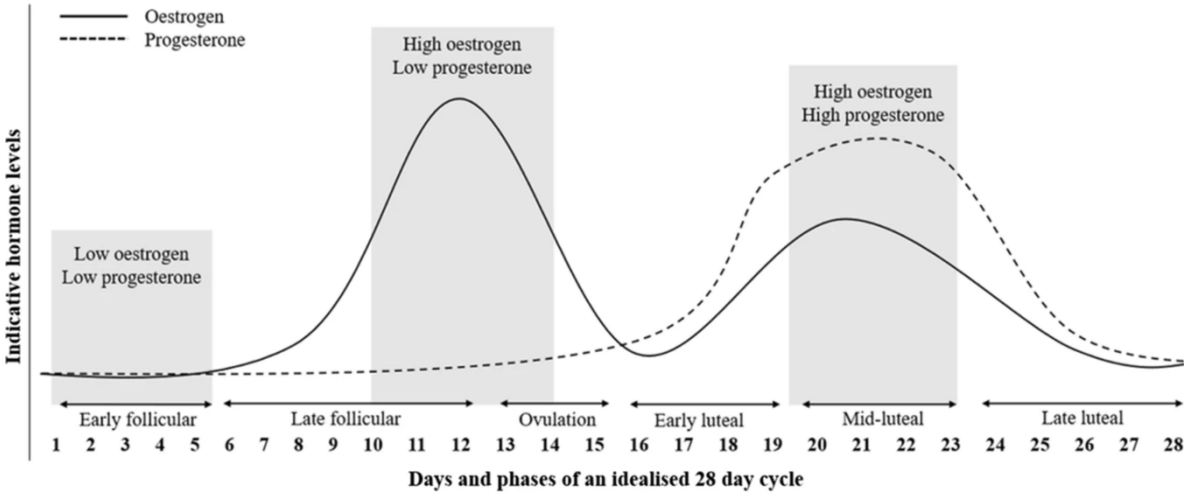

Figure 1. Fluctuations of oestradiol and progesterone across a conventional 28-day menstrual cycle. Figure from McNulty et al. (2020).

Besides these hormonal variations, women also tend to experience fluctuations in several physical and emotional symptoms such as bloatedness, fatigue, irritability and anxiety over the course of the menstrual cycle (Pfleeger et al., 1997; Alonso et al., 2004). Of importance to this thesis, recent experimental and clinical data have also shown changes in both chronic pain and acute experimental pain across the menstrual cycle (Martin, 2009; Hassan et al., 2014).

2.2.2. Acute Experimental Pain and the Menstrual Phase

An early meta-analysis by Riley et al. (1999) and a subsequent review by Martin (2009) both concluded that there are modest effects of the menstrual phase on experimental pain

sensitivity in healthy, pain-free women, although the direction of effects reported by both groups of authors are contradictory. Riley et al. (1999) found *lower* pain sensitivity in the follicular compared to luteal phase, whereas Martin (2009) concluded that pain sensitivity was *higher* in the follicular phase. In contrast, Sherman and LeResche (2006) reported that the available findings are largely equivocal. Similarly, a recent review of 42 studies by Iacovides et al. (2015a) concluded that the current body of research does not paint a definitive picture on whether the menstrual phase affects experimental pain sensitivity. The conflicting findings are largely due to the numerous methodological inconsistencies across studies, such as differences in the experimental pain stimuli used and definition of menstrual phases. Moreover, many studies did not confirm that participants had ovulated or measure blood concentrations of 17ß-oestradiol and progesterone to verify menstrual phases, which could have affected the results or the interpretation of those findings. These various methodological inconsistencies and limitations have been comprehensively reviewed by Sherman and LeResche (2006), which readers are referred to. It is interesting to note that, in the most recent review by Iacovides et al. (2015a), a slightly greater proportion (64%) of the studies reviewed found variations in experimental pain sensitivity across the menstrual cycle. However, the authors noted that the better controlled studies mostly did not find an effect of the menstrual phase on pain sensitivity, although those studies were not without some of the methodological limitations as highlighted by Sherman and LeResche (2006). To the best of the candidate's knowledge, nine studies have been published since the time of the previous review in 2015 (Bartley et al., 2015; Iacovides et al., 2015b; Cankar et al., 2016; Palit et al., 2016; Alves et al., 2017; Nayak et al., 2017; Jasrotia et al., 2018; Payne et al., 2019; Pogatzki-Zahn et al., 2019). The results of these studies are also inconsistent, with a roughly

equal number of studies that did (5/9 studies), or did not (4/9 studies), observe variations in pain sensitivity across the menstrual cycle.

A small number of studies have investigated whether the menstrual phase affects experimental pain sensitivity in women with chronic pain conditions. Like the research in healthy, pain-free women, the findings of these studies are also largely inconsistent; three studies found variations in experimental pain sensitivity across the menstrual cycle (Isselée et al., 2002; Sherman et al., 2005; Teepker et al., 2011), while four studies did not observe any variability (Alonso et al., 2004; Okifuji and Turk, 2006; Vignolo et al., 2008; Balter et al., 2013).

2.2.3. Experimental Pain Modulation and the Menstrual Phase

Some of the observed effects of the menstrual phase on experimental pain sensitivity could be related to endogenous pain modulation systems. These consist of pain inhibitory and facilitatory mechanisms that decrease or increase, respectively, the pain signals that are transmitted from the nociceptors to the brain, where pain is eventually produced. Pain modulation systems may partly explain why the amount of pain experienced is often not correlated with the size of the stimulus or severity of tissue damage (Olango and Finn, 2014). Pain modulation in humans can be studied experimentally using various methods, such as opioid analgesia, placebo analgesia and stress-induced hyperalgesia or analgesia (Fillingim et al., 2009). Of these, theConditioned Pain Modulation (CPM) paradigm is perhaps the most widely used in experimental settings (Yarnitsky et al., 2010; Lewis et al., 2012; Kennedy et al., 2016). The CPM, which activates the pain inhibition pathway, involves applying an experimental pain stimulus to one part of the body to dampen the pain produced by a different noxious stimulus applied to another body part (Damien et al., 2018).

Nine studies have examined experimental pain modulation across the menstrual cycle in healthy, pain-free women; seven of these studies assessed pain inhibition using the CPM paradigm (Tousignant-Laflamme and Marchand, 2009; Rezaii and Ernberg, 2010; Bartley and Rhudy, 2012; Rezaii et al., 2012; Wilson et al., 2013; Teepker et al., 2014; Palit et al., 2016), while two studies used an emotional picture-viewing paradigm that assesses both pain inhibition and pain facilitation. In this method, participants are shown a series of pictures that are intended to evoke negative or positive emotions, in order to increase or decrease, respectively, the perceived intensity of a noxious stimulus (Rhudy and Bartley, 2010; Rhudy et al., 2013). Most of the CPM studies (5/7 studies) did not observe changes in the magnitude of pain inhibition across the menstrual cycle. Similarly, the two studies that used the emotional-picture viewing paradigm also did not find any effect of the menstrual phase on emotional pain modulation.

In the only study that investigated pain modulation across the menstrual cycle in women with a chronic pain condition (migraine), no effect of the menstrual phase on CPM inhibition was observed (Teepker et al., 2014).

2.2.4. Chronic Pain Severity and the Menstrual Phase

In contrast to the uncertainty regarding the effects of menstrual phase on experimental pain sensitivity, studies examining the relationship between menstrual phase and chronic pain have produced more consistent results. There are currently two published reviews on this topic (Martin, 2009; Hassan et al., 2014). The authors of both reviews found robust evidence indicating that there is menstrual cycle-related variability in the severity of pain symptoms in women with various chronic pain conditions (e.g., migraine, temporomandibular pain disorder, fibromyalgia, rheumatoid arthritis, irritable bowel syndrome). Moreover, the

majority of the data appear to show a worsening of self-reported pain severity during the early follicular and/or late-luteal phase, when concentrations of 17ß-oestradiol are low (Martin, 2009; Hassan et al., 2014). However, much of the chronic pain research is also confounded by various methodological inconsistencies and limitations across studies. These include differences in the way pain symptoms were assessed, not controlling for comorbidities, not measuring circulating levels of ovarian hormones to verify menstrual phases, among others (Hassan et al., 2014).

2.2.5. Summary of Pain in Women

Despite the relatively large body of research on the effects of menstrual phase on experimental pain sensitivity, there is currently no agreement among researchers on whether the menstrual cycle does, or does not, affect experimental pain sensitivity in healthy women or those with chronic pain conditions. Regarding experimental pain modulation, the limited number of studies in this area mostly did not observe any effect of menstrual phase on CPM inhibition or emotional pain modulation. In contrast, the severity of pain symptoms for many chronic pain conditions has consistently been shown to vary across the menstrual cycle. Although incompletely understood, the potential mechanisms underlying the variations in pain across the menstrual cycle could be due to effects of the ovarian hormones on various pain pathways in both the central and peripheral nervous systems, responses to stress and inflammation, or neurotransmitters such as serotonin and γ-aminobutyric acid (GABA) (Marcus, 1995; Martin, 2009). However, a discussion on the mechanisms is beyond the scope of this literature review and interested readers are directed to excellent reviews on this topic (Fillingim and Maixner, 1995; Aloisi and Bonifazi, 2006; Amandusson and Blomqvist, 2013).

The overall ambiguity in this area of research is mostly due to the various methodological inconsistencies and limitations across many of the studies. While a handful of studies have sought to address some of these problems, such as measuring ovarian hormone concentrations in the blood and confirming ovulation, there is a paucity of such better-controlled studies. Moreover, none of the previous studies assessed the hydration status of participants, which could be a possible confound.

2.3. Hydration, Menstrual Phase and Pain

Euhydration is a state of *normal* body water content, whereas hypohydration refers to the state of *reduced* body water content that exceeds the normal daily fluctuations (>2% body mass loss) (Greenleaf, 1992; American College of Sports et al., 2007). While there is some variability across studies and between individuals, the commonly used biochemical thresholds for defining hypohydration are: serum osmolality (S_{osm}) > 290 mOsm.kg^{-1}, urine specific gravity (USG) > 1.020 and/or urine osmolality > 700 mOsm.kg^{-1} (Cheuvront and Sawka, 2005; American College of Sports et al., 2007; Armstrong et al., 2010; Armstrong et al., 2012). Dehydration, on the other hand, refers to the *process* of fluid loss that results in hypohydration (Akerman et al., 2016; Nuccio et al., 2017)

Hypohydration occurs when body fluid losses exceed fluid intake. Excessive fluid losses incurred through sweating (e.g., prolonged exercise, strenuous work, environmental heat exposure) is perhaps the most common way individuals become hypohydrated. This is especially prevalent among athletes, where approximately 75% of them are already hypohydrated upon arrival at their training sessions (Volpe et al., 2009; Arnaoutis et al., 2015; Magal et al., 2015). However, inadequate fluid intake during normal daily activities can also lead to hypohydration. Therefore, in addition to athletes, hypohydration is also a problem among the general public (Manz and Wentz, 2005; Chang et al., 2016).

2.3.1. Hypohydration and Pain

Hypohydration has been shown to negatively impact cognitive function, mood state and fatigue in women (Szinnai et al., 2005; Armstrong et al., 2011; Pross et al., 2013). These factors, in turn, can contribute to pain (Willoughby et al., 2002), therefore indicating a

possible relationship between hypohydration and pain. Indeed, recent research indicates that hypohydration can increase pain. Mild hypohydration of as little as a 1% loss in body mass, induced by a combination of fasting and exercise, invoked greater activity in pain-related regions of the brain during stimulation of cold pressor pain in men (Ogino et al., 2014). Participants also displayed greater pain sensitivity during the cold pressor task when they were hypohydrated, compared to when they were rehydrated. Similar observations were made in a later study, where a group of men dehydrated by restricting fluid intake for 24 hours (Bear et al., 2016). Mild hypohydration (1% body mass loss) was found to increase experimental pain sensitivity relative to the euhydrated condition (Bear et al., 2016). However, both studies were exclusively performed in men and it is not known whether hypohydration can also contribute to pain in women. In the only study that included female participants, Moyen et al. (2015) conducted a field study on 103 male and 16 female cyclists who were taking part in an ultra-endurance race. Cyclists who were hypohydrated before and during the race reported more intense pain in their leg muscles compared to the euhydrated cyclists. The authors also reported examining possible differences in the pain ratings between the male and female cyclists and did not find sex differences, indicating that hypohydration may also increase pain in women. Nonetheless, the effects of hypohydration on pain in women has not been formally investigated. This is important as the menstrual phase is associated with variations in body fluid regulation, in addition to their potential impacts on pain as discussed previously.

2.3.2. Hydration and the Menstrual Phase

One of the more prominent impacts of the menstrual phase on hydration is the osmotic control of arginine vasopressin (AVP) and thirst sensation (Spruce et al., 1985; Vokes et al.,

1988; Stachenfeld, 2008). Arginine vasopressin – also known as anti-diuretic hormone – is one of the primary hormones involved in body fluid regulation and its main effect in this context is to increase free-water retention in the kidneys (Baylis, 1987). Meanwhile, thirst sensation is a key regulator of fluid intake (McKinley and Johnson, 2004). Both AVP and thirst are primarily stimulated by an increase in serum/plasma osmolality (a biomarker of hydration status), such as during dehydration (Baylis, 1987; McKinley and Johnson, 2004).

The luteal phase has been associated with a lowering of the osmotic thresholds at which AVP is released and thirst is stimulated (Spruce et al., 1985; Vokes et al., 1988; Stachenfeld et al., 1999; Stachenfeld et al., 2001) – effects that are primarily attributed to 17ß-oestradiol (Calzone et al., 2001; Stachenfeld and Keefe, 2002). In other words, less of an osmotic stimulus is required to activate the AVP and thirst responses (and their respective effects on increasing renal water retention and fluid intake) during the luteal phase (Giersch et al., 2019). There could also be increased sodium (and thus, water) retention in the luteal phase, by way of the progesterone-related increase in plasma aldosterone concentrations during this phase (De Souza et al., 1989; Stachenfeld et al., 1999; Stachenfeld et al., 2001). Moreover, other studies have also observed an increase in body water content measured by bioelectrical impedance analysis during the luteal versus follicular phase (Bunt et al., 1989; Mitchell et al., 1993; Tomazo-Ravnik and Jakopič, 2006; Fruzzetti et al., 2007; Stachoń, 2016). Therefore, these findings indicate that women may be more protected against dehydration in the luteal compared to follicular phase. Yet, a decrease in plasma volume – the fluid component of the blood that is most affected by changes in hydration status – during the luteal phase is also commonly reported (Stephenson and Kolka, 1988; Stachenfeld et al., 1999; Stachenfeld et al., 2001). This is thought to be caused by the preferential movement of fluid from the intravascular space into the interstitium (Øian et al., 1987). Nevertheless, these findings

demonstrate that there are differences in body fluid regulation between menstrual phases, which could subsequently affect hydration status.

More important, research indicates that hypohydration is a common occurrence among women. A study by Malisova et al. (2016) showed that approximately 20% of women in Europe met the criteria for hypohydration (urine osmolality > 810 $mOsm.kg^{-1}$). In the United States, data from the third National Health and Nutrition Examination Survey on 7,855 women classified around half of the women as being hypohydrated (plasma tonicity > 295 $mOsm.kg^{-1}$) (Stookey, 2005). Another study on 958 women in Britain identified around 23% of them as having low total water intakes, defined as having a daily water intake of < 2.0 L and a water-to-energy intake ratio of < 1.0 $g.kcal^{-1}$ (Gibson and Shirreffs, 2013), which may increase their risk of becoming hypohydrated. Hypohydration appears to be especially common among the older population, in whom chronic pain conditions are also more prevalent (Rustøen et al., 2005; Tsang et al., 2008; Fayaz et al., 2016). Approximately 30% of older women (aged 50 years and above) were found to be markedly hypohydrated (plasma tonicity ≥ 300 $mOsm.kg^{-1}$), compared to approximately 10% in the younger women (Stookey, 2005). Similarly, Hooper et al. (2015) found that 40% of elderly women living in residential homes met the criteria for hypohydration (S_{Osm} > 295 $mOsm.kg^{-1}$), while another study identified 40% of elderly women to be hypohydrated upon admission to hospital (serum sodium > 150 $mg.dL^{-1}$ and/or ratio of blood urea nitrogen to creatine > 25) (Lavizzo-Mourey et al., 1988).

2.3.3. Implications of Hydration for Pain in Women

The findings of a hyperalgesic effect of hypohydration in men, and of the potential menstrual phase effects on pain and on hydration, may have several potential implications for both the

study and treatment of pain in women. First, hydration status could have confounded previous research on the effects of menstrual phase on pain sensitivity and therefore contributed to the conflicting findings in the literature. In this context, potential differences in hydration status between menstrual phases could either exaggerate or mask the menstrual phase effects on pain, leading to erroneous conclusions about the associations between menstrual phase and pain sensitivity. However, hydration status was not measured in previous studies on this topic. It may, therefore, be necessary for future research to measure and control for hydration status, in order to clarify some of the confusion regarding the effects of menstrual phase on pain sensitivity. Second, since both the menstrual phase and hypohydration can independently affect pain, they could have interactive effects on pain when combined. In this instance, hypohydration could have a more pronounced hyperalgesic effect in one menstrual phase compared to another. However, this hypothesis has not been tested. Lastly, hypohydration could reduce the efficacy of pain treatments. For example, Parker et al. (2012) assessed the effects of hypohydration (36 hr of fluid restriction) on the outcomes of an osteopathic manipulative treatment program in eight women and 11 men with chronic low back pain. Greater improvements in treatment outcomes were observed when participants attended the treatment sessions in a euhydrated versus hypohydrated state. Therefore, it may be important for clinicians and practitioners to assess the hydration status of pain patients to maximise the efficacy of treatments.

2.4. Other Female Populations

Although this literature review and thesis focuses on premenopausal, eumenorrheic (i.e., naturally cycling) women, it should be noted that they only make up approximately half of the female population. Given the influence of the ovarian hormones (and their fluctuations across a natural menstrual cycle) on pain and hydration, it is important to consider two groups of women whose hormonal milieu differ vastly from that of eumenorrheic women: oral contraceptive pill (OCP) users and postmenopausal women.

2.4.1. Oral Contraceptive Pill Users

The OCP is perhaps the most popular contraceptive method among women worldwide. Recent data from the United States showed that OCP users comprised 13% (6 million) of the 47 million women who were using contraception (Daniels and Abma, 2018). Similarly, in the United Kingdom, 16% of the 194,054 women who were surveyed were OCP users (Cea-Soriano et al., 2014).

A typical OCP cycle consists of a 21-day active phase where exogenous oestrogens and progestins are ingested daily, followed by a 7-day placebo phase where no hormones are ingested. During the active phase, the exogenous hormones in OCPs suppress the production of endogenous 17ß-oestradiol and progesterone, leading to chronically low levels of both hormones, which are similar to the levels during the early follicular phase of a natural menstrual cycle, but markedly lower than in the mid-luteal phase (Rechichi et al., 2009; Elliott-Sale et al., 2020). During the placebo phase, concentrations of endogenous 17ß-oestradiol increase slightly, whereas progesteron e levels remain suppressed. Therefore, compared to eumenorrheic women, those using OCPs are exposed to high concentrations of

exogenous oestrogens and progestins, have low levels of endogenous 17ß-oestradiol and progesterone, and do not experience the same marked hormonal fluctuations as eumenorrheic women (Elliott-Sale et al., 2020).

There is a paucity of research on the effects of OCPs on pain. Among the studies on acute experimental pain, a number of them found higher pain sensitivity in OCP users compared to eumenorrheic women (Drobek et al., 2002; Kowalczyk et al., 2006a; Kowalczyk et al., 2010; Ribeiro-Dasilva et al., 2011), whereas several others did not observe any difference in pain sensitivity between both groups of women (Veith et al., 1984; Koltyn et al., 2003; Rezaii and Ernberg, 2010). Furthermore, the menstrual cycle-related fluctuations in pain sensitivity seen in eumenorrheic women appear to be absent in OCP users (Vignolo et al., 2008; Kowalczyk et al., 2010; Barbosa et al., 2013). In studies on women with chronic pain conditions, the self-reported severity of pain symptoms was found to be less variable across the cycle in the OCP users compared to the eumenorrheic women (Dao et al., 1998; LeResche et al., 2003).

In addition to pain, body water regulation also differs between OCP users and eumenorrheic women. The use of OCPs mainly results in a lowering of the osmotic thresholds for the release of AVP and stimulation of thirst sensation, similar to that seen during the mid-luteal phase in eumenorrheic women (Stachenfeld et al., 1999; Stachenfeld, 2008). As such, when compared to eumenorrheic women in the early follicular phase, OCP users may exhibit a greater water-conservation response for a given body water deficit, which could potentially make them less susceptible to dehydration and the associated negative consequences (Giersch et al., 2019).

However, the research in OCP users is complicated by the numerous types (e.g., monophasic, biphasic, triphasic) and brands of OCPs, each containing different doses of synthetic

oestrogens and progestins. Additionally, the type of progestin (and their associated characteristics and effects) also varies across pill brands. Therefore, unless the pill brand is standardised across participants, the effects of OCPs on a given outcome cannot be conclusively determined.

2.4.2. Postmenopausal Women

Menopause, which generally occurs between the ages of 45 and 55 years (Brambilla and McKinlay, 1989; Luoto et al., 1994; Bromberger et al., 1997), is accompanied by the loss of ovarian function, cessation of menstruation and decreases in 17ß-oestradiol and progesterone concentrations (Davis et al., 2015). With an ageing population, it is estimated that by the year 2030, there will be approximately 1.2 billion menopausal and postmenopausal women in the world (Hill, 1996).

The transition from the pre- to postmenopausal period has been associated with changes in the incidence and severity of several chronic pain conditions. A review of seven studies investigating the relationship between menopause and low back pain concluded that the incidence and severity of low back pain was higher in menopausal and postmenopausal women compared to premenopausal women (Kozinoga et al., 2015). In contrast, the opposite pattern was found for temporomandibular disorders, where there appears to be a lower prevalence among the postmenopausal compared to premenopausal women (Locker and Slade, 1988; LeResche et al., 2003). For migraine, a review found that, compared to the premenopausal years, the prevalence of migraine generally increases during the menopausal transition and decreases in the postmenopausal period (Ripa et al., 2015). However, in women already suffering from migraine, there appears to be a worsening of migraine attacks after, compared to before, menopause (Ripa et al., 2015). The use of hormone replacement

therapy (HRT) – commonly used to alleviate vasomotor symptoms and other symptoms of menopause – in postmenopausal women must also be considered as it significantly alters the hormonal profile. The use of HRTs has been associated with an increased risk of several chronic pain conditions, including temporomandibular disorders (LeResche et al., 1997) and low back pain (Brynhildsen et al., 1998). Experimental pain sensitivity could also be impacted by HRTs, with one study showing greater pain sensitivity during noxious heat stimulation in postmenopausal women who are on HRT, compared to the non-HRT users (Fillingim and Edwards, 2001).

Differences in body water regulation between premenopausal and postmenopausal women have also been observed (Stachenfeld, 2008; 2014). While administration of synthetic oestrogen reduced the osmotic threshold for AVP release in both groups of women, only the postmenopausal women experienced an associated increase in water and sodium retention (Stachenfeld, 2008; 2014).

An important consideration when examining the research in postmenopausal women is the influence of age. Ageing independently affects pain sensitivity and body water regulation (Kenney and Chiu, 2001; Arnaud, 2002; Gibson and Farrell, 2004; Lautenbacher et al., 2017). Therefore, the older age of postmenopausal women could also contribute to their different pain and body fluid regulatory responses when compared to the younger, premenopausal women.

2.5. Conclusions

There is currently no definitive conclusion regarding the effects of menstrual phase on pain sensitivity. Apart from the various methodological limitations (e.g., lack of hormonal verification of menstrual phase, not confirming ovulation) and differences (e.g., type of experimental pain stimulus used, definition of menstrual phases) across studies that could explain the conflicting findings, hydration status could have also contributed to the equivocal results in the literature. Hypohydration was shown to increase pain sensitivity in men, but whether this occurs in women has not been formally examined. Furthermore, menstrual phase may influence hydration status, therefore indicating that menstrual cycle-related changes in hydration may have also confounded previous research on pain in women. This also suggests possible interactions between hydration status and the menstrual phase on pain.

As such, the primary focus of this thesis is to investigate the effects of hypohydration on pain in women across different menstrual phases. The findings from this research have several important implications: it could (i) help us better understand and interpret previous research on pain in women, particularly as it relates to the menstrual phase, (ii) assist in designing more high-quality research in this area, and (iii) aid in developing strategies to improve the treatment and management of pain in women. The importance and necessity of this research is further underscored by the common occurrence of hypohydration among women. Lastly, while the focus of this thesis is on premenopausal, eumenorrheic women, it is also important to address these questions in OCP users and postmenopausal women, whose different hormonal profiles could influence their pain responses and/or hydration status.

CHAPTER THREE

3.0. Research Aims and Hypotheses

Chapter 2 laid out the current state of the literature and highlighted some of the gaps in the knowledge, which subsequently provided the basis for the objectives of this thesis and the development of the experimental study (**Chapter 4**). Emerging evidence has shown that hypohydration increases pain sensitivity in men, but whether this occurs in women is not known. While there is a considerable body of research investigating the effects of menstrual phase on body fluid regulation and on pain responses separately, there has been no attempt to synthesise these three areas of research. Therefore, the primary purpose of this thesis is to examine the interactions between hypohydration, menstrual phase and pain sensitivity (Primary Aims). In addition, since previous research suggests a strong association between thirst and pain sensitivity (Farrell et al., 2006; Geuter et al., 2016), the candidate explored whether acute water ingestion, which reduces thirst, could reverse the hyperalgesic effects of hypohydration (Secondary Aim).

3.1. Aims

The Primary Aims were to investigate the effects of hypohydration on pain sensitivity in women, and whether these effects vary as a function of menstrual phases. Given the independent effects of hypohydration and the menstrual phase on pain sensitivity, the objective was to examine whether both factors could interact to influence pain. The findings from the Primary Aims then led to the Secondary Aim, which was to investigate whether acute water ingestion could reverse the negative effects of hypohydration on pain.

The Primary and Secondary Aims can be split into more specific objectives as presented below:

Primary Aims:

1) Investigate the effects of hypohydration on pain sensitivity in eumenorrheic women during the early-follicular and mid-luteal phases of the menstrual cycle, with accurate verification of menstrual phases and minimization of potential confounders (e.g., dietary and lifestyle factors, dysmenorrhea).

2) Investigate whether the effects of hypohydration on pain sensitivity differ between the early-follicular and mid-luteal phase.

Secondary Aim:

1) Explore the potential efficacy of acute water ingestion as a countermeasure against the hyperalgesic effects of hypohydration.

Taken together, the findings of General Aims I and II would improve the understanding of pain in women and potentially inform the development of more effective pain treatments and

management strategies. This thesis could also serve as a foundation for future research on hypohydration, menstrual phase and pain.

3.2. Hypotheses

1) Hypohydration will increase pain sensitivity in women in both menstrual phases.

2) The magnitude of the increase in pain sensitivity due to hypohydration will be greater in the mid-luteal compared to early-follicular phase.

