Factors Influencing the Exertional Heat Stress Response in Athletic Females

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ABSTRACT

Climate change continues to expose an increasing proportion of the global population to more frequent extreme heat events. Concurrently, our society has seen an increase in the number of women that participate in physically demanding leisure time (exercise and sport) and occupational activity. Yet, limited research has been conducted to understand the female physiological responses to exercise-heat stress, especially when considering their various ovarian hormone profiles. This thesis expands our understanding of the female physiological responses to heat stress from different perspectives. Firstly, previous evidence has demonstrated that ambient heat stress amplifies the increase in the cytokine interleukin-6 following exercise, an up-regulator of hepcidin - the hormone that downregulates iron metabolism. In **Chapter Five** iron sufficient females' serum iron parameters and hepcidin levels following a self-paced cycling work trial were compared in temperate and hot conditions, and different menstrual phases. IL-6 and hepcidin both increased post-exercise (198% and 38%, respectively), interestingly, neither were affected by ambient temperature or menstrual phase (all $p>0.15$). **Chapter Six** determined the measurement error of a 30-min self-paced cycling protocol in moderate, warm-dry and warm-humid environments using thirty-three athletic women distinguished by their ovulatory status and ovarian hormone concentrations. With an ICC=0.90, $p<0.01$, and a mean CV of 4.7%, SEM of 3.8 kJ (2.1 W) and reliable bias of -2.1 kJ (-1.2 W), it was confirmed that this protocol has high test-retest reproducibility that is not influenced by ambient environment or a female's hormonal/ovulatory status. Finally, in order to better predict females' risk for exertional heat stress, **Chapter Seven** attempted to clarify the role of the ovarian hormones when explaining the variance of the core temperature response using the 30-min self-paced cycling protocol from **Chapter Six** in thirty-six trained women. It was found that estrogen contributes minimally, whilst baseline core temperature and

power output contribute the most to peak core temperature during exercise. Taken together, this thesis deepens our understanding of females' physiological responses and testing norms for current sport science practices. Specifically, it details iron metabolism responses when exposed to exercise-heat stress, elucidates the role of ovarian hormones regarding exertional heat strain and adds test-retest norms specific to athletic women to the available literature.

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First, I would like to thank all my supervisors: Associate Professor Toby Mündel, Dr Claire Badenhorst, Professor James Cotter, and Professor Narihiko Kondo, for their consistent guidance, support, and input throughout my PhD journey. Especially to Toby, I cannot thank you enough for being an awesome supervisor all the time, supporting me not only as your student but also as a woman being away from home by herself for the first time. To our lab manager Adam, thank you for bearing with all my requests and fixing all the equipment I broke!

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LIST OF ABBREVIATIONS

A

ANO	Anovulatory
A_D:mass	Body surface area to mass ratio
ANOVA	Analysis of variance
ATP	Adenosine triphosphate

B

BSA	Body surface area

C

° C	Degree centigrade
C	Cycling
CI	Confidence interval
CV	Coefficient of variation

D

DRY	Warm-dry environmental heat

E

E_2	Estrogen
EF	Early follicular phase

G

g	Gram

g	Gravity

H

Hb	Hemoglobin
h	Hour
HR	Heart rate
HUM	Warm-humid environmental heat
HOT	Hot-humid environmental heat

I

ICC	Intraclass correlation
IL-6	Interleukin-6
ID	Iron deficiency

K

kg	Kilogram
kJ	Kilojoule
kpa	Kilopascal

L

L	Litre
LoA	Limits of agreement
LSR	Local sweat rate

M

m	Metre

MF	Mid-follicular phase
mg	Milligram
min	Minute
ML	Mid-luteal phase
Mo	Month
MOD	Moderate environmental heat
mRNA	*Messenger RNA*

O

O_2	Oxygen
OCP	Oral contraception pill
OVU	Ovulatory

P

P_4	Progesterone
Post-ex	Post-exercise
PPO	Peak power output
Pre-ex	Pre-exercise
PV	Plasma volume

Q

*q*EF	*Quasi*-early follicular
*q*F	*Quasi*-follicular
*q*L	*Quasi*-luteal

R

R	Running
r	Pearson's correlation coefficient
$\bar{R}^2$	Residual variance
RH	Relative humidity
RPE	Rate of perceived exertion

S

s	Second
SD	Standard deviation
SE	Standard error
SEM	Standard error of measurement
SF	Serum ferritin
SI	Serum iron
SR	Sweat rate
sTfr	Soluble transferrin receptor

T

ΔT_{core}	Change in core temperature
T_{base}	Baseline core temperature
T_c/T_{core}	Core temperature
T_{peak}	Peak core temperature
T_{rec}	Rectal temperature

T_{sk}	Skin temperature
$\overline{T}_{sk}$	Area weighted mean skin temperature
TIBC	Total iron-binding capacity
TS	Transferrin saturation
TTE	Time to exhaustion

V

$\dot{V}O_2max$	Maximal oxygen uptake

W

W	Watt
W_{max}	Peak aerobic power
WARM	Warm environmental heat
WBSR	Whole-body sweat rate
WBGT	Wet-bulb globe temperature
WK	Week

Y

Y	Year

LIST OF PUBLICATIONS

Chapter Five

Zheng, H., Badenhorst, C. E., Lei, T. H., Liao, Y. H., Che Muhamed, A. M., Fujii, N., Kondo, N., & Mündel, T. (2021). Menstrual phase and ambient temperature do not influence iron regulation in the acute exercise period. *Am J Physiol Regul Integr Comp Physiol,* 320(6):R780-R790.

Chapter Six

Zheng, H., Badenhorst, C. E., Lei, T. H., Che Muhamed, A. M., Liao, Y. H., Amano, T., Fujii, N., Nishiyasu, T., Kondo, N., & Mündel, T. (2021). Measurement error of self-paced exercise performance in athletic women is not affected by ovulatory status or ambient environment. *J Appl Physiol,* 131(5):1496-1504, 2021.

Chapter Seven

Zheng, H., Badenhorst, C. E., Lei, T. H., Che Muhamed, A. M., Liao, Y. H., Fujii, N., Kondo, N., & Mündel, T. (2022). Do E_2 and P_4 contribute to the explained variance in core temperature response for trained women during exertional heat stress when metabolic rates are very high?. *Eur J Appl Physiol*. Advance online publication. https://doi.org/10.1007/s00421-022-04996-2.

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iron before exercise, and ferritin after exercise (middle, n = 32); and between hepcidin after exercise, IL-6 before exercise, and rectal temperature (T_{rec}) before exercise (bottom, n = 32). Values are all common individual data points, analyzed using Pearson's correlation coefficient and all $P < 0.05$.

Figure 9. Diagram of experimental overview. Three distinct groups were determined according to their hormonal status (A), with each participant performing trials under two distinct ambient environments (B) and repeated 2 wk later according to their (*quasi*-) follicular and luteal phases (A). Each 30- min self-paced work trial was preloaded with either a 12 or 20 min fixed-intensity period (C). 1 participants for Lei et al. (2017); 2 participants for Lei et al. (2019); 3 participants for Zheng et al. (2021). DRY, warm-dry; HUM, warm-humid; MOD, moderate; OCP, oral contraceptive pill; $\dot{V}O_2max$, maximal oxygen consumption**.**

Figure 10. Brinley (top) and Bland–Altman (bottom) plots displaying individual work trial results. Two identified outliers circled red. For Bland–Altman plot, solid black line, line of equality; red line, bias; dashed black lines, limits of agreement; gray lines, confidence limits; shaded area, confidence limits for mean.

Figure 11. Brinley plots displaying individual work trial results and grouped by hormonal status (top, OVU, ovulatory; ANO, anovulatory; OCP, oral contraceptive pill), ambient condition (middle, MOD, moderate; DRY, warm-dry; HUM, warm-humid), and preload duration (bottom).

Figure 12. Bland–Altman plots displaying individual work trial results and grouped by hormonal status (top, OVU, ovulatory; ANO, anovulatory; OCP, oral contraceptive pill), ambient condition (middle, MOD, moderate; DRY, warm-dry; HUM, warm-humid), and preload duration (bottom).

Figure 13. Diagram of experimental overview. Ovulatory (OVU), anovulatory (ANO) and oral contraceptive pill (OCP) users performed trials in their (*quasi*-) early follicular (EF) and/or mid-luteal (ML) phases in warm-dry (DRY) and/or warm-humid (HUM) environmental heat. *n*=23 completed 4 trials and *n*=10 completed 2 trials; whereas *n*=3 completed only 1 trial due to scheduling difficulties and drop-out.

Figure 14. a: Bivariate associations between independent variables and peak T_{core} (T_{peak}) on all common data points. **p*<0.05. **b**: The percentage of explained and unexplained (residual) variance ($\overline{\boldsymbol{R}}^2$) for explaining T_{peak}.

Figure 15. Bivariate associations between peak core temperature (T_{peak}) during exercise and core temperature at the start of the work trial (T_0; top row, *n*=115); between T_{peak} and mean power output during the work trial (middle row, *n*=115); between T_{peak} and E_2 concentration measured before exercise (bottom row, *n*=104). Values are all common individual data points, analyzed using Pearson's correlation coefficient and Spearman's Rho, respectively.

Figure 16. a: Bivariate associations between independent variables and mean power output on all common data points. **p*<0.05. **b**: The percentage of explained and unexplained (residual) variance ($\overline{\boldsymbol{R}}^2$) for explaining mean power output.

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Chapter One

1.0 Introduction

With the continuing development of global warming, there has been an increase in the duration, intensity, and frequency of heat weaves (Perkins et al., 2012). A likely result of this is that a larger percentage of the population is exposed to severe heat weaves on a regular basis, accompanied by an increase in morbidity and mortality rates because of sustained high temperatures (Perkins-Kirkpatrick & Gibson, 2017, Dosio et al., 2018). At the same time, current research has suggested that there is a higher portion of females that are heat intolerant, and that females may be at a higher risk of exertional heat illness compared to males (Alele et al., 2020). Despite numerous research projects being conducted on the human thermoregulatory responses to heat exposure(s), as well as possible interventions to mitigate the detrimental impact of heat stress, female only cohorts have been underrepresented in this research area. One of the reasons for this is the alterations in their thermoregulation due to the fluctuations in ovarian hormone levels. However, with the promotion of gender equality, ever increasing numbers of females are taking part in sports events and undertaking physical activities at their workplace. In New Zealand, the percentage of females in the labour force has been increasing and reached 47.7% in 2021 (*Labor force, female – New Zealand*, 2022). Gender equality at the Olympic Games was nearly achieved at Tokyo 2020, with 48.8% of athletes identifying as female, and is expected to reach full gender equality in 2024 Paris Olympic Games (International Olympic Committee, 2021). Based on the changes in both environmental and social climates, the candidate has endeavoured to deepen the understanding of females'

physiological responses to exercise-heat stress, especially while regarding their different ovarian hormonal status.

To better understand females' thermoregulatory responses to heat exposures and reduce their risk of heat illness, previous research has attempted to explain the variance in females' core temperature by performing regression analysis using morphological, physiological, functional as well as environmental factors (e.g., Havenith et al., 1998, Notley et al., 2019b). Although the conclusions were consistent between studies and suggested that heat load played a key role in explaining the variance of core temperature (Havenith et al., 1998, Notley et al., 2019b), none of them took ovarian hormone levels into consideration. This lack of inclusion is an evident limitation as reproductive hormones significantly affect females' thermoregulation. Therefore, one of the purposes of this thesis is to elucidate the role of ovarian hormones when predicting the risk of heat illness for females with distinct hormonal profiles in different phases of their menstrual cycle.

To attenuate the perception of heat strain and improve performance during acute heat exposure, sports scientists have been working on developing different intervention strategies, such as pre-cooling and heat acclimation. Evidently, when assessing the effect of an intervention on performance, it is very important that an ecological protocol with high reproducibility is used. This ecological validity ensures that any detected difference in performance reflects the effect of the intervention rather than random error. However, little has been done regarding this concern, especially in females. Thereby, one of the purposes of this thesis was to assess the reproducibility of a commonly used exercise protocol when performed by physically active females indifferent hormonal states under different environmental conditions.

Finally, whilst females' thermoregulatory responses to heat stress have been relatively well demonstrated, the impact of heat exposures on other physiological outcomes has not. One such example of this would be the impact of heat stress on iron metabolism, a nutrient critical for

athlete physical performance, as well as their wellbeing. Exercise has been demonstrated to negatively affect iron status through several well-established ways (Sim et al., 2019). As a result, there are documented higher prevalence rates of iron deficiency among active females compared to sedentary females in the general population (Sim et al., 2019). Furthermore, hepcidin, a critical iron regulatory hormone discovered in the last two decades, has been demonstrated to negatively regulate iron levels (Nemeth et al., 2004). In athletes increases in by IL-6 following exercise elicits increases in hepcidin levels post exercise proposing another way that exercise negatively affects iron status (Peeling et al., 2008). Interestingly, the greater the increase in core temperature during exercise under heat stress has been found to amplify the increase in IL-6 (Jones et al., 2010). Whether this means performing physical activity in hot environments is more stressful to iron status, due to exaggerated increases in hepcidin activity, is another research question addressed in this thesis.

This thesis starts with a brief introduction of the research background and questions (**Chapter One**); then moves on to address the above questions by firstly reviewing relevant literature in three sub-sections: 1.) Females' iron metabolism regarding their menstrual cycle, exercise, and ambient heat stress (**Chapter Two 2.1**); 2.) Females' performance in the heat and their reliability (**Chapter Two 2.2**); 3.) Factors determining females' core temperature response under exercise-heat stress (**Chapter Two 2.3**). Based on this, research aims, and hypotheses were outlined in the following chapter (**Chapter Three**) accordingly. Due to the different methods used in each experimental chapter, **Chapter Four** briefly illustrates the participants characteristics, the overall thesis design as well as experimental protocols with visualized diagrams. Detailed methods specific to each experimental chapter are explained accordingly in the following experimental chapters. **Chapter Five – Seven** are experimental chapters that correspond to the three research questions derived from the three sub-sections in **Chapter Two**. Each experimental chapter is formatted as a standard paper for publication, which consists of

Abstract, Introduction, Methods, Results, Discussion, Considerations, and Perspectives sections. Lastly, **Chapter Eight** provides a general discussion for this thesis, as well as its limitations and special considerations. Directions for future research are proposed before ending with conclusions by revisiting the Aims and Hypothesis that were presented in **Chapter Three**.

Finally, it is worth mentioning the candidate's original plan for her PhD here. As originally planned, the candidate would conduct two studies, one as demonstrated in **Chapter Five**, followed by a heat acclimation study looking at the cumulative effect of exercise-heat stress on females' iron metabolism while considering their menstrual cycle. However, as the candidate started data collection for the second study, the COVID pandemic hit New Zealand and the experiment was put on hold. The New Zealand government had been proactively restricting the spread of the virus with several national lockdowns, thereby the candidate reassessed the thesis and decided to replace the heat acclimation study with a retrospective analysis of data from two studies already collected in our lab. Hence, the literature review was changed (addition) and the thesis in general has seen organic/forced change.

Chapter Two

2.0 Review of Literature

This chapter succinctly summarizes the current literature, from which the research questions are developed, in the three sub-sections. Each section focuses on the research question investigated in the corresponding experimental chapter, specifically **Chapters Five-Seven**. Although the research questions addressed by the three experimental chapters are relatively independent, they share one rational background which is the physiological alterations over a female's menstrual cycle. The candidate firstly reviewed the literature on iron metabolism over the menstrual cycle, with a focus on exercise and ambient heat stress. Following this, the second and third sections of this chapter are based on the reproductive endocrinologic alterations over the menstrual cycle, with a focus on the interaction between menstrual cycle, thermoregulation and exercise performance. It is well established that different ovarian hormone levels over the menstrual cycle lead to a series of thermoregulatory changes, typically represented by a ~0.3 °C elevation in females' basal temperature following ovulation (Figure 1). For more comprehensive reviews on each of these topics, please refer to Sim et al. (2019), Charkoudian & Stachenfeld (2014) and Giersch et al. (2020).

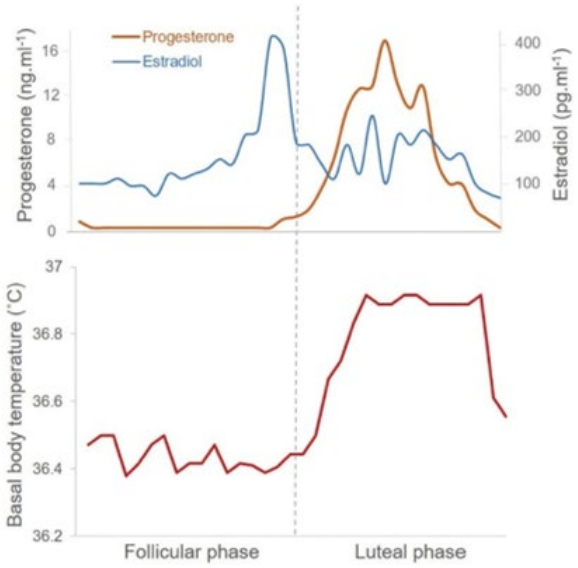

Figure 1. Reproductive hormone and basal body temperature changes across an ovulatory menstrual cycle (Adopted from Baker et al., 2020).

2.1 Non-Reproductive Functions of Ovarian Hormones

The menstrual cycle starts on the first day of menstruation and ends with the onset of the subsequent menstrual bleed. Ovulation usually occurs around the 14th day of the menstrual cycle, for an average menstrual cycle length of around 28 days. It is well-known that females' thermoregulatory responses to acute exercise-heat exposures vary over their menstrual cycle, because of the fluctuation of estrogen and progesterone levels (Mihm & Muttukrishna, 2011, Charkoudian & Stachenfeld, 2014). The early follicular phase (EF), usually days 1-7 of the menstrual cycle, and the mid-luteal phase (ML), usually days 18-24 of the menstrual cycle, are the most investigated phases in eumenorrheic females. A practice that is a result of differences in ovarian hormones being the greatest between these two phases. The EF is characterized by low concentrations of both estrogen (E_2) and progesterone (P_4), whereas the ML is characterized by increased/moderate concentrations of E_2 and high concentration of P_4 (Figure

1). It has been shown that E_2 lowers the thermoregulatory operating point hence facilitates heat dissipation and lowers the basal body temperature (Tankersley et al., 1992). While P_4 has the opposite effect (Stachenfeld et al., 2000), and as a result, the luteal phase is characterized by a ~0.3 °C elevation in body temperature.

Research has demonstrated that over half of the female athlete population take the oral contraceptive pills (OCP) (Rechichi et al., 2009). Researchers tend to agree that the thermoregulatory effects of the endogenous hormones persist over OCP cycles (Grucza et al., 1993; Martin & Buono, 1997; Charkoudian & Johnson, 1997; Rogers & Baker, 1997; Tenaglia et al., 1999; Sunderland & Nevill, 2003; Lei et al., 2019). It was observed that similar to the luteal phase in an eumenorrheic menstrual cycle, an elevated core temperature (T_{core}) was observed in the *quasi*-luteal phase (*q*L, second and third week taking the pill) of the OCP cycle, compared to the *quasi*-early follicular phase (*q*EF, when participants were ingesting the placebo pill) (Martin & Buono, 1997; Charkoudian & Johnson, 1997; Rogers & Baker, 1997; Tenaglia et al., 1999). Although the thermoregulatory effects of ovarian hormones have been well-investigated in naturally menstruating females and females taking OCP, the impact of the subsequent thermoregulatory responses on other aspects of physiological and health outcomes is still an active area of investigation.