3) Acute water ingestion will reduce pain sensitivity when participants are hypohydrated but will not affect pain when participants are euhydrated.

It should be noted that these are the planned analyses. Further exploratory analyses, such as correlations and regressions, would likely be performed to gain further insight into the main results.

CHAPTER FOUR

4.0. General Methodology

The purpose of this chapter is to provide a description of the study design, experimental procedures and measures used, as well as a critical appraisal of these. This chapter will begin with a critical appraisal of the methods and measures used in this study, followed by a description of the study design and experimental procedures.

4.1. Experimental Pain Assessment

4.1.1. Experimental Pain Stimulus

Ischaemic pain was the choice of experimental pain stimulus in this study. There are several reasons that influenced this decision. First, it appears to be more clinically relevant compared to other experimental pain stimuli such as noxious heat, mechanical pressure and electrical stimulation (Smith et al., 1966). The pain sensations evoked by the ischaemic pain stimulus mimics aspects of clinical and chronic pain such as its duration (sustained), severity (moderate-to-severe), depth (deep-muscle level) and quality (large affective component) (Rainville et al., 1992; Fillingim and Ness, 2000; Graven-Nielsen et al., 2003). Ischaemic pain measures were also shown to be correlated with the self-reported severity of clinical pain symptoms (Fillingim et al., 1996; Edwards et al., 2001). Moreover, the ischaemic pain test may also be useful in predicting pain-related treatment outcomes among women (Edwards et al. 2003). These findings provide further evidence of the clinical relevance of the ischaemic pain stimulus and may suggest shared mechanisms with clinical and chronic pain.

Second, the ischaemic pain stimulus does not appear to have a "ceiling effect". The perceived intensity of the ischaemic pain stimulus increases linearly over time until participants can no longer tolerate the pain (Pretovaara et al. 1984, Rainville et al. 1992, Maxiner and Humphrey 1993). This would allow meaningful assessment of pain tolerance (see Section 4.1.3.1). This contrasts with the cold pressor test, where the perceived intensity of the pain stimulus increases to a peak within approximately 2 min, then declines gradually as the test progresses (Harris and Rollman, 1983; Rainville et al., 1992; Bear et al., 2016). This means that participants who can tolerate the pain for a reasonable duration would ultimately be able to tolerate it until the imposed time limit, thereby precluding meaningful statistical analyses of

pain tolerance as an outcome measure (Bear et al., 2016). Indeed, several studies using the cold pressor task have reported a considerable proportion of participants who were able to endure to the pain until the cut-off time (Hellstrom and Lundberg 2000, Stening et al. 2007, Bear et al. 2016). A similar issue with pressure algometry pain has also been reported (Harris and Rollman, 1983).

Lastly, the ischaemic pain stimulus may be better able to detect menstrual phase effects compared to other experimental pain stimuli. In the study by Fillingim et al. (1997), women displayed variations in displayed menstrual-phase related variations in their responses to ischaemic pain, whereas their pain responses to noxious heat stimulation were not influenced by menstrual phase. This is perhaps because the ischaemic pain responses are thought to be mediated by the endogenous opioid system, (Pertovaara et al., 1982), which in turn, may be modulated by the ovarian hormones (Craft et al., 2004; Smith et al., 2006). For example, differences in the magnitude of morphine analgesia between the follicular and luteal phase have been observed (Ribeiro-Dasilva et al., 2011). (Fillingim et al., 1997). In addition, there is also evidence from animal studies that the LH surge may reduce the sensitivity of opiate receptors in the brain (Berglund et al., 1988), which may lead to an increase in pain sensitivity during the luteal phase.

4.1.2. Ischaemic Pain Test

The ischaemic pain test consisted of the Submaximal Effort Tourniquet Procedure (Smith et al., 1966; Moore et al., 1979), which produces ischaemic pain in the arm by occluding blood flow to the forearm muscles and voluntarily contracting them (Maixner et al., 1990; Svensson and Arendt-Nielsen, 1995). Briefly, in this study, a pneumatic cuff was positioned on the participant's non-dominant arm and inflated to 250 mmHg to occlude blood flow.

Participants then performed 25 handgrip exercises at 30% of their pre-determined maximal handgrip strength (see Section 4.8.1). The pneumatic cuff was removed when the participant could not tolerate the pain any longer, or when the maximum time limit of 20 min had elapsed, whichever came first. A more detailed description of the protocol for the ischaemic pain test can be found in Section 4.10.1.

The protocol for the ischaemic pain test was conceived based on what was used in previous studies (e.g., Maixner and Humphrey, 1993; Fillingim et al., 1997; Klatzkin et al., 2010). Pilot data also showed that the selected test variables were not excessively fatiguing, as the required force output could be maintained throughout the exercises. A diagram of a participant's force output across the 25 handgrip exercises can be seen in Figure 2. This is important as the perception of fatigue could influence the participant's perception of pain. Additionally, the intensity of the pain produced by this protocol was not too mild such that participants could easily reach the 20-min time limit.

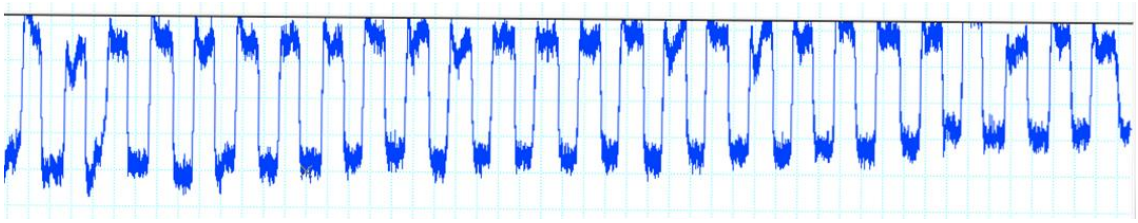

Figure 2. Force output form a participant across the 25 handgrip exercises. Black horizontal line represents the target force (i.e., 30% of maximum strength).

The maximum time limit of 20 min was selected based on what was used in previous studies (e.g., Pfleeger et al., 1997; Klatzkin et al., 2007). Moreover, in previous studies where a similar protocol for the ischaemic pain test was used, the average pain tolerance displayed by women was between 8 and 15 min (Maixner and Humphrey, 1993; Fillingim et al., 1997; Klatzkin et al., 2010), which is well within the 20-min time limit. Therefore, since the

likelihood of participants tolerating the pain beyond this time limit is low, it would allow meaningful analysis of pain tolerance.

Also, participants performed the ischaemic pain test using their non-dominant arm so that their dominant arm could be used for the venepuncture procedure (see Section 4.10). The dominant arm was chosen for blood sampling since the antecubital vein on this arm is usually more visible and prominent than that of the non-dominant arm, therefore increasing the likelihood of success on the first attempt. Furthermore, since the venepuncture procedure inherently produces pain (Cason and Grissom, 1997; Usichenko et al., 2004; Basaranoglu et al., 2006; Ott et al., 2012), it is important not to perform the venepuncture on the same arm that will be used for the ischaemic pain test to avoid confound.

The ischaemic pain test has been shown to be a valid experimental pain model in humans. Several studies have observed a linear stimulus-response relationship during the ischaemic pain test, where the ratings of perceived pain intensity increases linearly with the duration of the arm under ischaemic conditions (Moore et al., 1979; Pertovaara et al., 1984; Hagenouw et al., 1986; Rainville et al., 1992; Maixner and Humphrey, 1993). The ischaemic pain test is also sensitive to the analgesic effects of opioids like morphine and pentazocine, across different populations (e.g., men and women) (Posner, 1984; Fillingim et al., 2004; Fillingim et al., 2005; Ribeiro-Dasilva et al., 2011) and drug concentrations (Smith et al., 1966; Smith et al., 1968; Posner, 1984). Furthermore, a dose-response relationship between the magnitude of morphine-induced analgesia and ischaemic pain measures has been shown, where ischaemic pain tolerance increased linearly as the concentration of morphine administered increased from 0 mg (placebo) to 15 mg (Smith et al., 1968). In fact, the ischaemic pain test

682 is often regarded as the most sensitive pain model for evaluating opioid-induced analgesia
683 (Fillingim et al., 2004; Ribeiro-Dasilva et al., 2011; King et al., 2013).

684 The ischaemic pain test has also been found to be reliable. In one study, the authors evaluated
685 the reliability of morphine and pentazocine analgesia across three experimental pain models,
686 including muscle ischaemia (King et al., 2013). The analgesic index scores between repeated
687 sessions were more consistent for the ischaemic pain measures (Pearson's correlation
688 coefficient, r = 0.52-0.68), compared to noxious heat (r = 0.28-0.48) and mechanical pressure
689 (r = 0.06-0.72) pain. Moreover, the linear stimulus-response relationship of the ischaemic
690 pain test, as described earlier, has been consistently observed by several researchers, despite
691 variations in the exact protocol used (i.e., number, duration and intensity of handgrip
692 exercises, and inflation pressure of pneumatic cuff).

693 ***4.1.3. Ischaemic Pain Measures***

694 The experience of pain is recognised to be bi-dimensional, comprising of both a
695 sensory/discriminative and affective/motivational component (Auvray et al., 2010; Talbot et
696 al., 2019). There are several outcome measures that can be used to quantify a person's pain
697 responses, with each measure capturing a different aspect of pain. Furthermore, a given
698 intervention can have differential effects on these measures. Therefore, it is necessary to use a
699 range of pain outcome measures to obtain a more comprehensive assessment of pain. In this
700 study, ischaemic pain sensitivity was quantified by two measures: (i) pain tolerance and (ii)
701 pain intensity and pain unpleasantness ratings.

4.1.3.1. Pain Tolerance

Pain tolerance refers to the maximum amount of pain an individual is able to, or is willing to endure. In this study, pain tolerance was operationally defined as the total duration, measured in seconds, that participants were willing to keep their arm under ischaemic conditions. In other words, it is the duration from when the cuff was inflated to when it was deflated (see Section 4.10.1).

Pain tolerance is a commonly measured variable in many experimental pain studies that largely reflects the affective/motivational component of pain (Weisenberg et al., 1975; Price, 2002). Pain tolerance is a measure of a person's behavioural response or reaction to a painful stimulus, which indicates his/her attitude towards the pain and motivation to avoid the pain (Melzack and Casey, 1968; Weisenberg et al., 1975). This may be especially relevant to chronic pain patients. For example, chronic pain patients who displayed lower ischaemic pain tolerance also reported a greater severity of clinical pain symptoms and pain-related disability, compared to patients with higher pain tolerances (Edwards et al., 2001). In another study on women with chronic pain, those who had higher ischaemic pain tolerance experienced greater reductions in the severity of their pain symptoms after a treatment program, compared to those with lower pain tolerance (Edwards et al., 2003).

The validity and reliability of pain tolerance as a measure of ischaemic pain sensitivity has been demonstrated (Smith et al., 1966; Smith et al., 1968; Posner, 1984). Ischaemic pain tolerance increases with the administration of morphine in a dose-dependent manner (see Section 4.1.2) (Smith et al., 1968), whereas it decreases when naloxone – an antagonist of the opiate receptor – is administered (Schull et al., 1981).

However, pain tolerance may be susceptible to practice effects with repeated sessions as participants become more familiar and habituated with the pain test (Von Graffenried et al., 1978; Chapman et al., 1985). The increased familiarity may also indirectly affect pain tolerance by reducing state anxiety levels (Chapman and Feather, 1973; Von Graffenried et al., 1978). One study observed an increase in ischaemic pain tolerance across three session days, with a corresponding linear decrease in state anxiety (Von Graffenried et al., 1978). That said, the practice and anxiety effects can be minimised by familiarising participants with the pain test prior to the experimental sessions (see Section 4.8).

4.1.3.2. Pain Intensity and Pain Unpleasantness Ratings

Pain intensity refers to the magnitude of pain an individual perceives, and it reflects the sensory aspect of pain. Whereas, pain unpleasantness captures the affective dimension of pain. In other words, pain intensity measures the perceived *quantity* of pain, while pain unpleasantness measures the *quality* of pain.

Pain intensity and pain unpleasantness ratings were each measured using a 10 cm horizontal Visual Analogue Scale (VAS) (Haefeli and Elfering, 2006). For pain intensity, "no pain at all" (score of 0) and "most intense pain imaginable" (score of 10) defined the extreme ends of the scale, while the phrases "not unpleasant at all" (score of 0) and "extremely unpleasant" (score of 10) were used for pain unpleasantness. For each scale, participants responded by placing a mark along the horizontal line. The distance, measured in cm, between the end corresponding to a score of 0 and the mark drawn by participants, was defined as their pain intensity or pain unpleasantness. To ensure participants understood the difference between pain intensity and pain unpleasantness, a set of instructions adapted from a previous study (Price et al., 1983) was read to participants before each pain test.

Participants rated their pain intensity and pain unpleasantness twice each during the ischaemic pain test: (i) 1 min after the handgrip exercises (approximately 3 min after the cuff was inflated), and (i) at pain tolerance, just prior to deflating the cuff. The purpose of the first set of pain ratings was to assess the participant's subjective feelings of pain in response to a fixed stimulus intensity, while the pain ratings obtained at pain tolerance was to ensure that participants had reached their maximal ability to tolerate the pain when they terminated test.

We chose to obtain the first set of pain ratings 1 min after the handgrip exercises, instead of immediately after, to minimise possible confounding effects of exercise-induced fatigue and pressure pain due to inflation of the pneumatic cuff (Estebe et al., 2000). Moreover, this time point – which occurred around 3 min after the cuff was inflated – was also not too far into test such that it would exceed the pain tolerances of most participants. This is supported by previous data showing that the average ischaemic pain tolerance of women was between 10 and 12 min (Maixner and Humphrey, 1993; Fillingim et al., 1997; Pfleeger et al., 1997). If the pain ratings were obtained at a later point during the test, it increases the possibility of participants reaching pain tolerance before then, thereby reducing the data collected.

Many studies have obtained pain intensity and pain unpleasantness ratings at the point where participants report intolerable pain. However, the participants in most of these studies did not display differences between experimental conditions in their pain intensity and pain unpleasantness ratings at pain tolerance (Fillingim et al., 1997; Pfleeger et al., 1997; Girdler et al., 2005), This is perhaps unsurprising, since pain intensity and pain unpleasantness ratings at pain tolerance are expected to be maximal, regardless of how long participants had tolerated the pain for. Therefore, the pain ratings obtained at pain tolerance were to verify

that participants terminated the test because the perceived level of pain had become unbearable, rather than due to other factors (e.g., boredom).

The VAS is a valid and reliable tool to measure pain intensity and pain unpleasantness in healthy pain-free individuals (Price et al., 1983; Rosier et al., 2002; Ferreira-Valente et al., 2011), as well as in patients with chronic pain conditions (Price et al., 1983; Bijur et al., 2003; Alghadir et al., 2018; Thong et al., 2018). The VAS is also strongly correlated with other commonly used pain-rating scales, such as the Numerical Rating Scale (NRS) and Verbal Rating Scale (VRS) (Ferreira-Valente et al., 2011; Thong et al., 2018). Studies have also reported that the VAS is more sensitive to changes in pain ratings, compared to the NRS and VRS, both of which have a fixed number of response options (Joyce et al., 1975; Ohnhaus and Adler, 1975; Rosier et al., 2002). Pain ratings measured using the VAS also appear to display minimal variation across multiple experimental sessions over a 2- to 4-week period (Rosier et al., 2002; Quiton and Greenspan, 2008).

However, unlike the VRS which can be administered either verbally or via paper, the VAS can only be assessed via paper (Jensen et al., 1986), although electronic forms of the VAS have been developed and used in some studies (Jamison et al., 2001; Jamison et al., 2002). The VAS may also be more difficult to understand and to use compared to NRS or VRS, especially among the elderly (Kremer et al., 1981; Williamson and Hoggart, 2005). One study reported a failure rate of 11% on the VAS, which was higher than that for the NRS (2%) and VRS (0%) (Kremer et al., 1981). Participants who failed to complete the VAS were also generally older (mean age of 75 years) than those who successfully completed it (mean age if 55 years). Lastly, as scores on the VAS must be measured manually by the researchers, the potential for human error cannot be discounted.

4.2. Hydration Status

4.2.1. Dehydration Method

In this study, hypohydration was induced by 24 hr of fluid restriction. Specifically, participants were asked to restrict fluid intake and refrain from consuming foods that have a high-water content (e.g. soups, fruits) for 24 hr before the hypohydrated trials. Although several studies have reported a high incidence of underhydration and suboptimal fluid intakes among free-living individuals (Braun et al. 2019, Malisova et al. 2016, Ali et al. 2019), specific fluid intake amounts were not prescribed for the euhydrated trials. Instead, participants were asked to drink *ad libitum* and were also encouraged to ingest fluids regularly throughout the 24-hr period before the trial. As this would be more representative of the participant's habitual fluid consumption, it would maximise the ecological validity of the study.

Several studies have successfully used 24 hr of fluid restriction to achieve mild hypohydration in healthy female subjects (Moyen et al., 2016; Caldwell et al., 2018; Stachenfeld et al., 2018; Giersch et al., 2020). In these studies, body mass loss after fluid restriction averaged around 1%. Several biomarkers of hydration status, including serum/plasma osmolality and USG, were also increased when compared to either the euhydrated condition (Moyen et al., 2016; Caldwell et al., 2018; Giersch et al., 2020), or the *ad libitum* fluid intake condition (Stachenfeld et al., 2018).

Fluid restriction was chosen as the dehydration method as it closely mimics the common way many individuals become hypohydrated – by not drinking enough fluids throughout the day. It also isolates the effects of hypohydration on the measured outcomes of the study. In

814 contrast, the commonly used dehydration methods of exercise and heat exposure both
815 introduce additional stressors (e.g., elevated body core temperature and heart rate, fatigue)
816 that can confound the observed results. Moreover, as described previously, exercise has
817 independent effects on pain sensitivity, state anxiety levels and mood state (see Section 4.4).
818 Heat exposure alone may also degrade mood (Holland et al., 1985; McMorris et al., 2006;
819 Caldwell et al., 2018), or increase state anxiety levels (Burr et al., 1990; Tawatsupa et al.,
820 2010). It is possible that when heat exposure and/or exercise is combined with
821 hypohydration, they may exaggerate or attenuate the effects of hypohydration alone
822 (Lieberman, 2007; Adams et al., 2019a). For example, since exercise is known to reduce
823 pain sensitivity (Naugle et al., 2012), it may antagonise the potential hyperalgesic effects of
824 hypohydration, leading to an underestimation of the true effects of hypohydration *per se* on
825 pain sensitivity (Lieberman, 2007).

826 The time required to achieve a hypohydrated state also differs across the various methods. A
827 given level of hypohydration can be achieved more rapidly through heat exposure and/or
828 exercise compared to fluid restriction alone. Only 2 hr of environmental heat exposure (45ºC
829 and 70% relative humidity) (Cian et al., 2001) or moderate treadmill exercise (Cian et al.,
830 2001; Backhouse et al., 2007) is needed to reduce body mass by 2.8%, whereas at least 28 hr
831 of fluid restriction is required to achieve a similar reduction in body mass (Shirreffs et al.,
832 2004; Szinnai et al., 2005). The prolonged duration of the dehydration period with fluid
833 restriction may itself be a stressor and may also increase levels of fatigue and boredom
834 (Lieberman, 2010).

835 The physiological effects of the different dehydration methods also vary. Passive heat
836 exposure or administration of diuretics causes large amounts of both electrolytes and water to

be lost from the body, resulting in a substantial decrease in plasma volume (Caldwell et al., 1984; Jimenez et al., 1999). In contrast, exercise or fluid restriction produces hyperosmotic hypovolemia, where the loss in plasma volume is often minimal. For example, in a study where participants were dehydrated until they lost 2.8% of their body mass, either through exercise or through passive heat exposure, the decrease in plasma volume during exercise was only 3.8%, whereas it decreased by 12% during heat exposure (Jimenez et al., 1999).

4.2.2. Measures of Hydration Status

Hydration status in humans can be assessed using several methods, ranging from blood, urinary, salivary to perceptual markers (Kavouras, 2002; Armstrong, 2007). Many researchers state that there is currently no "gold standard" for measuring hydration status (Kavouras, 2002; Cheuvront and Sawka, 2005; Armstrong, 2007; Baron et al., 2015). This is because the accuracy and validity of each method is heavily influenced by the context in which it is applied. Thus, the current recommendation is to combine the use of two or more hydration markers (Cheuvront and Sawka, 2005; Armstrong, 2007; Armstrong et al., 2013b; Cheuvront and Kenefick, 2016). In this study, hydration status was assessed using five different markers: (i) S_{osm}, (ii) plasma copeptin, (iii) USG, (iv) changes in body mass, and (v) thirst sensation ratings. The reasons for selecting each of these hydration markers, as well as their advantages and disadvantages, will be discussed in the following sections.

4.2.2.1. Serum Osmolality

Serum/plasma osmolality refers to the concentration of solutes in the extracellular fluid space. It is said to correspond to intracellular osmolality as changes in the solute concentration of one fluid compartments creates an osmotic gradient that causes fluid to shift

from the area of high to low concentration. For example, a decrease in extracellular water during (hypertonic) dehydration causes an increase in plasma osmolality (P_{osm}), which in turn leads to a movement of fluid from the intracellular to extracellular space in order to preserve plasma volume (Nose et al., 1988). It should be noted that S_{osm} and P_{osm} are often used interchangeably as they are almost identical (Seifarth Christian et al., 2004; Bezuidenhout et al., 2016). However, while there does not appear to be a preference for either measure among researchers, clinicians usually measure S_{osm} (instead of P_{osm}) when diagnosing fluid-balance disorders (Faria et al., 2017). Therefore, S_{osm} may be considered to be more clinically relevant than P_{osm} and was subsequently chosen as the main hydration marker in this study. In this study, S_{osm} was measured using freezing-point depression osmometry (see Section 4.11.2.2), which is the mostly commonly used method to measure plasma/serum osmolality (Armstrong, 2007). Furthermore, this method is also thought to be more precise and reliable than the vapour pressure method (Mercier et al., 1978; Lord, 1999).

Serum/plasma osmolality is the primary regulated variable in fluid-electrolyte homeostasis. Normal values of serum/plasma osmolality in euhydrated individuals range from 275 to 295 $mOsm.kg^{-1}$ (Senay, 1979; Cheuvront et al., 2010; Hew-Butler et al., 2018). The excess loss of fluids during dehydration, either through sweating or insufficient fluid intake, causes serum/plasma osmolality to increase. An increase in P_{osm} by around 5 $mOsm.kg^{-1}$ has been proposed as the threshold for identifying hypohydration (Cheuvront et al., 2010; Cheuvront et al., 2011).

Serum/plasma osmolality is considered one of the most valid and precise markers of hydration status under well-controlled laboratory conditions (i.e., posture, environmental exposure, diet, fluid intake and physical activity are controlled across experimental trials) and

when body fluids are stable (Armstrong, 2007). As such, it is often used as the criterion measure of hydration status to evaluate the validity of other hydration biomarkers that do not sample directly from body fluid compartments (e.g., urinary and salivary indices) (Cheuvront and Sawka, 2005; Oppliger et al., 2005; Sommerfield et al., 2016).

Serum/plasma osmolality has been shown to be responsive to changes in body water during acute dehydration and rehydration in both men (Sawka et al., 1985; Popowski et al., 2001; Munoz et al., 2013) and women (De Souza et al., 1989; Stachenfeld et al., 1996; Stachenfeld et al., 1999; Armstrong et al., 2011). Popowski et al. (2001) monitored the changes in P_{osm} as men were progressively dehydrated by 5% of their body mass by exercising in the heat, and during a subsequent rehydration period. The authors noted that the changes in P_{osm} were proportional to the changes in body water (represented by percent change in body mass). Specifically, P_{osm} increased and decreased incrementally as body water was gradually lost (dehydration) and gained (rehydration), respectively. Similar findings have been reported in women, where P_{osm} increased and decreased linearly throughout 150 min of exercise in the heat (1.4% body mass loss) and a subsequent 180-min period of *ad libitum* water intake, respectively (Stachenfeld et al., 1999). Overall, the changes in P_{osm} appear to be linearly correlated with the magnitude of body water loss (represented by percent decrease in body mass), especially during exercise-induced dehydration (Sawka et al., 1996; Appel et al., 2005). More important to this thesis, the ability of serum/plasma osmolality to detect mild hypohydration in women subjected to 24 hr of fluid restriction has also been demonstrated (Moyen et al., 2016; Caldwell et al., 2018; Stachenfeld et al., 2018; Giersch et al., 2020). These studies observed a significant elevation in S_{osm} or P_{osm} of around 4 $mOsm.kg^{-1}$ from the euhydrated condition, despite only a 1.4% decrease in body mass.

However, despite the accuracy of serum/plasma osmolality in detecting acute body water changes in the laboratory and claims of it being a "gold standard" for hydration assessment (Cheuvront et al., 2010; Cheuvront et al., 2013), there are several limitations with using S_{osm} to evaluate hydration status (Bohnen et al., 1992; Armstrong, 2007; Armstrong et al., 2013d).

First, serum/plasma osmolality may not be an accurate indicator of hydration status under normal conditions of daily living. Due to the tight control of serum/plasma osmolality, it seldom deviates by more than 1 to 2% from its set-point value, despite daily variations in diet, physical activity and environmental exposure (Appel et al., 2005; Cheuvront et al., 2013; Hew-Butler et al., 2018). This is because small increases in serum/plasma osmolality of as little as 2% are enough to activate powerful compensatory mechanisms (i.e., secretion of AVP and stimulation of thirst sensation) to preserve fluid-electrolyte homeostasis (Armstrong, 2005). This tight regulation of serum/plasma osmolality is reflected by data showing remarkably similar resting values of serum/ plasma osmolality across men and women with vastly different habitual daily fluid intakes (Appel et al., 2005; Armstrong et al., 2013c; Perrier et al., 2013b; Johnson et al., 2015; Johnson et al., 2016). Moreover, both S_{osm} and P_{osm} also appear to be unresponsive to modest changes in daily fluid intake and water balance (Armstrong et al., 2013c; Perrier et al., 2013a; Johnson et al., 2015). When women who normally consume a high amount of fluids daily (around 3.34 L per day) reduced their daily fluid intake by 40% to 2 L for 4 days, S_{osm} remained constant throughout the intervention period despite a 0.8% decrease in body mass (Johnson et al., 2015). Therefore, serum/plasma osmolality may not be useful for identifying chronic hypohydration in free-living individuals. It may also have limited utility in assessing the effectiveness of water interventions that are targeted at improving hydration status.

928 Second, although there is generally a linear relationship between the changes in P_{osm} and the
929 changes in body water as mentioned previously, there is considerable inter-individual
930 variability in the response of P_{osm} to a given fluid intervention (Armstrong et al., 2013d). In a
931 study by Sollanek et al. (2011) where 30 participants drank a 500 ml bolus of water, the
932 changes in P_{osm} ranged from approximately -9 $mOsm.kg^{-1}$ to +4 $mOsm.kg^{-1}$. Data presented
933 by Sawka et al. (1996) also showed that a 5% decrease in body water resulted in highly
934 variable changes in P_{osm} that ranged from around -6 to +8 $mOsm.kg^{-1}$.

935 Third, there are several factors outside of changes in body water balance that can affect the
936 accuracy of both S_{osm} and P_{osm} measurements. Examples include ingesting a large bolus of
937 fluid (Sollanek et al., 2011), posture of participants (Vokes et al., 1988) and the method of
938 dehydration (Munoz et al., 2013). One study in women found that P_{osm} was lower when it
939 was measured with participants in a seated versus recumbent position (Vokes et al., 1988).
940 Munoz et al. (2013) compared the S_{osm} responses to dehydration induced by exercise or
941 passive heat exposure and found that with a 1% decrease in body mass, S_{osm} was significantly
942 elevated from baseline (296 to 301 $mOsm.kg^{-1}$) in the exercise condition only, whereas it did
943 not change significantly during heat exposure. Various technical factors related to the
944 handling and storage of the blood samples prior to analysis can also alter the measured values
945 of both S_{osm} and P_{osm} (Bohnen et al., 1992; Seifarth Christian et al., 2004; Bezuidenhout et al.,
946 2016; Sureda-Vives et al., 2017; Sollanek et al., 2019). For example, the P_{osm} of blood
947 samples stored at room temperature (21 to 24ºC) decreased steadily throughout a 24 hr
948 period, whereas those stored at a cooler temperature of 4ºC decreased only during the first 3
949 hr and remained stable thereafter (Bohnen et al., 1992). Therefore, failure to adequately
950 control or account for these factors may lead to erroneous conclusions about a person's
951 hydration status.

Lastly, the measurement of serum/plasma osmolality is invasive (due to blood sampling), expensive and requires both a high level of technical expertise and specialised laboratory equipment. These characteristics make it inaccessible to the general public for daily monitoring of hydration status, or for estimating hydration status in field settings. As such, other techniques that are relatively simple and inexpensive, whilst still provide reasonably accurate information about hydration status, may have greater utility in such instances. These techniques include USG, changes in body mass and subjective ratings of thirst sensation.

4.2.2.2. Plasma Copeptin

Copeptin is a surrogate marker for AVP – a key hormone in body fluid regulation (see section 4.2.2.1) (Jochberger et al., 2006; Morgenthaler et al., 2008). Arginine vasopressin is synthesised in the hypothalamus and then transported to the posterior pituitary gland where it is stored (Robertson et al., 1976). It is released into the circulation primarily in response to small increases in serum/plasma osmolality (2 to 3%), such as during dehydration, and subsequently produces an antidiuretic effect via its actions on the kidneys (Stachenfeld, 2008). Decreases in plasma volume (~10%) can also stimulate AVP secretion, however it is a less potent signal compared to osmolality (Stachenfeld, 2008).

Plasma AVP concentrations are strongly and linearly correlated with P_{osm} (Robertson et al., 1973; Robertson and Athar, 1976; Robertson et al., 1976; Vokes et al., 1988). For example, AVP increases in parallel with the increase in P_{osm} during dehydration or hypertonic saline infusion (Robertson and Athar, 1976; Hammer et al., 1980; Vokes et al., 1988). Therefore, measuring circulating levels of AVP can provide useful information on the body's hydration status (Stachenfeld et al., 1996; Melin et al., 2001).

974 However, obtaining reliable and accurate measurements of plasma AVP is known to be
975 notoriously challenging (Morgenthaler et al., 2008). One reason is due to the instability of
976 AVP in plasma. The measured plasma AVP concentrations decreased by 60% after 3 weeks
977 of storage at -20°C, compared to its initial concentration in fresh plasma (Robertson et al.,
978 1973). It also has a short half-life of up to 20 to 24 min (Robertson et al., 1973; Baumann and
979 Dingman, 1976), meaning that it is rapidly cleared from the bloodstream after being secreted.
980 Normal concentrations of plasma AVP among healthy individuals under daily living
981 conditions is also low, averaging around 0.5 to 2 $pg.ml^{-1}$ in most studies (Stachenfeld et al.,
982 2001; Jochberger et al., 2006; Perrier et al., 2013b; Johnson et al., 2016). Yet, most of the
983 currently available assays for determining plasma AVP levels are not sensitive enough to
984 detect such low levels of AVP (Norsk and Epstein, 1988; Morgenthaler et al., 2008). In one
985 study for example, AVP levels were undetectable in 18% (30/168) of the plasma samples
986 analysed, even though the radioimmunoassay used had a relatively low detection limit of 0.1
987 $pg.ml^{-1}$ (Hammerum et al., 1998). Furthermore, most of the AVP circulating in the blood is
988 bound to platelets (Preibisz et al., 1983), which can lead to an overestimation of the true
989 plasma AVP levels if care is not taken to fully remove the platelets from the plasma before
990 the samples are analysed (Preibisz et al., 1983; Kluge et al., 1999). The AVP concentration in
991 plasma samples that contained platelets was reported to be 10 times higher than that in
992 platelet-free plasma (Preibisz et al., 1983).

993 Copeptin and AVP are both derived from the precursor molecule, prepro-vasopressin (Land
994 et al., 1982). Copeptin makes up the C-terminal part of prepro-vasopressin and is released
995 into the circulation together with AVP in a 1:1 ratio (Fenske et al., 2018b). As such, copeptin
996 has been proposed as to be a suitable proxy marker for AVP (Morgenthaler et al., 2006;
997 Morgenthaler et al., 2008). With the recent development of a sensitive assay for determining

plasma copeptin concentrations (Struck et al., 2005; Morgenthaler et al., 2006), measuring copeptin as an alternative to direct measurement of plasma AVP has been gaining traction among researchers (Hew-Butler et al., 2011; Lemetais et al., 2018; Chang et al., 2020; Giersch et al., 2020).

There are several advantages with measuring copeptin (Morgenthaler et al., 2008). First, unlike AVP, copeptin is remarkably stable in both plasma and serum samples (Struck et al., 2005; Morgenthaler et al., 2006). Measured copeptin levels decreased by less than 10% of its initial concentration after storage at room temperature and 4°C for 7 and 14 days, respectively (Morgenthaler et al., 2006), while no decrease occurred after storage at -20°C for four weeks (Struck et al., 2005). Second, the assay for measuring copeptin is highly sensitive and can detect plasma copeptin concentrations in 97.5% of healthy individuals (Morgenthaler et al., 2006). Lastly, the process for measuring copeptin is relatively simpler and less time-consuming than that for AVP (Morgenthaler et al., 2006; Morgenthaler et al., 2008).

Recent data indicate that copeptin is a suitable proxy marker for AVP (Jochberger et al., 2006; Morgenthaler et al., 2006; Szinnai et al., 2007; Balanescu et al., 2011). A study by Balanescu et al. (2011) measured plasma copeptin, plasma AVP and P_{osm} in healthy individuals at various hydration states. Drinking a large bolus of water (20 $ml.kg^{-1}$ within 30 min) led to a decrease in P_{osm} which was mirrored by decreases in both plasma copeptin and AVP, whereas all three variables increased in parallel during a subsequent period of hypertonic saline infusion (Balanescu et al., 2011). Plasma copeptin also correlated strongly with both plasma AVP ($r = 0.8$) and P_{osm} ($r = 0.77$) across all hydration states. Another study reported similar findings, showing a strong correlation between plasma copeptin and S_{osm} ($r = 0.58$) during water deprivation and hypotonic saline infusion in both men and women

(Szinnai et al., 2007). More recently, data from Giersch et al. (2020) showed a significant correlation between plasma copeptin and percent body mass loss (r = -0.56) following 24 hr of fluid restriction in men and women. Therefore, these findings indicate that copeptin may be a suitable marker of hydration status in healthy individuals.

There is also considerable research on the clinical utility and validity of plasma copeptin as a diagnostic marker for various diseases related to body fluid imbalance (Nickel et al., 2012). Most notably, copeptin demonstrated high diagnostic accuracy in diagnosing diabetes insipidus (Katan et al., 2007; Fenske et al., 2018a; Winzeler et al., 2019). Copeptin has even been shown to be more accurate than the standard water deprivation test in diagnosing this condition (Katan et al., 2007; Fenske et al., 2018a). In cross-sectional studies, elevated copeptin levels have been observed in various patient populations such as those with sepsis (Struck et al., 2005; Jochberger et al., 2006), systemic inflammatory response syndrome (Jochberger et al., 2006), lower respiratory tract infection (Müller et al., 2007), diabetes mellitus (Enhorning et al., 2010; Enhörning et al., 2013) and metabolic syndrome (Enhorning et al., 2011; Enhörning et al., 2013; Eltabakh et al., 2018). Furthermore, longitudinal data have shown associations between elevated plasma copeptin levels and an increased risk of developing various medical disorders such as diabetes mellitus (Enhorning et al., 2010; Wannamethee et al., 2015), chronic kidney disease (Roussel et al., 2015; Tasevska et al., 2016) and abdominal obesity (Enhörning et al., 2013).

4.2.2.3. Urine Specific Gravity

The kidneys play a vital role in body fluid regulation due to their ability to vary urinary output in response to changes in total body water. In the case of a body water deficit, the increase in serum/plasma osmolality triggers the release of AVP, which causes the kidneys to

retain more water and excrete less in the urine. Consequently, urine volume decreases while urine concentration increases. Conversely, when total body water increases beyond normal, the kidneys get rid of the excess water by increasing urine output, resulting in the production of a large volume of dilute urine. Therefore, changes in urine concentration result from the body's compensatory response to the initial perturbation in body fluid balance. As such, measures of urine concentration (i.e., urine osmolality, USG and urine colour) can be used as an indirect marker of hydration status. In addition to the non-invasive nature and relative ease of obtaining urine samples, urinary indices are used extensively across various research, field and clinical settings to measure hydration status.

Urine specific gravity refers to the density of urine relative to that of pure water, which has a specific gravity of 1.000 (Stuempfle and Drury, 2003; Armstrong, 2005). It reflects the number and weight of solutes in the urine. Normal USG values in healthy, euhydrated individuals range from 1.007 to 1.030 (Cheuvront et al., 2010; Armstrong, 2012). Hypohydration is typically defined by USG values greater than 1.020 (Armstrong et al., 1994; Association, 2003; Cheuvront and Sawka, 2005; American College of Sports et al., 2007).

In this study, USG was measured via refractometry (Clinical Refractometer MASTER-SUR/NM, Atago Co. Ltd., Tokyo, Japan), as this is generally considered the "gold standard" for determining USG and is the most frequently used method (Chadha et al., 2001; Popowski et al., 2001; Fernández-Elías et al., 2014; Perrier et al., 2017). Refractometry has been shown to be the most accurate and reliable method for assessing USG compared to reagent strips or hydrometry (Brandon, 1994; de Buys Roessingh et al., 2001; Stuempfle and Drury, 2003; Costa et al., 2010). Using urine osmolality as the criterion measure, de Buys Roessingh et al.

(2001) compared the accuracy of USG values obtained with a refractometer and a dipstick. Urine osmolality was more strongly correlated with the refractometer values (r^2 = 0.9) than with the dipstick values (r^2 = 0.6), even after the dipstick values were corrected for pH. Furthermore, when compared with refractometry, USG values obtained with reagent strips were lower and resulted in up to 39% false negatives (i.e., incorrectly classifying an individual as being euhydrated when he/she is hypohydrated based on refractometry) (Adams, 1983; Abbey et al., 2014).

There is substantial data demonstrating the validity and reliability of USG measured with a handheld refractometer (Popowski et al., 2001; Fernandez-Elias et al., 2014; O'Neal et al., 2018). In one study where men were dehydrated by 4% of their body mass through exercise and were subsequently rehydrated, USG was found to accurately reflect the changes in body water (Armstrong et al., 1998). Specifically, USG increased and decreased in accordance with the decrease (dehydration) and increase (rehydration) in body mass, respectively. Urine specific gravity has also been shown to be sensitive to mild dehydration (around 1% body mass loss) induced by 24 hr of fluid restriction in women (Pross et al., 2013; Moyen et al., 2016; Caldwell et al., 2018; Giersch et al., 2020). Moreover, unlike serum/plasma osmolality, USG is responsive to moderate changes in daily fluid intake and can be used to distinguish between individuals with high and low habitual fluid intakes. (Perrier et al., 2013a; Johnson et al., 2015).

Although urine osmolality is considered the "gold standard" for measuring urine concentration (Chadha et al., 2001; Fernández-Elías et al., 2014; Souza et al., 2015), USG was used in this study because it can be measured immediately, therefore enabling quick confirmation that participants arrived at the laboratory in the intended hydration state (i.e.,

either euhydrated or hypohydrated). Furthermore, compared to urine osmolality, the measurement of USG is more practical, simple, requires less technical expertise and is less costly, thus making it the most popular urinary hydration index in field studies (Osterberg et al., 2009; Volpe et al., 2009) and in those that involve large populations (Polat et al., 2006; Malisova et al., 2016; Kavouras et al., 2017; Bialecka-Debek and Pietruszka, 2019). There is also less inter- and intra-individual variability in USG values (1.0% and 0.4%, respectively) compared to urine osmolality (57.9% and 28.3%, respectively) (Cheuvront et al., 2010). For example, urine osmolality has been shown to differ considerably across countries (Manz and Wentz, 2003; Malisova et al., 2016), with one study showing higher urine osmolality among the German (860 $mOsm.kg^{-1}$) compared to Polish (392 $mOsm.kg^{-1}$) population (Manz and Wentz, 2003). As such, there is currently no universally accepted hypohydration cut-off value for urine osmolality that is generalizable across all cultures (Baron et al., 2015; Perrier et al., 2015).

Another advantage of USG is that it only requires a small sample of urine (i.e., two to three droplets), making it easier to obtain an adequate amount of urine for sampling during hypohydrated trials when urine volume would normally be reduced. Lastly, USG is strongly correlated with urine osmolality across a range of hydration states (r = 0.8-0.9), indicating that USG may be used in place of urine osmolality (Oppliger et al., 2005; Armstrong et al., 2010; Fernandez-Elias et al., 2014).

However, there are some limitations with using USG as a hydration marker. First, the USG of a single urine sample provides a snapshot of hydration status and therefore may not be representative of 24-hr fluid balance (Armstrong et al., 2010; Cheuvront et al., 2015). Many researchers assess USG from urine samples collected immediately upon waking (i.e., "first-

morning" void), since the period of overnight fast can help minimise confounding effects of food consumption, fluid ingestion and exercise. However, compared to the USG of afternoon urine samples, the USG of urine samples collected in the morning is usually higher because no fluids were ingested during the overnight fast (Bottin et al., 2016; Suh et al., 2019). As the day progresses and individuals have had the opportunity to ingest fluids, USG tends to decrease. Morning urine samples also have a higher USG compared to 24-hr urine samples, which is considered the most accurate way to assess 24-hr fluid balance via urine concentration (Armstrong et al., 2010; Perrier et al., 2013a; Bottin et al., 2016). However, the collection of 24-hr urine samples is inconvenient and impractical in many situations and is therefore not widely used.

Second, USG alone, without information about urine volume, may lead to erroneous conclusions about hydration status. Since USG reflects the mass per unit volume, an increase in USG can occur due to either an increase in solute load (due to increased dietary solute intake), decrease in urine volume (due to dehydration), or both. One study investigating the effects of dietary protein intake on USG found that USG was higher when men were on a high protein diet, compared to when they were on a eucaloric diet with a lower protein intake (Martin et al., 2006). Fluid intake and fluid balance were not different between diets. In support of this finding, another study reported a strong and positive association (r = 0.92) between urinary protein metabolites and USG. These findings indicate that an elevated USG alone may not necessarily indicate hypohydration, unless urine volume is simultaneously reduced.

Lastly, USG appears to respond more slowly to changes in total body water compared to blood markers like serum/plasma osmolality, particularly when the change in body water is

rapid and substantial, for example during exercise in a hot environment or drinking a large amount of hypotonic fluids quickly (Armstrong, 2007; Munoz et al., 2013; Sommerfield et al., 2016). During 3 hr of exercise-induced dehydration that led to a 5% decrease in body mass, a significant increase in USG was not observed until body mass decreased by 3% , whereas P_{osm} became significantly elevated at only 1% body mass loss (Popowski et al., 2001). Similar observations were made during a subsequent 60-min rehydration period where participants replaced the fluid lost by drinking water – USG remained elevated whereas P_{osm} returned to baseline (Popowski et al., 2001). This is because a certain amount of time is required for the kidney's water conservation mechanisms to be activated during dehydration, as well as for body fluids to equilibrate during oral rehydration. However, when dehydration is induced more slowly and is less severe (approximately 1% body mass loss), such as by 24 hr of fluid restriction, USG has repeatedly been shown to be a valid index of hydration status (Pross et al., 2013; Caldwell et al., 2018; Stachenfeld et al., 2018; Giersch et al., 2020).

4.2.2.4. Changes in Body Mass

Measuring the changes in body mass is a simple, practical and highly accessible method to estimate hydration status. A decrease in body mass of ≥ 2% body mass is often used as the cut-off for hypohydration (American College of Sports et al., 2007). This technique assumes that the acute changes in body mass are entirely due to changes in body water content (Shirreffs, 2000; Maughan et al., 2007), whereby a decrease or increase in body mass of 1 kg equates to a loss or gain of 1 L of water, respectively (Shirreffs, 2003). This assumption holds true when dehydration is induced over a relatively short period of time (i.e., < 4 hr), for example during exercise and/or heat exposure, because body water is the only component of the body that can be lost in such a short time (Shirreffs, 2003; Armstrong, 2005). Changes in

body mass have therefore been widely used in many laboratory and field studies to quantify the severity of hypohydration and sweat losses due to exercise and/or heat stress (Kavouras, 2002). It is also often used as the criterion measure to evaluate the validity of other hydration markers during short-term dehydration (Armstrong et al., 1998; Popowski et al., 2001).

However, the accuracy of this technique as an indicator of changes in body fluid balance and hydration status decreases as the duration between body mass measurements increases beyond several hours (e.g., 24 hr) (Armstrong, 2005; Maughan et al., 2007). This is because it becomes increasingly difficult to control for other factors that may alter body mass, independently of changes in hydration status (Maughan et al., 2007). Some of these factors include the consumption of food, ingestion of fluids, urine and faecal losses, respiratory water loss, metabolic mass loss and trapped sweat in clothing(Cheuvront et al., 2002; Kavouras, 2002; Maughan et al., 2007; Baron et al., 2015). Another limitation of using changes in body mass to estimate hydration status is that knowledge of a euhydrated baseline body mass is required. However, this information is often unavailable in clinical settings or in free-living conditions. Additionally, due to natural daily fluctuations in body mass of around 0.66%, it is important to accurately determine baseline body mass by calculating the average of three consecutive body mass measurements that are taken upon waking (Cheuvront et al., 2004). This method of establishing baseline body has been used in some research studies to calculate the changes in body mass after dehydration (e.g., Moyen et al., 2016; Caldwell et al., 2018). However, this may not always be feasible as it can be quite costly to provide each participant with a bathroom scaleto take home. Moreover, it may also increase participant burden.

4.2.2.5. Thirst Sensation Ratings

Assessing an individual's perception of thirst is another quick, easy and cheap way to estimate hydration status, especially when there is no access to any equipment (Armstrong, 2005). Thirst sensation was quantified using a VAS (Rolls et al., 1980; Adams et al., 2019b), similar to that used for pain intensity and unpleasantness ratings (see Section 4.1.3.2). The ends of the line were defined as "not thirsty at all" (score of 0) and "extremely thirsty" (score of 10). Participants responded to the question "how thirsty do you feel now?" by drawing a mark along the scale. Similar to the pain ratings, the distance (in cm) from the zero end of the scale to the mark drawn by participants defined their perception of thirst (Kenefick, 2018).

Subjective ratings of thirst sensation can provide useful information on a person's hydration status as it is highly responsive to changes in serum/plasma osmolality. The onset of thirst generally occurs when P_{osm} increases by as little as 2%, or when body water stores decrease by approximately 2% of body mass (Greenleaf, 1992; Kenefick, 2018). Moreover, the perceived intensity of thirst sensation is linearly correlated with P_{osm}, and with the severity of hypohydration (Engell et al., 1987; Vokes et al., 1988; Armstrong et al., 2013a). Therefore, subjective ratings of thirst sensation can distinguish between hypohydrated and euhydrated states with relative accuracy (Armstrong et al., 2013a).

However, when hypohydrated individuals ingest fluids, thirst is satiated rapidly and drinking stops before sufficient fluids are ingested to restore body water balance (Adams et al., 2019b), resulting in a phenomenon known as "involuntary dehydration" (Greenleaf, 1992). In one study where a group of men underwent exercise-induced dehydration followed by 60 min of recovery where they drank *ad libitum*, thirst sensation increased when the men became hypohydrated by 3% body mass and immediately returned to baseline as they began drinking

water (Adams et al., 2019b). However, the men remained hypohydrated (2% body mass loss) at the end of the 60-min recovery period although they no longer felt thirsty. Additionally, a person's subjective feeling of thirst is easily influenced by numerous non-physiological factors, such as various characteristics of the ingested fluid (e.g., temperature, carbonation, taste) (Szlyk et al., 1989; Rolls et al., 1990; Peyrot des Gachons et al., 2016), the volume of fluid ingested (Rolls et al., 1990), macronutrient composition and sodium content of foods consumed (de Castro, 1991), gastric distension and perceived mouth dryness (Armstrong and Kavouras, 2019).

4.3. Hormonal Status

4.3.1. Selection of Menstrual Phases

Participants in this study were tested across two different phases of their menstrual cycle: (i) the early follicular (EF) phase and the (ii) mid-luteal (ML) phase. The EF phase was defined as days 3 to 7 of the menstrual cycle, with day 1 representing the first day of menses (Allen et al., 2016). The ML phase was defined as 6 to 10 days after obtaining a positive urinary LH test result (see below), or approximately days 19 to 23 of a conventional 28-day menstrual cycle (Allen et al., 2016).

The EF and ML phases were chosen due to their divergent hormonal profiles across the menstrual cycle (see Figure 1). The EF phase is characterised by low circulating levels of 17ß-oestradiol and progesterone, whereas both hormones are elevated during the ML phase, with progesterone reaching a peak and 17ß-oestradiol rising to a secondary peak. Therefore, comparing the EF and ML phases would maximise the differences in 17ß-oestradiol and progesterone concentrations between phases. Furthermore, many of the studies that investigated the effects of menstrual phase on pain sensitivity or on hydration have, at a minimum, included the EF and ML phases (Maughan et al., 1996; Riley et al., 1999; Stachenfeld et al., 1999), thereby facilitating comparison with the findings of previous studies.

4.3.2. Menstrual Phase Verification

A "three-step method" was used to estimate and verify the EF and ML phases (Allen et al., 2016; Schaumberg et al., 2017). This involves the combined use of three different methods: (i) self-report of menses onset, (ii) urinary LH testing and (iii) measurement of serum 17ß-

oestradiol and progesterone concentrations. This method has been shown to have a 90% success rate in correctly identifying the ML phase in eumenorrheic women (Schaumberg et al., 2017) and has been recommended in previous reviews (Allen et al., 2016; De Jonge et al., 2019).

First, the EF phase was prospectively identified using self-report of menses onset. Participants were asked to inform researchers once menstrual bleeding starts. The EF sessions took place within the third to seventh day of menses onset. Self-report of menses onset is a reliable method for identifying the EF phase, as the occurrence of menses is easily discerned and signifies the start of the follicular phase (Allen et al., 2016; De Jonge et al., 2019). Advantages of this method include its ease of use, zero cost and minimal participant burden. However, ovulation or the ML phase cannot be accurately determined with this method alone, as regular menses does not indicate that cycles are always ovulatory (Wideman et al., 2012; Schaumberg et al., 2017; De Jonge et al., 2019). This is important as there is a high prevalence of anovulation or luteal phase deficiency among regular cycling women (De Souza et al., 1998; De Souza et al., 2010; Schaumberg et al., 2017). For example, a study that evaluated 1,545 premenopausal women with regular menstrual cycles found that 37% of them had non-ovulatory cycles at the time of testing (Prior et al., 2015). In another study where 286 women were studied over a 3-month period, 18% of the women experienced at least one non-ovulatory cycle during the study period (Metcalf et al., 1983).

Ovulation is important as it is required to trigger the production of progesterone during the subsequent ML phase. Unlike ovulatory cycles, there is no observable mid-luteal peak in progesterone levels in non-ovulatory cycles (Hambridge et al., 2013; Lynch et al., 2014; Schliep et al., 2014). In this study, the day of ovulation was prospectively estimated via

urinary LH testing. Each participant was provided with a home-based ovulation prediction kit (EasyCheck® Ovulation Test, Phoenix Medcare Ltd, Auckland, New Zealand), which detects the rise of LH in the urine. Following the instructions provided by the manufacturer, participants began LH testing 16 days prior to the end of a typical cycle, based on their self-reported menstrual cycle length and day of menses onset. For each test, participants were required to collect a small urine sample in a cup and dip a test strip in it. Participants were asked to take a photograph of the test result and send it to the researcher for visual inspection. Participants performed one test daily until a positive LH test result – represented by a test line that is dark red in colour – was obtained. This indicates the onset of the LH surge and ovulation is presumed to occur within the next 24 to 48 hr (Gudgeon et al., 1990; Pearlstone and Surrey, 1994; Miller and Soules, 1996). The estimated ML phase was determined as the sixth to tenth day after obtaining the positive LH test result (Hoeger Bement et al., 2009; Ribeiro-Dasilva et al., 2011; Schaumberg et al., 2017).

Urinary LH testing is commonly used in research studies to predict ovulation and hence, prospectively identify the ML phase. The use of urinary LH tests has been shown to be an accurate and reliable indicator of impending ovulation (Corson, 1986; Gudgeon et al., 1990; Miller and Soules, 1996; Roos et al., 2015). One study compared the accuracy of urinary LH tests in predicting ovulation with transvaginal ultrasound – the "gold standard" for direct evidence of ovulation (Miller and Soules, 1996). The authors found that ovulation was correctly identified within 48 hours of a positive LH test result in 92% of the cycles examined. Moreover, the peak in urinary LH levels was shown to correlate well with the peak in serum LH (Miller and Soules, 1996; Tanabe et al., 2001). A review also stated that urinary LH tests are more accurate than other ovulation-prediction methods such as monitoring basal body temperature and checking for cervical discharge (Eichner and Timpe, 2004). In addition

to its accuracy, urinary LH tests are practical, easy to use, relatively inexpensive and have a low participant burden. However, there are several drawbacks with using urinary LH tests. First, test results can sometimes be ambiguous and difficult to interpret. An example is obtaining a dark pink line that borders between a negative and positive result. Second, the profile of the LH rise has considerable inter-individual variability (Park et al., 2007; Direito et al., 2013). Although a typical LH surge is characterised by a single spike, one study found that only 42% of the 43 women in the study exhibited this typical pattern of LH surge (Park et al., 2007). A biphasic pattern was observed in 44% of women, where there were two LH peaks interspersed with a momentary dip. The other 14% of women displayed a plateau in the LH peak that lasted over 2 to 3 days. Third, a positive LH test does not always guarantee an ovulatory cycle. A study that evaluated 26 eumenorrheic women found that 30% (8 women) did not have an adequate mid-luteal rise in progesterone (< 6 $ng.ml^{-1}$) that signifies ovulation, despite obtaining a positive LH test result (Schaumberg et al., 2017). In another study where urinary LH tests were performed twice daily, all 10 women in the study exhibited the increase in progesterone during the ML phase, but one woman did not have the LH surge (Nulsen et al., 1987). Urinary LH tests are therefore seldom used in isolation to predict ovulation and identify the ML phase. A more common practice is to combine urinary LH testing with measurement of serum 17ß-oestradiol and progesterone concentrations.