2.2 Is Additional Heat Stress During Exercise Detrimental to Female's Iron Status?

2.2.0 Overview

Iron is a functional nutrient in metabolic pathways as it is involved in the synthesis of many important proteins and enzymes. These mainly include: 1) Hemoglobin (Hb) and myoglobin, which are responsible for transporting oxygen in the bloodstream and within the muscle respectively; 2) Cytochromes, part of the respiratory chain, and have an essential role in electron transport and ATP production (Dallman, 1986; McClung & Murray-Kolb, 2013). In addition, iron is also of great importance for the functioning of immune and neural systems (Beard, 2001). Thus, a normal iron status is very important for human bodies' wellbeing and physical performance.

Iron is stored in the human body mainly as ferritin and hemosiderin; whilst transferrin proteins transport iron in the bloodstream (Dallman, 1986). It is hard to select a best parameter when describing iron status, as different iron parameters have different implications. The commonly used laboratory assessment of iron status includes Hb levels, serum iron (SI) and transferrin saturation (TS), with serum ferritin (SF) commonly used as an indicator of iron storage (McClung & Murray-Kolb, 2013). The development of iron deficiency (ID) consists of three stages, but it is not detrimental to athletic performance until stage 3 (Iron Deficiency Anaemia, SF<12 $\mu g.L^{-1}$) where there is a reduction in Hb (Dallman, 1986).

Factors that affect the iron status have been widely investigated by many researchers. Diet, which is the main source of iron, is the primary factor that affects iron levels. Red meat products are the main source of heme iron, which can be absorbed very efficiently by the human body (Barr & Rideout, 2004). For this reason, vegetarians are suggested to have a greater risk of ID

diagnosis (Snyder et al., 1989; Barr & Rideout, 2004). Hepcidin, the iron regulatory hormone, is another factor that has a pervasive influence on iron levels (McClung & Murray-Kolb, 2013). A potent up-regulator of hepcidin is inflammation, which triggers the synthesis of hepcidin and subsequently down-regulates the iron metabolism (McClung & Murray-Kolb, 2013).

Females, especially premenopausal females, are likely to have a poor iron status because of blood loss during menstruation and potentially habitual diets with low iron intake (Heath et al., 2001; Harvey et al., 2005). Maintaining normal and healthy iron status is of great importance especially for athletes who are aiming at optimizing exercise performance. Severely depleted iron status has been shown to diminish physical performance including $\dot{V}O_{2max}$ and aerobic work capacity, as well as cognitive function (Schumacher et al., 2002a, McClung & Murray-Kolb, 2013; Pedlar et al., 2018). Despite this, iron deficiency is found to be the most prevalent micronutrient deficiency disorder in the world (McClung & Murray-Kolb, 2013). It also has been reported that athletes commonly have poor iron status, especially among endurance athletes. This could be due to the iron lost through cumulative mechanisms including haemolysis (Selby & Eichner, 1986), haematuria (Siegal et al., 1979), sweating (Brune et al., 1986) and gastrointestinal bleeding (Stewart et al., 1984) during intense exercise. Inflammation induced by the exercise results in an increase in hepcidin post-exercise (Peeling et al., 2008). This is another likely factor attributing to athletes' poor iron status, due to reductions in iron absorption and recycling post-exercise with elevated hepcidin activity (Peeling et al., 2008).

2.2.1 Variation in Iron Status over the Menstrual Cycle

Serum iron-related parameters vary over the menstrual cycle because of: (1) the transient hemodilution and hemoconcentration in the premenstrual and post-ovulation phase, due to the hormone-mediated fluid shifts (Vellar 1974); (2) blood loss during menstruation. It is reported that for healthy females with normal blood loss during menstruation, approximate 1 mg of iron

is lost during each cycle; for those with heavy menstrual bleeding, this value can be 5-6 times higher (Napolitano et al., 2014).

The majority of the results regarding iron parameters on this topic are consistent. Hb concentration, which is the most frequently investigated parameter, has been shown to be significantly associated with the menstrual phases: Hb concentration is relatively low in follicular phase and peaks post-ovulation, then decreases towards the end of the menstrual cycle (Vellar 1974; Kim et al., 1993). Other iron-status parameters, including SI, SF and TS, have also been investigated and the results are relatively conclusive. SI and TS showed the same pattern as Hb, with their nadir in the menstrual period and peak in luteal phase (Kim et al., 1993; Inoue & Sugiyama, 1998; Angeli et al., 2016; Lainé et al., 2016). Similar fluctuations have also been observed with SF in some studies (Kim et al., 1993; Inoue & Sugiyama, 1998). Whilst total iron-binding capacity (TIBC) showed the opposite, a nadir in the luteal phase and peak in the follicular phase (Kim et al., 1993).

Conversely, Belza et al. (2005) monitored the iron parameters of 12 iron-depleted but non-anaemic women throughout their menstrual cycle and found no significant difference in iron parameters. This could be due to the small sample size ($n<20$), with Inoue & Sugiyama (1998) similarly not finding any significant changes in iron parameters over the menstrual cycle of females with normal iron status ($n=15$). However, they reported a significant difference in iron status between different menstrual phases in females with marginal ID ($n > 23$). Surprisingly, their Hb and SF levels were higher in follicular phase (Inoue & Sugiyama, 1998). This discrepancy potentially indicates that females' iron status fluctuates in different patterns throughout their menstrual cycle, but degree of change may be due to their different baseline iron levels.

Hepcidin, the iron regulatory hormone, has drawn a lot of researchers' attention. It was reported that serum hepcidin level fluctuates over the menstrual cycle like SI. Research has shown that

hepcidin decreases during menses, then rebounds and plateaus in luteal phase (Angeli et al., 2016; Lainé et al., 2016). The role of hepcidin in iron metabolism will be discussed in detail later.

2.2.2 The Effects of Exercise on Iron Status

2.2.2.1 Hepcidin

Hepcidin is a peptide hormone produced by liver, which plays a key role in systemic iron metabolism (Nemeth et al., 2004b). It is well established that hepatic iron level and inflammation are two primary factors that trigger the secretion of hepcidin (Peeling et al., 2008). Hepcidin down-regulates iron levels mainly by inducing the internalization of ferroportin, which is the only iron exporter responsible for iron absorption in the duodenum. In addition, it also interacts with ferroportin on the surface of macrophages and hepatocytes, thus inhibits the release and recycling of iron in the plasma (Nemeth et al., 2004b). High hepcidin levels have been suggested to attribute to hypoferremia and anemia of inflammation. Nemeth et al. (2004a) demonstrated that IL-6, a cytokine commonly used for the assessment of inflammation, is the mediator between inflammation and hepcidin production. Banzet et al. (2012) demonstrated that rats treated with cyclosporine A, which blunts the IL-6 increase, had a lower increase in hepcidin mRNA after exercise. Research in athletes has demonstrated that IL-6 increases hepcidin levels after exercise through increasing the hepcidin mRNA expression (Peeling et al., 2008). It was also observed that hepatic hepcidin mRNA levels started to increase immediately after exercise and peaked at ~2 hours later, with hormone levels peaking ~3 hours post-exercise (Peeling et al., 2008). Hence inflammation responses, specifically, IL-6, are the direct trigger of hepcidin secretion.

As it has been well established that physical exercise results in inflammatory responses (Peeling, 2010), Peeling et al. (2008) hypothesised that exercise-induced increases in IL-6 and

subsequent increases in hepcidin could affect iron metabolism in athletes. Therefore, in recent years, most studies regarding exercise and iron metabolism have been focusing on exercise-induced inflammation (IL-6) accompanied by the increase in hepcidin concentration.

2.2.2.2 Iron Metabolism after Acute Exercise

Many studies have investigated the effects of acute exercise bouts on iron status. Serum free Hb, SI, SF, IL-6 and hepcidin levels before, immediately and hour(s) after exercise have been typically measured to detect the effects of an acute exercise bout on iron metabolism. Studies investigating females' iron metabolism are summarized in Table 1.

Table 1. Females' iron parameters and inflammation responses after acute exercise.

Author	Gender/Phase	Protocols	Iron	IL-6	Hepcidin
Roecker et al., 2005	Female	Marathon race	N.A.	N.A.	*URINE* hepcidin peaked one day after the race.
Peeling et al., 2009b	6 males and 2 females (EF)	60 min high intensity running	Increased immediately post exercise, lower than the control group 6 and 24 h post exercise.	Peaked immediately after exercise and returned to baseline at 3 h post exercise.	*URINE* hepcidin was significantly higher 3 h post exercise than immediately post exercise. It remained higher at 6 h post exercise but could be due to diurnal variation.
Newlin et al., 2012	Females in MF	1 or 2 h running at 65% $\dot{V}O_2max$	Lowest iron level was observed at 9 h post exercise.	Peaked immediately after exercise.	*SERUM* hepcidin peaked at 3 h post exercise; significantly higher post the 2 h run compared to 1 h.
Bauer et al., 2018	Female	3 consecutive high-intensity boating bouts	Significantly diminished at 3 h post exercise.	N.A.	*SERUM* hepcidin was significantly elevated at 3 h post exercise.
Barba-Moreno et al., 2022	Females in EF, MF and L	40 min running at 75% $\dot{V}O_2peak$	Lower in EF but no effect of exercise.	Peaked 3 h post exercise; higher in L.	*SERUM* hepcidin peaked immediately after exercise.

EF, early follicular phase; **MF**, mid follicular phase; **L**, luteal phase; **$\dot{V}O_2max$/ $\dot{V}O_2peak$**, maximal/peak rate of O_2 uptake.

A transient increase in serum free Hb (Schumacher et al., 2002b; Peeling et al., 2009b; Sim et al., 2012), SI (Schumacher et al., 2002b; Peeling et al., 2009a; Peeling et al., 2009b; Troadec et al., 2009; Sim et al., 2012; Badenhorst et al., 2016) and SF (Schumacher et al., 2002b; Peeling et al., 2009b; Troadec et al., 2009; Newlin et al., 2012; Peeling et al., 2014; Badenhorst et al., 2016) immediately after exercise bouts were observed by several studies on females and/or males. This increased serum free Hb and SI likely results from haemolysis induced by exercise. It has been demonstrated that exercise accelerates the destruction of red blood cells, thus more Hb and iron are released into the plasma following exercise (Peeling et al., 2008). Based on this, it was further demonstrated that high-impact, weight-bearing exercise (e.g., running) can elicit higher levels of haemolytic activity than non-weight bearing exercise (e.g., cycling), due to more severe destruction of red blood cells (Telford et al., 2003; Sim et al.,2013). This greater increase in SF following high-impact weight-bearing exercise was suggested to be a result of 1) ferritinbeing recognized as one of the acute phase proteins; 2) the damage of cells where ferritin is stored, such as hepatocytes; so that more ferritin is released into plasma (Pattini et al., 1990).

Many studies observed a remarkable increase in IL-6 immediately after prolonged or intense exercise (e.g., Peeling et al., 2009a; Peeling et al., 2009b; Newlin et al., 2012; Sim et al., 2012; Sim et al., 2013; Comassi et al., 2015; Badenhorst et al., 2016), indicative of the exercise-induced acute inflammatory response. It was further demonstrated that IL-6 concentration reaches its peak immediately after exercise and then declines (Peeling et al., 2009b; Newlin et al., 2012). In non-athletes, Kemna et al. (2005) observed that the urinary hepcidin level peaked ~2-3 hours after the peak of IL-6 level in healthy participants who had been injected with lipopolysaccharide, an inflammation trigger. Similarly, an elevated hepcidin response post exercise has been observed by many researchers (Roecker et al., 2005; Peeling et al., 2009b, Newlin et al., 2012; Peeling et al., 2014; Badenhorst et al., 2016; Peeling et al., 2017). Roecker

et al. (2005) observed a transient increase in hepcidin level after a marathon in women, which peaked 1 day after the exercise. Whilst a later study by Peeling et al. (2009b) observed a significant increase in hepcidin level 3 h post a 60 min high intensity running session that peaked at 6 h post exercise. The discrepancy in the time course of the fluctuation in hepcidin level could be due to the different intensity/duration of exercise (Newlin et al., 2012). Notably, although the 6 h post exercise hepcidin level was highest in the study by Peeling et al. (2009b), it was not significantly higher than the value of the control group (seated rest, without any exercise intervention) at the same time point. Thus, the further increase could be due to diurnal variation of hepcidin (Kemna et al., 2007). Despite the different time frames of the exercise-induced increases in hepcidin, it could be concluded that a 3 h gap post exercise is enough to see a significant increase in hepcidin induced by IL-6, within participants who have a normal/healthy iron status. As such, 3 h post exercise has been used by many studies investigating post exercise peak hepcidin levels since then.

Several factors have been shown to affect the elevation in hepcidin levels after exercise. Baseline iron status was demonstrated to be a primary factor that affects hepcidin levels by many studies (Roecker et al., 2005; Peeling et al., 2009b; Newlin et al., 2012; Peeling et al., 2014; Peeling et al., 2017). These studies reported that in participants with low SI and SF, there was no/attenuated post exercise increase in hepcidin. This indicates that insufficient baseline iron levels counteract the effect of exercise on hepcidin levels. Newlin et al. (2012) also observed a longer elevation period of IL-6 levels in healthy participants compared to iron insufficient participants. However, the sample size was too small to conclude that the shorter elevation of IL-6 attributed to the unchanged hepcidin level. Further studies are required to investigate the underlying mechanisms of hepcidin activity post-exercise in various cohorts (e.g., iron sufficient/insufficient).

As most studies on this topic used running in their exercise protocol, Sim et al. (2013) compared IL-6 and hepcidin levels after running (R) and cycling (C) at the same intensity and duration. It was observed that the increase in IL-6 and hepcidin levels after exercise were lower in C compared to R. This could be due to the lower haemolytic responses induced by non-weight bearing exercise (cycling). Other factors also found to augment the increase in hepcidin levels include longer exercise duration (Newlin et al., 2012), and higher exercise intensity (Peeling et al., 2009a; Sim et al., 2013).

As mentioned above, hepcidin inhibits the absorption and release of recycled iron from macrophages. Consequently, after the transient increase in SI, a corresponding reduction in SI following the increase in hepcidin was detected by many studies (e.g. Peeling et al., 2009; Bauer et al., 2018). The SI has been found to be lower than pre-exercise values during the recovery period after exercise (Peeling et al., 2009b; Robson-Ansley et al., 2011).

Soluble transferrin receptor (sTfr) is another parameter that has frequently been used in recent years. It mediates the transfer of iron from transferrin to erythroid cells; therefore, its concentration reflects the rate of erythropoiesis (Thorstensen & Romslo, 1993). It was suggested that unlike SF, sTfr is not an acute phase protein and stays relatively stable after moderate exercise (Malczewska et al., 2000; Schumacher et al., 2002b, Nikolaidis et al., 2003). However, a decrease in sTfr at 3 and 24 hours post a 90 min sub-maximal running was reported by Sim et al. (2012), indicating that there was a decrease in red blood cell production. This discrepancy could be due to the longer duration/higher intensity of exercise involved in the study by Sim et al. (2012), which may suggest that the significant increase in hepcidin was enough to impair post-exercise erythropoiesis activity. Unfortunately, the hepcidin levels were not measured in these other studies (Malczewska et al., 2000; Schumacher et al., 2002b, Nikolaidis et al., 2003). More research is needed to elucidate the relationship between iron, hepcidin and erythropoiesis activity following exercise.

2.2.2.3 Iron Metabolism in Different Menstrual Phases

As previously discussed, the menstrual cycle has an influence on iron status and potentially hepcidin level. Several studies have demonstrated the effect of acute and repeated exercise bouts on females' iron regulation, however, they either measured exercise responses within the same phase (Newlin et al., 2012; Peeling et al., 2009b; Peeling et al., 2014), or did not standardize the menstrual phase at all (Roecker et al., 2005; Auersperger et al., 2012). There is only one study demonstrating females' iron metabolism responses to exercise in different menstrual phases (Barba-Moreno et al., 2022). It was observed that following 40 min treadmill running at 75% $\dot{V}O_2$peak, IL-6 increased and peaked at 3 h post exercise, whilst hepcidin peaked immediately after exercise. Menstrual phase was demonstrated to have an effect on the IL-6 response, such that the peak value was higher in luteal phase compared to EF and mid-follicular phase (MF). However, this did not further result in fluctuations in hepcidin over the menstrual cycle. As there are no conclusive findings on this topic, considering the high-prevalence of ID among female athletes, more studies are required to help female athletes prevent ID with menstrual phase-specific strategies.

2.2.3 The Effects of Exercise-Heat Stress on Iron Status

To date, there is no literature available on this topic on females. However, there is some evidence suggesting that exercise plus heat stress can potentially diminish iron status to a greater extent than exercise itself. This may be due to the increase in iron loss through sweating, and possibly higher levels of inflammatory responses and hepcidin secretion.

2.2.3.1 Sweat

Sweating is the most efficient thermoregulatory way for heat dissipation during exercise (Powers & Howley, 2007). Exercise in the heat can remarkably increase an athletes' sweat rate to maintain body temperature within the normal range. Fortunately, a decrease in sweat iron

concentration during an exercise bout as exercise proceeds has been reported (Waller & Haymes, 1996; DeRuisseau et al., 2002). Waller & Haymes (1996) measured the sweat iron concentrations of 9 male and 9 female athletes during 1 h of exercise at 50% $\dot{V}O_2$max. They found that the mean sweat iron concentration decreased significantly during exercise in the hot environment compared to neutral environment (0.14 mg/min and 0.22 mg/min respectively). As a result, although the sweat rate during exercise in the heat was much higher, the estimated whole body iron loss was lower during exercise in a hot environment (0.077 $mg \cdot m^{-2} \cdot h^{-1}$ and 0.08 $mg \cdot m^{-2} \cdot h^{-1}$ in neutral and hot environments, respectively). DeRuisseau et al. (2002) suggested that there is a possible iron conservation mechanism helping the body conserve iron during prolonged exercise. Thus, based on the currently available evidence, it is inappropriate to conclude that one-off exercise-heat exposure results in greater iron loss compared to exercise in neutral condition. However, whether consecutive exercise-heat exposures (e.g., heat acclimation) will have an impact on sweat iron concentration, is still unknown.

2.2.3.2 Inflammation and Hepcidin

As discussed above, hepcidin is a central down-regulator of iron metabolism and one of its primary triggers is inflammation, to be more exact, IL-6. It is well established that exercise elicits inflammatory responses including an increase in IL-6. Mündel et al. (2010) reported that there was an additional effect of heat on circulating IL-6 concentration during exercise. In their study, eight male participants completed two 60 min cycling trials at 70% ṾO2max in either thermoneutral (CON) or hot (HOT) conditions. Plasma IL-6 concentration was higher in HOT after 30min of exercise and remained elevated until 30 min-post exercise. The increase in circulating IL-6 concentration in HOT was approximately two-times that of the CON trial, which is consistent to a previous study using a similar protocol (Starkie et al., 2005). A significant positive correlation was observed between IL-6 concentrations and core temperatures. Therefore, it could be hypothesised that the augmented increase in IL-6 during

exercise in hot conditions may result in further or sustained increases in hepcidin level. As a result, there could be a greater decrease in iron regulation after prolonged exercise in hot conditions.