To verify that the EF and ML phases were correctly identified, serum 17ß-oestradiol and progesterone concentrations were measured retrospectively. The ML phase was verified by a conservative progesterone concentration limit of > 5 $ng.ml^{-1}$ (16 $nmol.L^{-1}$) to minimise the likelihood of including anovulatory or luteal phase-deficient cycles (Landgren et al., 1980; Stricker et al., 2006; Schaumberg et al., 2017; De Jonge et al., 2019).

Direct measurement of circulating 17ß-oestradiol and progesterone concentrations is the most valid and accurate way to determine menstrual phases. This is especially important if the research question involves determining effects of the female sex hormones on the outcome measures. However, this method can only be used retrospectively to confirm the menstrual phases and cannot be used to prospectively estimate these phases for the purpose of scheduling experimental sessions. Furthermore, the analysis of blood samples is time-consuming, requires a high level of technical expertise, is invasive and is costly.

4.4. Psychological Variables

Hypohydration is known to induce negative changes in psychological variables and mood state (Armstrong et al., 2011; Ely et al., 2013; Benton et al., 2016), which, in turn, may influence pain sensitivity (Turk and Okifuji, 2002; Linton and Shaw, 2011; Fillingim, 2017b). In addition to hypohdyration, the menstrual phase has also been associated with various psychological and mood changes (Janowsky et al., 1973; Sutker et al., 1983; Gonda et al., 2008). Therefore, in this study, state anxiety and mood state were measured to examine whether they could explain any observed effects of hypohydration and/or menstrual phase on pain sensitivity. Depressive symptomatology was also measured since it may vary across the menstrual cycle and could be a source of confound.

4.4.1. Depressive Symptomatology

Depressive symptomatology was measured using Centre for Epidemiologic Studies Depression Scale (CES-D) (Radloff, 1977). The CES-D is a self-report scale that measures how often an individual has experienced symptoms of depression over the past week (i.e., 7 days). The CES-D consists of 20 items, or depressive symptoms, that are grouped into four factors: depressed affect, positive affect, somatic activity and interpersonal relations. Participants were asked to rate each item, based on how they felt over the past week including the day of assessment, on a 4-point scale from 0 [rarely or none at all (less than 1 day)] to 3 [most or all of the time (5 to 7 days)]. The scores for each item were summed to give a total score that ranges from 0 to 60, where a higher score indicates a greater occurrence of depressive symptoms. Since the two experimental trials within each menstrual phase occurred within 2 to 5 days of each other (well within the 1-week response time of the CES-

D), the CES-D was administered only once in each menstrual phase, at the start of the euhydrated trials.

The CES-D is perhaps one of the most popular and frequently used self-report measure of depression, due to its brevity and excellent psychometric properties (Radloff, 1977; Smarr and Keefer, 2011; Carleton et al., 2013; Vilagut et al., 2016). It has been extensively validated as a tool to measure and screen for depression in various clinical and non-clinical populations and was shown to have high reliability and sensitivity (Radloff, 1977; Roberts, 1980; Zich et al., 1990; Morin et al., 2011; Cosco et al., 2017). For example, a recent analysis concluded that the CES-D is, in general, accurate in identifying major depression in both the general population and in patients (Vilagut et al., 2016). Radloff (1991) also showed that the CES-D differentiated well between depressed patients and healthy individuals. Scores on the CES-D also decreased over time with treatment, providing support for its reliability (Radloff, 1977). Additionally, the CES-D is comparable with clinical and diagnostic interviews and other widely used self-reported measures of depression, such as the Beck Depression Inventory and Patient Health Questionnaire-9 (Radloff, 1977; Zich et al., 1990; Haringsma et al., 2004; Shean and Baldwin, 2008; Amtmann et al., 2014).

There is some evidence that the level of depressive symptoms a woman experiences may fluctuate across the menstrual cycle (Hamilton et al., 1989; Gonda et al., 2008; Reed et al., 2008; Sakai and Ohashi, 2013). For example, a study by Sakai and Ohashi (2013) found that scores on the CES-D were higher in the luteal compared to follicular phase. Similar findings were reported in other studies where depression was measured using the Beck Depression Inventory or Zung Self-Rating Depression Scale (Gonda et al., 2008; Reed et al., 2008).

However, several others did not find effects of the menstrual phase on CES-D scores (Maki et al., 2002; Mordecai et al., 2008; Tu et al., 2013; Maki et al., 2015).

The level of depressive symptoms may also affect pain sensitivity. A recent meta-analysis comparing pain sensitivity between patients diagnosed with depression and healthy individuals found higher pain thresholds (i.e., lower pain sensitivity) in the former compared to latter group (Thompson et al., 2016), which supports the conclusion of an earlier meta-analysis (Dickens et al., 2003). Interestingly, the effects appear to be dependent on the experimental pain modality. While depressed patients appeared to be less sensitive to pain stimuli applied to the skin (e.g., noxious heat or cold) compared to healthy individuals, the converse was found for pain stimuli that affect the deep-tissue level (e.g. ischaemic pain) (Thompson et al., 2016). On the other hand, there appears to be a relatively high prevalence of clinical depression among chronic pain patients. A review found that 52% of chronic pain patients had a concurrent diagnosis of major depression (Bair et al., 2003). The same review also found that chronic pain patients presenting with depression experienced more pain symptoms, more intense pain, longer-lasting pain and more functional disability compared to the non-depressed pain patients (Blair et al., 2003).

Among healthy individuals, there appears to be a positive association between self-reported depression symptomatology and pain sensitivity (Walsh et al., 1998; Sullivan et al., 2001; Geisser et al., 2003). For example, Sullivan et al. (2001) found a positive correlation between the severity of self-reported depression symptoms and pain intensity ratings during a cold pressor task. Similarly, another found higher pain sensitivity during noxious mechanical pressure stimulation in individuals who reported more depressive symptoms (Walsh et al., 1998). However, contradictory findings have also been reported, where a negative association

between the level of depressive symptoms and pain sensitivity was found (Geisser et al., 2003). Yet, there are also several others that did not find associations between depressive symptoms and measures of pain sensitivity in healthy individuals (Wise et al., 2002; Sherman et al., 2004; Bulls et al., 2015).

4.4.2. State Anxiety

State anxiety refers to the degree of anxiety an individual feels at the present moment in response to a particular situation (Spielberger, 2010; 2013). It comes and goes frequently and changes in intensity over time and is therefore a momentary state (Spielberger, 2013). State anxiety is distinct from trait anxiety – another dimension of anxiety – which refers to an individual's general tendency to feel anxious (Spielberger, 2013). Trait anxiety reflects a personality trait of an individual and is relatively stable across time. As such, trait anxiety is unlikely to be perturbed acutely and was therefore not measured in this study.

State anxiety was assessed using the State scale of the State-Trait Anxiety Inventory (STAI-S) (Spielberger et al., 1983). The STAI-S is a self-report questionnaire that is one of the most popular and widely used instruments for assessing state anxiety in both research and clinical settings (Blumenthal et al., 1982; VanDyke et al., 2004; Kvaal et al., 2005; Cimpean and David, 2019). The state anxiety subscale consists of 20 items that are each scored on a 5-point Likert scale from 0 (not at all) to 4 (extremely). Participants respond to each item based on how they were feeling presently. The total score ranges from 20 to 80, with a higher total score indicating a higher level of state anxiety. Numerous studies have shown that the STAI-S is a valid and reliable tool for measuring state anxiety across a range of contexts (Metzger, 1976; Spielberger et al., 1983; Vitasari et al., 2011). Scores on the STAI-S increase in stressful situations (Metzger, 1976; Crocker and Grozelle, 1991; Shiloh et al., 2003) and

decrease in response to activities that promote relaxation, such as listening to music (Smith, 2008), aerobic exercise (Crocker and Grozelle, 1991; Vancampfort et al., 2011) and meditation (DeBerry, 1982; Anderson et al., 1999).

State anxiety was measured in this study for several reasons. First, state anxiety can be impacted by hypohydration (Benton et al., 2016; Young and Benton, 2018; Cousins et al., 2019). For example, one study showed that mild hypohydration (0.7% body mass loss) induced by passive heat exposure increased anxiety levels (Cousins et al., 2019). These findings agree with those reported by Benton et al. (2016), who found that hypohydration (0.7% body mass loss) was associated with an increase in anxiety, via an increase in subjective thirst ratings. In addition, state anxiety levels assessed by the STAI-S may also be influenced by menstrual phase among healthy women (Golub, 1976; Layton, 1989; Gonda et al., 2008; Walder et al., 2012), although the findings are not universal. Among the studies that found an effect of menstrual cycle phase on state anxiety levels, many observed an increase in state anxiety levels during the luteal compared to follicular phase (Golub, 1976; Layton, 1989; Gonda et al., 2008). However, the converse has also been reported, where state anxiety levels were higher during the follicular phase instead (Walder et al., 2012). Meanwhile, several other studies that did not find any effect of the menstrual cycle phase on state anxiety levels (Abplanalp et al., 1977; Golub and Harrington, 1981; Lahmeyer et al., 1982).

Second, state anxiety has been shown to influence pain sensitivity in both healthy individuals (Dougher et al., 1987; Ploghaus et al., 2001; Tang and Gibson, 2005; Metzger et al., 2019), as well as in chronic pain patients (Kain et al., 2000; Feeney, 2004; Lerman et al., 2015). Cimpean and David (2019) found that higher levels of state anxiety (STAI-S) were associated

with lower cold pressor pain tolerances (i.e., higher pain sensitivity). However, this association was mediated by participants' expectations of their ability to tolerate the pain – i.e., higher state anxiety levels were correlated with lower positive expectations, which in turn correlated with lower pain tolerances. In women, an association between higher state anxiety levels (STAI-S) and lower cold pressor pain thresholds (i.e., higher pain sensitivity) was also found (Jones and Zachariae, 2004). However, there were no correlations between state anxiety levels and pain tolerance or ratings of pain intensity and unpleasantness. Interestingly, in men, state anxiety levels correlated negatively with pain tolerance, but was not associated with pain threshold (Jones and Zachariae, 2004).

4.4.3. Mood State

Mood state has been described by Lane and Terry (2000) as a fleeting set of feelings that vary in intensity and duration and comprises one or more emotions. This definition implies that mood and emotions are intertwined and are usually indistinguishable.

Mood state was assessed using the Brunel Mood Scale (BRUMS) (Terry et al., 1999). Formerly known as the Profile of Mood States-Adolescents (POMS-A), the BRUMS is an abbreviated version of the original POMS (McNair et al., 1971). The BRUMS is a self-report questionnaire consisting of 24 items that measure six dimensions of mood: Tension, Depression, Anger, Confusion, Fatigue and Vigour. It is important to highlight that the Depression subscale of the BRUMS measures depressed mood at the present moment (Terry et al., 2003), and is distinct from the assessment of depressive symptoms by the CES-D (see Section 4.4.1). Each of the six subscales consists of four items. Participants rated each item on a 5-point point Likert scale from 0 (not at all) to 4 (extremely), based on how they were feeling presently. Each subscale was scored by summing the scores on each of its four items,

thus giving a score that ranges from 0 to 16. The score for each subscale was then used to calculate Total Mood Distress (TMD) using the formula: (Tension + Depression + Anger + Confusion + Fatigue) -Vigour. It should be noted that TMD is not an all-encompassing measure of overall mood (Suh et al., 2020). Thus, it is necessary to analyse and report the score on each subscale of the BRUMS individually.

The BRUMS is among the most widely used measure of mood state in various research and clinical settings (Moyen et al., 2015; Caldwell et al., 2018; El Tassa et al., 2018; Andrade et al., 2019). While several shortened versions of the original POMS exist (Shacham, 1983; Cella et al., 1987; Grove and Prapavessis, 1992; Terry et al., 1999), the BRUMS is perhaps the most extensively and rigorously validated version (Terry et al., 1999; Terry et al., 2003). Although initially developed in adolescents, the BRUMS has been validated numerous times in adult populations (Terry et al., 2003; Lane et al., 2007; Lan et al., 2012; Brandt et al., 2016). Scores on the BRUMS also appear to correlate well with the POMS (Terry et al., 2003), indicating its suitability as an alternative to the POMS. Major advantages with using the BRUMS are its efficiency and reduced participant burden. While the POMS contains 65 items and takes around 3 to 7 min to complete (Curran et al., 1995), the BRUMS only contains 24 items and can be completed in 1 to 2 min.

Studies using the BRUMS or POMS have consistently observed detrimental effects of hypohydration on mood state (Armstrong et al., 2011; Ely et al., 2013; Burchfield et al., 2014; Moyen et al., 2015; Caldwell et al., 2018). A recent review of 21 studies found that the dimensions of mood that appear to be the most impacted by hypohydration are alertness and fatigue, where a decrease in alertness and increase in fatigue were reported by majority of studies (Benton and Young, 2015).

There is also some evidence of fluctuations in mood state across the menstrual cycle. For example, in one study where mood state was measured using the POMS (Reed et al., 2008), scores on the subscales that measure negative mood (i.e., Tension, Depression, Anger, Fatigue and Confusion) were higher during the luteal compared to follicular phase, whereas there were no phase differences for scores on the positive mood subscales (i.e., Positive Mood, Elation, Vigour, Friendliness and Arousal). Findings from a separate study where participants rated their daily mood also indicate the presence of mood fluctuations across the menstrual cycle; however, the pattern of the variations appear to be unique to each participant (Lorenz et al., 2017). In a review of 47 studies, almost two-thirds (62%; 29/47) of the studies observed some effect of menstrual phase on mood state (Romans et al., 2012). Most of these studies reported more negative mood during the perimenstrual phase, compared to other phases of the menstrual cycle. However, due to several methodological shortcomings in most of the studies reviewed, the authors surmised that the effect of the menstrual phase on mood state is inconclusive (Romans et al., 2012).

Effects of mood state on pain sensitivity has been demonstrated repeatedly in several studies (Zelman et al., 1991; Willoughby et al., 2002; Loggia et al., 2008; Wagner et al., 2009). Compared to the control condition where mood state was not manipulated, experimentally induced depressive mood increased pain sensitivity during a cold pressor task, whereas elative mood reduced pain sensitivity (Zelman et al., 1991). Similarly, studies using noxious heat stimuli found that inducing unpleasant or depressed mood led to higher pain sensitivity compared to the condition where mood was neutral (Loggia et al., 2008; Berna et al., 2010).

4.5. Ethical Approval

The study was approved by the Massey University Human Ethics Committee: Southern A (18/66) and performed according the Declaration of Helsinki.

4.6. Participant Characteristics

Based on conventional α (0.05) and β (0.80) values and an effect size of 0.50 as in a previous meta-analysis on the effects of menstrual phase on ischaemic pain tolerance (Riley et al., 1999), an *a priori* power analysis (G*Power version 3.1.9.4; Heinrich Heine University Düsseldorf, Düsseldorf, Germany) showed that a minimum of 27 participants were required. However, the studies included in the meta-analysis only tested participants once in each menstrual phase, whereas participants in the current study performed two trials within each menstrual phase (see Section 4.7). Whereas, based on the study by Bear et al. (2016) on the effects of hypohydration on cold pressor pain that had a power (1- β) of 0.79 and an effect size of 0.53, a minimum of 17 participants were required. However, participants in this study were only tested once while euhydrated and once while hypohydrated, whereas participants in the current study performed two euhydrated and two hypohydrated trials (see Section 4.7). As such, the sample size required for the current study was estimated to be between 17 and 27 participants.

A total of 23 healthy, eumenorrheic women were initially recruited for this study. Participants were recruited from the Massey University Manawatu campus via posters or word-of-mouth. However, due to various reasons (see Section 5.0), fourteen participants successfully completed all trials in the correct menstrual phases and were included in the final analyses. The characteristics of these 14 participants are displayed in Table 1.

All participants received an information sheet, provided written informed consent and completed a health screening questionnaire prior to participation in the study. Participants reported having regular menstrual cycles that were, on average, 21 to 35 days in length (Creinin et al., 2004; Fehring et al., 2006; Bull et al., 2019). Menstrual cycle length was defined as the number of days from the first day of menses up until the start of the next menses (Fehring et al., 2006; Small et al., 2007). All participants were non-smokers and had not used hormonal contraceptives within the past six months (Schaumberg et al., 2017). Except for two participants (i.e., Subjects 11 and 14), all other participants had never been pregnant or given birth. Individuals with a pre-existing or history of any medical, psychiatric pain-related, and/or menstrual-related disorders, or who regularly use pain-relieving and/or over-the-counter medications were excluded from the study. Lastly, participants who are currently experiencing, or have a history of, dysmenorrhoea (i.e., menstrual pain) were also excluded. Dysmenorrhea was defined as recurrent menstrual pain in the past 6 months, with a pain intensity rating of greater than 3 out of 10 on a numerical rating scale, with 0 denoting "no pain at all" and 10 representing "worst imaginable pain" (Vincent et al., 2011; Iacovides et al., 2015b). Participants were compensated with a $50 gift voucher upon completion of all four experimental trials, or on a pro-rated basis.

Table 1. Characteristics of each participant

Subject no.	Age (y)	Height (cm)	Body Mass (kg)	Body Mass Index ($kg.m^2$)	Self-reported Menstrual Cycle Length (no. of days)	Self-reported Physical Activity (min per week)
1	25	164	67	25	28	200
2	42	152	67.9	29	26	300
3	33	157	59.9	24	26	60
4	25	171	65.8	23	28	180
5	30	167	76	27	26	150
6	20	162	58	22	27	75
7	20	173	74.7	25	25	200
8	25	159	57.9	23	29	530
9	25	162	50.9	19	30	240
10	28	156	54.7	22	30	135
11	45	174	90.3	30	28	60
12	29	158	67.6	27	25	0
13	19	162	73.8	28	28	120
14	30	172	76	26	27	160
Mean (standard deviation)	28 (7.7)	163 (7)	67.2 (10.5)	25 (3)	27 (2)	145 (130)

Note. Menstrual cycle length was defined as the number of days from the first day of menses up until the start of the next menses. Body mass was obtained during the first familiarization session. BMI, body mass index.

4.7. Experimental Overview

Participants visited the laboratory on six occasions: two familiarisation sessions, followed by four experimental trials. The four experimental trials were a full crossover of menstrual phase (EF and ML) and hydration status [euhydrated (EUH) and hypohydration (HYPO)], whereby participants performed two trials (EUH and HYPO) within one menstrual phase, followed by another two trials within the other menstrual phase. The Phase-Hydration order of all experimental trials was randomised and counterbalanced; however, the order of the EUH and HYPO trials for each participant was the same in both menstrual phases. Unless scheduling difficulties occurred, testing occurred in consecutive menstrual phases, meaning that participants who performed the EF trials first would perform their ML trials within the same menstrual cycle, approximately 14 days later (Figure 3A). For participants who started their trials in the ML phase, their EF trials would take place in the next menstrual cycle, approximately 7 days later (Figure 3B).

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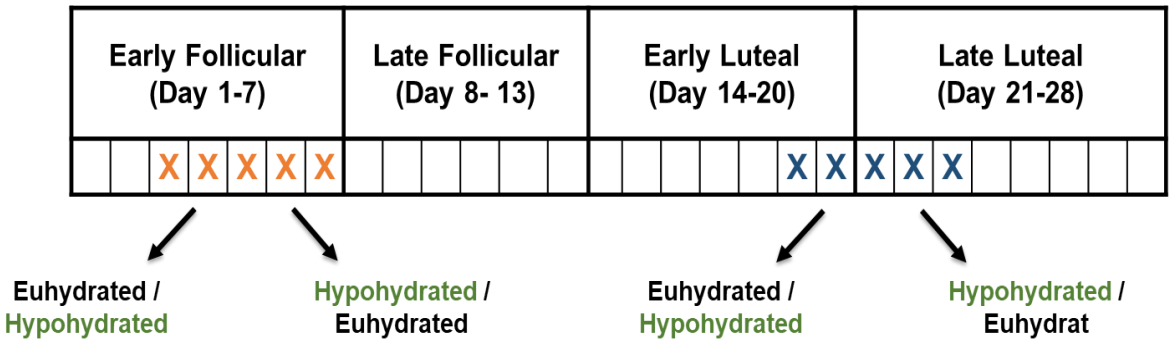
A)



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B)

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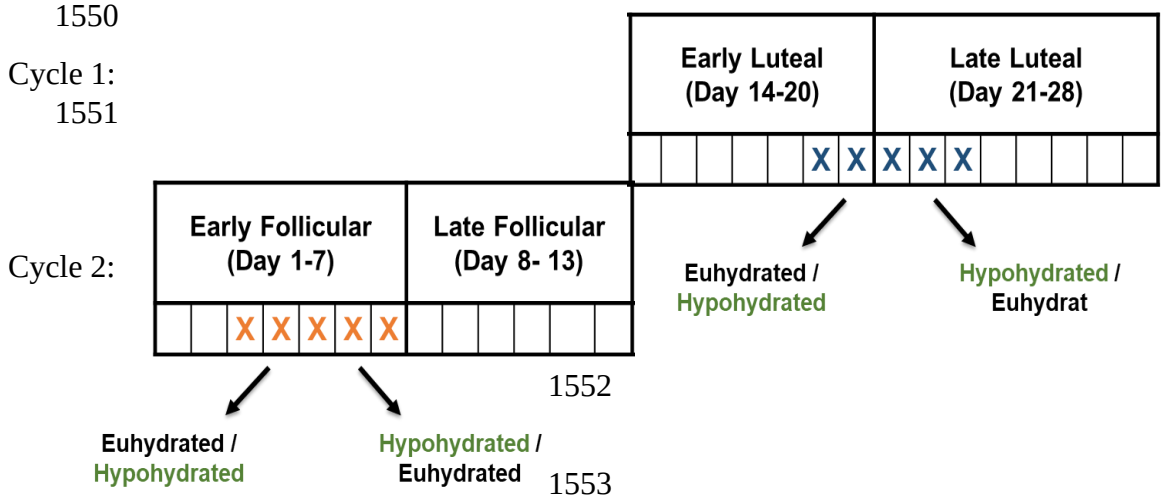


1554 Figure 3. Schematic diagram of study design, along with the hormonal profile during each phase, for
1555 participants who started their trials in the (A) early follicular or (B) mid-luteal phase.

4.8. Familiarisation

Participants completed two familiarisation sessions before beginning the experimental trials. The purpose of this was to acquaint them with the laboratory setting and experimental procedures, in order to reduce potential 'learning' and anxiety effects. The number of familiarisation trials was determined by preliminary assessment of the test-retest reliability of the experimental pain measures (see Section 4.8.2).

During the first familiarisation session, the participant's height and body mass were measured. The participant's body mass measured in this session was used to calculate her fluid volume for the acute water ingestion (see Section 4.10). Next was the assessment of maximal handgrip strength to determine the absolute intensity for the handgrip exercises during the ischaemic pain test. Participants then competed the CES-D, BRUMS, STAI-S and thirst sensation VAS, followed by the ischaemic pain test. The second familiarisation session followed the same procedure, but without the anthropometric measurements and maximal handgrip strength testing.

4.8.1. Maximal Handgrip Strength Assessment

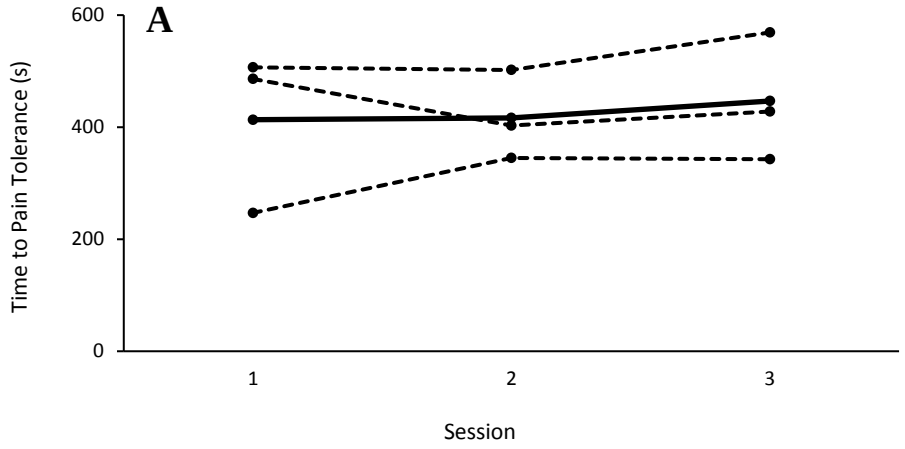
Using their non-dominant hand, participants performed three maximal voluntary contractions (MVCs) using a handgrip dynamometer (MLT003 Grip Force Transducer, ADInstruments Inc., CO, USA), with 1 min of rest between each MVC (Hoeger Bement et al., 2009). During each MVC, participants were asked to squeeze the dynamometer maximally and hold the contraction for 5 s. The force output from the dynamometer was recorded (PowerLab, ADInstruments, Dunedin, New Zealand) and displayed in real-time (LabChart Pro, ADInstruments, Dunedin, New Zealand). Participants received visual feedback of their force

output on a laptop screen and were verbally encouraged to produce maximal force. Maximal handgrip strength was determined by calculating the mean of the peak values for the three MVCs.

The maximal handgrip strength assessment was only conducted once during the first familiarisation session, rather than before each experimental trial, because performance of the MVCs may subsequently influence pain sensitivity (Hoeger Bement et al., 2009). Moreover, menstrual phase does not appear to influence grip strength (De Jonge et al., 2001; Fridén et al., 2003; Nicolay et al., 2008). Similarly, mild hypohydration (< 2% body mass loss) is also unlikely to compromise muscle strength in women (Gutiérrez et al., 2003; Kraft et al., 2012). In addition, a previous study showed that MVC performance in women is stable over time, with maximal force fluctuating by no more than 5% (Hunter et al., 2000).

4.8.2. Test-retest Reliability of Ischaemic Pain Measures

Three female volunteers were recruited to perform the ischaemic pain test three times each across three consecutive days. Each participant performed all three sessions at the same time of day (± 1 hr). Pain tolerance (seconds) and ratings of pain intensity and unpleasantness were measured in each session. Overall, pain tolerance ($P = 0.64$), pain intensity ($P = 0.47$) and pain unpleasantness ($P = 0.21$) ratings were similar across the three sessions (Figure 4). Thus, the data seem to indicate that a minimum of two familiarisation sessions would be necessary to lessen the practice or anxiety effects.



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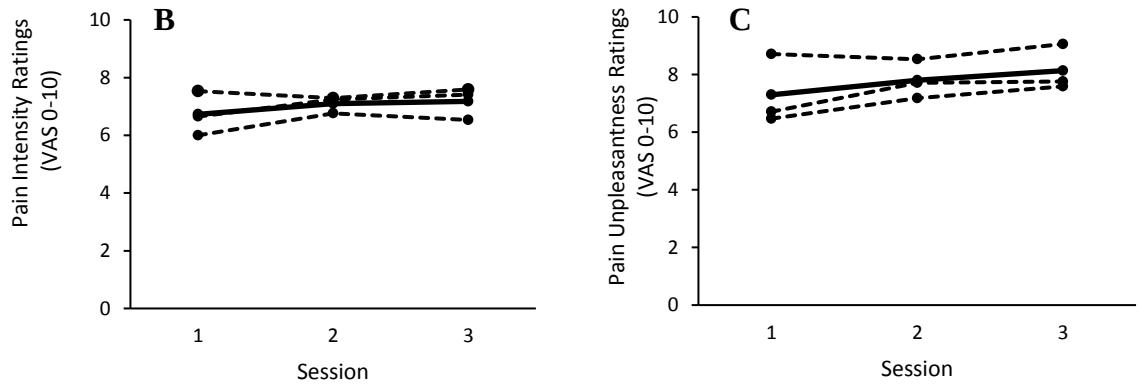


1598 .

1599 Figure 4. Individual and mean (A) pain tolerance, (B) pain intensity ratings and (C) pain
1600 unpleasantness ratings across three separate sessions.

1601 *Note.* VAS, visual analogue scale. Dashed lines represent individual data from each participant. Solid
1602 lines represent the mean data for all three participants.

4.9. Pre-Experimental Controls

Participants were asked to abstain from pain-relieving medications, alcohol intake and strenuous exercise at least 12 hr before the start of each experimental trial. Participants recorded their diet, physical activity and sleep duration over the 24-hr period before their first trial and were asked to replicate these for subsequent trials. Participants were also asked to maintain their habitual caffeine intake over the 24-hr period preceding each trial. This is important because acute caffeine intake has been shown to reduce experimental pain sensitivity in women (Keogh and Witt, 2001; Keogh and Chaloner, 2002), as well as decrease subjective ratings of muscle pain intensity during exercise (Gliottoni and Motl, 2008). Moreover, caffeine intake can alter several psychological factors that are related to pain sensitivity, such as mood state (Amendola et al., 1998; Peeling and Dawson, 2007) and cognitive function (Ruxton, 2008; McLellan et al., 2016). To prevent caffeine withdrawal during the hypohydrated trials where participants were required to restrict their fluid intake (see Section 4.2.1), participants who normally consume caffeinated beverages were provided with a caffeine tablet (No-Doz Awakeners, Novartis Consumer Health Inc.) to take in place of the beverages. To verify adherence to these protocols, participants completed a self-report diary at the start of each trial, where they wrote down all food items consumed, fluids ingested, and physical activity performed over the preceding 24-hr period. Participants also recorded their time to bed and wake, from which sleep duration was calculated. These data were looked through by the candidate to ensure they were similar to the participant's previous trials(s).

This dietary and lifestyle control was implemented to minimise possible confounding influences on the hydration markers, psychological variables and ischaemic pain measures.

For instance, dietary sodium intake can acutely increase S_{osm} (Kanbay et al., 2018), while a diet high in protein is associated with higher P_{osm} and USG compared to a low-protein diet (Martin et al., 2006). The consumption of food can also reduce experimental pain sensitivity and the effect may be modulated by the macronutrient composition of the ingested meal (Zmarzty et al., 1997; Anjana and Reetu, 2014).

Regarding the influence of exercise, there is consistent data showing a hypoalgesic (i.e., decrease in pain) effect of an acute bout of exercise in healthy, pain-free individuals and in some chronic pain patients (Naugle et al., 2012). In addition, post-exercise improvements in both mood state (Maroulakis and Zervas, 1993; Berger and Motl, 2000) and decrease in state anxiety levels have also been reported (Petruzzello et al., 1991; Ensari et al., 2015).

Lastly, sleep restriction has been associated with increased pain sensitivity in healthy individuals (Haack et al., 2007; Tiede et al., 2010; Matre et al., 2015), with the effects being apparent after only a single night of sleeping less than usual (Tiede et al., 2010; Lee et al., 2013). Moreover, negative effects of reduced sleep duration on state anxiety levels (Wu et al., 2008; Motomura et al., 2013) and mood state (Baum et al., 2014; Iacovides et al., 2017) have also been reported. Conversely, sleep duration may also affect hydration status, with a recent study reporting higher USG values and increased likelihood of becoming inadequately hydrated in individuals who slept 6 hr per night, compared to those who slept 8 hr per night (Rosinger et al., 2018). This finding is relevant to the current study, inasmuch as participants may inadvertently arrive for their euhydrated trials in a mildly hypohydrated state if they had little sleep the night before.

Each participant reported to the laboratory at the same time of day (± 1 hr) for all their experimental trials to minimise diurnal variations in pain sensitivity (Aviram et al., 2015) and

ovarian hormone concentrations (Fujimoto et al., 1990; Rahman et al., 2019). All trials were separated by at least 48 hr to ensure complete recovery of the venepuncture wound and the arm that was used for the ischaemic pain test. The ambient temperature in the laboratory was maintained at approximately 22ºC for all trials, as was the intensity of light at around 600 lux.

4.10. Experimental Protocol

Twenty-four hours before each trial, participants either drank *ad libitum* or restricted fluid intake. Upon arrival at the laboratory, a blood sample was obtained from the antecubital vein of the dominant arm via venepuncture. Participants then provided a urine sample for measurement of USG to confirm euhydration (USG < 1.020) or hypohydration (USG ≥ 1.020) (American College of Sports et al., 2007), after which body mass was measured. Participants then completed the dietary and physical activity record sheet, CES-D (for euhydrated trials only; see Section 4.4.1), STAI-S, BRUMS and thirst sensation VAS, after which they performed the ischaemic pain test.

Immediately after the ischaemic pain test, participants were asked to ingest a glass of water (5 $ml.kg^{-1}$ of body mass) and to finish it within 5 min. For a female weighing around 65 kg, this volume of water equated to around 325 ml, which is similar to that contained in a standard soft-drink can (330 ml) and therefore, can (presumably) be ingested easily in one bolus without causing major discomfort. This would also maximise the ecological validity of the water intervention. Moreover, a previous study showed that drinking a similar amount of water (4.3 $ml.kg^{-1}$ of body mass) produces an immediate decrease in subjective thirst ratings in hypohydrated participants (Takamata et al. 1995). The acute water ingestion was followed by 30 min of quiet rest, during which participants remained seated in the laboratory and did their activity of choice (e.g., reading, watching videos), which was kept the same for all experimental trials. The duration of the rest period was determined based on previous studies indicating that at least 30 min was required between repeated pain tests to minimise carryover effects (Hoeger Bement et al., 2008; Hoeger Bement et al., 2009). At the end of the 30 min break, participants once again completed the, STAI-S, BRUMS and thirst sensation VAS,

then repeated the ischaemic pain test. A schematic diagram of the experimental procedure is shown in Figure 5.

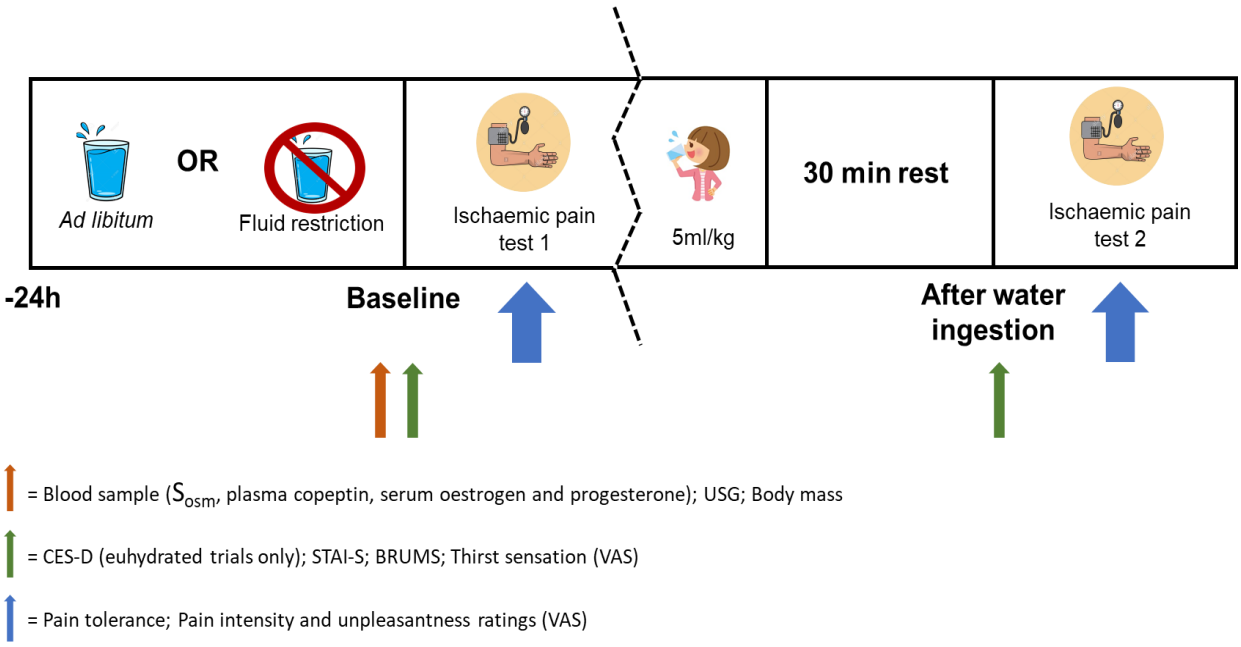


Figure 5. Schematic diagram of the experimental protocol.

Note. S_{osm}, serum osmolality; USG, urine specific gravity; CES-D, Centre for Epidemiologic Studies Depression scale; STAI-S, State scale of the State-Trait Anxiety Inventory; BRUMS, Brunel Mood Scale; VAS, visual analogue scale.

4.10.1. Ischaemic Pain Test Protocol

A pneumatic cuff was positioned on the participant's non-dominant arm, just above the elbow joint. The participant's arm was passively raised to a vertical position for 30 s to promote venous drainage. The pneumatic cuff was then inflated to 250 mmHg and a stopwatch was started, signifying the start of the pain test. The participant's arm was lowered to a horizontal position on the table, such that the forearm rested on the table with the elbow joint at an approximately 90º angle. To promote forearm ischaemia, participants then performed 25 handgrip exercises at 30% of their pre-determined maximum handgrip strength (see Section

4.8.1). Each contraction was sustained for 2 s and was followed by 2 s of rest. Auditory signals were played on an iPhone application (Tabata HIIT Interval Timer) to indicate the start and end of each contraction. Participants received continuous visual feedback of their force output on a laptop screen, with a black line indicating the target force to hit (see Figure 5). The force output was recorded and displayed in real-time using data acquisition software described previously (see Section 4.8.1).

Upon completion of the handgrip exercises, a neutral nature documentary was played while the cuff remained inflated on the participant's arm. The volume of the video was kept at 30% for all trials. The purpose of the video was to standardise each participant's attention during the pain test. One minute after the handgrip exercises, participants rated their pain intensity and pain unpleasantness on a 10-cm VAS (see Section 4.1.3.2). Participants were asked to keep their arm still and remain quiet throughout the test. Participants were also asked to indicate, by raising their opposite hand and saying "stop," when they are no longer able or willing to endure the pain (i.e., pain tolerance; see Section 4.1.3.1). At this point, the stopwatch was stopped, signifying the end of the pain test. Just before the cuff was deflated, participants rated their pain intensity and unpleasantness again. A maximum time limit of 20 min was enforced, which participants were not aware of.

4.11. Measurements

4.11.1. Anthropometric

Each participant's height and body mass were measured using a stadiometer (Seca, Hamburg, Germany; accurate to 0.1 cm) and electronic scale (Model X3M, HIWEIGH Technologies Limited, Shanghai, China; accurate to 10 g), respectively. These values were used to calculate body mass index (BMI) using the formula: body mass (kg)/height $(m)^2$.

Percent change in body mass change following 24 hr of fluid restriction was calculated from the body mass measured during the EUH trial within each menstrual phase.

4.11.2. Blood Analyses

Venous blood was collected into two 10 ml vacutainer tubes (Becton-Dickson, Oxford, UK) One tube contained clot activator for analysis of S_{osm} and serum 17ß-oestradiol and progesterone concentrations, while the other tube contained EDTA for analysis of plasma copeptin. The tube containing clot activator was inverted gently and left to clot for a minimum of 30 min. The whole blood in both tubes was then centrifuged (Centrifuge 5804 R, Eppendorf AG, Hamburg, Germany) at 4ºC and 260 *g* for 15 min to separate serum and plasma from whole blood. Aliquots of serum and plasma were then separated into three Eppendorf tubes (Genuine Axygen Quality, USA) each and stored at -80ºC for further analysis.

Commercially available enzyme-linked immune assays (ELISA) were used to measure concentrations of 17β-oestradiol (Demeditec Diagnostics, Kiel, Germany) and progesterone (IBL International, Hamburg, Germany) in serum samples, with a sensitivity of 6.2 pg.ml-1

and 0.045 ng.ml-1, respectively, and an intra-assay variation of < 6 and < 7%, respectively. The ratio of progesterone to 17ß-oestradiol (P_4:E_2 ratio) was then calculated (Elgindy, 2011). A progesterone concentration of > 5 ng.mL^{-1} was adopted for confirmation of an ovulatory cycle and correct identification of the ML phase (Schaumberg et al., 2017; De Jonge et al., 2019). Participants who did not meet this criterion were excluded from the main results. Additionally, the EF phase was confirmed by lower 17ß-oestradiol and progesterone concentrations in the EF compared to ML phase.

Serum osmolality was measured using a freezing-point depression osmometer (Digimatic osmometer Model 3D2, Advanced Instruments Inc., Norwood, MA, USA). Measurements were made in duplicate and the average value was calculated. If the two measurements differed by more than ~5%, a third measurement was taken and the average of the two closest values was calculated.

Plasma copeptin concentrations were measured with an automated immunofluorescent Copeptin proAVP KRYPTOR assay (B·R·A·H·M·S KRYPTOR compact PLUS analyser, Thermo Fisher Scientific, Henningsdorf, Germany), with a sensitivity of 1.08 pmol.L^{-1}.

4.12. Statistical Analyses

All statistical analyses were performed with SPSS software for windows (IBM SPSS Statistics 20, NY, USA) with *a priori* statistical significance set at $P \leq 0.05$. Descriptive values are reported as means ± standard deviation, unless otherwise stated. Data were assessed to ensure they were approximately normally distributed and for sphericity, with no corrections needed. Data for S_{osm}, plasma copeptin, USG, body mass, 17β-oestradiol, progesterone and the P_4:E_2 ratio were assessed using a two-way (Phase x Hydration) repeated measures analysis of variance (ANOVA). For thirst sensation ratings, BRUMS, STAI-S, pain tolerance, pain intensity ratings and pain unpleasantness ratings, the data were analysed by three-way (Phase x Hydration x Water) repeated measures ANOVA. When main or interaction effects occurred for the two- or three-way ANOVA, *post hoc* pair-wise analyses were conducted using paired-samples *t* tests with Bonferroni correction, where appropriate. *A priori* statistical significance was set at $P \leq 0.05$. Data for CES-D and percent body mass change were analysed using a paired-samples *t* test to compare the means between menstrual phases.

Bivariate Pearson's correlations were performed to examine the direction and strength of relationships between the main independent (i.e., hydration markers and ovarian hormone concentrations) and dependent (i.e., ischaemic pain measures) variables. Effect sizes are reported as partial eta-squared (η_p^2) with demarcations of small (< 0.06), medium (> 0.06 and < 0.14) and large (> 0.14) effects, respectively (Cohen, 1988).

CHAPTER FIVE

5.0. Results

A total of 23 participants were recruited for this study and all completed both familiarisation sessions. Five participants dropped out prior to the experimental trials due to scheduling difficulties (n = 3) and COVID-19 (n = 2). As such, 18 participants proceeded with the experimental trials. Out of these 18 participants, one participant dropped out after only performing the ML phase trials due to scheduling difficulties. Therefore, 17 participants completed all four experimental trials in both the EF and ML phases.

Following blood analysis for serum 17ß-oestradiol and progesterone concentrations however (see Section 5.2.1), five participants (29%) did not meet the progesterone threshold of > 5 $ng.ml^{-1}$ for the ML phase in one (n = 2) or both trials (n = 3), despite obtaining positive urinary LH tests. Of these five participants, only two participants successfully repeated one (n = 1) or two trials (n =1), where they subsequently met the > 5 $ng.ml^{-1}$ progesterone criterion for verification of the ML phase (see Section 4.3.2). One participant only completed one of the two repeat trials before early (unexpected) onset of menses prevented completion of the other trial. For the remaining two participants, attempts to reschedule their trials were unsuccessful. The data from these three participants were subsequently excluded from the main results. Therefore, the results that follow were analysed for 14 participants, all of whom met the > 5 $ng.ml^{-1}$ progesterone criterion for both trials in the ML phase.

The order of the experimental trials in terms of menstrual phase (EF and ML) and hydration condition (EUH and HYPO) was initially counterbalanced for the 23 recruited participants; however, the unexpected dropouts and hormone-based exclusions resulted in an unequal

number of participants in each Phase-Hydration order sequence. Six participants were tested during the EF phase first (n = 4 performed the EUH trial first and n = 2 performed the HYPO trial first), while eight participants started with the trials in the ML phase (n = 5 performed EUH trial first and n = 3 performed the HYPO trial first).

5.1. Self-Reported Sleep Duration and Depressive Symptomatology

Mean sleep duration across trials was 7.4 ± 1.6 hr (mean ± standard deviation), which was not different between hydration conditions (EUH: 7.6 ± 1.6 vs. HYPO: 7.2 ± 1.6 hr; $P = .15$, $\eta_p^2 = .15$) or menstrual phases (EF: 7.4 ± 1.4 vs. ML: 7.4 ± 1.7 hr; $P = 0.87$, $\eta_p^2 = 0.002$). There was also no Phase x Hydration interaction for sleep duration ($P = 0.29$, $\eta_p^2 = 0.09$).

Incomplete data were collected for depressive symptomatology assessed by the CES-D (n = 13). There was no main effect of menstrual phase on CES-D scores ($P = .55$, $\eta_p^2 = .03$) (Figure 6). Therefore, participants were in a similar psychological state in both menstrual phases.

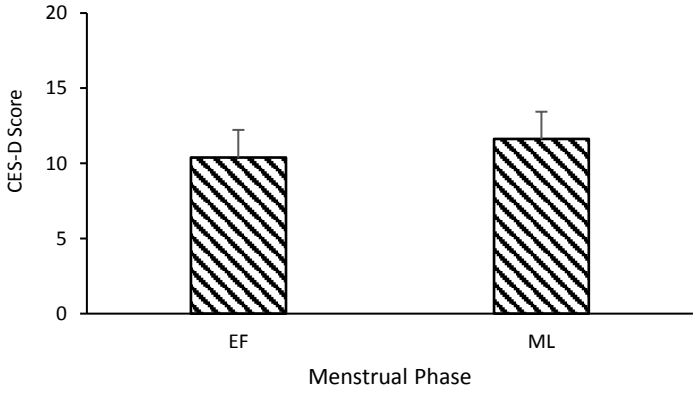


Figure 6. Mean CES-D score (n = 13) in each menstrual phase.

Data are presented as means + standard error. CES-D, Centre for Epidemiologic Studies Depression scale; EF, early follicular; ML, mid-luteal.

5.2. Menstrual Phase Identification and Verification

For the EF phase, the EUH and HYPO trials were performed on days 4 ± 2 (range: day 2-8) and 5 ± 2 (range: day 2-8) following the start of menses, respectively.

For the ML phase, the EUH and HYPO trials were performed 7 ± 2 days (range: 4-10 days) and 8 ± 2 days (range: 5-12 days) after a positive ovulation test, respectively. Assuming a conventional 28-day menstrual cycle, this corresponded to around days 21 (EUH) and 22 (HYPO) after menses onset.

5.2.1. Ovarian Hormone Concentrations

Mean ovarian hormone concentrations are presented in Table 2. Due to difficulty in obtaining blood samples in one or more trials for 3 participants, incomplete data were obtained for 17ß-oestradiol and progesterone concentrations (n = 11 each). Progesterone (P = .009, η_p^2 = 0.51) and 17ß-oestradiol (P = .001, η_p^2 = .66) concentrations were significantly higher in the ML compared to EF phase. Accordingly, the P_4:E_2 ratio was also higher in the ML compared to EF phase (P = .005, η_p^2 = .57). There was no main effect of hydration status on progesterone (P = .46, η_p^2 = .06), 17ß-oestradiol (P = .58, η_p^2 = .03), or the P_4:E_2 ratio (P = .76, η_p^2 = .01). There was also no Phase x Hydration interaction for any of these measures (all $P \geq .46$, $\eta_p^2 \leq .06$).

Table 2. Mean ovarian hormone concentrations in each hydration condition and menstrual phase.

		Early Follicular		Mid-luteal	
	n	EUH	HYPO	EUH	HYPO
17ß-oestradiol ($pg.ml^{-1}$)*	11	49 ± 23 (range: 33 – 101)	56 ± 38 (range: 12 – 127)	106 ± 67 (range: 50 – 273)	107 ± 54 (range: 45 – 209)
Progesterone ($ng.ml^{-1}$)*	11	0.5 ± 0.3 (range: 0.2 – 0.9)	0.5 ± 0.3 (range: 0.2 – 0.9)	23 ± 25.5 (range: 6 – 91.7)	28.6 ± 31.3 (range: 5.6 – 107)
P_4:E_2 Ratio*	11	11 ± 7	11 ± 7	265 ± 272	287 ± 265

Note. Data are presented as means ± SD. EUH, euhydrated; HYPO, hypohydrated; P_4, progesterone; E_2, 17ß-oestradiol.

* Significantly higher in the mid-luteal compared to early follicular phase, $P < 0.05$.

5.3. Hydration Status

5.3.1. Hydration Markers

Biochemical markers of hydration status obtained at baseline (i.e., before water ingestion) indicate that 24 hr of fluid restriction was successful in eliciting mild dehydration (Table 3). Absolute body mass was lower in HYPO than in EUH (P = .006, η_p^2 = .45), independent of menstrual phase (Phase x Hydration interaction, P = .31, η_p^2 = .08). Therefore, the average percent change in body mass from EUH to HYPO was -0.9 ± 1.3%. There was no main effect of menstrual phase on absolute body mass (P = .51, η_p^2 = .04) or percent body mass change (P = .27, η_p^2 = .29).

Due to difficulty in obtaining blood samples, incomplete data were collected for S_{osm} (n = 10) and plasma copeptin (n = 9). S_{osm} was higher in HYPO than in EUH (P = .001, η_p^2 = .72), as well as in the ML compared to EF phase (P = .03, η_p^2 = .43). However, there was no Phase x Hydration interaction for S_{osm} (P = .22, η_p^2 = .17).

Plasma copeptin was also higher in HYPO than in EUH (P = .04, η_p^2 = .44). However, there was no main effect of menstrual phase (P = .11, η_p^2 = .28) or a Phase x Hydration interaction effect for plasma copeptin (P = .40, η_p^2 = .09).

Similarly, USG was higher in HYPO than in EUH (P < .001, η_p^2 = .84); but there was no main effect of menstrual phase (P = .57, η_p^2 = .03) or a Phase x Hydration interaction for USG (P = .63, η_p^2 = .02).

In accordance with the significant increases in these blood and urinary hydration markers, participants also reported feeling thirstier during HYPO compared to EUH (see Figure 7).

Table 3. Hydration markers at baseline (i.e., before water ingestion) in each hydration condition and menstrual phase.

		Early Follicular		Mid-luteal	
	n	EUH	HYPO	EUH	HYPO
Body Mass (kg)‡	14	67.1 ± 10.6	66.3 ± 10.2	67.1 ± 10.7	66.6 ± 10.2
Body Mass Change, % [a]*	14		-1.1		-0.7
Serum Osmolality ($mOsm.kg^{-1}$)*#	10	283 ± 6	286 ± 6	279 ± 6	284 ± 5
Plasma Copeptin ($pmol.L^{-1}$)*	9	4.8 ± 6.5	7.1 ± 2.6	3.3 ± 3.0	7.1 ± 3.8
USG*	14	1.009 ± 0.01	1.025 ±0.005	1.009 ± 0.008	1.023 ± 0.004
Baseline Thirst Sensation (cm)*	13	1.9 ± 1.9	6.6 ± 2.2	2.2 ± 2	7.3 ± 2.4

Note. Data are presented as means ± SD. USG, urine specific gravity; BM, body mass; EUH, euhydrated; HYPO, hypohydrated; VAS, visual analogue scale.

[a] Body mass change was calculated relative to EUH.

* Significantly greater in HYPO compared to EUH, $P < .05$.

‡ Significantly lower in HYPO compared to EUH, $P < .05$.

Significantly lower in the mid-luteal compared to early follicular phase.

5.3.2. Thirst Sensation Ratings

Incomplete data were collected for thirst sensation ratings (n = 13). Thirst sensation ratings were higher during HYPO than in EUH ($P < .001$, $\eta_p^2 = .89$) (Figure 7). There was also a main effect of menstrual phase, with thirst sensation ratings being higher in the ML compared to EF phase ($P = .04$, $\eta_p^2 = .30$). However, there was no Phase x Hydration interaction for thirst sensation ratings ($P = .67$, $\eta_p^2 = .02$).

After water ingestion, thirst ratings decreased from baseline by a larger extent during HYPO than in EUH (HYPO: Δ 4.0 ± 2.3 vs. EUH: Δ 0.6 ± 1.4 cm; $P < .001$, $\eta_p^2 = .65$). However, these effects were not dependent on menstrual phase (Phase x Hydration x Water interaction: $P = .47$, $\eta_p^2 = .05$).

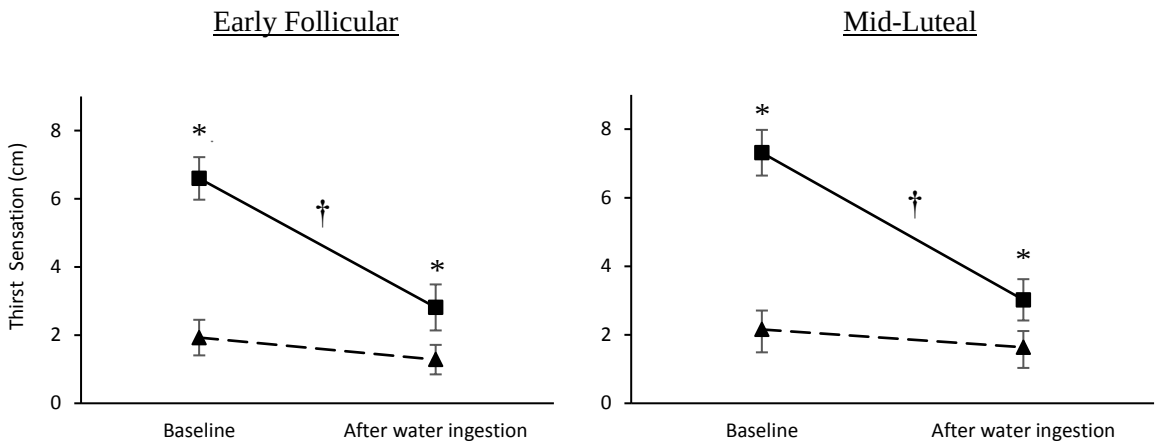


Figure 7. Mean ratings of thirst sensation ($n = 13$) at baseline (i.e., before water ingestion) and after water ingestion in each hydration condition and menstrual phase.