The studies investigating the relationship between exercise-heat stress and hepcidin levels are sparse. The study by Bloomer et al. (2014) demonstrated that two-repeated-heat exposures stimulated an increase in hepcidin mRNA expression in aged rats, compared to control. In young rats' hepcidin mRNA expression was not affected by exercise in the heat. More recently, Hayashi et al. (2020) found that additional heat stress in a hypoxic environment had no effect on IL-6 and hepcidin responses following 60 min of cycling at 60% of $\dot{V}O_2$max. Whilst McKay et al. (2021) demonstrated that when absolute intensity was matched, treadmill running induced greater increases in IL-6 in a hot environment compared to cold, however no difference was observed in the post-exercise hepcidin response. Unfortunately, neither of the two previous studies monitored T_{core} during the trial. More studies are warranted to elucidate the relationship between T_{core} and hepcidin responses following exercise, especially on females.

2.2.4 Summary

This section of the review looked at two primary factors that affect females' iron status: menstrual cycle and exercise. It has been demonstrated that females' iron parameters are lower in their follicular phase mainly due to menstrual blood loss. Exercise affects iron status via haemolysis, sweat and most importantly, inflammation. Inflammation, specifically increases in IL-6, and negatively affects iron regulation via triggering the secretion of hepcidin. Although it has been demonstrated that ambient heat stress augments the inflammation induced by exercise, it remains unknown whether the increase in hepcidin will be amplified post-exercise in females. Thereby, the purpose of the first study is to investigate the effects of the additional ambient heat stress on females' iron metabolism, specifically, in their different menstrual

phases. It is hypothesized that for females with normal iron status, exercising in the heat will result in greater increases in IL-6 and hepcidin, compared to exercise in temperate conditions. In addition, their pre-exercise hepcidin levels will be higher in luteal phase compared to follicular phase.

2.3 Can Females Perform Reliably in the Heat?

2.3.0 Overview

Performance has always been one of the important measures when sports scientists evaluate the efficacy of treatments (e.g., training programs). Therefore, it is critical to demonstrate that the observed change in performance is a result of the intervention rather than a result of measurement error. Although it is well-established that aerobic exercise performance is impaired under heat stress, few studies have assessed the reliability of a protocol when used in different ambient conditions. Even fewer research studies have carried out this type of investigation with female participants. More caution should be taken when interpreting performance data of females, especially when considering the potential effects of their menstrual cycle. This section firstly reviews the literature on females' performance under heat stress over their menstrual/OCP cycle, and is followed by summarizing the limited literature looking at the reliability of time trials used to measure aerobic exercise performance.

2.3.1 Female's Performance Under Thermal Stress Over Their Menstrual / OCP Cycle

During competitions in real life, athletes are required to complete a set amount of work in the shortest time possible. Hence, from an ecological validity point of view, as much as the candidate thinks it is critical to use consistent-duration test (participants complete as much

work as possible in a set period of time) or constant-work test (participants complete a set amount of work as quickly as possible) when measuring participants' performance, almost all of the available studies (n=9) on this topic used constant-power protocols, with only two studies including a self-paced time-trial (consistent-work test). Nevertheless, to elucidate the potential impact of menstrual/OCP phase on females' performance under heat stress, all the currently available literature in this area was reviewed in this section regardless of their test protocols.

Eight studies investigated eumenorrheic females' performance under heat stress throughout their menstrual cycle (Table 2). A decreased mean tolerance time in luteal phase was reported by two studies (Avellini et al., 1980; Tenaglia et al., 1999). Avellini et al. (1980) observed a significantly shorter mean tolerance time in the post-ovulation phase during exercise in humid heat, due to the delayed sweating onset. The delay resulted in a greater heat storage and a higher elevation of T_{core}, but once sweating initiated, the sweat rate (SR) / T_{core} was similar between phases. The study by Tenaglia et al. (1999) reported that during light intermittent exercise under uncompensable heat stress, the increasing rate of T_{core} was similar in EF and ML, but T_{core} reached the safe limit earlier in ML because of the higher resting value. However, in another study that also used uncompensable heat stress (Kolka & Stephenson, 1997), no difference was observed in mean tolerance time between menstrual phases. This could possibly be because of the different exercise protocols (intermittent vs. continuous) used between studies. Meanwhile, it is worth noting that Kolka & Stephenson (1997) terminated the heat tolerance tests when subjects had "high HR and high internal temperature", with no specific description provided. A study by Janse et al. (2012) compared participants' time to exhaustion (TTE) during an incremental exercise test, following 60 min of submaximal exercise in humid heat. The TTE during the ML was 5.7% shorter than during EF, with no significant difference in final T_{core}. Despite a higher T_{core} during rest and exercise, the increasing rate of T_{core} during fixed-intensity exercise was also higher in the ML. By contrast, no difference in body heat

storage and evaporative heat loss were observed over the menstrual cycle, when performing fixed-intensity exercise at different intensities (Lei et al., 2017). Similar findings were also observed in dry hot conditions, with Notley and his colleagues (2019a) demonstrating that females' dry and evaporative heat exchange did not differ between menstrual phases, irrespective of exercise intensity.

The study by Lei et al. (2017), which is the only one that included a self-paced exercise protocol, found that there was no difference in exercise performance in hot environments over the menstrual cycle. In this study, well-trained female athletes performed 6 min cycling at 125 W and 150 W each, followed by a 30 min self-paced time trial. No difference was observed in the work completed during self-paced time trial between menstrual phases, but there was a reduction of performance in humid hot environments, compared to dry. Sunderland & Nevill (2003), also found no difference in their maximal intermittent 15 m sprint times and running distance at exhaustion in dry-heat environments. However, in Lei et al. (2017) even though the work capacity was similar in different menstrual phases, it was observed that participants dropped their power more rapidly in the ML phase during exercise in humid hot environments. This along with the aforementioned study by Janse et al. (2012), suggests that in luteal phase, although female athletes' performance is not compromised during a 30min self-paced time trial, they may still suffer from greater heat strain during exercise in humid hot environments.

Only 3 studies have investigated OCP users' exercise performance under heat stress (Table 2). One observed better performance during a high intensity intermittent running in *q*L (Sunderland & Nevill, 2003), whilst the other two reported that OCP phase did not have an effect on exercise performance (Tenaglia et al., 1999, Lei et al., 2019). It is worth noting that the study by Lei et al. (2019) used the same protocol as their earlier study on eumenorrheic participants (Lei et al., 2017). Similarly, participants' performance during a self-paced time trial was affected by environmental conditions but not OCP phases.

Table 2. Females' performance under heat stress over their menstrual / OCP cycle.

Authors	Subjects' $\dot{V}O2max$	Phases	Exercise protocols	Environment	Performance
Avellini *et al*.,1980	57.0 ± 1.5 mL min^{-1} kg^{-1}	Pre-OVU Post-OVU	3h heat stress test: walk on a treadmill at 5.6km/h with 2% gradient	Humid heat T_{db}: 36 °C T_{wb}: 30 °C	Shorter mean tolerance time in post-OV.
Frye & Kamon, 1981	54.08 ± 4.27 mL min^{-1} kg^{-1}	Pre-OVU Post-OVU	3h heat stress test: walk on a treadmill at 25-30% $\dot{V}O_2max$	Dry heat T_{db}: 48 °C T_{wb}: 25 °C	No significant differences were observed between different phases.
Kolka & Stephenson, 1988	2.42 ± 0.18 L min^{-1}	L/F	Cycled at 80% $\dot{V}O_2peak$ until T_{es} had increased by 0.8 °C	T_{db}=50.4 °C AH=1.61kPa	Exercise time did not differ between phases.
Kolka & Stephenson, 1997	2.66 ± 0.30 L min^{-1}	EF/LF/ML	Treadmill exercise at 40% peak aerobic power output, for up to 75min	Clothing with high thermal resistance	Exercise time was not different between menstrual phases.
Tenaglia et al., 1999	~43 mL min^{-1} kg^{-1}	EF/ML *q*EF/*q*ML	Participants alternated between 15min of walking on a treadmill at 4km/h and 15 min of seated rest, up to 300 min	40 °C, 30% RH Wearing protective clothing ensemble	Within group: mean tolerance time was longer in EF than in ML but similar between *q*EF and *q*ML.
Sunderland & Nevill, 2003	~50 mL min^{-1} kg^{-1}	EF/ML *q*F/*q*L	Repeated LIST, 3min rest during each set	31 °C, 23.1% RH	No difference in the distance run during the LIST between F and L, but longer in *q*L than in *q*F.
Janse *et al*., 2012	40.0 ±6.9 mL min^{-1} kg^{-1}	EF/ML	Fixed-intensity exercise at 60% $\dot{V}O_2max$ for 60 min followed by an incremental performance test to volitional fatigue.	Humid heat: 32 °C, 60% RH	Time to exhaustion was shorter in ML.

Lei *et al.*, 2017	58 ± 9 mL min^{-1} kg^{-}	EF/ML	20 min resting 6 min at 125 W 6 min at 150 W 30 min self-paced time trial	HUM: 29 °C, 81% RH DRY: 34 °C, 41% RH	Work capacity and mean power output were similar between menstrual phases; but higher in DRY than in HUM.
Lei *et al.*, 2019	~56 mL min^{-1} kg^{-1}	*q*F/*q*L	Replicated as above	Replicated as above	Similar work capacity and mean power output between OCP phases; but higher in DRY than in HUM.

F, follicular phase; **L**, luteal phase; **EF**, early follicular phase; **LF**, late follicular phase; **ML**, mid luteal phase; ***q* (E)F**, *quasi*-(early) follicular phase; ***q*(M)L**, *quasi*-mid-luteal phase; **Pre-OVU**, pre-ovulation; **Post-OVU**, post-ovulation; **$\dot{V}O_2max$/ $\dot{V}O_2peak$**, maximal/peal rate of O_2 uptake; **T_{es}**, oesophageal temperature; **T_{db}**, dry bulb temperature; **T_{wb}**, wet bulb temperature; **AH**, absolute humidity; **RH**, relative humidity; **LIST**, Loughborough Intermittent Shuttle Test.

2.3.2 Reliability of Exercise Performance During A Self-Paced Time Trial

Although there are a few studies comparing females' performance in different menstrual phases under heat stress, it is not certain that the observed difference in performance was a result of the intervention (in this case, hormonal status, and environmental conditions), other than (or above the) measurement error. In this section, the candidate reviews the current literature assessing the reliability of the time trial protocol used to measure athletes' aerobic exercise (≥30min) performance. Due to scarce female studies on this topic, male studies are also reviewed in this section (Table 3).

2.3.2.1 Methods Assessing Measurement's Reliability

It is well established that reliability is classified into two types: relative and absolute reliability; different methods are used to access different types of reliability correspondingly. Relative reliability, assessed by either Pearson's correlation coefficient (*r*) or intraclass correlation (ICC), reflects how stable the individuals' rank is amongst a sample over repeated tests. As Pearson's correlation is bivariate, it can only be used when comparing two repeated tests; whilst ICC can be used to compare more than two repeated retests. For both *r* and ICC, a value close to 1 demonstrates good reliability of the measurement. However, statisticians have found that the correlations are greatly affected by the heterogeneity of the sample, in other words, the range of values in the sample. For example, a sample with high level of heterogeneity and large measurement error can still produce high *r*/ICC (Atkinson & Nevill, 1998; Hopkins et al., 2001). Hence, as the relative reliability itself does not tell the systemic bias, it should not be used alone when assessing the reliability of the measurement (Atkinson & Nevill, 1998). Having said that, it is still worth mentioning that studies using *r*/ICC to assess the reliability of the time trial protocols have all demonstrated very good reliability, with *r*/ICC values ≥ 0.92, regardless of the participants' sex (Bishop, 1997; Schabort et al., 1998; Doyle & Martinez,

1998; Marino et al., 2002; Laursen et al., 2003; Abbiss et al., 2008; Peiffer & Losco, 2011; Driller, 2012; Sharma et al., 2015; Che Jusoh et al., 2015).

As mentioned above, in addition to relative reliability, absolute reliability is always recommended by statisticians when assessing the reliability of the measurement. This defines the actual fluctuation of the individuals' measurements over repeated tests. Traditionally, standard error of measurement (SEM, also known as typical error) and coefficient of variation (CV) are used to evaluate absolute reliability. SEM expresses within subjects' variation in the actual units of the measurement, hence its value is unique to the protocol. By contrast, CV expresses SEM as a percentage of the mean -hence it is dimensionless. Therefore, CV has been used the most as it enables the comparison between different studies, even if measurements are in different units. Unlike r/ICC, there is not an agreed range of SEM/CV which indicates the degree of reliability, other than the smaller the SEM/CV is, the more reliable the measurements are. Previous studies on this topic have reported CV of the self-paced time trial performance varying between 0.6 – 4.2, which scientists take as an indication of good reliability (e.g., Jeukendrup et al., 1996; Bishop 1997; Che Jusoh et al., 2015). Nevertheless, although CV provides a very practical way to compare the repeatability of different protocols, it only accounts for 68% of the measurement error (Atkinson & Nevill, 1998).

Considering the disadvantages of these methods, Bland & Altman (1983) proposed another method to assess the absolute reliability, limits of agreement (LoA). Specifically, given the premise that the mean differences between two repeated measurements are normally distributed, LoA can be thought of as the 95% confidence interval of the mean differences. Thereby, it is calculated as mean±1.96SD, where mean and SD are the mean and SD of the differences between two repeated trials. In this circumstance, the mean difference is called bias and ±1.96 SD denotes the 95% tolerance interval of the random error (for more detailed explanations of LoA, please refer to the original research article by Bland & Altman, 1983 and

the review article by Atkinson & Nevill, 1998). The associated Bland-Altman plot, where the individual differences between two trials are plotted against their mean, along with the 95% limits of agreement, provide scientists with a schematical way to show the systematic bias and random error. More recently, it was proposed by Giavarina (2015) that if the line of equality (y=0) lies within the 95% CI of the mean of the differences, the systemic bias is not significant hence the measurement is reliable. In the candidate's opinion, SEM and LoA serve more as descriptive characteristics of a protocol, as they themselves do not tell the reliability of the protocol. However, they can be used as references when the same protocols are used by a similar population, e.g., the effect of the intervention should be expected to be greater than SEM/beyond the LoA.

2.3.2.2 Factors Affecting Measurement Reliability

As mentioned above, from the ecological validity point of view, self-paced time trial is a better option when monitoring athletes' performance. It is also at an advantage in terms of obtaining a reliable measurement, compared to time to exhaustion protocols (Jeukendrup et al., 1996). Apart from exercise protocol, there are also other factors affecting measurement's reliability, such as exercise mode, participants' sex, and training status (Hopkins et al., 2001).

As it has long been recognized that a self-paced time trial (either constant work or constant duration) is much more reliable than time to exhaustion protocol when assessing athletes' aerobic exercise performance (Jeukendrup et al., 1996), more recently, a self-paced time trial preloaded with a bout of fixed-intensity exercise started to become popular among sports scientists (e.g., Lei et al., 2017). With this "hybrid" protocol, scientists can not only observe participants' physiological responses during steady-state exercise but also assess participants' aerobic exercise performance within the time trial. Previous studies have demonstrated good reliability of preloaded time trial protocols with CV $\leq$ 4.4, regardless of exercise mode (Doyle & Martinez, 1998; Russell et al., 2004; Swell & McGregor, 2008; Driller, 2012). In addition,

although it has been clearly demonstrated that ambient heat stress impairs exercise performance (e.g., Tatterson et al., 2000), it was found that it does not necessarily have a negative effect on the reproducibility of the exercise protocol, when monitoring endurance exercise performance (Marino et al., 2002; Tyler & Sunderland, 2008; Che Jusoh et al., 2015).

Moreover, it is worth noting that almost all the studies summarized above were carried out on males. Untrained females were suspected to be less reliable than untrained males, possibly because they are less active (Hopkins et al., 2001). Nevertheless, the two studies assessing well-trained females aerobic exercise performance demonstrated good reliability (Bishop, 1997; Russell et al., 2004). Bishop (1997) is the first and the only study to specifically look at females in this area. He reported a CV of 2.7% and ICC of 0.97 after observing 20 female athletes performing two repeated 1 h self-paced cycling time trials, which demonstrated high reproducibility. More recently, Russell et al. (2004) recruited four female and four male athletes to complete two preloaded 10 km treadmill runs. They found the protocol extremely reliable with CV of 1.26% (females) and 0.54% (males). However, caution needs to be taken here when interpreting the results considering their small sample size. Furthermore, even though these two studies were conducted on females, Bishop (1997) did not give any information regarding participants' menstrual status. While Russell et al. (2004) scheduled two repeated trials, one trial per menstrual cycle, to control for their participants' menstrual status. Therefore, their results cannot reveal any potential effects of menstrual cycle on the reproducibility of the exercise protocols.

Table 3. The reliability of the time trial protocol used to measure athletes' aerobic exercise (≥30min) performance.

Author(s)	**Exercise mode**	**Exercise protocol**	**Performance measurement**	**CV%**	**r**	**ICC**	**SEM**	**Other**
Jeukendrup et al., 1996	C	TT *6	Time	3.35				
Bishop, 1997	C	1h TT *2	Power output	2.7		0.97	3.4	Female participants only
Schabort et al., 1998	R	1 h TT *3	Distance	2.7		0.90		
Doyle & Martinez, 1998	C/R	Pre-load TT *4	Time	C 3.5 R 4.4	0.93			Two female participants
Marino et al., 2002	C	TT interspersed with sprints under warm condition *3	Distance	1.34		0.93		LoA
Laursen et al., 2003	C	40 km TT *3	Time	0.9	0.96			B&A plot
Russell et al., 2004	R	Pre-load TT *2	Time	M 0.54 F 1.26			M 0.998 F 0.985	Four male and four female participants
Tyler & Sunderland, 2008	R	Pre-load TT in MOD and HOT conditions *6	Time	MOD 2.4 HOT 2.7				
Swell & McGregor, 2008	C	Pre-load TT *4	Power output	3.4				One female participant; B&A plot
Driller, 2012	C	Pre-load TT *3	Power output	0.7		1.00	2.1kJ	
Che Jusoh et al., 2015	C	Pre-load TT *3	Work	3.6		0.96		

C, cycling exercise; **R**, running exercise; **TT**, time trial; **MOD**, moderate condition; **HOT**, hot condition; **CV%**, coefficient of vibration; **r**, correlation coefficient; **ICC**, intraclass coefficient; **SEM**, standard error of measurement; **LoA**, limit of agreement; **B&A plot**, Bland & Altman plot.

2.3.3 Summary

Although it has been reasonably well reported that menstrual/OCP cycle does not affect females' performance under heat stress, there is no protocol which has been shown to be reliable when monitoring females' performance in different environmental conditions. Thereby, an ecologically valid protocol with high reproducibility when performed by female athletes is urgently required. Note that when considering females', it is critical to consider different hormonal status over a menstrual cycle and different menstrual status throughout their life when conducting research on this topic.