Note. Data are presented as means ± standard error. EUH, euhydrated; HYPO, hypohydrated; VAS, visual analogue scale.

* Significantly higher in HYPO than in EUH at the respective time point, $P < .05$.

† Significant decrease from baseline to after water ingestion, $P < .05$.

5.4. Ischaemic Pain Measures

5.4.1. Pain Tolerance

One participant was able to tolerate the ischaemic pain until the maximum time limit of 20 min (1200 s) in one trial (EUH trial in the ML phase). Her pain tolerance in this trial was therefore recorded as 1200 s.

Pain tolerance during HYPO was lower compared to EUH (P = .02, η_p^2 = .37), independent of menstrual phase (Phase x Hydration interaction, P = .15, η_p^2 = .15) (Figure 8). There was no main effect of menstrual phase on pain tolerance (P = .43, η_p^2 = .05). Pain tolerance was also unaffected by acute water ingestion (P = .75, η_p^2 = .01), independent of hydration status (Hydration x Water interaction, P = .92, η_p^2 = .001) or menstrual phase (Phase x Water interaction, P = .85, η_p^2 = .003).

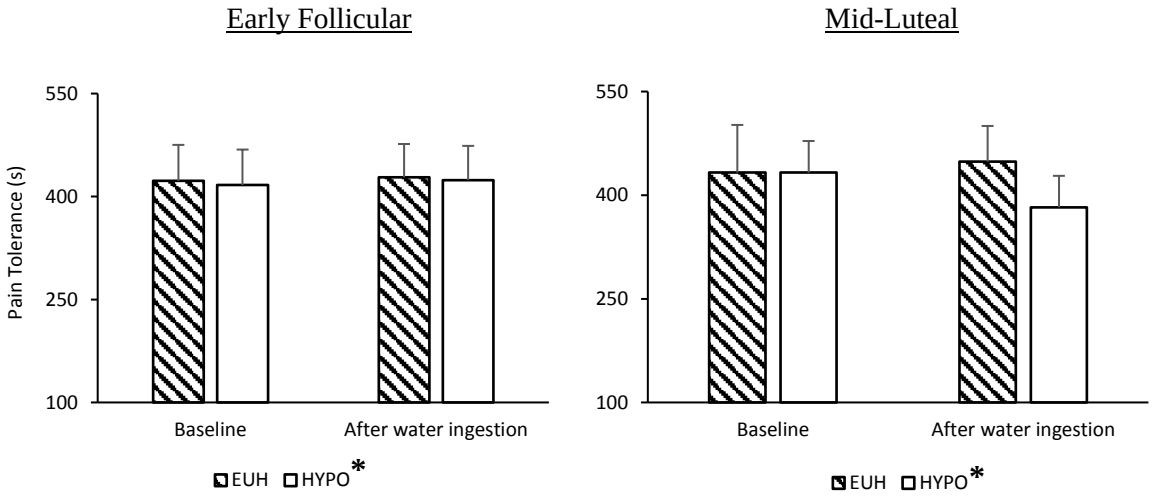


Figure 8. Mean pain tolerance (n = 14) at baseline (i.e., before water ingestion) and after water ingestion in each hydration condition and menstrual phase.

Note. Data are presented as means + standard error. EUH, euhydrated; HYPO, hypohydrated.

***** Significantly lower in HYPO (i.e., higher pain sensitivity) than in EUH, P < .05

5.4.2. Pain Intensity and Pain Unpleasantness Ratings

Incomplete data were collected for pain intensity and pain unpleasantness ratings (n = 12 each) at 1 min after handgrip exercise as two participants terminated the pain test before reaching this time point.

At 1 min after handgrip exercise, both pain intensity (P = .004, η_p^2 = .55) (Figure 9A) and pain unpleasantness (P = .02, η_p^2 =.40) (Figure 9B) ratings were higher during HYPO than in EUH, independent of menstrual phase (Phase x Hydration interaction: P = .42, η_p^2 = .06 and P = .15, η_p^2 = .18, for pain intensity and pain unpleasantness, respectively). However, there was no main effect of menstrual phase for either variable (P = .98, η_p^2 < .001 and P = .76, η_p^2 = .01 for pain intensity and pain unpleasantness, respectively). After water ingestion, both pain intensity (P = .004, η_p^2 = .54) and pain unpleasantness (P = .04, η_p^2 = .34) ratings increased significantly from baseline, with these effects being independent of hydration status (Hydration x Water interaction, all $P \geq .50$, $\eta_p^2 \leq .04$) or menstrual phase (Phase x Water interaction, all $P \geq .53$, $\eta_p^2 \leq .04$).

At pain tolerance however, participants did not rate their pain intensity (Figure 10A) or pain unpleasantness (Figure 10B) differently between hydration conditions (P = .13, η_p^2 = .17 and P = .33, η_p^2 = .07 respectively) or between menstrual phases (P = .82, η_p^2 = .004 and P = .98, $\eta_p^2 \leq .001$, respectively). There was also no Phase x Hydration interaction for either pain intensity (P = .96, $\eta_p^2 \leq .001$) or pain unpleasantness (P = .68, η_p^2 = .01) ratings at pain tolerance. After water ingestion, ratings of pain intensity at pain tolerance increased significantly from baseline (P = .04, η_p^2 = .29), independent of hydration status (Hydration x Water interaction: P = .99, $\eta_p^2 \leq .001$) or menstrual phase (Phase x Water interaction: P = .55, η_p^2 = .03). For pain unpleasantness ratings at pain tolerance however, there was no main

effect of water ingestion (P = .10, η_p^2 = .20), regardless of hydration status (Hydration x Water interaction: P = .11, η_p^2 = .19) or menstrual phase (Phase x Water interaction: P = .79, η_p^2 = .01).

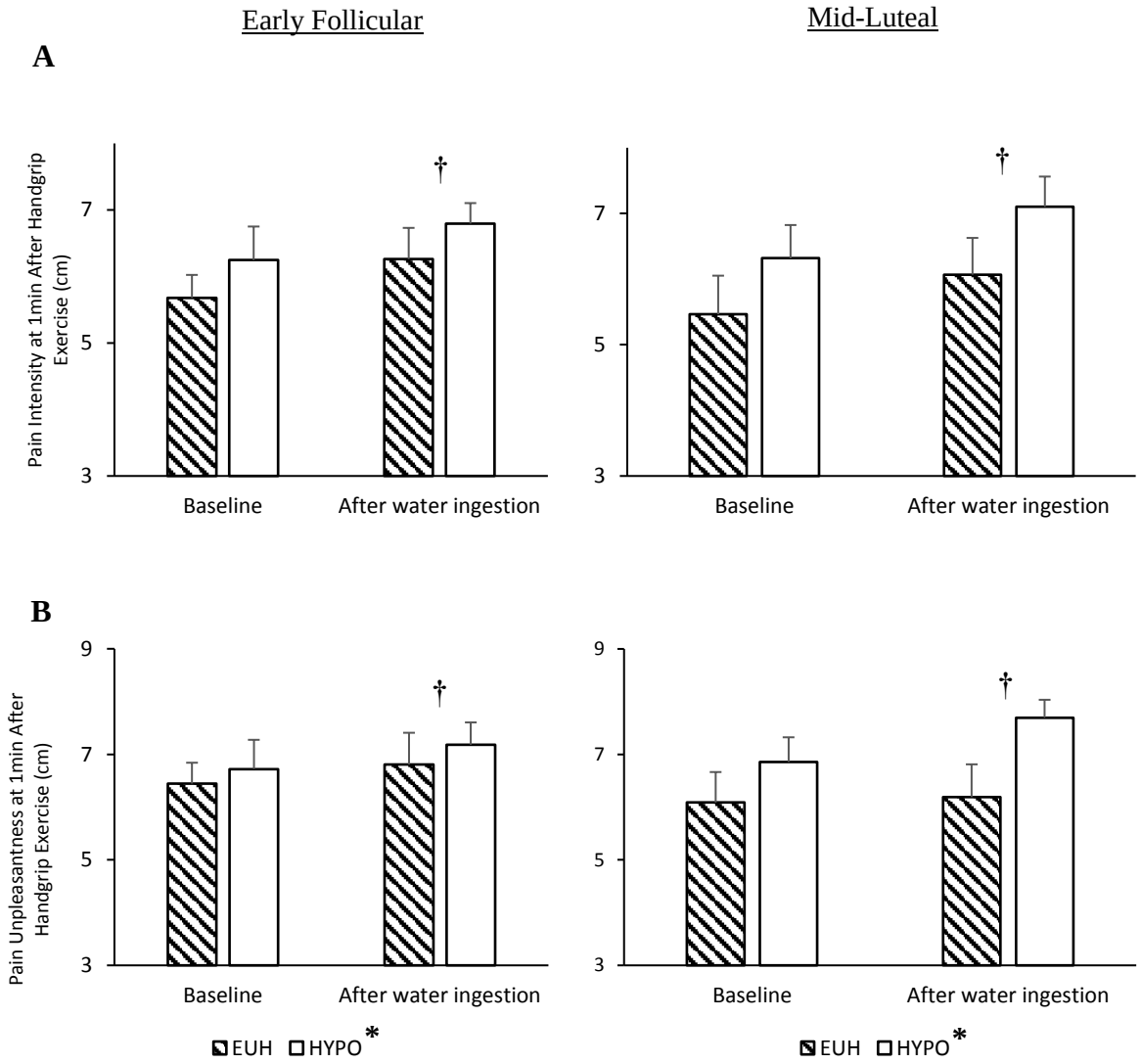


Figure 9. Mean ratings of (A) pain intensity (n = 12) and (B) pain unpleasantness (n = 12) at 1 min after the handgrip exercises, at baseline (i.e., before water ingestion) and after water ingestion in each hydration condition and menstrual phase.

Note. Data are presented as means + standard error. EUH, euhydrated; HYPO, hypohydrated.

***** Significantly higher in HYPO compared to EUH, P < .05.

† Significantly higher after water ingestion compared to baseline, P < .05.

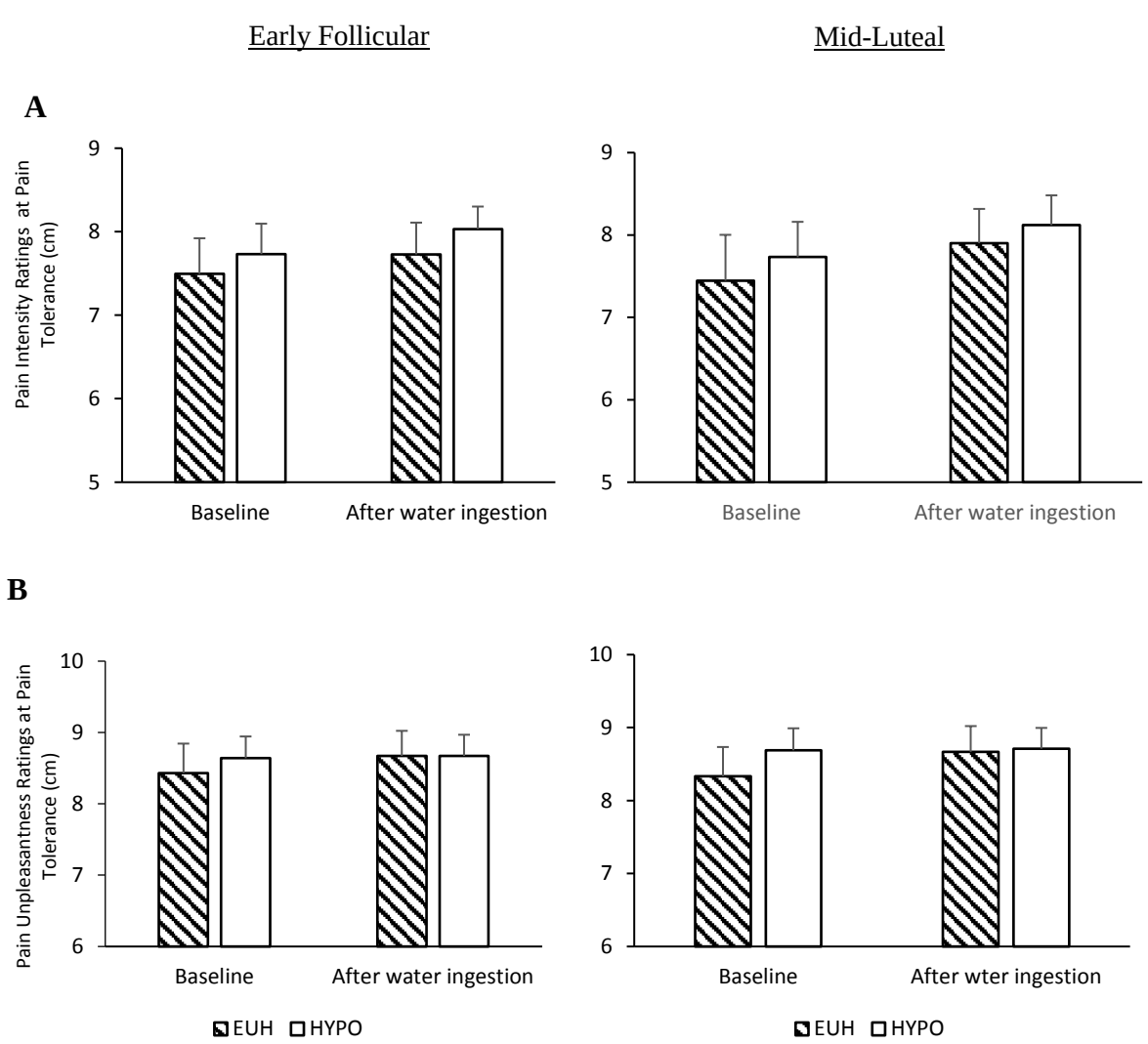


Figure 10. Mean ratings of (A) pain intensity (n = 14) and (B) pain unpleasantness (n = 14) at pain tolerance, at baseline (i.e., before water ingestion) and after water ingestion in each hydration condition and menstrual phase.

Note. Data are presented as means + standard error. EUH, euhydrated; HYPO, hypohydrated.

5.5. Psychological Variables

5.5.1. State Anxiety

State anxiety assessed by the STAI-S was not different between hydration conditions (P = .59, η_p^2 = .02) or menstrual phases (P = .82, η_p^2 = .004) (Figure 11). However, there was a main effect of water ingestion on state anxiety, since it was lower after water ingestion compared to baseline (P = .006, η_p^2 = .45), irrespective of hydration status (Hydration x Water interaction, P = .16, η_p^2 = .15) or menstrual phase (Phase x Water interaction, P = .06, η_p^2 = .26).

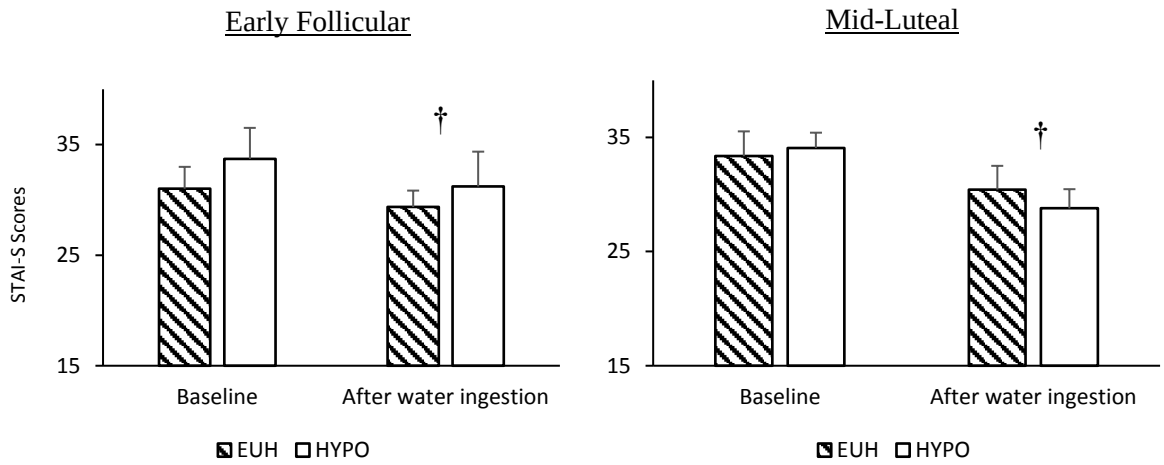


Figure 11. Mean scores on the STAI-S (n = 14) at baseline (i.e., before water ingestion) and after water ingestion in each hydration condition and menstrual phase.

Data are presented as means + standard error. STAI-S, State scale of the Spielberger State-Trait Anxiety Inventory; EUH, euhydrated; HYPO, hypohydrated.

† Significantly lower after water ingestion compared to baseline, P < .05.

5.5.2. Mood State

Scores on each BRUMS subscale and the TMD scores are shown in Table 4. There was no main effect of hydration status (all $P \geq .17$, $\eta_p^2 \leq .14$) or menstrual phase (all $P \geq .28$, $\eta_p^2 \leq .09$) for any of the BRUMS subscales (i.e., Tension, Depression, Anger, Fatigue, Confusion and Vigour). Total Mood Disturbance scores also did not differ between hydration conditions ($P = .88$, $\eta_p^2 = .002$) or menstrual phases ($P = .54$, $\eta_p^2 = .03$).

There were main effects of water ingestion for Tension ($P = .03$, $\eta_p^2 = .32$) and Anger ($P = .05$, $\eta_p^2 = .26$), with both being lower after water ingestion compared to baseline. However, there was no Hydration x Water interaction ($P = .55$, $\eta_p^2 = .03$ and $P = .20$, $\eta_p^2 = .12$ for Tension and Anger, respectively), or Phase x Water interaction ($P = .82$, $\eta_p^2 = .004$ and $P = .61$, $\eta_p^2 = .02$ for Tension and Anger, respectively) for either variable.

On the other hand, the magnitude of the decrease in Depression from baseline to after water ingestion was larger during the ML compared to EF phase (ML: Δ 0.5 ± 0.9 vs. EF: Δ 0.1 ± 1.0; $P = .02$, $\eta_p^2 = .34$). The same pattern was observed for TMD scores, where it decreased by a larger extent from baseline to after water ingestion during the ML compared to EF phase (ML: Δ 3.9 ± 6.2 vs. EF: Δ 1.2 ± 4.6; $P = .04$, $\eta_p^2 = .28$).

There was no main effect of water ingestion, no Hydration x Water interaction and no Phase x Water interaction for Fatigue, Confusion or Vigour (all $P \geq .06$, $\eta_p^2 = .26$).

Table 4. Mean scores on each subscale of the BRUMS (all $n = 14$) and the TMD scores ($n = 14$) at baseline (i.e., before water ingestion) and after water ingestion in each hydration condition and menstrual phase.

	Early Follicular				Mid-Luteal			
	EUH		HYPO		EUH		HYPO	
	Baseline	After water ingestion	Baseline	After water ingestion	Baseline	After water ingestion	Baseline	After water ingestion
Tension	1.6 ± 2.5	0.8 ± 1.4	1.4 ± 2.9	0.9 ±2.7	1.7 ± 2.8	1.1 ± 1.8	1.4 ± 1.7	0.7 ± 1.1
Depression	0.4 ± 0.9	0.1 ± 0.3	0.7 ± 1.9	0.9 ± 2.9	1.1 ± 1.8	**0.6 ± 1.9**	0.6 ± 0.8	**0.1 ± 0.3**
Anger	0.8 ± 1.6	0.2 ± 0.6	0.4 ± 0.8	0.5 ± 1.6	0.8 ± 1.7	0.4 ± 1.3	0.6 ± 0.9	0.3 ± 0.8
Fatigue	3.2 ± 3.3	3.6 ± 2.8	3.7 ± 2.8	3.4 ± 2.8	4.2 ± 4.5	3.4 ± 4.3	4.9 ± 3.5	3.7 ± 4
Confusion	1.1 ± 1.5	0.6 ± 1.4	0.6 ± 1.6	0.6 ± 1.7	1.6 ± 2.8	1.1 ± 2.3	0.6 ± 1.3	0.3 ± 0.8
Vigour	9.9 ± 3.1	9.7 ± 2.9	8.1 ± 3.3	8.2 ± 3.3	8.2 ± 2.5	8.9 ± 2.8	7.7 ± 2.8	8.9 ± 3.4
TMD	-2.7 ± 8.7	-4.4 ± 5.5	-1.3 ± 10	-2 ± 10.9	1.2 ± 12.7	**-2.4 ± 11.7**	0.4 ± 7.8	**-3.8 ± 8.3**

Note. Data are presented as means ± SD. Significant differences ($P > .05$) are in boldface.

BRUMS, Brunel Mood Scale; TMD, Total Mood Disturbance; EUH, euhydrated; HYPO, hypohydrated.

5.6. Correlations

To further examine the strength and direction of the linear relationships between the independent and dependent variables, linear correlation analyses were performed between the hydration variables, ovarian hormone concentrations and ischaemic pain measures (see Table 5). For thirst sensation ratings and ischaemic pain measures, only the baseline (i.e., before water ingestion) data were entered into the correlational analyses.

5.6.1. Hydration Variables and Ischaemic Pain Measures

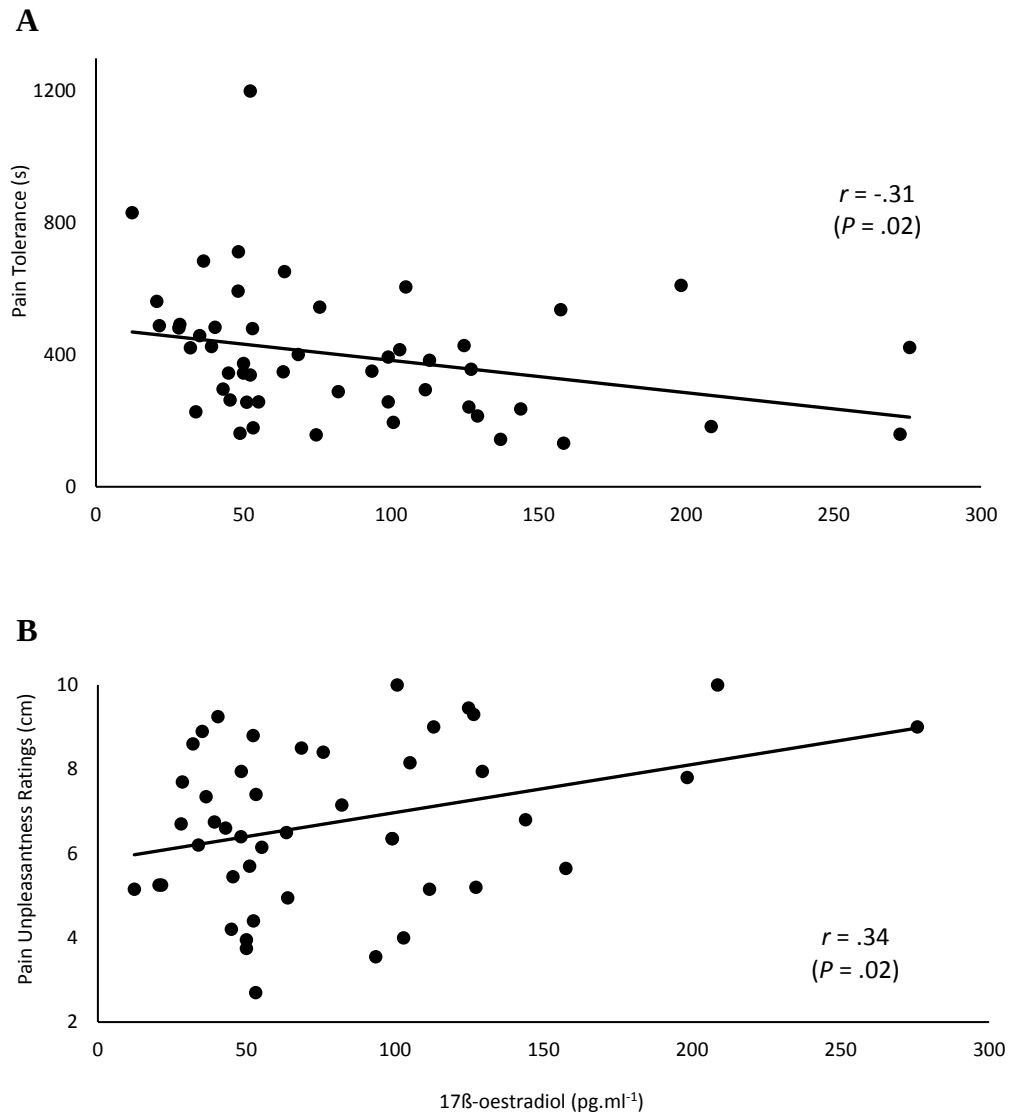
There were no significant correlations between any of the hydration variables (i.e. S_{osm}, plasma copeptin, USG and baseline thirst sensation ratings) and the ischaemic pain measures (i.e., pain tolerance, pain intensity ratings and pain unpleasantness ratings) (all $r \leq .20$ and $P \geq .17$) (Table 5).

5.6.2. Ovarian Hormones and Ischaemic Pain Measures

Serum concentrations of 17ß-oestradiol were negatively correlated with pain tolerance (r =-.31, P = .02) (Figure 12A) and were positively correlated with pain unpleasantness ratings (r = .34, P = .02) (Figure 12B). However, there was no significant correlation between 17ß-oestradiol concentrations and pain intensity ratings (r = .23, P = .13) (Table 5).

There were no significant correlations between progesterone concentrations and any of the ischaemic pain measures (all $r \leq$ -.14, $P \geq$ .31). Similarly, the P_4:E_2 ratio was also not significantly correlated with any of the ischaemic pain measures (all $r \leq$ -.20, $P \geq$ .17) (Table 5).

1991



1992

1993

1994

1995 Figure 12. Linear relationship between 17ß-oestradiol concentrations and (A) pain tolerance and (B)
1996 pain unpleasantness ratings.

1997 VAS, visual analogue scale.

1998 Table 5. Pearson correlation coefficients, *r*, for linear relationships between hydration markers,
1999 ovarian hormone concentrations and ischaemic pain measures.

	n	Pain Tolerance (s)	Pain Intensity Ratings (cm)	Pain Unpleasantness Ratings (cm)
Serum Osmolality ($mOsm.kg^{-1}$)	10	.17	.21	.11
Plasma Copeptin ($pmol.L^{-1}$)	9	.05	-.06	-.05
USG	14	.03	.12	.18
Baseline Thirst Sensation (cm)	13	.09	.20	.16
17ß-oestradiol ($pg.ml^{-1}$)	10	**-.31**	.23	**.34**
Progesterone ($ng.ml^{-1}$)	10	-.14	-.11	-.11
P_4:E_2 Ratio	10	-.06	-.17	-.22

2000 *Note*. Significant correlations ($P < .05$) are in boldface. USG, urine specific gravity; VAS, visual
2001 analogue scale; P_4, progesterone; E_2, 17ß-oestradiol.