2.4 What Affects Female's Core Temperature?

2.4.0 Overview

As there has been more and more females taking part in sports events and performing physical activities at the workplace, females' thermoregulation under heat stress has been relatively well-investigated. Previously, scientists have attempted to explain the variance of core temperature during exercise–heat exposures, through morphological, physiological, functional, and environmental factors. These investigations provide us with insight on how to prevent people from getting heat illness, which is of great importance, especially for females. It has been demonstrated that females are at a higher risk of exertional heat illness compared to males. In addition, the percentage of females that are heat intolerant is higher (Alele et al., 2020). In this section, the candidate reviews the literature with two focuses: females' physiological thermoregulatory responses to exercise-heat stress, and factors that determine the core temperatures during exercise-heat exposures. Male studies are also included in the second part as there is limited research conducted on females.

2.4.1 Physiological Thermal Regulation During Acute Heat Exposure

It is well investigated that females' thermoregulatory responses to acute exercise-heat exposures vary over their menstrual cycle, because of the fluctuations of E_2 and P_4 levels. The EF (usually 1^{st}-7^{th} day of the menstrual cycle) and ML (usually 18^{th}-24^{th} day of the menstrual cycle) phases are mostly investigated, as the differences in ovarian hormones levels are the greatest between these two phases. The EF is characterized by low concentrations of both E_2 and P_4, whereas ML is characterized by moderate concentrations of E_2 and high concentrations of P_4 (Figure 1). It has been shown that E_2 facilitates heat dissipation hence lowers the basal body temperature (Tankersley et al., 1992) while P_4 has the opposite effect (Stachenfeld et al., 2000).

It should not be overlooked that over half of the female athlete population is using the OCP (Rechichi et al., 2009). Therefore, it is also worth mentioning the effects of chronic OCP administration on female's thermoregulation.

2.4.1.1 Eumenorrheic Females with Regular Menstrual Cycle

Several studies have compared females' responses to acute exercise-heat exposure in different menstrual phases. Significant findings reported by these studies are listed in Table 4. It has been well established that the basal body temperature is elevated by ~0.3-0.5 °C throughout the luteal phase (Janse de Jonge, 2003). Eight of these studies, all of which compared the responses between EF and ML, support this conclusion (Stephenson & Kolka, 1985; Kolka & Stephenson, 1989; Kolka & Stephenson, 1997; Tenaglia et al. 1999; Stachenfeld et al., 2000; Janse de Jonge et al., 2012; Lei et al., 2017; Notley et al., 2019a). Nevertheless, Avellini et al. (1980) and Frye & Kamon (1981) found no significant difference in T_{core} between pre- and post-ovulation phase. A study by Garcia et al. (2006) reported that significant differences between pre- and post-ovulation resting core temperatures was only observed in the group of

participants whose serum P_4 levels were higher than 3 ng/mL. In the other group of participants whose P_4 levels were lower than 3ng/mL, body temperature increased after the ovulation, but there was no significant difference in resting T_{core} between menstrual phases. This potentially explains the inconsistent results on T_{core} as the serum P_4 concentration was not measured in those studies. Thus, it is essential to verify the menstrual phase by measuring the serum P_4 levels when conducting female thermoregulatory research especially when considering their menstrual cycle.

There are 3 studies investigating the responses during exercise in humid hot environments (Garcia et al., 2006, Janse de Jonge et al., 2012; Lei et al., 2017). According to the study by Lei et al. (2017), the higher T_{core} in the ML phase during rest and fixed-intensity exercise persisted during self-paced exercise in humid hot environments (29 °C, 81% RH). Whereas, it was not significantly different between menstrual phases in dry heat. The study by Janse de Jonge et al. (2012) reported that the increasing rate of T_{core} was higher in ML during fixed-intensity exercise in humid hot environments (32 °C, 60% RH), compared to EF. However, in the study by Garcia et al., which was also conducted in humid hot environments (32 °C, 80% RH), and used the same exercise protocol, females' T_{core} during exercise in their luteal phase was similar to that in follicular phase, due to their higher SR (Garcia et al., 2006). This is also the only study which reported higher SR in luteal phase, the reason for this discrepancy was suspected to be a result of different testing protocols.

Among these studies, higher T_{skin} was observed in the ML during rest (Tenaglia et al., 1999) and exercise (Kolka & Stephenson, 1988; Tenaglia et al., 1999). Lei et al. (2017) also reported higher end-exercise T_{skin} in the ML phase after exercise in humid hot environments, whereas it was the opposite in dry heat.

Ten out of 20 studies found no difference in thermoeffectors and cardiovascular variables over the menstrual cycle. However, a higher T_{core} threshold of sweating (Stephenson & Kolka, 1985; Kolka & Stephenson, 1989; Stachenfeld et al., 2000) and cutaneous vasodilation (Stephenson & Kolka, 1985) in the ML phase were reported by some studies. This discrepancy could be explained by the fact that in the luteal phase, there is a lower elevation in P_4, sweating and cutaneous vasodilation threshold in physically well-trained females (Kuwahara et al., 2005). The participants in the aforementioned four studies were described as "healthy" or "recreationally active", indicating that their aerobic fitness levels might be lower compared to elite athletic females. After the initiation of sweating, SR (Stephenson & Kolka, 1988; Kolka & Stephenson, 1997; Stachenfeld et al., 2000) and thermosensitivity (the slope of the SR/ T_{core}) have been shown to be similar between menstrual phases (Kolka & Stephenson, 1985; Kolka & Stephenson, 1989; Lei et al., 2017; Notley et al., 2019a). A HR elevation in the luteal phase was also observed during rest (Stephenson & Kolka, 1985) and submaximal exercise, in humid hot environments (Janse et al., 2012). Notably, in the study by Lei et al. (2017), the differences in thermoeffectors and cardiovascular variables during rest and fixed-intensity exercise between environments/menstrual phases was non-existent during self-paced exercise. This suggests that during self-paced exercise, behavioral thermoregulation plays a greater role than physiological thermoregulation. Finally, a study by Stephenson & Kolka (1988) specifically investigated the plasma volume (PV) response during high intensity exercise-heat stress in women over the menstrual cycle. It was observed that PV decreased faster in the follicular phase, but the end exercise PV was similar to M values, due to the higher baseline value in the follicular phase.

2.4.1.2 OCP Users

There are a few studies on this topic, and it is difficult to make a direct comparison between them as different preparations and brands of OCP were used (i.e., monophasic and triphasic). Nevertheless, there are some relatively conclusive findings on this topic. Similar to the luteal phase in a regular menstrual cycle, an elevated T_{core} was observed in the *q*L of the OCP cycle, compared to the *q*EF (during which participants take the placebo pill) (Martin & Buono, 1997; Charkoudian & Johnson, 1997; Rogers & Baker, 1997; Tenaglia et al., 1999). Two studies that compared the T_{core} between day ~5 and day ~20 of OCP use (*quasi* mid/late follicular and *quasi* mid/late luteal phase), also reported an elevated T_{core} in the second half of the OCP cycle (Sunderland & Nevill, 2003; Lei et al., 2019). Researchers tend to agree that the effects of the endogenous hormones persist over OCP cycles (Grucza et al., 1993; Martin & Buono, 1997; Charkoudian & Johnson, 1997; Rogers & Baker, 1997; Tenaglia et al., 1999; Sunderland & Nevill, 2003; Lei et al., 2019). Though interestingly, Lei et al. (2019) reported the same concentrations of both endogenous (measured) and exogenous (by design) hormones in different phases of OCP users. The underlying mechanisms for these T_{core} changes between OCP phases remain unclear. Since blood exogenous hormone levels cannot be easily measured, results need to be interpreted cautiously in these cases (Burrows & Peters, 2007).

Along with the elevated T_{core}, similar to the thermoregulatory shift that happens in the luteal phase, delayed sweating and cutaneous vasodilation threshold were observed in *q*L (pill-on), compared to *q*EF (Grucza et al. 1993; Charkoudian & Johnson, 1997; Rogers & Baker, 1997; Lei et al., 2019). Blunted sudomotor and vasomotor responsiveness during OCP use has also been reported (Martin & Buono, 1997). However, once initiated, thermosensitivity of cutaneous vasodilation and sweating were similar between different OCP phases (Grucza et al., 1993; Charkoudian & Johnson, 1997). Research would suggest that this is not a result of alternations in the body fluid balance (Rogers & Baker, 1997; Stachenfeld et al., 2000) or

peripheral effects on cutaneous vessels (Charkoudian & Johnson, 1997). In fact, it is well accepted that ovarian hormones affect thermoregulatory responses during heat stress through central mechanisms. Hence, although OCP use elicits a shift in sudomotor and vasomotor threshold and responsiveness, their thermosensitivity is not affected.

When comparing chronic OCP users with eumenorrheic females, impaired skin blood flow (Minahan et al., 2017) and sudomotor responses (Grucza et al., 1993; Lei et al., 2019) have been reported. However, exercise performance in OCP users has not been shown to be compromised during exercise in hot environments, compared to those who normally menstruate (Sunderland & Nevill, 2003, Lei et al., 2019).

Table 4. Females' physiology responses to acute exercise-heat exposure over their menstrual / OCP cycle.

Authors	Subjects	Phases	Environment	T_{core}	Sweating
Avellini et al., 1980	Fit women	Pre-OVU/Post-OVU	Humid heat T_{db}: 36 °C T_{wb}: 30 °C	No difference between phases during resting, but higher at 90 min during exercise in post-OVU.	WBSR was similar between phases; sweating onset was delayed in post-OVU but not significant.
Frye & Kamon, 1981	Well-trained females	Pre-OVU/Post-OVU	Dry heat T_{db}/T_{wb}: 48/25 °C	No significant differences were observed between phases.	
Kolka & Stephenson, 1985	Healthy women	F/L	35 °C	Higher in L during resting and exercise.	Higher sweating threshold in L, but thermosensitivity was not significantly different.
Kolka & Stephenson, 1988	Healthy women	F/L	T_{db}=50.4 °C AH=1.61kPa	Higher in L during resting and exercise.	Similar between menstrual phases.
Grucza et al., 1993	Healthy women	F/L *q*F/*q*L	24 °C, 50% RH	Tc were higher in L/*q*L compared to F/*q*F	OC users had lower sweat rate in *q*L compared to non-users in L.
Martin & Buono, 1997	Healthy women	P/N	30 °C, 50% RH	T_c was higher during P compared to N, and the differences became larger as exercise proceeded.	WBSR and LSR were similar between two phases.
Charkoudian & Johnson, 1997	Healthy women	P/N	Passive heating	Baseline T_c was higher in P.	Sweating threshold was higher in P.
Kolka & Stephenson, 1997	Recreationally active females	EF/LF/ML	Clothing with high thermal resistance	End exercise T_c: ML > EF > LF. Change in T_c was similar between menstrual cycle.	WBSR was similar between menstrual phases.
Rogers & Baker, 1997	Healthy women	P/N	22 °C, 39% RH	Higher in P.	Sweating threshold was higher in P.

Tenaglia et al., 1999	Recreationally active females	EF/ML *q*EF/*q*ML	40 °C, 30% RH Wearing protective clothing ensemble	Higher in ML during resting and exercise, similar at the end of exercise. Change in T_c was higher in EF.	No significant difference between phases.
Stachenfeld et al., 2000	Healthy women	EF/ML	35 °C	Higher in ML during resting and exercise.	Higher sweating threshold in ML; no difference in SR.
Stachenfeld et al., 2000	Healthy women	P_4 only or combined E+P_4 use for 4 weeks	35 °C	Higher in P_4 than E+P_4.	Sweating onset and sweat rate were similar across all trials.
Sunderland & Nevill, 2002	Well-trained females	EF/ML	31 °C, 23.1% RH	T_c tended to be higher in L at resting. Similar between F and L and increased at a similar rate during exercise.	Similar between menstrual phases.
Sunderland & Nevill, 2003	Well-trained females	F/L *q*F/*q*L	31 °C, 23.1% RH	Resting and exercise T_c was higher in *q*L. The increasing rate of T_c was higher in *q*F. No difference between non-OC users and OC users.	Estimated WBSR was similar between menstrual/OCP phases.
Garcia et al., 2006	Healthy females	F/L	32 °C, 80% RH	Resting T_c was higher in L, no difference during exercise.	WBSR was higher in L.
Janse et al., 2012	Recreationally active females	EF/ML	32 °C, 60% RH	Higher in ML during resting and submaximal exercise. No difference at exhaustion. Higher increase rate from rest to 45 min in ML.	N.A.
Minahan et al., 2017	Recreationally active females	EF and P	35 °C	Resting T_c was higher in P compared to EF; the difference disappeared as exercise proceeding.	N.A.
Lei et al., 2017	Well-trained females	EF/ML	WBGT 27 °C HUM: 29 °C, 81% RH DRY: 34 °C, 41% RH.	Higher in ML during resting and fixed-intensity exercise; these differences persisted in HUM during self-paced exercise. The rise in T_c was higher in ML.	WBSR, sweat onset threshold and thermosensitivity

					was similar between menstrual phases during self-paced time trial.
Lei et al., 2019	Well-trained females	*q*F/*q*L	Replicated as above	Resting T_c was higher in *q*L than in *q*F, the difference disappeared as exercise proceeding.	WBSR, sweat onset threshold and thermosensitivity was similar between OCP phases during self-paced time trial.
Notley et al., 2019a	Recreationally active females	EF/LF/ML	40 °C, 15% RH	Baseline T_c was higher in ML compared to EF and LF, however they were not different during exercise.	N.A.

F, follicular phase; **L**, luteal phase; **EF**, early follicular phase; **LF**, late follicular phase; **ML**, mid luteal phase; ***q* (E)F**, *quasi*-(early) follicular phase; ***q*(M)L**, *quasi*-mid-luteal phase; **Pre-OVU**, pre-ovulation; **Post-OVU**, post-ovulation; **T_c**, core temperature; **E**, estrogen; **P_4**, progesterone; **OC(P)**, oral contraceptive (pills); **P/N**, pill on/off phase of the oral contraceptive cycle; **SR**, sweat rate; **WBSR**, whole body sweat rate; **WBGT**, Wet Bulb Globe Temperature; **T_{db}**, dry bulb temperature; **T_{wb}**, wet bulb temperature; **AH**, absolute humidity; **RH**, relative humidity

2.4.2 Explaining Core Temperature During Exercise

To diminish people's risk of getting heat injury and improve their productivity under heat stress, scientists have been investigating factors that affect people's heat stress responses. To elucidate the role of different factors during heat exposures researchers rely on an integrated approach to explain the variance of individual heat stress responses (e.g., T_{core}). To do so they use a heterogenous samples and perform correlation/regression analysis with independent variables of interest.

Factors related to heat stress responses can be classified into four categories: morphological (body mass, surface area and % fat etc.), physiological (metabolic rate or heat production, whole-body or local sweat rates etc.), functional (aerobic fitness and power) and environmental (ambient temperature and humidity). Amongst all the factors, morphological characteristics are the most frequently investigated. It is well established that higher body mass has a positive effect on heat tolerance (Havenith et al., 1998). Currently, there are no conclusive results on the effect of body surface area and body surface area to mass ratio (A_D:mass) (Foster et al., 2020). Higher aerobic fitness level (indicated by $\dot{V}O_2max$) is another factor that has been proven to have a positive effect on heat tolerance (Foster et al., 2020). Scientists have further demonstrated that the effects of individual characteristics on heat stress responses are dependent on environmental conditions (Havenith et al., 1998) and metabolic heat production (Havenith et al., 1998; Cramer & Jay, 2015; Notley et al., 2019b). As such, heat production/power output expressed in Watts per kilogram have been found to be the strongest factor when predicting T_{core} (Cramer & Jay, 2014; Gibson et al., 2017).

However, once again most of the studies in this area were completed in male-only or a mix of males and females participant cohorts. The only study looking at gender difference demonstrated that during exercise at low/moderate intensities in humid/dry heat conditions,

body fat percentage, A_D:mass, sweating set point and $\dot{V}O_2$max explain ~60% of the variance in T_{core} in males and females. There was also no difference between males and females if $\dot{V}O_2$max and anthropometric variables were included in the regression analysis (Havenith & Middendorp, 1990). More recently, the only study that has used a female-only sample demonstrated that during exercise in dry heat, the influence of aerobic fitness on females' heat loss capacity is dependent on metabolic heat production (Lamarche et al., 2017). This result is in agreeance with what has been found in males (Cramer & Jay, 2015).

So far, no gender difference has been found when predicting heat stress responses with related individual factors. However, none of the previous studies have accounted for the females' menstrual cycle. The fluctuation of ovarian hormones leads to a series of thermoregulatory alterations. Therefore, to elucidate the role of E_2 and P_4 when explaining the variance in females' heat stress responses, it is necessary to include these reproductive hormone levels when performing correlation/regression analysis. It is still worth mentioning that Notley et al. (2019b) evaluated females' whole-body heat loss during exercise in dry heat with the direct calorimetry method, and no difference was observed between different menstrual phases. However, the participants in their study were non-endurance-trained, thereby the conclusion should not be applied to well-trained/elite athletic females.

2.4.3 Summary

Investigating females' thermoregulation over the menstrual cycle is not something new, the thermoregulatory effects of ovarian hormones have been relatively well-demonstrated. However, when predicting heat stress responses during exercise under heat stress, ovarian hormones have not been taken into consideration. Therefore, studies are required to develop a model predicting T_{core} specifically for females, which accounts for their different menstrual and hormonal status.

Chapter Three

3.0 Research Aims and Hypotheses

The purpose of this thesis is to: 1) clarify whether environmental heat stress and menstrual phase have any effect on female athletes' iron metabolism; 2) assess whether environmental heat stress and ovarian hormones moderate the reliability of the exercise protocol used to monitor female athletes' performance; 3) identify the potential effect of ovarian hormones in terms of explaining the power output and peak T_{core} during exercise in thermally stressful environments. To address these research questions, firstly, comparisons of exercise performance and iron-related parameters in different ambient conditions and menstrual phases will be conducted (General Aim I). However, this raises the following question: does the change in measured performance truly reflect an intervention effect beyond the measurement error? Thus, General Aim II is to retrospectively evaluate the reproducibility of the pre-loaded work trial protocol used on female athletes. Participants with varying hormonal and ovulatory status are included for a representative response of the female athlete cohort. The trials used also covered a range of different ambient conditions. Finally, in order to maximise female athletes' performance and minimise their risk of exertional heat injury and illness during acute heat exposures, it is necessary to elucidate the role of ovarian hormones. Ovarian hormone levels along with other previously investigated factors, will help determine the peak T_{core} response and power output profile during exercise in thermally stressful conditions (General Aim III). Taken together, this thesis fills a much-needed gap in the female athlete specific literature in heat stress responses.

3.1 Aims

The primary objective of General Aim 1 is to determine the acute effects of environmental heat stress on post-exercise iron metabolism of female athletes in different menstrual cycle phases. Specifically, to clarify whether a greater ambient temperature amplifies the detrimental impact of exercise on iron status by eliciting a higher inflammatory and hepcidin response (**Chapter Five**). Following this, the objective of General Aim II is to ascertain the measurement error in performance using the pre-loaded work trial by retrospectively analysing data collected from female athletes with different hormonal profiles (**Chapter Six**). Finally, General Aim III retrospectively investigated the contribution of different factors (morphological, physiological, functional, and environmental) explaining the individual variation of peak core temperatures and power output during exercise in thermally stressful conditions (**Chapter Seven**).

General Aim I, General Aim II and General Aim III can be split into more specific objectives listed below:

General Aim I

1.) Investigate whether environmental heat stress during exercise amplifies the inflammatory response and subsequently triggers a greater rise in hepcidin post-exercise;
2.) Investigate whether the effect of environmental heat stress on iron metabolism differs between menstrual phases.

General Aim II

1.) Evaluate the reproducibility of a pre-loaded self-paced work trial when performed by athletic females with different ovulatory and hormonal status in different ambient conditions;

2.) Add athletic female-specific exercise performance norms to the literature.

General Aim III

1.) Investigate which factors determine female athletes' peak T_{core} during exercise-heat stress;

2.) Investigate which factors determine female athletes' work output during heat stress.

3.2 Hypotheses

1) Environmental heat stress will impair female athletes' performance and elicit a greater inflammatory response. This will result in a greater increase in hepcidin, compared to moderate temperature conditions (**Chapter Five**). No hypothesis is made regarding menstrual cycle phase and iron status parameters as there are limited and conflicting findings.

2) The pre-loaded 30 min self-paced work trial is a reliable protocol to monitor female athletes' exercise performance under different ambient conditions, regardless of their hormonal and ovulatory status (**Chapter Six**).

3) The ovarian hormones, along with other factors that have been investigated, contribute significantly to the individual variance in T_{core} when female athletes' exercise under heat stress (**Chapter Seven**).

Chapter Four

4.0 General Methodology

The purpose of this chapter is to provide a brief overview of the experimental procedures and protocols shared by all experimental chapters included in this thesis. Detailed methods of each study are further described in the following experimental chapters (**Chapter Five** to **Seven**). As **Chapters Six** and **Seven** included data previously collected in our lab, all relevant methods of the original studies were included in corresponding experiment chapters. Irrelevant measures which are unnecessary for our retrospective analysis were not included in this thesis. Due to the retrospective nature of the second and third studies (**Chapters Six** and **Seven**), this chapter specifically focusses on clarifying which participants/trials were included in each study.

4.1 Overview of the Experimental Protocol

A total of 36 aerobically trained female athletes were recruited for the purposes of this thesis (Table 5). Among them, 10 participants were taking OCPs, whilst the rest were further identified as ovulatory/anovulatory by verification of ovarian hormone concentrations. After the preliminary tests, participants completed two experimental trials in their *q*F and *q*L phases respectively, in two of warm-dry (DRY), warm-humid (HUM), moderate (MOD) or hot-humid (HOT) ambient conditions. Each experimental trial consisted of a pre-loaded fixed-intensity warm-up followed by a 30 min self-paced time trial (Figure 2).

Table 5. Participants anthropometric characteristics. Data are expressed as mean ± SD.

Characteristics	Mean (SD)	Min	Max
Age (y)	32 (9)	19	51
Height (cm)	166 (7)	148	179
Mass (kg)	64 (8)	46	82
A_D (m^2)	1.70 (0.12)	1.37	1.98
A_D:mass	0.027 (0.002)	0.023	0.030
% fat	23 (6)	13	37.2
$\dot{V}O_2max$ ($L \cdot min^{-1}$)	3.4 (0.6)	2.3	5.0
$\dot{V}O_2max$ ($mL \cdot min^{-1} \cdot kg^{-1}$)	53 (9)	37.3	72.6
Wmax (W)	276 (35)	225	392
Wmax ($W \cdot kg^{-1}$)	4.4 (0.6)	3.0	5.7
Training history (y)	6.3 (3.9)	1	16

A_D, Dubois body surface area; **$\dot{V}O_2max$**, maximal rate of O_2 consumption; **Wmax**, peak aerobic power. Values are Mean (SD).

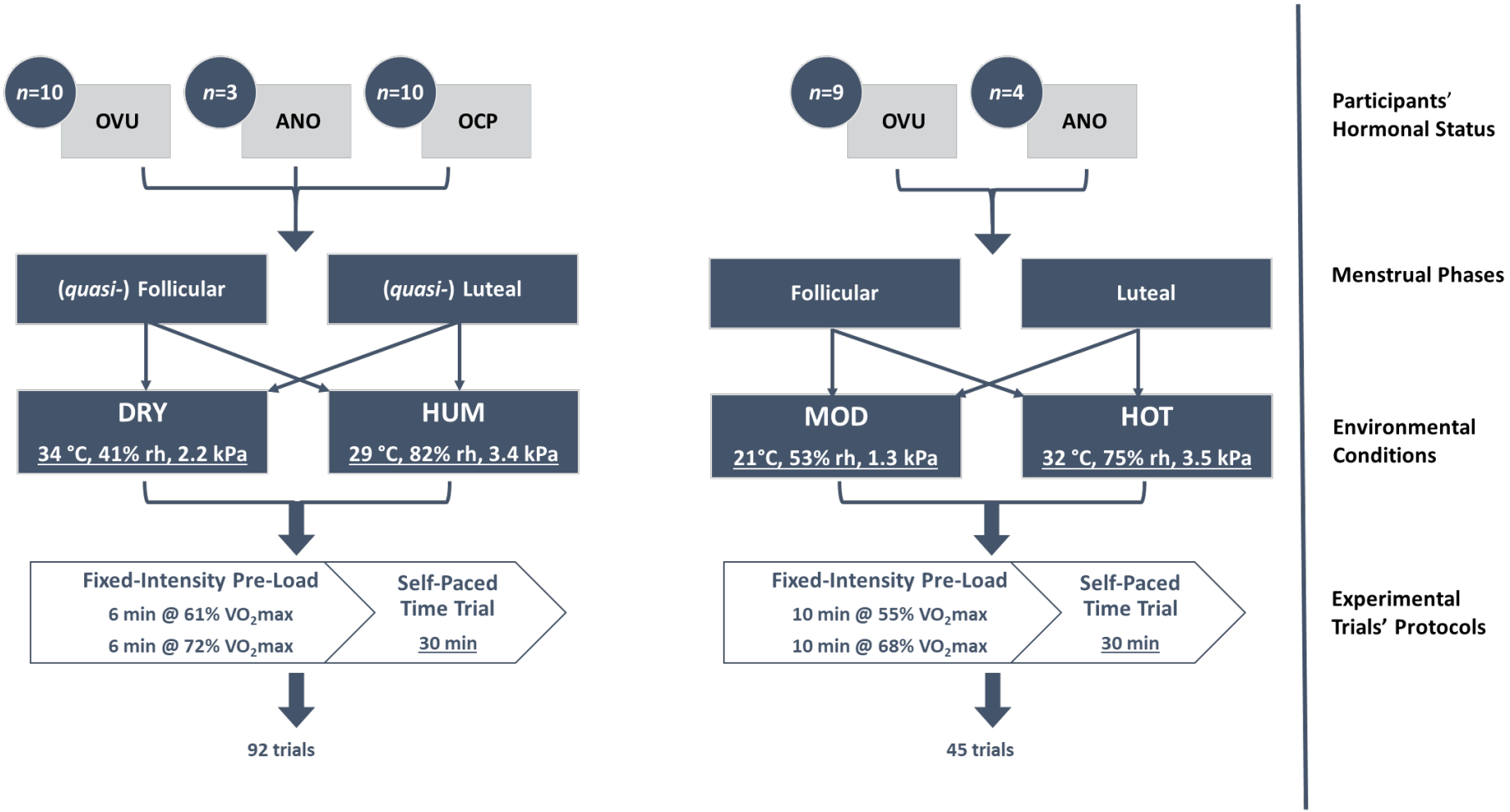

Figure 2. Diagram of experimental overview. Ovulatory (OVU), anovulatory (ANO) and oral contraceptive pill (OCP) users performed trials in their (*quasi*-) early follicular (EF) and/or mid-luteal (ML) phases in warm-dry (DRY) and warm-humid (HUM) or moderate (MOD) and hot (HOT) environmental heat. Note: some participants did not complete all 4 trials due to scheduling difficulties and drop-out.

4.2 General Aim I

To achieve General Aim I, thirteen aerobically trained females were recruited (right panel in Figure 2). Participants were asked to complete two experimental trials in their EF and ML phases respectively, in either MOD/HOT conditions (upper panel in Figure 3). All experimental trials used the identical exercise protocol, the only difference was the ambient conditions. Nine participants were retrospectively verified to have ovulated, with 8 of them completing all four trials. Thereby, considering the purpose of General Aim I, data of n=8 were included for analysis.

As indicated in the lower panel Figure 3, the experimental trial started with a two-stage warm-up at 100 W and 125 W, 10 min each. This was immediately followed by a 30 min self-paced work trial. Venous blood samples were collected before, immediately after, and 3 hours post exercise. For a detailed description of the methods, please refer to **5.2**.

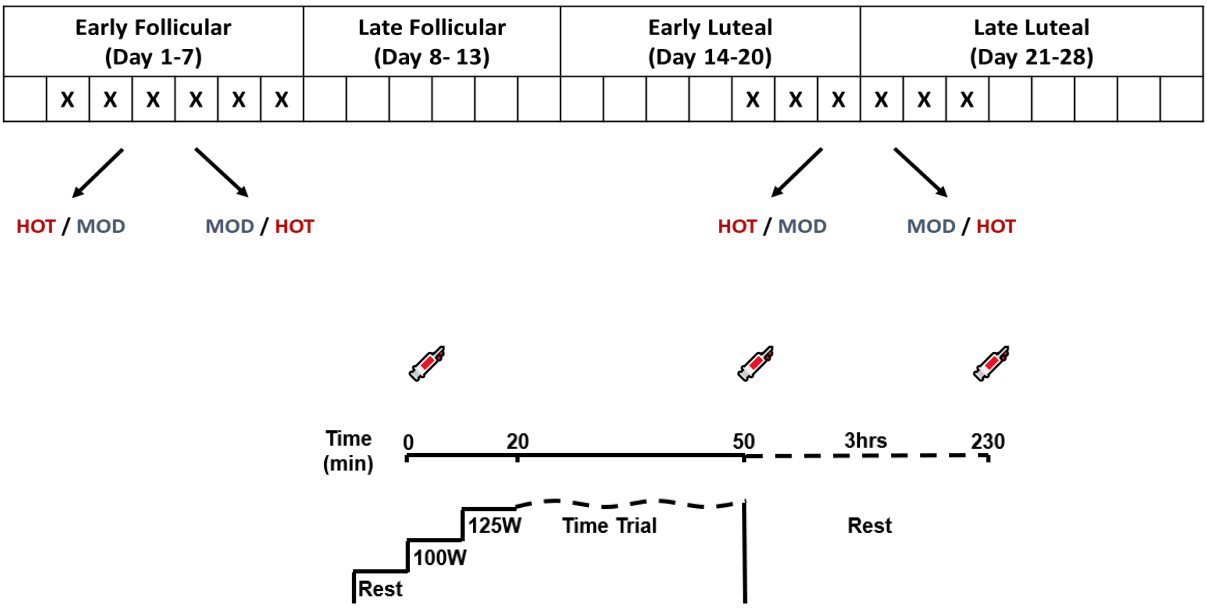

Figure 3. Schematic diagram of General Aim I study design and experimental procedure.

4.3 General Aim II

To achieve General Aim II, retrospective analysis of data collected for three research studies in our laboratory was completed. In addition to the participants from General Aim I, another 23 participants from two previous studies were included (left panel in Figure 2). Evidence from previous studies, states that ambient heat stress has a significant effect on female athletes' performance whilst menstrual phase does not (Lei et al., 2017). The reliability analysis was based on the two trials completed under the same ambient conditions in different menstrual phases. Thereby, data from participants who completed both trials at least under one type of ambient condition were included in **Chapter Six**. This gave us a total of 130 trials, performed by 33 participants to analyze for this study.

The study design of the two previous studies was identical to what has been described in **4.2**. Except trials occurred in their *q*EF/*q*ML for OCP users (Figure 4) and different environmental conditions (humid/dry heat instead of moderate/hot) were applied. The experimental trial started with a 12 min warm-up instead of 20 min, at 125 W and 150 W, each of 6 min. Venous blood samples were only collected before the start of the experimental trials.

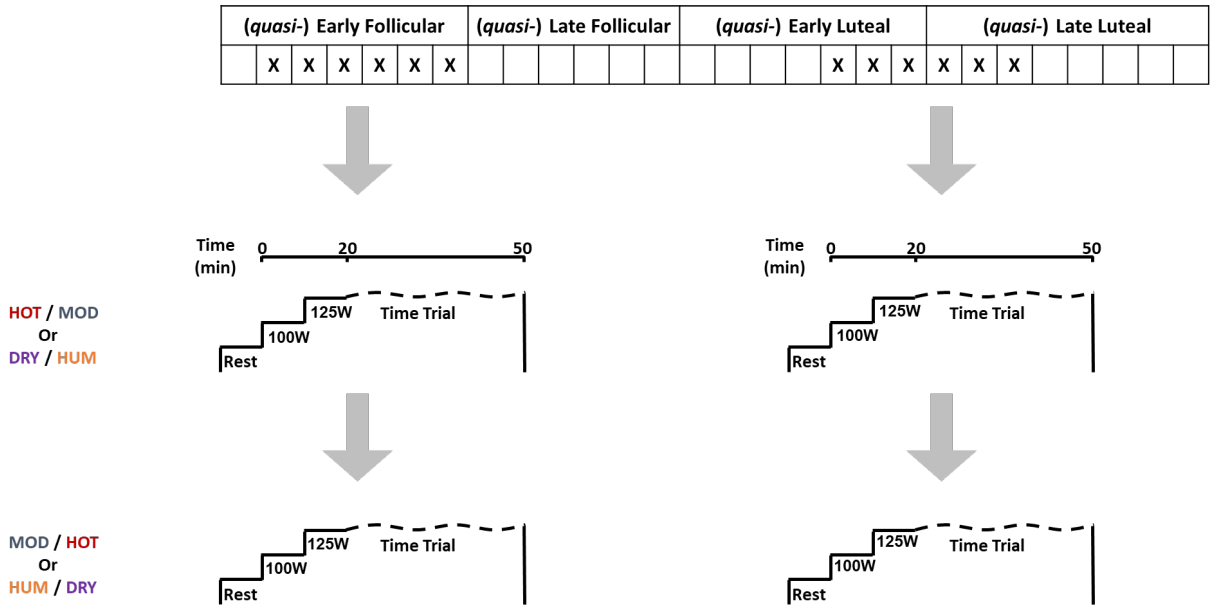

Figure 4. Schematic diagram of General Aim II study design and experimental procedure.

4.4 General Aim III

Similarly, to that explained in **4.3**, to achieve General Aim III, a retrospective analysis of the data from the same studies, but different experimental trials were included due to a different research purpose. As illustrated in **Chapter Three**, with a focus on explaining the risk for exertional heat strain in female athletes exercising under heat stress, only trials completed in hot conditions were included for analysis. A total of 115 trials performed by 36 participants (indicated as HOT, DRY, and HUM in Figure 5) was included for this analysis. Study design and experimental protocols are identical to those described in **4.3** and are illustrated in Figure 5; for more detailed descriptions, please refer to **Chapter Seven**.

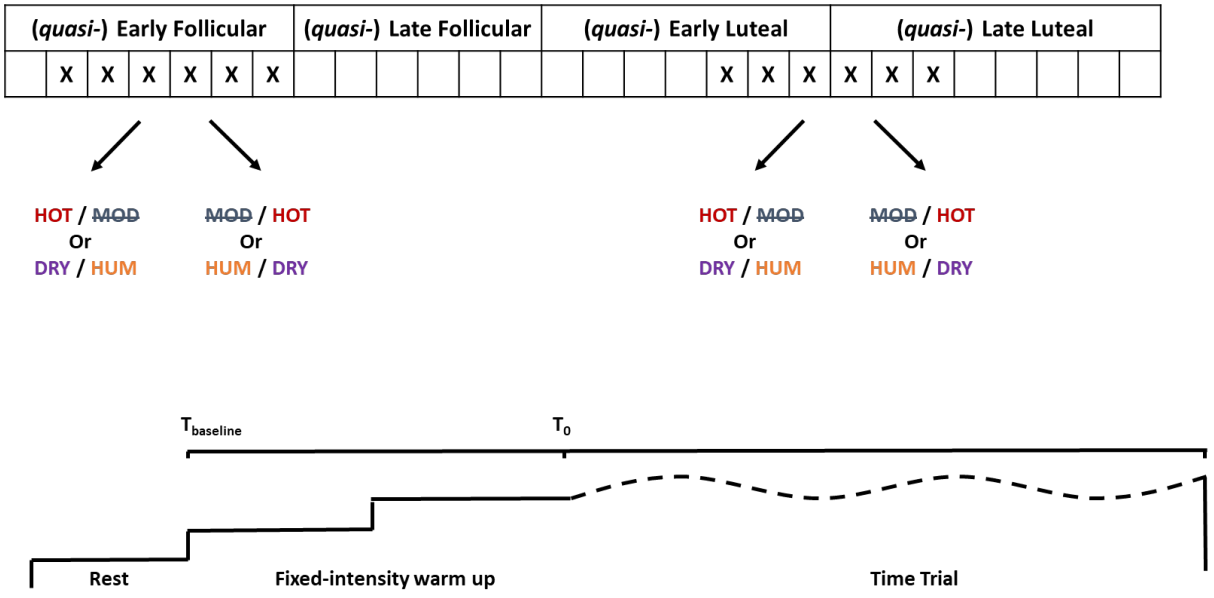

Figure 5. Schematic diagram of General Aim III study design and experimental procedure.

Chapter Five

5.0 Menstrual Phase and Ambient Temperature Do Not Influence Iron Regulation in the Acute Exercise Period

The formatting of this chapter is consistent with the accepted publication: Zheng, H., Badenhorst, C. E., Lei, T. H., Liao, Y. H., Che Muhamed, A. M., Fujii, N., Kondo, N., & Mündel, T. (2021). Menstrual phase and ambient temperature do not influence iron regulation in the acute exercise period. *Am J Physiol Regul Integr Comp Physiol,* 320(6):R780-R790.

Abstract

The current chapter investigated whether ambient heat augments the inflammatory and post-exercise hepcidin response in women, and if menstrual phase and/or self-pacing modulated these physiological effects. Eight trained females (age: 37±7 y; $\dot{V}O_2$max: 46±7 mL·kg^{-1}·min^{-1}; peak power output: 4.5±0.8 W·kg^{-1}) underwent 20 min of fixed-intensity cycling (100W and 125 W) followed by a 30-min work trial (≈75% $\dot{V}O_2$max) in a moderate (MOD: 20±1 °C, 53±8% relative humidity) and warm-humid (WARM: 32±0 °C, 75±3% relative humidity) environment in both their early follicular (days 5±2) and mid-luteal (days 21±3) phases. Mean power output was 5±4 W higher in MOD than in WARM (p=0.02) trials such that the difference in core temperature rise was limited between environments (-0.29±0.18 °C in MOD, p<0.01). IL-6 and hepcidin both increased post-exercise (198% and 38%, respectively), however, neither were affected by ambient temperature or menstrual phase (all p>0.15). Multiple regression analysis demonstrated that the IL-6 response to exercise was explained by leukocyte and platelet count (r^2=0.72, p<0.01) and the hepcidin response to exercise was explained by

serum iron and ferritin (r^2=0.62, p<0.01). During exercise participants almost matched their fluid loss (0.48±0.18 kg·h^{-1}) with water intake (0.35±0.15 L·h^{-1}) such that changes in body mass (-0.3±0.3%) and serum osmolality (0.5±2.0 mOsm·kg^{-1}) were minimal or negligible, indicating a behavioral fluid-regulatory response. These results indicate that trained, iron sufficient women suffer no detriment to their iron regulation in response to exercise with acute ambient heat stress or between menstrual phases on account of a performance-physiological trade-off.