CHAPTER SIX

6.0. Discussion

The current thesis sought to determine the independent and combined effects of hypohydration and menstrual phase on pain sensitivity. Specifically, the pain responses to hypohydration were compared between the early follicular and mid-luteal phases of the menstrual cycle. Additionally, the potential efficacy of acute water ingestion as a remedy to the deleterious impact of hypohydration on pain sensitivity was explored. The main findings of this thesis were that: (i) mild hypohydration increased pain sensitivity in healthy, eumenorrheic women when compared to euhydration, (ii) menstrual phase did not affect pain sensitivity, nor did it influence the effect of hypohydration on pain, and (3) acute water ingestion did not reduce pain sensitivity, but this finding may be confounded by carryover effects from the baseline (i.e. before water ingestion) pain test. Additionally, higher levels of 17ß-oestradiol were correlated with higher sensitivity to pain. Mood state and state anxiety levels were not affected by hypohydration or menstrual phase. Furthermore, menstrual phase had no effect on hydration status, either in a euhydrated or hypohydrated state. Overall, the findings support the first hypothesis that hypohydration will increase pain sensitivity in both menstrual phases, but refute the second and third hypotheses that hypohydration will increase pain sensitivity by a larger extent during the ML compared to EF phase, and that acute water ingestion would reverse the hyperalgesic effect of hypohydration, respectively.

6.1. Hypohydration and Pain in Women

Independent of menstrual phase, mild hypohydration achieved by 24 hr of fluid restriction reduced pain tolerance (- 7.8%) and increased pain intensity (+ 12.7%) and pain unpleasantness (+ 11.5%) ratings during ischaemic pain stimulation, when compared to euhydration.

These findings are consistent with those of previous studies that showed negative effects of mild hypohydration on experimental pain sensitivity in men (Ogino et al., 2014; Bear et al., 2016). Ogino et al. (2014) observed a decrease in pain thresholds (i.e., increased pain sensitivity) following a combination of exercise-induced dehydration and fluid restriction, whereas Bear et al. (2016) found an increase in pain intensity ratings but no difference in pain thresholds after 24 hr of fluid restriction. However, women were not examined in either study. Therefore, the current findings extend the results of these earlier studies to women by showing that mild hypohydration also increases experimental pain sensitivity in women. The previous studies also did not include pain measures that reflect the affective/emotional experience of pain (i.e., pain tolerance or pain unpleasantness ratings), which may be important for chronic pain conditions, whereas both pain threshold and pain intensity ratings reflect the sensory/discriminative aspect of pain (Price et al., 1987; Price, 2002). Although Bear et al. (2016) measured pain tolerance, the nature of the stimulus-response relationship for the cold pressor task – whereby the perceived intensity of the pain stimulus plateaus and gradually declines over time, instead of increasing linearly (Rainville et al., 1992) – resulted in a large proportion (around two-thirds) of participants who were able to tolerate the pain until the 4-min cut-off time, thereby precluding meaningful analyses of the results. This issue was not apparent with the ischaemic pain test used in the current study, since there was only

one instance where the 20-min time limit was breached (see Section 5.4.1). Therefore, the current findings further add to the previous data by showing that mild hypohydration also negatively impacts the affective/emotional aspect of pain, as evidenced by the decrease in pain tolerance and increase in pain unpleasantness ratings compared to EUH.

Notably, the level of dehydration achieved in the current study (0.9% change in body mass from EUH to HYPO) was slightly lower than in these previous studies in men (≥ 1% body mass loss); however, an increase in pain sensitivity was still observed. This suggests that, in terms of acute experimental pain, women may be more sensitive to the adverse effect of hypohydration compared to men. Szinnai et al. (2005) found that hypohydration resulted in slower reaction times only in women but not in men, although both sexes were similarly hypohydrated (2.6% body mass loss). Similarly, another group of authors observed mood decrements during exercise-induced dehydration only in women, whereas men were not affected (Armstrong et al., 2011; Ganio et al., 2011). This was in spite of the slightly larger degree of hypohydration in the men (1.6% body mass loss) compared to the women (1.4 % body mass loss).

The exact mechanisms underlying the hyperalgesic effect of hypohydration cannot be determined from the current data. However, one potential mechanism through which hypohydration may increase pain sensitivity is through subjective thirst, which was higher during HYPO compared to EUH. Findings from Geuter et al. (2016) showed that an increase in thirst sensation – induced by consumption of salty snacks followed by 5 hr of fluid deprivation – led to an increase in pain intensity ratings during noxious heat stimulation. Conversely, subsequent *ad libitum* fluid intake, which reduced thirst sensation, resulted in a decrease in pain sensitivity. In another study, stimulation of thirst sensation via hypertonic

saline infusion increased pain intensity ratings during noxious pressure stimulation, which were associated with stronger activation of brain regions that are known to be involved in the perception of pain – namely, the anterior cingulate cortex and insula (Farrell et al., 2006). These brain regions were previously shown to be activated by the perception thirst, even in the absence of a painful stimulus (Egan et al., 2003; Farrell et al., 2006; Saker et al., 2020). Similar findings were reported by Ogino et al. (2014), who found that the lowered cold pressor pain thresholds in hypohydrated men were accompanied by greater activity in these pain-related brain regions. However, it is not apparent whether the increased pain sensitivity and pain-related brain activity were due to thirst *per se*, or the physiological consequences of hypohydration, such as intracellular dehydration consequent to elevated plasma osmolality and/or reduced blood volume. Future studies that simultaneously measure brain activity and pain responses in hypohydrated participants, both before (high thirst) and after (low thirst) drinking water, would be useful in untangling the relative contributions of thirst and physiological hypohydration to the increased pain responses and associated increase in pain-related brain activity.

Nonetheless, these findings are linked to the concept that thirst and pain, along with hunger and temperature sensation, are motivational states that govern behaviour in order to maintain homeostasis (Berridge, 2004; Fields, 2006; Geuter et al., 2016). For example, perturbations to fluid-electrolyte balance generate the sensation of thirst, which then encourages fluid intake to restore fluid homeostasis. However, when two or more of these motivational states occur simultaneously and both demands cannot be met with a single behaviour, the brain prioritises the sensation it perceives to pose a greater immediate threat to survival, depending on the prevailing circumstances. This is supported by the observation that there are brain structures that were only activated when thirst and pain were experienced concurrently, but not with

either sensation in isolation, suggesting that these brain regions serve to integrate the two sensations and decide which to prioritise (Farrell et al., 2006). In the context of the current study, the experience of pain was prioritised over thirst; therefore, pain sensitivity was enhanced so that participants would respond to it first. This may reflect a basic survival instinct – the experience of pain signals impending or existing tissue damage with immediate implications for survival, whereas moderate thirst and mild hypohydration, such as that experienced by participants in the current study, may be relatively more benign and inconsequential in the short-term. Conversely, it is possible that pain sensitivity may decrease if participants were more severely hypohydrated and thirst sensation was sufficiently intense such that it cannot be ignored (e.g., not drinking water for several days). This was demonstrated in a study where mice displayed a *decrease* in pain responses (i.e., increased pain threshold) during noxious heat stimulation (via hot-plate test) after they were deprived of water for 72 hr (Konecka et al., 1985). Interestingly, overall thirst ratings were higher during the ML compared to EF phase (i.e., main effect of Phase), yet there was no phase difference in pain sensitivity (see below). However, the difference in thirst ratings between the menstrual phases was relatively small compared to the difference between hydration conditions, which may explain the lack of menstrual phase effect on pain sensitivity.

6.2. Menstrual Phase and Pain Sensitivity

Menstrual phase did not modulate the hyperalgesic effect of hypohydration, nor did it independently affect pain sensitivity. The lack of a menstrual phase effect on ischaemic pain sensitivity is consistent with the findings of most studies that previously examined ischaemic pain responses across the menstrual cycle (Straneva et al., 2002; Sherman et al., 2005; Klatzkin et al., 2010; Ribeiro-Dasilva et al., 2011; Bartley and Rhudy, 2013). In contrast, studies by Fillingim et al. (1997) and Pfleeger et al. (1997) observed differences in ischaemic pain sensitivity across menstrual phases, with both studies showing greater pain sensitivity during the luteal compared to follicular phase. However, blood levels of 17ß-oestradiol and progesterone were not measured in the study by Pfleeger et al. (1997). Furthermore, the specific definition of the luteal phase in the study Filliingim et al. (1997) differed from that in the current study. The current study examined the mid-luteal phase, when concentrations of both progesterone and 17ß-oestradiol are elevated (Figure 1). In contrast, Fillingim et al. (1997) included the late-luteal period in their classification of the luteal phase, during which levels of progesterone and 17ß-oestradiol are declining rapidly and are less stable compared to the ML phase. Given that phases characterised by fluctuating or unstable 17ß-oestradiol levels have been associated with increased severity of various chronic pain conditions (Hassan et al. 2014), this may explain why phase differences in pain sensitivity were observed by Fillingim et al. (1997) but not in the current study.

The overall body of evidence regarding menstrual phase effects on pain has yielded equivocal and inconclusive results (Sherman and LeResche, 2006; Iacovides et al., 2015a). One of the major limitations in most studies is the failure to measure blood levels of 17ß-oestradiol and progesterone to verify menstrual phases. This makes it difficult to draw conclusions about the

relationship between menstrual phase and pain sensitivity. Furthermore, hydration status was not accounted for in previous studies, which, as shown in this study, can influence pain sensitivity in women. This study showed that after measuring and controlling for hydration status (i.e., participants were in the same hydration state at the time of testing), properly verifying menstrual phases as per pervious recommendations (Schaumberg et al., 2017; De Jonge et al., 2019) and minimising several potential confounders (e.g., dietary and lifestyle factors, dysmenorrhea), there was no difference in ischaemic pain sensitivity between the EF and ML phases. This suggests that the hormonal fluctuations within the menstrual cycle do not seem to greatly influence pain sensitivity.

The absence of a phase difference in pain sensitivity may be somewhat surprising, given the interactions between the ovarian hormones and various pain processing mechanisms (Martin, 2009; Amandusson and Blomqvist, 2013; Iacovides et al., 2015a). However, despite the similarity in pain sensitivity between menstrual phases, higher 17ß-oestradiol levels were correlated with higher pain sensitivity when collapsed across cycle phases. Specifically, concentrations of 17ß-oestradiol correlated negatively with pain tolerance and positively with pain unpleasantness ratings. Since both pain tolerance and pain unpleasantness ratings assess the affective/motivation dimension of pain, whereas pain intensity ratings relate to the sensory/discriminatory aspect, the lack of correlation between 17ß-oestradiol concentrations and pain intensity ratings suggest that 17ß-oestradiol mainly influences the affective component of pain.

Taken together, the current data suggest a difference in the effects of acute versus prolonged exposure to high levels of 17ß-oestradiol on pain, such that women with higher baseline levels of 17ß-oestradiol may be more sensitive to pain compared to women with lower 17ß-

oestradiol levels (i.e., between-subject effect), whereas within each woman, the acute increases in 17ß-oestradiol levels during the menstrual cycle (i.e., within-subject effect) does not influence pain sensitivity, regardless of their baseline 17ß-oestradiol levels. This proposition is supported by evidence of increased pain sensitivity in women taking HRT or OCPs, who are chronically exposed to high levels of exogenous oestrogens that exceed the endogenous levels (Burrows and Peters, 2007; Sims and Heather, 2018), compared to the non-users. For example, Fillingim and Edwards (2001) found that postmenopausal women taking HRT were more sensitive to noxious heat stimuli compared to postmenopausal women who were not on HRT. Similar findings were reported in another study that induced pain using the cold pressor task (Gautam et al., 2012). However, pain sensitivity during electrical stimulation does not appear to be influenced by HRT use (France et al., 2004). Among premenopausal women, some studies also reported higher sensitivity to noxious pressure or cold pressor pain in OCP users compared to the non-users (Kowalczyk et al., 2006b; Kowalczyk et al., 2010; Ribeiro-Dasilva et al., 2011). Moreover, epidemiological studies suggest an increased risk of temporomandibular disorders among women taking HRT or OCP (LeResche et al., 1997). In another study on postmenopausal women with orofacial pain, those taking HRT reported a greater severity of pain symptoms compared to non-HRT users (Wise et al., 2000). However, it is important to note that the synthetic, exogenous oestrogens contained in HRT and OCPs may have different effects on pain compared to endogenous 17ß-oestradiol. Therefore, it is possible that the increased pain sensitivity observed in women on HRT or OCPs may be due to specific effects of the exogenous oestrogens, rather than of oestrogens (natural or synthetic) in general.

One factor that may explain why this apparent pronociceptive effect of 17ß-oestradiol did not result as differences in pain sensitivity between the EF and ML phases, despite the marked

difference in 17ß-oestradiol levels, is progesterone. Concentrations of 17ß-oestradiol and progesterone are elevated simultaneously during the ML phase and animal studies show that progesterone may antagonise the pronociceptive effects of 17ß-oestradiol (Ji et al., 2005; Kuba et al., 2006). Furthermore, progesterone is thought to oppose the effects of 17ß-oestradiol on various physiological systems (Frankovich and Lebrun, 2000). However, this hypothesis seems unlikely given that the P_4:E_2 ratio did not correlate significantly with any pain measure (Table 5). Another possible explanation for the discordance between the results from the ANOVA and correlational analyses is that the magnitude of menstrual cycle-related fluctuation in 17ß-oestradiol levels is not large enough to influence pain sensitivity. In the current data set, 17ß-oestradiol levels increased by roughly 2-fold from the EF to ML phase. In comparison, the inter-individual difference in 17ß-oestradiol levels, collapsed across menstrual phases, varied by approximately 10-fold, yet 17ß-oestradiol levels were only weakly correlated with pain sensitivity (r = 0.3) (Table 2). It is also worth mentioning that levels of 17ß-oestradiol do not reach its highest point in the menstrual cycle during the ML phase; instead, the peak in 17ß-oestradiol occurs during the late-follicular phase. Therefore, the selection of the EF and ML phases in the current study may have reduced the ability to detect phase differences in pain sensitivity, because 17ß-oestradiol levels during the ML phase are not as high as they would be during the late follicular phase.

6.3. Psychological Variables

Both state anxiety levels and mood state were unaffected by mild hypohydration. As such, it is unlikely that the increased pain sensitivity during HYPO was explained by differences in state anxiety and/or mood state. The negative impact of mild hypohydration on mood state in both men and women have been reported in several studies (Shirreffs et al., 2004; Ganio et al., 2011; Pross et al., 2013). Pross et al. (2013) subjected women to 24 h of fluid restriction and found negative impacts on several mood parameters, including feelings of fatigue, confusion and vigour. However, the findings were likely confounded by the series of cognitive tests that participants had to complete throughout the fluid restriction period (Pross, 2017). Also, the level of hypohydration in most studies that observed mood decrements was around 2% body mass loss, compared to approximately 0.9% in the current study. Therefore, similar to some studies (Turner et al., 2017; Caldwell et al., 2018; Stachenfeld et al., 2018), the level of hypohydration in the current study may have been insufficient to induce mood impairments. There also appears to be a temporal aspect to the negative mood effects of hypohydration, such that the mood changes may only be evidenced at certain time points. Pross et al. (2013) assessed mood state at several time points across 24 hr of fluid restriction. Overall, several mood states assessed by the POMS, including fatigue, confusion and vigour, were negatively affected by fluid restriction. However, these effects were only observed after 19 and 21 hr of fluid restriction, but not after 24 hr. Therefore, it is possible that participants in the current study experienced mood degradations initially during fluid restriction, but they gradually adapted to these mood changes such that when they were assessed 24 hr later, their mood state was not different compared to the euhydrated trial.

Mood state and state anxiety levels also did not differ between the EF and ML phases, consistent with the conclusion of a recent review that did not find robust evidence of changes in mood across the menstrual cycle (Romans et al., 2012). In contrast, several previous studies have reported mood alterations or changes in anxiety levels across the menstrual cycle, with most reporting a worsening of mood or increase in anxiety levels during the late-luteal compared to follicular phase (Moos et al., 1969; Gonda et al., 2008; Reed et al., 2008). However, the current study compared the EF and ML phases and did not include the late-luteal phase, which may explain why no menstrual phase effects on mood state or anxiety levels were found. That said, even when differences in mood state or anxiety levels across the menstrual cycle were found, those differences tend to be marginal or limited to only a few of the mood parameters measured (Gonda et al., 2008; Schwartz et al., 2012; Romans et al., 2013). For instance, in the study by Romans et al. (2013) where mood was measured daily over six months, only four of the 14 mood parameters varied across the menstrual cycle, with all four mood items being worse during menses compared to the follicular or luteal phases . Likewise, another study found that daily urinary concentrations of 17ß-oestradiol and progesterone metabolites were correlated with only five of the 12 mood parameters assessed (Schwartz et al., 2012). More important, both studies found that physical health and perceived stress were more influential determinants of mood compared to menstrual phase (Schwartz et al., 2012; Romans et al., 2013).

6.4. Hydration Status

Twenty-four hours of fluid restriction successfully induced mild hypohydration, as evident from the significantly higher S_{osm}, plasma copeptin, USG and thirst ratings during HYPO when compared to EUH. The increase in these hydration variables, as well as the percent change in body mass from the EUH to HYPO trials, were similar between menstrual phases. The data therefore suggest that menstrual phase does not appear to influence the hydration effects of 24 hr of fluid restriction. These findings are similar to previous studies that showed similar body fluid losses during exercise between menstrual phases (Stachenfeld et al., 1999; Rodriguez-Giustiniani and Galloway, 2019; Nose et al., 2020).

Fluid retention is commonly thought to occur during the luteal phase of the menstrual cycle (Moos et al., 1969; White et al., 2011), possibly due to the increase in resting plasma AVP concentrations (Forsling et al., 1981). In the current study, S_{osm} was lower during the ML compared to EF phase in both hydration conditions; however, plasma copeptin (a surrogate marker of AVP), USG and body mass were similar between menstrual phases. Furthermore, the decrease in body mass during HYPO when compared to EUH was similar between menstrual phases. Therefore, the lower S_{osm} during the ML phase does not appear to reflect increased fluid retention. Instead, the data suggest that the menstrual phase does not acutely influence hydration status, either in a euhydrated state or when mildly hypohydrated after 24 hr of fluid restriction.

These findings are similar to previous studies that did not find differences in fluid balance at rest, indicated by urinary indices of hydration status or body mass, between menstrual phases (Maughan et al., 1996; Giersch et al., 2020). The most probable explanation for the lower S_{osm} during the ML phase is that the osmotic set point for body fluid regulation is reset to a

lower plasma/serum osmolality. Similar to the current study, a previous study by Stachenfeld et al. (1999) observed a lower resting P_{osm} during the ML compared to EF phase, with no difference in plasma AVP concentrations or body mass between phases. The increases in P_{osm} and AVP during exercise-induced dehydration were also similar between phases. The authors further demonstrated a lowering of the osmotic threshold for the release of AVP and thirst stimulation during exercise, without any effect on overall body fluid balance. Similar findings have been observed during hypertonic saline infusion, where the earlier release of AVP and thirst onset during the ML phase was not commensurate with greater fluid retention. It was subsequently concluded that body fluids are regulated around a lower operating point during the ML phase, thereby allowing the maintenance of a lower resting P_{osm} (Stachenfeld, 2008). Although the current study was not designed to examine the osmoregulation of AVP or thirst, it is possible that a similar phenomenon may have occurred.

6.5. Effects of Acute Water Ingestion

Give that thirst may be a potential mechanism linking hypohydration and increased pain sensitivity, it was hypothesised that having a drink of water while in a hypohydrated state would reduce pain sensitivity. To test this hypothesis, participants in the current study repeated the ischaemic pain test after consuming a moderate volume of water (5 $ml.kg^{-1}$ of body mass or approximately 335 ml), equivalent to that in a standard soft-drink can (330 ml). The goal of the acute water intervention was to reduce thirst sensation without rehydrating participants, in order to elucidate the relative contributions of thirst sensation and physiological hypohydration to pain sensitivity. As expected, water ingestion led to a significant decrease in thirst sensation during HYPO. However, contrary to the study's hypothesis that pain sensitivity would also decrease, pain tolerance was unaffected whereas both pain intensity and pain unpleasantness ratings *increased* instead. The increased pain ratings may appear counterintuitive – participants felt more pain although they were less thirsty after, compared to before, water ingestion. However, the unexpected findings may be due to carryover (sensitisation) effects from the baseline (i.e., before water ingestion) pain test. A previous study demonstrated that repeated pain stimulation within a single session produced a sensitisation effect, whereby pain ratings increased gradually with each repeated stimulation (May et al., 2012). Furthermore, the increased pain ratings after water ingestion occurred during EUH as well, even though thirst ratings were unaffected. Therefore, this sensitisation effect may have masked any potential positive effect of acute water ingestion on pain sensitivity. Also, it should be noted that thirst sensation ratings after water ingestion were still higher during HYPO compared to EUH, indicating that thirst was not fully satiated. This incomplete satiation of thirst may have attenuated any positive effect of acute water ingestion on pain sensitivity. Future research is warranted to determine whether drinking

water to satiety while hypohydrated may reduce pain sensitivity. Interestingly, the increased pain intensity and pain unpleasantness ratings were not accompanied by a decrease in pain tolerance. Although participants felt a greater level of pain, it did not provoke a response from them to withdraw from the pain stimulus earlier. This may be due to a familiarity effect from the baseline pain test, which may have reduced anxiety levels and caused participants to be less fearful of, and less averse to, the pain sensations during the subsequent pain test (Chapman and Feather, 1973; Von Graffenried et al., 1978). Indeed, participants reported feeling less anxious and having a better mood after they consumed water. Specifically, state anxiety levels (Figure 11), as well as Tension and Anger scores on the BRUMS (Table 4), decreased after water ingestion. These findings are consistent with previous studies that showed improvements in various mood states after acute water ingestion (Cian et al., 2001; Pross et al., 2013; Zhang et al., 2020), although these positive effects are not consistently observed (Edmonds et al., 2013a; Patsalos and Thoma, 2020). Interestingly, the improvements in TMD and Depression after water ingestion were only observed in the ML but not the EF phase (see Table 4). This could be due to the higher thirst ratings during the ML phase. For example, Edmonds et al. (2013b) found that the improvement in simple reaction time after acute water ingestion depended on subjective thirst. More specifically, reaction times were unaffected by water ingestion in participants who were not thirsty, whereas participants who were thirsty had faster reaction times after they drank water. In another study, acute water ingestion improved performance on a cognitive task in participants who were thirsty (Rogers et al., 2001). Conversely, in participants who were not thirsty, cognitive performance worsened after water ingestion. However, despite the observed improvements in state anxiety levels and mood state after water ingestion in the current

study, there were no beneficial effects on pain sensitivity, which suggest that the positive effects of acute water ingestion on pain sensitivity, if any, are likely to be minimal.

In conclusion, the current data suggest that hypohydration induces negative effects on pain sensitivity that may be not be reversed simply by having a drink of water. Although the findings may be confounded by carryover effects from the repeated pain tests, the acute water intervention did what it was intended to – i.e., reduce thirst sensation. Moreover, water ingestion also improved some aspects of mood and reduced state anxiety levels. Therefore, that pain sensitivity was not reduced despite these positive perceptual and psychological effects seem to suggest that physiological mechanisms may be more important in explaining the relationship between hypohydration and increased pain sensitivity. In the context of ischaemic pain, a potential physiological mechanism that could be explored in future research is that of reduced blood flow to the tissue or region affected by pain. The decrease in blood volume that commonly occurs with hypohydration (Shirreffs et al., 2004; Popkin et al., 2010) may decrease tissue perfusion, potentially resulting in a greater degree of ischaemia and pain. A study on patients with peripheral arterial disease found that increasing daily water intake from 1.1 L to 2.7 L for 6 weeks led to an improvement in measures of leg blood flow (e.g., ankle-to-brachial pressure index, maximum walking distance) and a decrease in the intensity of pain symptoms (Fernández et al., 2018). This suggests that the degree of tissue perfusion, and therefore (ischaemic) pain, may be related to a person's level of hydration. Future studies that obtain measures of tissue blood flow (via near-infrared spectroscopy or doppler ultrasound) during pain stimulation in hypohydrated individuals are required to test this hypothesis. Altogether, these findings underscore the importance of maintaining adequate hydration throughout the day via regular fluid intake to avoid becoming hypohydrated and the consequent hyperalgesia. Indeed, there is some evidence that increasing daily fluid intake

may improve clinical pain symptoms (Spigt et al., 2005; Fernández et al., 2018; Torkan et al., 2021). For example, patients with chronic migraine reported feeling less intense headaches after increasing their daily water intake by around 1 L for 12 weeks (Spigt et al., 2005). More recently, in a study by Torkan et al. (2021), women with primary dysmenorrhea who increased their daily water intake from 1.6 L to 2 L over two menstrual cycles reported decreases in both the severity of their menstrual pain, as well as the number of pain-relievers consumed.However, objective markers of hydration status were not measured in these studies; thus, it is unclear whether the patients were hypohydrated or euhydrated before the fluid intervention, and whether hydration status improved after the intervention.

6.6. Considerations and Limitations

Given that the prioritisation of one motivational state over another is thought to depend on situational factors (Farrell et al., 2006; Fields, 2006), there is one notable factor that may have influenced the prioritisation of pain over thirst in the current study, resulting in the increased pain sensitivity during HYPO. Participants knew that they would be provided with water after the baseline pain test, which may have caused them to direct their attention away from thirst toward the pain, because they have the assurance that their thirst would be taken care of later. This hypothesis is supported by studies showing reduced pain sensitivity in the presence of distractions (Villemure et al., 2003; Buhle and Wager, 2010; Sprenger et al., 2012). For example, in one study where participants were simultaneously exposed to odorants during stimulation of noxious heat pain, pain intensity ratings were higher when participants focused their attention on the pain, compared to when focused on the odorant (Villemure et al., 2003). The expectation of receiving water may have also reduced the perceived threat and importance of thirst to survival relative to that of pain, causing the pain experience to dominate the thirst sensation and resulting in the increased pain sensitivity during HYPO compared to EUH. Conversely, it may be argued the heightened anticipation of ingesting water during HYPO may have caused participants to terminate the pain test prematurely, resulting in the lower pain tolerance that was observed. However, the similarity of pain intensity and pain unpleasantness ratings at pain tolerance across trials indicate that participants terminated the pain test at the same level of perceived pain (see Figure 9B).

It should be noted that this study only examined the EF and ML phases, which were selected due to their distinct hormonal profiles, as explained previously (see Section 4.3.1). However, another phase with a unique hormonal environment is the late follicular phase. Unlike the EF

and ML phases where levels of both 17ß-oestradiol and progesterone are low and high, respectively, the late follicular phase is characterised by high 17ß-oestradiol and low progesterone levels (see Figure 1). Moreover, levels of 17ß-oestradiol during the late follicular phase are higher than that during the ML phase. Given the positive correlation between 17ß-oestradiol levels and pain sensitivity found in the current study, as well as previous research suggesting that the changes in body fluid regulation are mostly related to 17ß-oestradiol (Calzone et al., 2001; Stachenfeld and Keefe, 2002), it would behove future researchers to investigate whether the hyperalgesic effect of hypohydration may be different during the late follicular phase. However, the late follicular 17ß-oestradiol peak is relatively brief, lasting around one day, whereas 17ß-oestradiol and progesterone levels remain elevated for several days during the ML phase. Therefore, the practical importance of the late follicular phase is unclear. Furthermore, testing during the late follicular phase may be logistically challenging. In order to capture the 17ß-oestradiol peak, or as close to it as possible, testing would have to occur on the day of the LH surge (De Jonge et al., 2019). However, participants (and researchers) may not always be available for testing at such short notice. This is especially so for the current study, where the fluid restriction intervention requires 24 hr. Furthermore, the crossover design of the current study makes testing during the late follicular phase challenging. The short window (i.e., approximately one day) of the 17ß-oestradiol peak makes it difficult to schedule two trials (i.e., EUH and HYPO) on non-consecutive days within this period.