5.1 Introduction

Iron deficiency is the most common micronutrient disease with a global prevalence estimated at ≥15% (World Health Organization, 2020). When compared to males, females have a higher prevalence of iron deficiency due to suboptimal iron intake and menstrual blood loss, with the largest population group affected being women of reproductive age (World Health Organization, 2020). Compromised iron status is frequently observed in female athletes, especially endurance. Prevalence of iron deficiency for those women exercising regularly (15-35%) is reported to be at least three times greater than their male counterparts (3-11%), and substantially higher than in the general population (10-14%) (Weaver et al., 1992; Sim et al., 2019). For these exercising women, iron depletion is a consequence of several exercise-associated factors including hemolysis, hematuria, gastro-intestinal bleeding and sweating (Weaver et al., 1992; Beard & Tobin, 2000), and dietary factors such as suboptimal iron intake, likely associated with low energy intake (Castell et al., 2019; Sim et al. 2019). However, over the last decade research has focused on the exercise-induced response of the liver-derived iron regulatory hormone, hepcidin, in the post-exercise period (Peeling et al., 2008). This key regulator of iron status ensures homeostatic regulation of iron in response to systemic iron stores, by degrading the iron-export protein ferroportin located in the intestine, on macrophages

and hepatocytes, thereby reducing dietary iron absorption, recycling from senescent erythrocytes, and sequestering of iron in cells (Nemeth et al., 2004a; Nemeth et al., 2004b). Exercise acutely increases hepcidin with a peak observed at 3h post-exercise (Reeling et al., 2009; Newlin et al., 2012), with the primary mediator of this upregulation being the inflammatory cytokine IL-6 (Nemeth et al., 2004a; Banzet et al., 2012), and the magnitude of the hepcidin response mediated by the individual's current iron stores (Peeling et al., 2014).

The increase in IL-6 during and immediately following a bout of exercise is part of the acute phase/inflammatory response characterized by a leukocytosis and cytokinemia alongside the release of other acute phase proteins (e.g., C-reactive protein) (Pedersen et al., 2000; Walsh et al., 2011). The magnitude of the inflammatory response is attributed to both the duration and intensity of the exercise secondary to the hormonal profile due to neuroendocrine-immune crosstalk (Pedersen et al., 2000; Walsh et al., 2011). The primary ovarian steroids estrogen and progesterone, which fluctuate between menstrual phases, have been shown to exert an influence on the inflammatory response in the exercise period. Previous research has demonstrated a greater leukocytosis following 90 min of cycling exercise at intensities of 50-65% maximal O_2 uptake ($\dot{V}O_2max$) during the luteal compared to the follicular phase (Timmons et al., 2005; Hashimoto et al., 2014). However, of these two studies only Timmons et al. (2005) reported a change in IL-6 concentrations following exercise that was greater in eumenorrheic than oral contraceptive pill users in the luteal phase, a time when endogenous progesterone and estrogen were higher in the former. In a recent study, females running at 75% maximal aerobic speed during the luteal phase produced significantly greater IL-6 following exercise when compared to the post-exercise period in the follicular phase (Barba-Moreno, et al., 2020). This limited research indicates that during the luteal phase, when estrogen and progesterone are raised, the inflammatory/IL-6 response to exercise appears to be amplified. However, to the candidate's knowledge only one study has determined whether this augmented

response might influence iron metabolism in a thermoneutral environment (Barba-Moreno, et al., 2020). Therefore, further investigation into whether the phase of the menstrual cycle influences the acute phase response to exercise, and whether this mediates any effect on hepcidin and iron metabolism, is required.

Ambient heat stress has been shown to exacerbate the IL-6 response to exercise. For example, when heat stress is applied via air or water the greater rise in core body temperature (T_{core}) results in augmented concentrations of hypothalamic-pituitary-adrenal hormones that in turn cause greater leukocytosis and an increased IL-6 response in exercising men (Rhind et al., 1999; Rhind et al., 2004; Mündel et al., 2010). However, whether the combination of exercise and heat stress affects hepcidin and iron metabolism through its effect on IL-6 has not been investigated. It should be noted that only one study has investigated the IL-6 response to exercise in the heat in women. Larsen et al. (2018) had women in their follicular phase cycle in 22 °C or 35 °C and observed neither an increase due to exercise nor heat stress. It is probable that a threshold for an exponential increase in hypothalamic-pituitary-adrenal hormones required to trigger the inflammatory response, including IL-6, was not reached (Radomski et al., 1998) because of an insufficient heat load (combined metabolic and environmental). Additionally, the females were not tested in the post-ovulatory period when T_{core} is regulated higher and the above-mentioned changes in estrogen and progesterone have exerted their effect.

One final consideration is that all above-mentioned studies have utilized exercise protocols that are fixed intensity in nature, whereby the physiological responses are essentially ‘clamped’. Not only does this method lack face-validity where exercisers can, and expect to, vary their intensity especially during competitions, but during exercise with ambient heat stress this denies the exerciser of their most effective and powerful form of temperature regulation: behavior (Parsons, 2014). Compared to autonomic thermoregulatory responses (e.g., eccrine sweating or changing cutaneous vasomotor tone), behavior’s capacity is limitless (Benzinger

et al., 1969). For example, (behaviorally) reducing power output minimizes the physiological/thermoregulatory strain women encounter under more thermally challenging ambient heat at the expense of exercise performance (Lei et al., 2017, Lei et al., 2019).

Due to the very limited, and partially conflicting, female-specific literature a hypothesis-driven study was deemed inappropriate, and an exploratory approach taken. However, by extrapolating the literature from studies in men the following was speculated:

1. Data using fixed-intensity exercise in men indicates that a greater ambient temperature during exercise would cause greater thermoregulatory strain and increased inflammation (i.e., leukocytes and IL-6) with a proportionate rise in hepcidin.
2. Data using variable-intensity exercise in men indicates that a greater ambient temperature during exercise would cause greater reductions in exercise intensity to minimize the above-mentioned physiological strain but at the expense of performance.

There is insufficient data to determine whether a greater ambient temperature during exercise would interact with the menstrual cycle i.e., during EF characterized by a net iron loss due to menses, and/or during ML when T_{core} and the inflammatory response is increased.

5.2 Methods

5.2.1 Ethical Approval

This study was approved by the Massey University Human Ethics Committee (Southern A) and performed in accordance with the latest revision of the *Declaration of Helsinki*, with each participant providing informed, written consent.

5.2.2 Participants

An *a priori* power analysis determined that based on conventional α (0.05) and β (0.80) values, and an effect size of 0.62 as in Lei et al. (2017), a minimum of five participants was required. However, to allow comparison to Lei et al. (2017, *n*=10 following exclusion of *n*=3 due to anovulation), thirteen healthy and eumenorrheic females that cycled regularly for this study were recruited. All self-reported a regular menstrual cycle 21-35 days in length for the three months prior to the study, and no hormonal contraception or iron supplementation for greater than six months. Unfortunately, due to *a posteriori* determination of an anovulatory cycle (*n*=3) or disclosure of iron supplementation (*n*=1), and incompletion due to factors unrelated to the study (*n*=1) data are reported for *n*=8 (see Table 6). No participants presented as iron deplete/deficient (i.e., serum ferritin <30 $\mu g \cdot L^{-1}$) to minimize a non-response for hepcidin in the post-exercise measurement (Peeling et al., 2014).

Table 6: Participant characteristics upon entry to the study. All variables were collected during the early-follicular phase of a menstrual cycle, with blood-borne measures taken in a rested state i.e., morning, non-exercised.

Participant	**Age (y)**	**Height (cm)**	**Weight (kg)**	**Body Fat (%)**	**BSA (m^2)**	**$\dot{V}O_2max$ ($L \cdot min^{-1}$)**	**Wmax (W)**	**Fer ($\mu g \cdot L^{-1}$)**	**Tf ($g \cdot L^{-1}$)**	**Fe ($\mu mol \cdot L^{-1}$)**	**Sat (%)**	**IBC ($\mu mol \cdot L^{-1}$)**
1	25	157	51	18	1.49	2.5	236	45	2.9	13	19	67
2	45	179	67	20	1.84	2.7	256	51	2.5	21	38	56
3	32	158	58	22	1.58	3.2	294	43	2.7	14	23	62
4	43	159	72	37	1.75	2.6	232	125	2.2	20	40	50
5	30	179	69	19	1.87	3.9	392	74	2.3	11	21	53
6	43	168	63	19	1.72	3.2	313	58	2.5	21	36	57
7	39	164	66	24	1.72	2.9	268	34	3.0	19	28	69
8	38	166	58	19	1.65	2.3	250	41	2.6	13	22	59
Mean±SD	**37±7**	**166±9**	**63±7**	**22±6**	**1.70±0.13**	**2.9±0.5**	**280±53**	**59±29**	**2.6±0.3**	**17±4**	**28±8**	**59±6**

BSA, body surface area; **Fe**, iron; **Fer**, ferritin; **IBC**, iron-binding capacity; **Sat**, iron saturation; **Tf**, transferrin; **$\dot{V}O_2max$**, maximal rate of O_2 uptake; **Wmax**, maximal aerobic power.

5.2.3 Experimental Overview

Data collection was conducted excluding the Southern Hemisphere summer (i.e., March-November) where the average daily temperature did not exceed 22 °C, nor had participants spent any time in a warmer climate for at least one month prior to the study. All participants attended the laboratory on six occasions: (1) preliminary submaximal and maximal aerobic capacity test, (2) experimental familiarization, (3-6) experimental trials. The four experimental trials were a full crossover of menstrual phase (EF and ML) and ambient temperature (moderate [MOD, 20±1 °C, 53±8% relative humidity] and warm [WARM, 32±0 °C, 75±3% relative humidity]). To best deduct the order effect, the order of the trials was randomized and counterbalanced except the order of the ambient temperature was consistent in different phases within participants. Experimental trials were conducted in the morning (±1 h) and following >24 h of dietary and exercise control. Each trial consisted of 20 min of fixed intensity 'warm-up' immediately followed by a 30 min self-paced work trial where only percentage of time elapsed (every 20% or 6 min) was provided to the participant. All exercise was performed on an electronically braked cycle ergometer (Lode Excalibur, Groningen, The Netherlands), with handlebars, seat height and pedal preference standardized according to individual preference. Participants completed the preliminary testing and familiarization (separated by 3-7 days) during the follicular phase, with half of the participants starting their experimental trials the following luteal phase (i.e., 14 days later) and the other half the following follicular phase (i.e., 28 days later), with within-phase experimental trials of the different ambient condition separated by 3-4 days (see *Menstrual Cycle Phase and Ambient Conditions* below).

5.2.4 Preliminary Testing and Familiarization

All preliminary testing was conducted in the EF phase of each participant's menstrual cycle to minimize the potential effects of menstrual phase on their physiological and performance

responses during the tests. Following anthropometric measurements (height, weight, body composition), a 24-min steady-state submaximal exercise test was conducted in a temperate laboratory environment (18-22 °C) with a fan-generated airflow of 19 $km \cdot h^{-1}$ facing participants. The submaximal exercise test consisted of four consecutive six-minute stages with power outputs of 100 W, 125 W, 150 W and 175 W at comfortable but constant cadence. O_2 consumption was measured during the last two minutes of each stage. Following 10 min rest from the submaximal test, a $\dot{V}O_2max$ test was performed. The initial workload began at 100 W and increased by 25 W every minute, until volitional exhaustion. The exercise intensity during the self-paced exercise was based on 75% of an individual's $\dot{V}O_2max$, which was derived from the linear relationship between the power output and the O_2 consumption during both the steady-state submaximal exercise test and maximal aerobic capacity test. Following at least 24 hours rest from the preliminary session, a familiarization trial was conducted to ensure all participants were familiar with the testing procedures and to minimize the learning effect during trials. This trial replicated entirely the experimental trials outlined below.

5.2.5 Dietary and Exercise Control

Diet and physical activity during the 48 hours prior to the first experimental trial were recorded and participants instructed to repeat these for the following trials. The day of and prior to any experimental trial was marked by abstinence from alcohol, exercise, and only habitual caffeine use (as abstinence would confound from withdrawal effects). This dietary and exercise control minimized variation in pre-trial metabolic state. Fluid intake was encouraged to ensure a euhydrated state i.e., <1.020 (Sawka et al., 2007).

5.2.6 Menstrual Cycle Phase and Ambient Conditions

Participants were tested during the EF and ML phases, to maximize differences in estrogen and progesterone concentrations and permit comparison with results of previous studies (Timmons et al., 2005; Hashimoto et al., 2014; Lei et al., 2017; Barba-Moreno et al., 2020). Testing was scheduled using the three-step method (Allen et al., 2016) whereby self-reported menses onset and urinary luteinizing hormone testing (EasyCheck® Ovulation Test, Phoenix Medcare Ltd, Auckland, New Zealand) prospectively identified EF and ML, whilst measurement of serum 17β-estradiol and progesterone retrospectively confirmed ML. To ensure ovulation had occurred, participants with progesterone <5 $ng \cdot ml^{-1}$ were excluded from the final analyses (Lei et al., 2019; Che Muhamedet al., 2016). Testing occurred on days 3±1 and 7±2 (EF), and 20±2 and 22±3 (ML) following start of menses.

The ambient conditions for MOD were set to mimic standard laboratory conditions without ambient heat stress, whilst WARM was set to mimic the likely conditions awaiting female athletes at the forthcoming Tokyo Summer Olympics (Garrett et al., 2019), equivalent to a wet-bulb globe temperature (WBGT) of 29 °C.

5.2.7 Experimental Procedure

These four trials were conducted in the same environmental chamber with a fan-generated airflow of 19 $km \cdot h^{-1}$. Upon their arrival at the laboratory (by 09:00), participants voided, producing a urine sample to confirm a urine specific gravity <1.020 (42; 1.012±0.007). Following this, nude body weight was recorded and participants self-inserted a rectal thermistor 12 cm beyond their anal sphincter. A blood sample was obtained from an antecubital vein after participants had rested seated for 15 min. Participants entered the environmental chamber wearing only cycling shorts and top, shoes and socks. Participants rested seated on the ergometer for 20 min during which they were instrumented, and baseline measurements were

recorded. Participants then completed 10 min of cycling at each of 100 and 125 W, to allow sufficient warm-up and fixed-intensity responses to be recorded. Physiological measurements taken during the final 2 min of each intensity included expired gas, heart rate (HR) and rating of perceived exertion RPE), whilst rectal (T_{rec}) and skin (T_{sk}) temperatures were measured continuously. Immediately on completion of the 125 W bout, the ergometer was set to linear mode and participants were instructed to perform as much work as possible over 30 min. During this 30 min self-paced period, work completed (kJ), HR and RPE were recorded every 6 min, whilst T_{rec} and T_{sk} were measured continuously and tap water at 20 °C was provided to drink *ad libitum* throughout to minimize dehydration. Total work completed was used as our performance criterion, whereas the time profile of power output was used to determine pacing (Lei et al., 2017). After the completion of the 30 min self-paced exercise, a post-exercise blood sample was taken followed by the participant towel-drying and the recording of nude weight, before a final blood sample 3 h following the end of exercise.

5.2.8 Measurements

5.2.8.1 Anthropometric

Participant height and weight were measured using a stadiometer (Seca, Germany; accurate to 0.1cm) and scale (Jadever, Taiwan; accurate to 0.01 kg), from which surface area was estimated (Dubois & Dubois, 1916). Body composition was measured using multi-frequency bioelectrical impedance analysis (InBody 230, Korea) using a standard procedure (Kyle et al., 2014).

5.2.8.2 Cardio-Respiratory

Expired respiratory gases were collected from a mixing chamber and analyzed for O_2 consumption using an online, breath-by-breath system (VacuMed Vista, Turbofit, Ventura, CA, USA) using a 30-s average. This system was calibrated before each trial using a zero and

β-standard gas concentrations, and volume (VacuMed 3L Calibration Syringe). HR was recorded from the detection of R–R intervals (Polar Vantage XL, Polar Electro, Kempele, Finland).

5.2.8.3 Body Temperatures and Sweat Loss

T_{core} was indexed from T_{rec} measured with a rectal thermistor (Covidien Mon-a-Therm, USA; accurate to 0.1 °C). $\bar{T}_{sk}$ was measured at four sites using calibrated skin thermistors (Grant Instrument Ltd, Cambridge, UK; accurate to 0.2 °C) secured on the calf, thigh, chest, and forearm using surgical tape (3M Healthcare, USA). Area-weighted T_{sk} ($\overline{T}_{sk}$) was calculated according to the equation of Ramanathan (1964). Core and skin temperatures were recorded continuously using TracerDAQ software (Measurement Computing Corporation, Norton, MA, USA). Whole-body sweat rate (WBSR) was estimated from nude body mass loss, corrected for fluid consumed.

5.2.8.4 Hematological Variables

Venous blood was collected by venipuncture into a vacutainer (Becton-Dickinson, Oxford, UK) containing either clot activator or anticoagulant (K_3EDTA). Whole blood was used to measure hemoglobin concentration (HemoCue® Hb+ 201 System, Ängelholm, Sweden). Analysis of leukocyte count and subsets was performed using an automated cell counter (Ac·T 5diff Hematology Analyzer, Beckman Coulter, USA). Once clotted (>30 min) the whole blood was centrifuged at 4 °C and 805 *g* for 15 min and aliquots of serum were transferred into Eppendorf tubes (Genuine Axygen Quality, USA) and stored at −80 °C until further analysis. Serum osmolality was measured using freezing-point depression (Digimatic osmometer Model 3D2, Advanced Instruments Inc., Norwood, MA, USA). Serum samples were analyzed using enzyme-linked immune assays for 17β-estradiol (Demeditec Diagnostics, Kiel, Germany) and

progesterone (IBL International, Hamburg, Germany) 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. Serum hepcidin and IL-6 concentrations were analyzed using enzyme-linked immunosorbent assays (R&D Systems, Minneapolis, MN, USA), with a sensitivity of 1.70 pg·ml^{-1} and 0.04 pg·ml^{-1}, respectively, and an intra-assay variation of <4 and <8%, respectively. Serum iron parameters (ferritin, transferrin, iron) were determined using an automated analyzer (Cobas E601, Roche Diagnostics, Auckland, New Zealand) with the following sensitivity and repeatability: 0.5 µg·L^{-1} and <4% (ferritin), 0.1 g·L^{-1} and <3% (transferrin) 0.9 µmol ·L^{-1} and <2% (iron). The following calculations determined iron binding and saturation: $\boldsymbol{iron\ binding = transferrin \times 22.8}$, and $\boldsymbol{iron\ saturation = \frac{iron \times 100\%}{iron\ binding}}$.

5.2.8.5 Perceptual Measures

RPE was measured using the 15-grade scale, from 6 to 20 (Borg, 1970).

5.2.8.6 Statistical Analysis

All statistical analyses were performed with SPSS software for windows (IBM SPSS Statistics 20, NY, USA). Descriptive values were obtained and reported as means and standard deviation (SD). Homogeneity of variance was examined by Levene's test whilst the normality of the data was examined by the Shapiro-Wilk Test, with no significant effects. Therefore, data were analyzed by using two-way ANOVA (menstrual phase × ambient condition) or three-way in response to exercise (menstrual phase × ambient condition × time-point). Sphericity was assessed and where the assumption of sphericity could not be assumed, adjustments to the degrees of freedom were made (ε>0.75=Huynh-Feldt; ε<0.75=Greenhouse-Geisser). Where appropriate, *post hoc* pairwise analysis was performed using a paired samples t test. For significant results, partial eta-squared (η_p^2) is reported as a measure of effect size, a value greater than 0.25 denoting large effects. Pearson's correlation coefficient was used to examine

the direction and strength of relationships between the independent (ovarian hormones and body temperature) and dependent (inflammatory and iron regulatory) variables. Finally, hierarchical multiple regression was used to predict the IL-6 and hepcidin responses using these variables. Statistical significance was set at $p<0.05$.

5.3 Results

5.3.1 Menstrual Phase Verification

Progesterone (EF: 0.6±0.3 *vs.* ML: 13.8±6.4 ng·mL^{-1}) and 17β-estradiol (EF: 79±62 *vs.* ML: 116±85 pg·mL^{-1}) concentrations were significantly higher in the luteal phase (both $p<0.05$, $\eta_p^2>0.45$) but were unaffected by ambient temperature (both $p>0.32$). Similarly, resting T_{rec} was elevated by 0.28±0.25 °C in the luteal phase ($p=0.02$, $\eta_p^2=0.58$) but was unaffected by ambient temperature ($p=0.37$). The progesterone/17β-estradiol ratio was significantly higher in the luteal phase (EF: 10±6 *vs.* ML: 154±79, $p<0.01$, $\eta_p^2>0.79$) but was unaffected by ambient temperature ($p=0.70$).

Apart from HR and $\bar{T}_{sk}$ (8±5 beats·min^{-1} and 4.6±0.7 °C greater in WARM, both $p<0.01$, $\eta_p^2>0.71$), no variables described below measured different at rest due to menstrual phase or ambient temperature (all $p>0.12$). Therefore, for T_{rec}, $\bar{T}_{sk}$ and HR data analyses were determined using change from rest (Δ).

5.3.2 Exercise Performance

Total work completed was not different between menstrual phases (EF: 279±74 *vs*. ML: 272±47 kJ, $p=0.65$) but was 3±5% higher in MOD than in WARM (280±57 *vs*. 272±59 kJ, $p=0.02$, $\eta_p^2=0.57$). Accordingly, mean power output was unaffected by menstrual phase (EF: 155±41 *vs*. ML: 151±26 W, $p=0.65$) but was 5±4 W higher in MOD than in WARM (156±32

vs. 151±33 W, p=0.02, η_p^2=0.59). When viewing performance as the self-paced exercise profile, power output differed across time as a function of ambient temperature (time × ambient temperature: p=0.01, η_p^2=0.40), whereby power output became greater in MOD than WARM between 18 and 30 min. However, this was not dependent on menstrual phase (p=0.64).

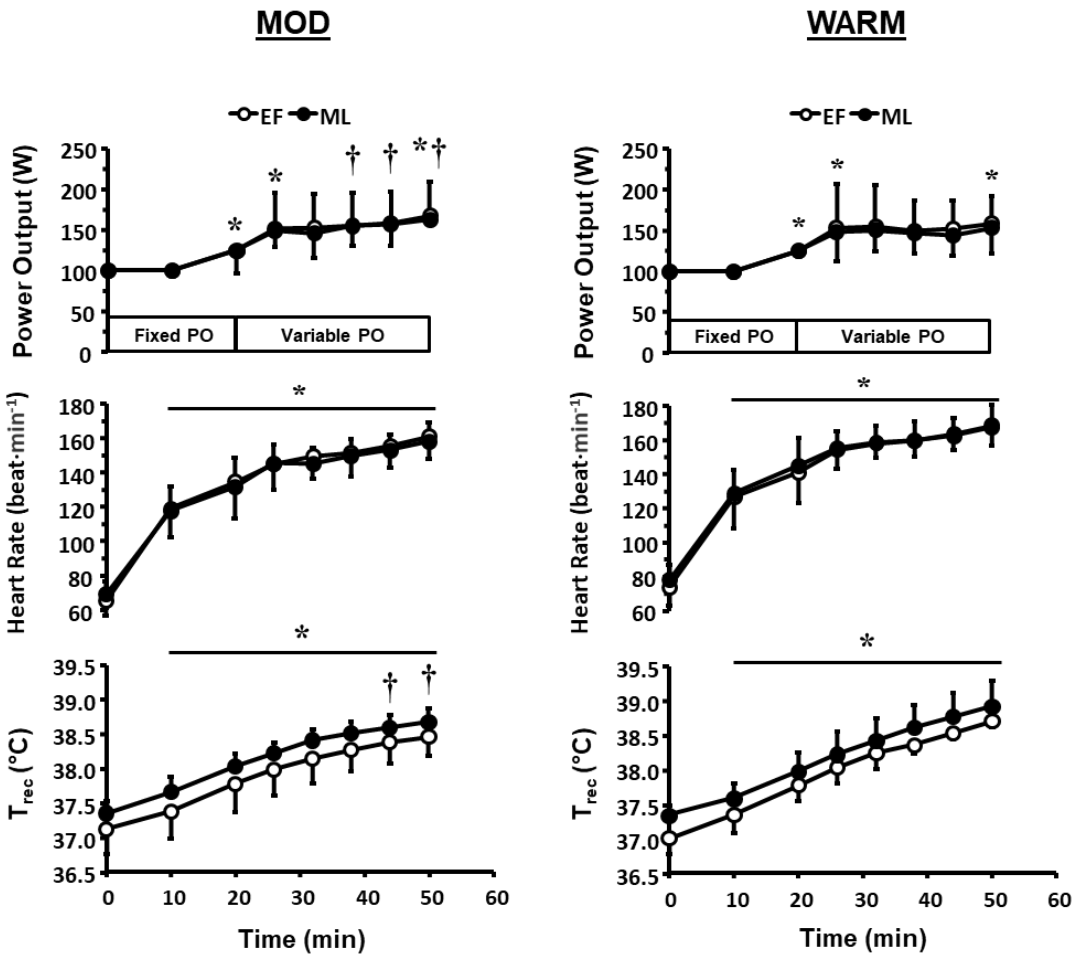

Figure 6. Power output, heart rate, and rectal temperature (T_{rec}) during 20-min cycling with fixed power output (PO) followed by a 30-min work trial with variable PO in a 20 °C (MOD) and 32 °C (WARM) environment during the early follicular (EF) and midluteal (ML) phase. Values are means ± SD (n=8) and analyzed using ANOVA, with post hoc pairwise analysis performed using a paired samples t test. *Significantly different to previous time-point; †significant difference between ambient temperatures.

5.3.3 Physiological Measures

5.3.3.1 Exercise Intensity

Participants exercised at 55±8% $\dot{V}O_2$max (100 W) and 68±9% $\dot{V}O_2$max (125 W) during the fixed-intensity warm-up, however the increase in exercise intensity differed as a function of ambient temperature (intensity × ambient temperature: p=0.03, η_p^2=0.52) i.e., the actual exercise intensity at 100 W MOD=100 W WARM<125 W MOD<125 W WARM.

From resting values (MOD: 68±7 *vs*. WARM: 76±9 beats·min^{-1}) the rise (Δ) in HR with exercise (p<0.01, η_p^2=0.85) continued until the end of the work trial, but this was not dependent on ambient temperature or menstrual phase (both p>0.61).

5.3.3.2 Body Temperatures and Fluid

From resting values (EF: 37.07±0.28 *vs*. ML: 37.35±0.11 °C) the rise (Δ) in T_{rec} during exercise differed as a function of ambient temperature (time × ambient temperature: p<0.01, η_p^2=0.81) such that the increase in T_{rec} during MOD was lower than during WARM (Δ1.33±0.26 *vs*. Δ1.62±0.26 °C), but this was not dependent on menstrual phase (p=0.13).

From resting values (MOD: 29.5±0.7 *vs*. WARM: 34.1±0.3 °C) the rise (Δ) in $\bar{T}_{sk}$ during exercise differed as a function of ambient temperature (time × ambient temperature: p=0.01, η_p^2=0.31) such that the increase in $\bar{T}_{sk}$ during MOD was lower than during WARM (Δ0.8±1.6 *vs*. Δ1.5±0.5 °C), but this was not dependent on menstrual phase (p=0.12).

The volume of water consumed during exercise was lower during MOD than WARM (p=0.01, η_p^2=0.68) but was not dependent on menstrual phase (p=0.40). WBSR was also lower during MOD than WARM (p<0.01, η_p^2=0.89) but was not dependent on menstrual phase (p=0.89), resulting in a smaller loss of body mass following MOD than WARM (p=0.01, η_p^2=0.61). Serum osmolality differed between menstrual phases as a function of ambient temperature

(menstrual phase × ambient temperature: p=0.03, η_p^2=0.54) such that values during MOD in the luteal phase were lower than at other times, but this was not dependent on exercise (p=0.59).

Table 7: Markers of fluid balance measured in 20 °C (MOD) and 32 °C (WARM) during the early follicular and mid-luteal phases. Values are mean±SD for *n*=8, and analyzed using ANOVA.

	Early Follicular		**Mid-Luteal**	
	MOD	*WARM*	*MOD*	*WARM*
WBSR ($g \cdot h^{-1}$) [a]	0.35±0.08	0.61±0.17	0.37±0.10	0.61±0.15
Water Consumption ($mL \cdot h^{-1}$) [a]	0.27±0.07	0.39±0.10	0.28±0.10	0.46±0.22
Δ Body Mass (%) [a]	-0.2±0.2	-0.5±0.4	-0.2±0.3	-0.3±0.3
S_{Osm} ($mOsm \cdot kg^{-1}$) [b] *Pre:*	289±4	289±3	288±3	289±2
Post:	290±2	289±2	288±2	290±3

Pre/Post, before/after exercise; **S_{Osm}**, serum osmolality; **WBSR**, whole-body sweat rate. [a] main effect of ambient temperature, [b] interaction effect menstrual phase × ambient temperature.

5.3.3.3 Markers of Inflammation

Due to machine malfunction, incomplete data were analyzed for leukocyte count (n=6). The change in leukocyte count following exercise differed as a function of ambient temperature (time × ambient temperature: p=0.01, η_p^2=0.77) such that the increase following MOD was only ~40% the magnitude following WARM, but this was not dependent on menstrual phase (p=0.90). Similarly, the change in neutrophil count following exercise differed as a function of ambient temperature (time × ambient temperature: p<0.01, η_p^2=0.97) such that the increase following MOD was only ~30% the magnitude following WARM, but this was not dependent on menstrual phase (p=0.77). Whereas, both lymphocyte (p=0.02, η_p^2=0.71) and monocyte (p<0.01, η_p^2=0.92) counts increased following exercise without being dependent on menstrual phase or ambient temperature (all p>0.28). Platelet count increased following exercise (p=0.01, η_p^2=0.81) and was lower in MOD than WARM (p=0.03, η_p^2=0.65), but this was not dependent on menstrual phase (p=0.64) (Table 8).

Table 8: Markers of inflammation measured before (Pre) and after (Post) exercise in 20 °C (MOD) and 32 °C (WARM) during the early follicular and mid-luteal phases. Values are mean±SD for *n*=6, and analyzed using ANOVA.

	Early Follicular				**Mid-Luteal**			
	MOD		*WARM*		*MOD*		*WARM*	
	Pre	**Post**	**Pre**	**Post**	**Pre**	**Post**	**Pre**	**Post**
Leukocytes ($10^9 \cdot L^{-1}$) [c]	7.5±3.4	8.8±4.8	7.0±2.6	9.1±4.5	7.1±1.7	7.8±1.9	7.1±1.5	9.8±2.3
Neutrophils ($10^9 \cdot L^{-1}$) [c]	4.1±1.7	4.7±2.5	4.0±2.0	5.4±2.7	3.9±0.9	4.4±0.9	4.3±1.2	6.4±1.6
Lymphocytes ($10^9 \cdot L^{-1}$) [a]	2.2±0.9	2.9±1.4	2.6±1.3	3.4±2.2	2.1±0.5	2.6±0.7	2.4±0.7	2.8±0.7
Monocytes ($10^9 \cdot L^{-1}$) [a]	0.5±0.3	0.7±0.3	0.4±0.2	0.7±0.3	0.4±0.1	0.6±0.3	0.4±0.1	0.7±0.2
Platelets ($10^9 \cdot L^{-1}$) [ab]	208±11	226±29	234±59	262±56	194±31	227±22	221±17	260±24

[a] main effect of exercise, [b] main effect of ambient temperature, [c] interaction effect exercise × ambient temperature.

IL-6 increased following exercise (p<0.01, η_p^2=0.77) but this was not dependent on menstrual phase or ambient temperature (both p>0.19) (Figure 7).

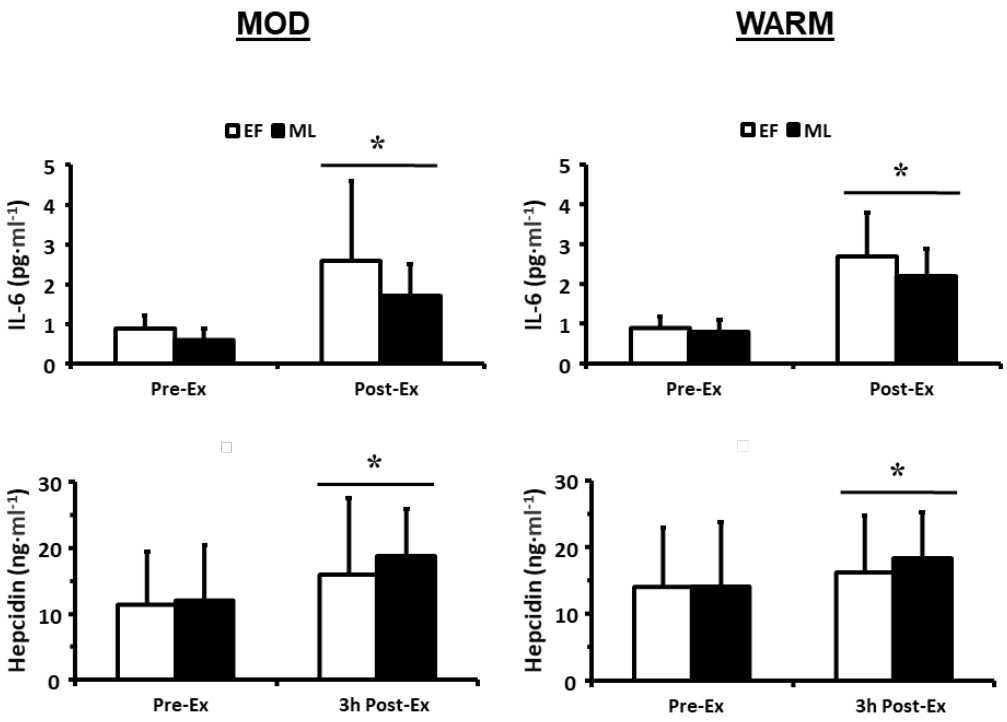

Figure 7. Interleukin-6 (IL-6) and hepcidin concentrations before (pre-ex), after (post-ex), and 3 h following exercise (3 h post-ex) in a 20 °C (MOD) and 32 °C (WARM) environment during the early follicular (EF) and midluteal (ML) phase. Values are means ± SD (n = 8) and analyzed using ANOVA. *Significantly different to pre-ex.

5.3.3.4 Markers of Iron Regulation

Hepcidin increased following exercise (p=0.01, η_p^2=0.61) but this was not dependent on menstrual phase or ambient temperature (both p>0.25) (Figure 7). Ferritin (p=0.01, η_p^2=0.62) and transferrin (p=0.01, η_p^2=0.64) increased following exercise whilst hemoglobin did not (p=0.10), with none of these variables affected by menstrual phase or ambient temperature (all p>0.28). Serum iron was unaffected by exercise, menstrual cycle, and ambient temperature (all p>0.29) (Table 9).

Table 9: Markers of iron regulation measured before (Pre) and after (Post) exercise in 20 °C (MOD) and 32 °C (WARM) during the early follicular and mid-luteal phases. Values are mean±SD for *n*=8, and analyzed using ANOVA.

	Early Follicular				**Mid-Luteal**			
	MOD		*WARM*		*MOD*		*WARM*	
	Pre	**Post**	**Pre**	**Post**	**Pre**	**Post**	**Pre**	**Post**
Hemoglobin (g·d L^{-1})	13.8±0.6	14.3±0.8	13.6±0.9	14.1±0.7	13.8±1.1	14.0±0.8	14.1±1.3	14.3±0.7
Ferritin (μg·L^{-1}) [a]	51±22	56±25	50±24	55±25	55±21	58±23	51±22	55±25
Transferrin (g·L^{-1}) [a]	2.5±0.3	2.6±0.3	2.5±0.3	2.6±0.3	2.6±0.3	2.7±0.4	2.6±0.4	2.7±0.5
Iron (μmol·L^{-1})	14.6±6.0	15.4±5.5	16.4±6.3	17.1±5.8	18.1±7.2	19.0±10.9	18.1±7.1	18.9±8.0

Notes: [a] main effect of exercise.

5.3.4 Perceptual

The rise in RPE during exercise (from 9.4±0.9 a.u., $p<0.01$, $\eta_p^2=0.84$) continued until 18 min during the work trial before plateauing (14.6±1.2 a.u.) and was lower in MOD compared to WARM (by 1.2±0.8 a.u., $p<0.01$, $\eta_p^2=0.72$), but this was not dependent on menstrual phase ($p=0.45$).

5.3.5 Correlations and Regressions

As IL-6 after exercise was associated with leukocyte count after exercise ($r=0.80$, $p<0.01$) and platelet count after exercise ($r=0.40$, $p=0.05$), they were entered into the regression equation to determine the IL-6 response after exercise. Leukocyte count ($\beta=1.12$, $p<0.01$) explained 63% of the variance with platelet count ($\beta=-0.44$, $p=0.02$) contributing another 9% independently (model: $r^2=0.72$, $p<0.01$).