Another important consideration is that participants in the current study were healthy, eumenorrheic women with regular menstrual cycles, which ignores postmenopausal women and OCP users. As mentioned previously (see Section 2.4), these two groups of women have vastly different hormonal profiles compared to eumenorrheic women. Therefore, it is possible

that hypohydration may affect pain sensitivity differently in these women compared to eumenorrheic women. However, since this study is the first of its kind, it is envisaged that this study would spur further research on the topic in postmenopausal women and OCP users.

Despite the methodological rigour of the current study, there are a few noteworthy methodological limitations. First, the effect of acute water ingestion on pain sensitivity could not be properly determined due to carryover effect of the baseline (i.e. before water ingestion) pain test. In the current study, the baseline pain test and post-water ingestion pain test were separated by a 30 min rest period, which was previously shown to be adequate for attenuating carryover effects from the first to second pain test (Hoeger Bement et al., 2008). However, the pressure pain test used in that study only lasted for 2 min, whereas the ischaemic pain test used in the current study lasted around 7 min. Also, while that study induced pain in the index finger, pain was induced in the arm in the current study. Therefore, the longer duration of pain stimulation and the larger amount of muscle mass involved in the current study possibly resulted in a stronger or more long-lasting carryover effect that was not eliminated after 30 min.

Second, blood samples were not obtained after water ingestion to confirm that hydration status was not altered. Although this was the original intention, it was subsequently decided against due to several reasons. The first reason is related to the nature of the ischaemic pain test and the fact that the venepuncture procedure itself produces pain. Since the pain induced by the ischaemic pain stimulus affects the entire arm, rather than being localised, it precluded the use of the left arm (i.e., the arm used for the ischaemic pain test) for obtaining blood samples to prevent confound from the venepuncture-induced pain. Therefore, the venipuncture could only performed on the right arm. Second, the time that elapsed from when

the baseline blood samples were drawn from the right arm to the post-water ingestion period was insufficient for adequate healing of the initial venepuncture wound to permit another insult on the same arm. This is because the arm may be more sensitive, which could result in substantial discomfort and pain if wounded again. Although this problem may be solved with the use of an intravenous cannula, it was decided against in this study as it could hinder participant recruitment and retention. Furthermore, there was much difficulty with obtaining the baseline blood samples in the first place. In some instances, two or three attempts were required, whereas in other cases, blood samples could not be obtained even after three attempts. Nevertheless, previous findings indicate that consuming a moderate volume of water does not acutely alter hydration status. Geelen et al. (1984) previously showed that in men, S_{osm} remained elevated above euhydrated values for up to 60 min after ingesting 10 $ml.kg^{-1}$ of water in a mildly hypohydrated state. In another study, no changes in USG were observed 30 min after hypohydrated participants drank 600 ml of water (Logan-Sprenger and Spriet, 2013). Since participants in the current study ingested a considerably smaller volume of fluid (5 $ml.kg^{-1}$ body mass, or an average of 335 ml) than that administered in these studies, it is conceivable that hydration status would not have changed in the current study as well. Additionally, the relatively short interval of 30 min between water ingestion and the subsequent pain test (see Figure 4) is probably insufficient for hydration status to be altered, because a previous study showed that at least 75 min is required for 300 ml of water to be completely absorbed into the bloodstream (Péronnet et al., 2012).

The third limitation is the relatively small sample size. Although 23 participants were initially recruited and familiarised, only 14 participants were included in the final analyses due to various reasons as described previously (see Section 5.0). Given the considerable inter-individual variability in the pain outcome measures, the small sample size may have reduced

the ability to detect statistically significant differences between menstrual phases. That said, based on the observed effect size for the pain measures and sample size of the current study, this study appeared to have a statistical power of 91%, which suggests a low probability that the results obtained were false negatives.

Fourth, although efforts were made to counterbalance the Phase-Hydration order of the trials, the unexpected participant exclusions resulted in an unequal number of participants in each Phase-Hydration order. Most notably, nine participants performed the EUH trials first, compared to only five participants who started with the HYPO trials. Therefore, it is possible that the data obtained during the HYPO trials may be confounded by practice effects (Kowalczyk et al., 2006b; Stening et al., 2007; May et al., 2012). The data for the ischaemic pain measures were subsequently re-analysed across consecutive trials, irrespective of menstrual phase or hydration status, to examine potential effects of trial order. The analyses revealed no statistically significant order effects pain tolerance ($P = 0.36$), pain intensity ratings ($P = 0.30$), or pain unpleasantness ratings ($P = 0.79$). As such, it is unlikely that the imperfect counterbalancing of the Phase-Hydration trial order had a major impact on the results of the current study.

Fifth, percent body mass change during HYPO was calculated from the body mass measured in the EUH trial (see Section 4.11.1). Since the HYPO and EUH trials within each menstrual phase were separated by at least 48 hr, it is likely that the body mass measurements were confounded by factors other than changes in body water content (e.g., consumption of food and fluids, urine and faecal losses) (see Section 4.2.2.4). Therefore, the decrease in body mass in the HYPO trial relative to the EUH trial may not accurately reflect fluid losses induced by 24 hr of fluid restriction. However, other physiological markers of hydration

status (i.e., S_{osm}, plasma copeptin and USG; see Table 3) indicate that participants were indeed hypohydrated during the HYPO compared to EUH trials. The calculation of percent body mass change would likely be more accurate if a baseline body mass was obtained at the start of the 24 hr of fluid restriction, or if a 3-day baseline body mass was established as described previously (Cheuvront et al., 2004) (see Section 4.2.2.4); however, neither was done in the current study due to logistical constraints and to minimise participant burden.

Lastly, since participants could not be blinded to the hydration intervention, the increased pain sensitivity observed during HYPO may have been confounded by nocebo effects (Reicherts et al., 2016; Blasini et al., 2017). Although participants were not aware of the study hypotheses, they were aware of the aims of the study, one of which is to investigate the effects of hypohydration on pain sensitivity. Furthermore, given the negative connotations of hypohydration or dehydration, it is conceivable that participants would expect hypohydration to increase pain sensitivity. This negative expectation may have subsequently influenced participants' actual responses to the ischaemic pain test.

6.7. Perspectives

The findings of this study may be especially relevant to individuals who are suffering from chronic pain. Ingesting adequate fluids regularly throughout the day to stay well-hydrated may be a cost-effective strategy to manage pain symptoms daily. This could reduce the overall level of pain, thereby improving physical function and quality of life. For patients who are currently receiving treatment for their chronic pain, the treatment may be more efficacious if they are in a well-hydrated versus hypohydrated state. One study in patients with low back pain receiving osteopathic manipulative treatment showed that treatment outcomes were poorer when patients were hypohydrated (via 36 hr reduced fluid intake), compared to when they were euhydrated (Parker et al., 2012).

The present findings may also be applicable to individuals who are about to undergo or recovering from surgical procedures. Patients would normally have to abstain from fluids (and food) for a prolonged period prior to and following the surgery, which may cause them to be hypohydrated. A previous study suggests that patients may lose up to 1 L of fluids after a period of preoperative overnight fasting (Keane and Murray, 1986). Along with the commonly reported side effects of hypohydration (e.g., thirst, dizziness, headaches), patients may also feel more intense pain after the operation, which may subsequently prolong their recovery. Indeed, in a study on more than 17,000 patients scheduled for a range of different surgeries, excessive pain was found to be one of the major determinants of prolonged hospital stay after the surgery (Chung and Mezei, 1999). Compared to patients who did not report excessive pain after surgery, those who experienced pain ended up staying in hospital for a 20 to 30% longer duration. Furthermore, the excessive pain after surgery may also require the use of more pain-relieving medications, which may result in nausea and vomiting and further

prolong recovery time (Chung and Mezei, 1999). In another study, intravenous fluid administration during the preoperative fasting period was found to decrease self-reported pain after surgery and less requirement for pain-relieving medications, compared to the control group who were given a smaller volume of intravenous fluid (Maharaj et al., 2005).

The current findings may also be important for the elderly, where the prevalence of hypohydration appears to be high (Stookey, 2005; Forsyth et al., 2008). Moreover, ageing is also associated with various physiological changes that may increase the risk of dehydration in older adults (Mack et al., 1994; Hooper et al., 2014). Furthermore, due to trouble walking or other physical disabilities, elderly individuals may intentionally reduce their fluid intake to reduce the frequency of bathroom visits, which further increases their likelihood of becoming hypohydrated. More important, several reports show a high prevalence of chronic pain among the elderly population (Rapo-Pylkkö et al., 2016; Stompór et al., 2019). Therefore, it is pertinent to design strategies that would prevent elderly individuals from becoming hypohydrated, as it could potentially improve their pain outcomes.

Lastly, another group to consider are workers in occupations that inherently predispose them to dehydration, due to environmental exposure, limited access to drinking water and/or the bathroom, mandatory personal protective equipment that may make regular drinking difficult (e.g., face masks or respirators) and increase heat stress (thereby increasing sweating and fluid loss), or a combination of these factors. Examples include, but are not limited to, construction workers, healthcare workers and firefighters (Walker et al., 2016; Piil et al., 2018; Foster et al., 2020). In addition to the deleterious physiological and cognitive consequences of hypohydration, these individuals may also experience more pain, which could further diminish their work performance or productivity.

6.8. Conclusions and Future Directions

The present study examined the effect of mild hypohydration on pain sensitivity during the EF and ML phases, in order to determine whether an interaction between hypohydration and menstrual phase on pain sensitivity exists. The data showed that, irrespective of menstrual phase, mild hypohydration induced by 24 hr of fluid restriction increases pain sensitivity; these effects do not seem to be reversed by acute fluid ingestion. Pain sensitivity was not affected by the acute hormonal fluctuations across menstrual phases; however, women with higher baseline oestrogen levels appeared to be more sensitive to pain than those with lower oestrogen levels. This suggests that the "tonic" effect of oestrogen may be a more important contributing factor to pain sensitivity than its "phasic" effect. Overall, the take-home messages from this study are that: (i) it is important for women to ingest adequate fluids throughout the day to maintain a well-hydrated state and prevent dehydration, especially if they are experiencing pain (e.g., recovering from injury or surgery) or are about to go through a painful experience (e.g., surgery), and (ii) future researchers investigating pain in women should measure and control for hydration status to prevent confound.

Since this is the first study, to the candidate's knowledge, that has investigated the combined effects of hypohydration and the menstrual phase on pain sensitivity, much remains to be known and understood about the potential interactions between hypohydration and the ovarian hormones on pain. Therefore, future research should:

1. Investigate the effects of hypohydration on pain sensitivity in OCP users and in postmenopausal women on HRT.

2. Examine the pain responses to hypohydration during the late follicular phase when 17ß-oestradiol concentrations are elevated independently of progesterone, and how may differ from the EF and ML phases.

3. Identify the mechanisms (i.e., physiological, perceptual and/or psychological) underlying the relation between hypohydration and increased pain sensitivity.

6.9. Reflections

As this thesis comes to an end, it seems befitting to take some time to reflect on the process and numerous challenges faced (and triumphs celebrated) over the last 3.5 years.

This journey began with a life-changing email that contained an acceptance to this doctoral program that aims to investigate the relation between hypohydration and pain. The first six months consisted of a great deal of reading and planning. It was then decided that this thesis would investigate the effect of hypohydration on pain in both eumenorrheic women and OCP users during different phases of their menstrual and OCP cycles, respectively. Specifically, the aim was to compare the hypohydration effects of pain between these two groups of women. In addition to examining the pain responses, the thermoregulatory responses during passive heat stress was also proposed. On paper, the plan appeared brilliant and feasible – following 24 hr of fluid restriction, participants would first have their pain responses measured, then they would enter the heat chamber, don a water-perfused jacket, soak their legs in a tub of hot water and begin 1 hr of passive heat stress, during which their sweating and skin blood flow responses would be measured. Also, to improve the practical relevance of the study, participants would perform an orthostatic challenge at the end of the passive heating, to determine the functional impact of hypohydration and heat stress. This idea of using a single experimental set-up to perform two studies could not have sounded more fantastic.

With this grandiose plan in hand and plenty of excitement, months of pilot testing promptly began to iron out the finer details of the experimental protocol. At the same time, a comprehensive review on the interaction between hypohydration and the menstrual and OCP cycles on pain was written, while a separate review on thermoregulation was started.

However, it quickly became apparent that, with only four years (maximum) to complete the study and one researcher on board, the plan was not going to work out. The thermoregulation portion of the study was therefore (sadly) abandoned. In the next year that followed, data collection for the eumenorrheic women was performed, which proved to be a tremendous undertaking (see below). After competing data collection for 12 participants (menstrual phases were not yet verified for half of them), the initial results looked promising. The original plan was therefore revised to its final state – rather than repeating the study in OCP users, this thesis would focus on performing a high-quality study on a larger sample (the goal was $n = 24$) of eumenorrheic women.

Although the study seems relatively simple and straightforward, the process to get to this point was replete with numerous challenges and obstacles. The first challenge was with participant recruitment. Unfortunately, the idea of subjecting oneself to pain and 24 hr of fluid restriction does not sound appealing to most people. Furthermore, the inclusion/exclusion criteria (e.g., eumenorrheic women, regular menstrual cycles) made the participant recruitment process even more challenging. The study also required considerable time commitment from the participants, since they were required to visit the laboratory for six sessions (or more), spanning across at least two months.

The second challenge was testing within specific menstrual phases, which significantly reduces flexibility with scheduling and increases amount of time required to complete data collection for one participant. There were several occasions where, due to other personal commitments, participants were not available when the opportune time for testing rolled around. This meant that testing would have to be delayed for at least another cycle. On other occasions, testing had to be delayed due to absence of a positive LH test result during one

menstrual cycle. These participants were also asked to repeat the ovulation testing procedure, which was conceivably burdensome for them.

The third challenge was with retrospective verification of menstrual phases. The most accurate way of verifying menstrual phases is by directly measuring oestrogen and progesterone concentrations in the blood. In addition, a minimum threshold for progesterone should be set *a priori* for verification of the mid-luteal phase to rule out luteal phase deficient cycles. However, analysing the blood samples takes time and can only be performed in batches. This means that menstrual phases can only be verified several weeks or months after participants have competed the study. This would not pose an issue if all participants were later confirmed to have ovulated; however, this is rarely the case. In this thesis for example, almost one-third of participants appeared to have experienced anovulation or luteal phase deficiency. Unfortunately, some of these participants could not repeat their trials when contacted. However, even for participants who agreed to repeat their trials, those trials were not always successful. For example, there was one participant where attempts to repeat her luteal phase trials were unsuccessful twice, because on both occasions, her period came (unexpectedly) before the scheduled date of the trials. This was in spite of obtaining positive LH tests during those two menstrual cycles.

Lastly, there was the COVID-19 pandemic which threw a spanner in the works. Data collection was halted abruptly for several months due to the nationwide lockdown and the subsequent restrictions that were implemented after the lockdown. Even after lab testing resumed and things seemingly went back to “normal” (for New Zealand), the reduced student presence on campus due to online lectures further hampered participant recruitment efforts. This was not helped by the increased focus on personal hygiene and physical distancing,

which further deterred individuals from taking part in a study that involves the collection of biological (blood and urine) samples. Notably, two participants ended up dropping out of the study due to hygiene and safety concerns, which were understandable.

Aside from the challenges, it is also worth acknowledging the little triumphs that were celebrated along the way. Certainly, the most notable victory was when the literature review was finally accepted for publication after two rejections. Other memorable triumphs include successfully obtaining a blood sample on the first attempt, when a participant obtains a positive LH test and when a new participant signs up for the study.

To sum it up, it has been a wild ride (to say the least) and the challenges mentioned here are just the tip of the iceberg. However, the journey to get to this point was no less rewarding. The effects of the ovarian hormones on various body systems are still poorly understood, partly due to the dearth of high-quality (i.e., with accurate verification of menstrual phases) menstrual cycle-related studies. It is imperative that the difficulties associated with performing such studies do not hinder advancements in this very important field of research. After all, as Theodore Roosevelt once said, “Nothing in this world is worth having or worth doing unless it means effort, pain (pun intended), difficulty.”

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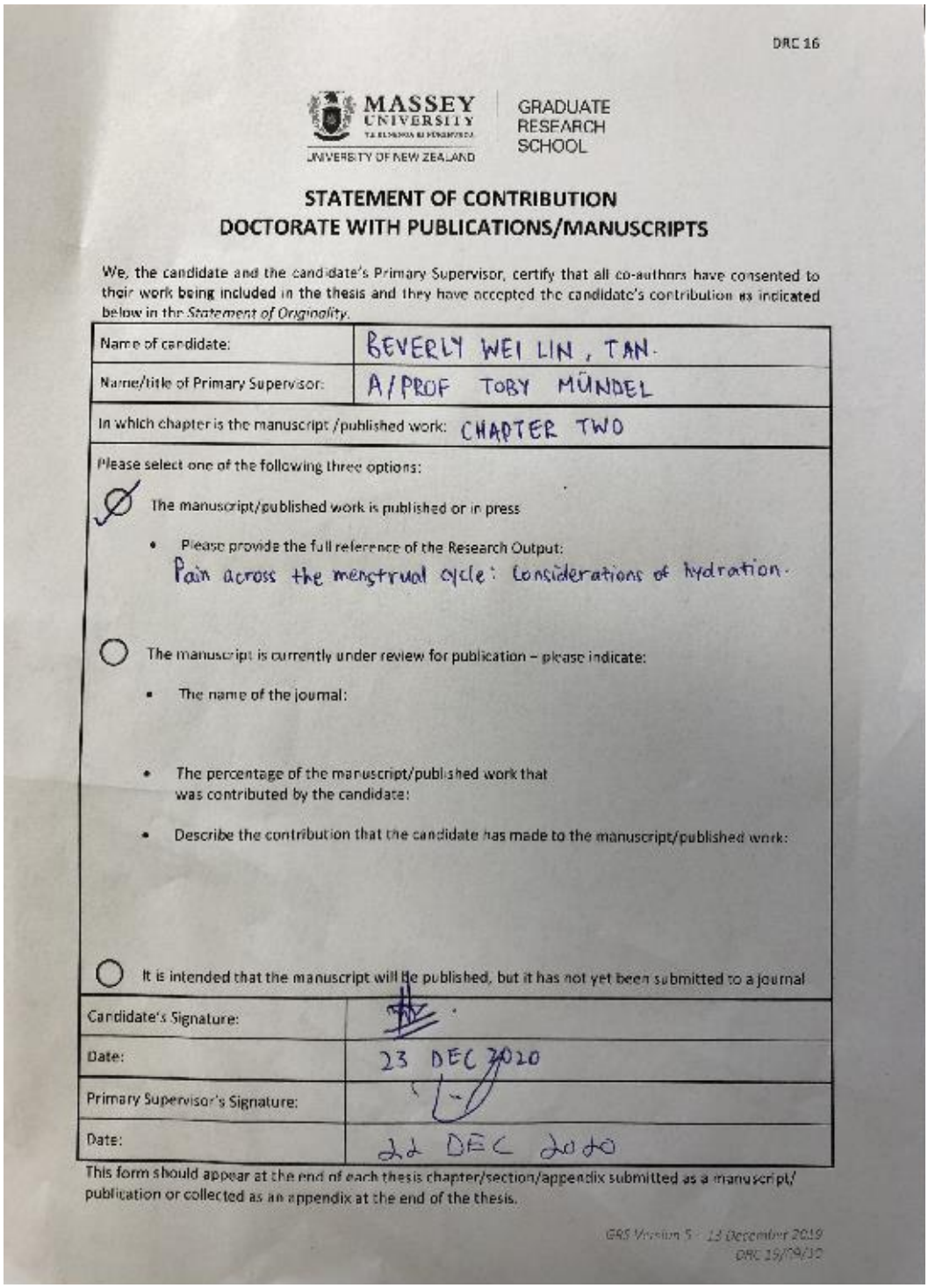
APPENDIX I

Statement of Contribution



DRC 16

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STATEMENT OF CONTRIBUTION
DOCTORATE WITH PUBLICATIONS/MANUSCRIPTS

We, the candidate and the candidate's Primary Supervisor, certify that all co-authors have consented to their work being included in the thesis and they have accepted the candidate's contribution as indicated below in the *Statement of Originality*.

Name of candidate:	BEVERLY WEI LIN, TAN.
Name/title of Primary Supervisor:	A/PROF TOBY MÜNDEL
In which chapter is the manuscript /published work:	CHAPTER TWO

Please select one of the following three options:

Ø The manuscript/published work is published or in press

- Please provide the full reference of the Research Output:
  Pain across the menstrual cycle: Considerations of hydration.

○ The manuscript is currently under review for publication – please indicate:

- The name of the journal:

- The percentage of the manuscript/published work that was contributed by the candidate:

- Describe the contribution that the candidate has made to the manuscript/published work:

○ It is intended that the manuscript will be published, but it has not yet been submitted to a journal

Candidate's Signature:	
Date:	23 DEC 2020
Primary Supervisor's Signature:	
Date:	22 DEC 2020

This form should appear at the end of each thesis chapter/section/appendix submitted as a manuscript/ publication or collected as an appendix at the end of the thesis.

GRS Version 5 – 13 December 2019
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APPENDIX II

Explanation of COVID-19 Impacts

MASSEY UNIVERSITY
UNIVERSITY OF NEW ZEALAND
GRADUATE RESEARCH SCHOOL

Note for Examiners of Doctoral Theses
Explanation of COVID-19 Impacts

The Doctoral Research Committee recognises the impacts of Covid-19 on research, particularly for doctoral candidates, and we appreciate the efforts made by supervisors and candidates to ensure timely completion of the doctoral thesis. We know that in some cases this has meant the project has needed to be changed in some way, including its final presentation. For students whose work has been impacted, we invite supervisors to provide a note for examiners explaining the circumstances.

Instructions for Supervisors:

The note is designed to enable you to communicate to examiners your desire for them to take account of certain factors in their assessment of a thesis to address delays and disruptions experienced by a thesis student as a result of the Covid-19 pandemic.

The attached form should be used to provide an explanation to the examiners on what to consider in their evaluation. It should detail how the project was altered or how the final product of the thesis has been affected as a result of the disruption. Statements should be clear and succinct for the benefit of the examiners and in fairness to the student and others in the student cohort.

The form should be signed by the student, the supervisor and the Head of Academic Unit, or nominee, and included in the information that is sent out with the thesis.

For doctoral candidates, the completed form should be inserted into the front of the thesis before the abstract by the candidate when submitting their digital thesis for examination in the Student Portal. At the completion of the examination, the amended form which excludes any confidential comments to the examiners, should be included in the appendices.

Please be sure to indicate whether a student has received a suspension of studies due to Covid-19 and/or an extension, as it is important to note if students have already had some special consideration.

Approved by DRC 10/Feb/2021
DRC 21/02/03

MASSEY UNIVERSITY
TE KUNENGA KI PŪREHUROA
UNIVERSITY OF NEW ZEALAND

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Thank you for taking the time to examine this thesis, which has been undertaken during the Covid-19 pandemic. The New Zealand Government's response to Covid-19 includes a system of Alert Levels which have impacted upon researchers. Our University's pandemic plan applied the Government's expectations to our research environment to ensure the health and safety of our researchers, however, research was impacted by restrictions and disruptions, as outlined below.

For a six-week period from March 26 to April 27 2020, New Zealand was placed under very strict lockdown conditions (Level 4 – Lockdown), with students and staff unable to physically access University facilities, unless they were involved in essential research related to Covid-19. All field work ceased and data collection with humans was restricted to online methods, if appropriate. The restrictions were partially lifted on April 27, but students and staff were not generally allowed back into University facilities until May 13.

Ongoing disruptions have also been encountered for some students due to uncertainties over the potential for future Covid-19-related restrictions on activities, and a Covid-19 cluster outbreak based in Auckland in New Zealand on 12 August 2020 led to the imposition of rolling Level 2 (Reduce) and Level 3 (Restrict) conditions until 23 September 2020. Auckland campus based students remained on Level 2 until 7 October 2020. This Alert Level system continues to be utilised throughout 2021.

These changing Alert Levels have meant that some research students had experimental, clinical, laboratory, field work, and/or data collection or analysis interrupted, and consequently may have had to adjust their research plans. For some students, the impacts of Covid-19 stretched far beyond the lockdown period in April/May 2020, as they may have had to significantly revise their research plans.

Overseas travel is not permitted by the University and restrictions have been placed on the New Zealand borders which are closed to non-New Zealand citizens and permanent residents. This meant that international students who were based offshore at the time of lockdown, were unable to return to New Zealand. A small number of offshore students were provided permission to return to New Zealand in early 2021. Many students have also suffered from anxiety and stress-related issues, and have had financial impacts, meaning their research progress has been significantly delayed.

This form, as completed by the supervisor and student, outlines the extent that the research has been affected by Covid-19 conditions.

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Please consider the factors listed below in your assessment of the work.

This statement has been prepared by the candidate's supervisor in consultation with the student and has been endorsed by the relevant Head of Academic Unit.

Student Name:	Beverly Tan	ID Number:	17205161
Supervisor Name:	Toby Mundel	Date:	12-Mar-21

Thesis title:

Effects of Hypohydration and Menstrual Phase on Pain

Considerations to be taken into account. Note: This statement will remain in the final copy of the thesis which will be available from the Massey University Library following the examination process.
[*Enter key considerations here for the examiners. This can include but is not limited to change of scope, scale, topic, focus; limitations in relation to data collection, access to necessary literature or archival materials, laboratories, field sites; disruptions as a result of lockdown and various alert levels, medical or health considerations etc*]

Due to the COVID-19 pandemic here in Palmerston North, New Zealand, we were unable to continue participant testing for approximately 3 months (March-May 2020) due to heightened health and safety restrictions. During this same time, we were also not allowed onto campus and as an international student (with all family in Singapore) Beverly was largely confined to her bedroom in her shared accommodation that significantly increased her levels of overall stress. Following this, although we were able to continue testing for her PhD, recruitment became a lot more challenging as most people were wary of their inter-person contact. Add to this a study that already required women to track across their menstrual cycles, induced pain and caused dehydration and I would estimate that approximately 6-9 months of data collection was lost or made much more difficult.

The overall consequence, beyond the obvious stated above, was that Beverly was unable to recruit or complete the intended n=24 that we had determined was likely necessary for her study. Obviously, this loss of data to some extent affected her thesis and eventual study outcome. However, I am also very proud of what Beverly DID achieve during her (just over) 3 years of study.

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Confidential for Examiners Only: *[Please enter any other considerations which are confidential for examiners only and should not be placed in the final thesis version submitted to Library following the examination process]*

Signed, confirming this is a fair reflection of the impact of Covid-19 on this research.

Student Beverly Tan Digitally signed by Beverly Tan Date: 2021.08.15 16:01:13 +13'00'

Supervisor Toby Mundel Digitally signed by Toby Mundel Date: 2021.08.19 12:40:01 +12'00'

Head of Academic Unit (or nominee) Andrew Foskett Digitally signed by Andrew Foskett Date: 2021.08.15 14:45:21 +12'00'

Approved by DRC 10/Feb/2021
DRC 21/02/03