As hepcidin after exercise was associated with iron before exercise ($r=0.59$, $p<0.01$), ferritin after exercise ($r=0.58$, $p<0.01$), IL-6 before exercise ($r=-0.46$, $p=0.01$) and T_{rec} before exercise ($r=0.39$, $p=0.03$), they were entered into the regression equation to determine the hepcidin response after exercise. Iron before exercise ($\beta=0.54$, $p<0.01$) explained 35% of the variance with ferritin after exercise ($\beta=0.53$, $p<0.01$) contributing another 28% independently (model: $r^2=0.62$, $p<0.01$), but IL-6 ($\beta=-0.21$, $p=0.08$) and T_{rec} ($\beta=0.22$, $p=0.06$) before exercise did not contribute independently.

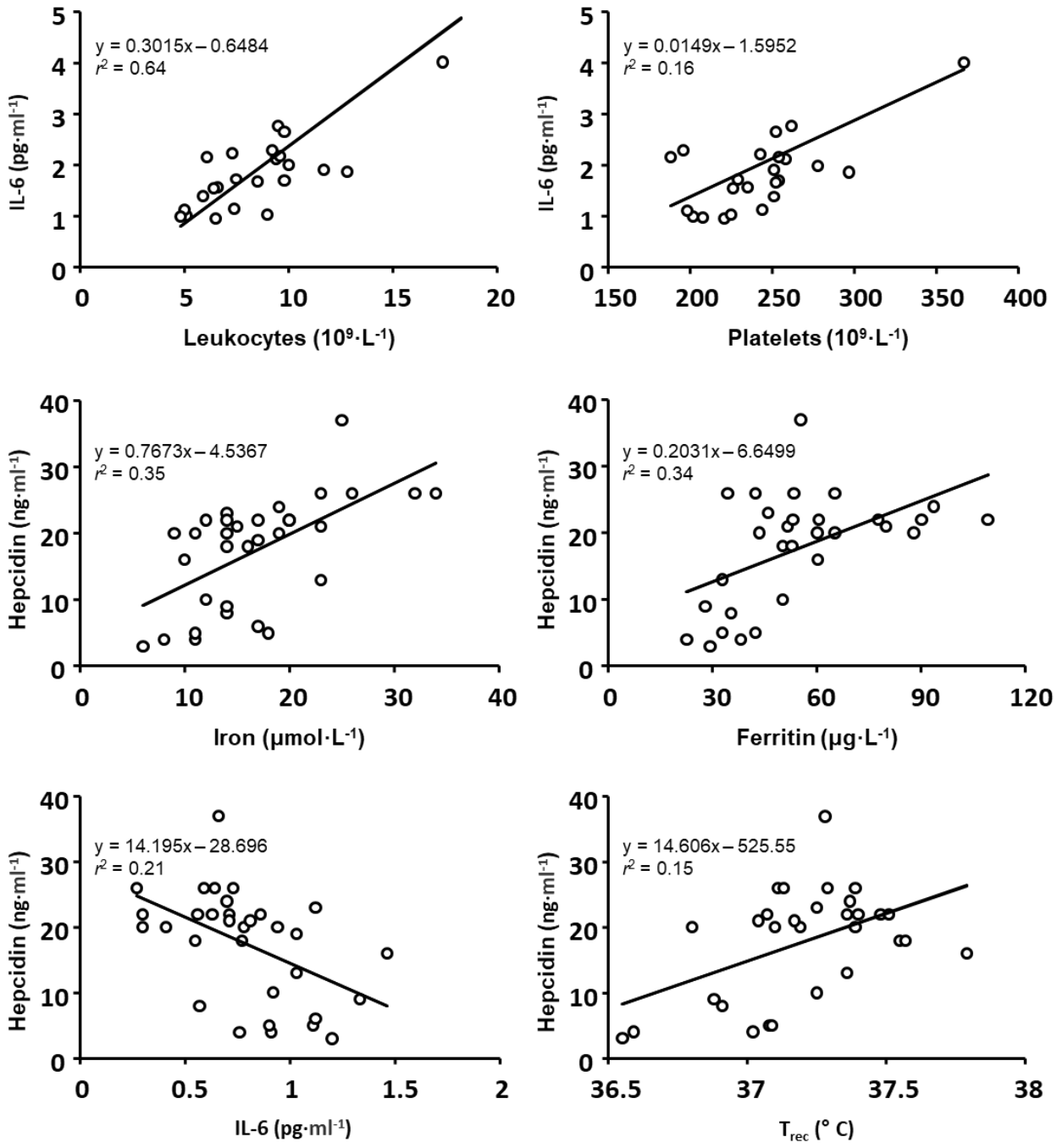

Figure 8. Bivariate associations between interleukin-6 (IL-6) after exercise, leukocyte count after exercise, and platelet count after exercise (top, n=24); between hepcidin after exercise, iron before exercise, and ferritin after exercise (middle, n = 32); and between hepcidin after exercise, IL-6 before exercise, and rectal temperature (T_{rec}) before exercise (bottom, n = 32). Values are all common individual data points, analyzed using Pearson's correlation coefficient and all $P < 0.05$.

5.4 Discussion

The important results from this chapter were that (1) ambient heat stress increased leukocyte, neutrophil and platelet counts following exercise, but despite this IL-6 and hepcidin responses post-exercise were not further augmented by the heat; (2) these effects were likely a consequence of T_{rec} only being 0.3 °C higher in the heat on account of a reduced workload and performance, but no measures were modulated by menstrual phase; (3) 72% of the variance in the post-exercise IL-6 response was explained by leukocyte and platelet count, and 62% of the variance in hepcidin after exercise was explained by iron before and ferritin after exercise. Collectively, these results indicate that trained, iron sufficient females suffer no detriment to their iron regulation in the acute exercise period due to ambient heat stress or menstrual phase because of a performance-thermoregulatory trade-off.

This is the first investigation to determine if adding ambient heat stress (32 °C) to exercise compromises iron regulation. We chose to allow our participants to self-pace during their 30-min work trial (75-80% $\dot{V}O_2$max) as this more accurately reflects how athletes behave in real-world racing (Montain et al., 2007) and laboratory simulation (Tatterson et al., 2000) with ambient heat stress. Under these conditions, although concentrations of hepcidin increased by 38% from baseline to 3 h post-exercise, ambient heat did not augment this response (Figure 7). In support of our results, Hayashi et al. (2020) demonstrated no effect of supplemented heat stress (32 *vs*. 23 °C) on hepcidin concentrations following 60 min of cycling at 60% $\dot{V}O_2$max in men; however, both trials were also conducted in a hypoxic environment (F_iO_2 = 14.5%) such that the independent effect of ambient heat was not able to be discerned. Nevertheless, these two studies do indicate that ambient heat stress may not acutely affect the hepcidin response to sub-maximal exercise of moderate duration i.e., 60-80% $\dot{V}O_2$max, ≤60 min.

The upregulation of hepcidin post-exercise is primarily determined by IL-6 (Nemeth et al., 2004a; Banzet et al., 2012), with ambient heat stress augmenting this exercise-induced cytokinemia due to a greater acute-phase response secondary to thermoregulatory strain (Mündel et al., 2016; Rhind et al., 1999). However, in the current study we observed that whilst IL-6 increased following exercise, ambient heat did not affect this response (Figure 7). This is most likely as a result of our participants being able to limit their difference in T_{rec} between environments to 0.3 °C on account of their reduced power output (*cf.* metabolic heat production) during WARM from approximately half-way through their work trial (Figure 6); in other words, a performance-physiological/thermoregulatory trade-off. Thus, although we observed an effect of ambient temperature on several other markers of inflammation (Table 8), these were not of a magnitude to affect IL-6. An individual's current iron stores are the primary mediator of the magnitude of the hepcidin response post-exercise (Peeling et al., 2014). Our results support this as we observed that 62% of the variance in hepcidin following exercise was explained by baseline serum iron and ferritin after exercise. Moreover, upon entry into the study, although purposely not iron deficient (i.e., ferritin >30 $\mu g \cdot L^{-1}$, see Table 6) there was an even split between the current females that would be described as having suboptimal (30-60 $\mu g \cdot L^{-1}$) and healthy (>60 $\mu g \cdot L^{-1}$) iron stores (Peeling er al., 2014). This may explain the comparatively lower post-exercise hepcidin response in our cohort to other recent studies (Peeling er al., 2014; Hayashi et al., 2020) i.e., 38% *vs*. 70-130%.

Although previous studies have demonstrated that menstrual phase can affect the inflammatory response to exercise (Timmons et al., 2005; Hashimoto et al., 2014; Barba-Moreno et al., 2020), in the current study no such effect was observed and certainly pales in comparison to the influence of exercise and ambient heat (Tables 7 and 8). Our results also support those from the only previous investigation into the effects of the menstrual cycle on hepcidin (Barba-Moreno et al., 2020), such that changes in ovarian hormones at physiologic concentrations

appear to have no effect on the exercise response. This contrasts with results from human studies using, for example, *in vitro* hepatoma cells and in women receiving *in vitro* fertilization (Yang et al., 2012; Lehtihet et al., 2015) in which supraphysiological levels of 17β-estradiol were used, thus making comparison inappropriate. Nevertheless, Alfaro-Magallanes et al. (2021) recently demonstrated that in women taking monophasic oral contraception concentrations of hepcidin were elevated both at rest and 3 h following an interval running exercise during pill withdrawal, a time when endogenous 17β-estradiol concentrations were no longer suppressed and had increased almost four-fold.

Janse de Jonge et al. (2012) previously demonstrated that following 60 min cycling at 60% $\dot{V}O_2$max, females' exhaustion time during an incremental test was 10% shorter in 32 °C *vs*. 20 °C, compared to being 3% shorter in the mid-luteal *vs*. early follicular phase. The present study used a protocol that more accurately reflects real-world competition/behavior and found that, under very similar environmental conditions, women suffered a performance disadvantage of 3% in 32 °C *vs*. 20 °C although with no effect of menstrual phase. Notably, this decrement in performance from a moderate to heat-stressful environment (3% ≡ 5 W) is significantly lower (p=0.01, independent t test) than the effect a more humid environment confers when compared to dry heat (7% ≡ 8 W) in a similar cohort of trained females i.e. 4.5±0.8 W·kg^{-1} *vs*. 4.2±0.4 W·kg^{-1} (Lei et al., 2017), on account of a reduced evaporative power of the body to the environment (Gagnon et al., 2013; Che Muhamed et al., 2016). Therefore, the current results support our previous studies, which have all utilized a 30-min work trial, in demonstrating that it is the (absolute) humidity of a heat-stressful environment that imposes a greater influence on performance than ambient temperature *per se* (Lei et al., 2017; Lei et al., 2019; Lei et al., 2020).

An unexpected but interesting finding was that fluid balance was maintained in spite of the potential stress caused by exercise (i.e., exercise-induced sudomotor, respiratory, renal and metabolic water loss) and acute perturbations of water and sodium regulation brought about by

changes in the ovarian hormones across the menstrual cycle (Stachenfeld, 2008). This was not a planned observation; rather an attempt to control for any confounding effect of dehydration whereby participants were simply allowed to consume water *ad libitum*. Our participants voluntarily/behaviorally adjusted their water intake during WARM to offset the increased sweat loss such that net body mass loss and increases in serum osmolality were either minimal or negligible. Although we observed a small effect of the menstrual phase on serum osmolality this is unlikely of physiological consequence as our women did not exhibit anywhere near the 2-3% change in tonicity or 10% plasma volume contraction required to initiate thirst or arginine vasopressin (Stachenfeld, 2008), although the latter requires confirmation as we were unable to accurately record this measure. Moreover, replication of this effect, including measures of thirst and arginine vasopressin, is warranted.

5.5 Considerations

The results observed in this study are valid only for the current sample, protocol, and conditions. It is acknowledged that many factors influence the IL-6 and hepcidin responses to exercise; notably mode, intensity and duration of exercise, and energy and carbohydrate availability (Pedersen & Hoffman-Goetz, 2000; Peeling et al., 2008) whilst iron status affects the hepcidin response (Peeling et al., 2014). Our approach captured the phases of lowest hormone exposure and peak progesterone but not the late-follicular (pre-ovulatory) phase when estrogen peaks without an increase in progesterone. However, this phase is normally limited to ~48h whereas women are in EF and ML for ≤50% of their reproductive lives. Another limitation was that an index of T_{core} with a known lag-time (*cf.* esophageal temperature; Mündel et al., 2016) was used. A final noteworthy consideration is that ~40% of participants in this study had to be excluded or did not complete. This number is consistent with the

recommendations by Janse de Jonge et al. (2019) and serves to highlight the importance of sufficient recruitment and the inherent challenge of research into the menstrual cycle.

5.6 Perspectives and Significance

It is well documented that acute and chronic exercise can influence iron homeostasis through the augmented hepcidin activity in the post-exercise period, a response driven by changes in the inflammatory cytokine IL-6. While research has established this timeline of hepcidin activity and altered iron regulation, most of this research has been conducted in moderate environmental conditions and where the exercise intensity has been clamped to exaggerate the inflammatory response; yet rarely does this exercise model translate well to real-life exercise and sport. Incidence of iron deficiency is high in female athletes, yet the hepcidin response - known to influence risk of iron deficiency - has not been well researched. The current results provide novel insight into the hepcidin response post-exercise utilizing a more ecologically-valid design, where a woman may be able to adjust her work-rate and hence moderate her physiological response in order to meet the demands of both exercise and the environmental conditions. Furthermore, the incidence of exercise-associated hyponatremia is greater for women than men (Hew-Butler et al., 2015), there is an increased mismatch of fluid intake *vs*. fluid loss during exercise in warm and humid environments (Sawka, 1992), and with high concentrations of 17β-estradiol (such as in the luteal phase or with estrogen-containing contraception) the osmotic threshold for arginine vasopressin and thirst is reduced during exercise (Stachenfeld et al., 1999). The current result of women maintaining fluid balance remarkably well by behaviorally matching their intake to loss provides important and novel evidence for the simultaneous prevention of (excessive) dehydration and minimizing risk for

hyponatremia (Hew-Butler et al., 2015), and should stimulate necessary further research in this area.

Chapter Six

6.0 Measurement Error of Self-paced Exercise Performance in Athletic Women is Not Affected by Ovulatory Status or Ambient Environment.

The formatting of this chapter is consistent with the accepted publication: Zheng, H., Badenhorst, C. E., Lei, T. H., Che Muhamed, A. M., Liao, Y. H., Amano, T., Fujii, N., Nishiyasu, T., Kondo, N., & Mündel, T. (2021). Measurement error of self-paced exercise performance in athletic women is not affected by ovulatory status or ambient environment. *J Appl Physiol,* 131(5):1496-1504, 2021.

Abstract

Measurement error(s) of exercise tests for women are severely lacking in the literature. The purpose of this chapter was to 1) determine whether ovulatory status or ambient environment were moderating variables when completing a 30-min self-paced work trial, and 2) provide test-retest norms specific to athletic women. A retrospective analysis of three heat stress studies was completed using 33 female participants (31±9 y, 54±10 mL min^{-1} kg^{-1}) that yielded 130 separate trials. Participants were classified as ovulatory (*n*=19), anovulatory (*n*=4) and oral contraceptive pill users (*n*=10). Participants completed trials ~2 weeks apart in their (*quasi*-) EF and ML in two of moderate (1.3±0.1 kPa, 20.5±0.5 °C, *18 trials*), warm-dry (2.2±0.2 kPa, 34.1±0.2 °C, *46 trials*) or warm-humid (3.4±0.1 kPa, 30.2±1.1 °C, *66 trials*) environments. We quantified reliability using limits of agreement, intraclass correlation coefficient (ICC), standard error of measurement (SEM) and coefficient of variation (CV). Test-retest reliability

was high, clinically-valid (ICC=0.90, $p<0.01$) and acceptable with a mean CV of 4.7%, SEM of 3.8 kJ (2.1 W) and reliable bias of -2.1 kJ (-1.2 W). The various ovulatory status and contrasting ambient conditions had no appreciable effect on reliability. These results indicate that athletic women can perform 30-min self-paced work trials ~2 weeks apart with an acceptable and low variability irrespective of their hormonal status or heat-stressful environments.

6.1 Introduction

Although *Title IX* and the NIH Revitalization Act provided a mechanism to prevent the exclusion of females as research participants and focus, there is still considerable bias against women in basic and pre-clinical biomedical research, including physiology (Beery & Zucker, 2011). The anatomic and physiologic characteristics that distinguish the exercise response in women from men indicate a need for recommendations and norms specific to women for exercise testing (Charkoudian & Joyner, 2004); yet these data have not been used to determine if sex-specific exercise prescription is necessary (Liguori, 2021). Estrogen and progesterone play important secondary (non-reproductive) roles that, according to a specific hormonal milieu, influence physiological systems differently in women, such as vascular, thermal and osmotic regulation (Charkoudian & Stachenfeld, 2014; Sims & Heather, 2018). However, women of reproductive age that exercise regularly are more likely to display anovulatory/luteal phase-deficient cycles (30-50%; De Souza et al., 2010; Schaumberget al., 2017), and prevalence of OCP use amongst physically active and athletic women is high (>50%; Rechichi et al., 2009; Martin et al., 2018). Therefore, when considering research on physically-active females it would be prudent to *include* rather than *exclude* these cohorts because of their

physiology (endocrinology), in addition to eumenorrheic women in order to make findings as representative and applicable to athletic females as possible.

Exercise performance is a common and important outcome measure used to assess the efficacy of treatment effects, such as training programs and other interventions (nutritional, pharmacological, physiological etc.). Due to the high inter- and intra-variability in menstrual cycle status, and the potential confounding influence on exercise performance, it seems necessary to ascertain if performance effects are due to treatments or due to measurement error (high test or within-/between-subject variability). Another advantage of knowing measurement error is that true differences can be determined without unrealistically large sample sizes, especially if the study design incorporates lifestyle standardization (e.g., diet, time of day etc.) and a within-subject design. Despite their use in providing mechanistic data during a physiological steady-state, constant power tests display inferior reliability and lower ecological validity than constant work or duration tests (Hopkins et al., 2001; Currell & Jeukendrup, 2008). When considering aerobic tests (i.e., > 20 min duration) only one study has previously determined the reliability of a protocol in females. Bishop (1997) had twenty female cyclists and triathletes complete two 60-min cycling work trials separated by a week and reported a coefficient of variation (CV) of 2.7%, standard error of measurement (SEM) of 3.4 W and intraclass correlation coefficient (ICC) of 0.97 for average power output. However, details about the participants' ovulatory/menstrual/hormonal status were not provided.

Given the arguments above, there appears to be a lack of literature describing typical variance of the most commonly utilized exercise testing used for assessing the female physiological response: laboratory cycle ergometry of >20 min duration. It has been previously reported that the (*quasi*-) menstrual phase did not influence performance of a 30-min work trial, whereas the ambient profile (increased temperature and humidity) reduced work performed by 3-5% (Lei et al., 2017; Lei et al., 2019; **Chapter 5**). Considering that the ~2 weeks separating trials

between (*quasi*-) menstrual phases did not affect exercise performance, we used this (within-environment) design to determine test-retest reliability in a homogenous sample of aerobically-trained women. This type of retrospective analysis has also been demonstrated to be a reliable method to detect whether there is a treatment effect or not (Salgado et al., 2020). The primary purpose of the current study was to determine whether ovulatory/hormonal status or ambient profile were moderating variables for measurement error of a 30-min self-paced work trial, whilst a secondary purpose was to add exercise performance norms specific to athletic women to the literature. Given that our previous studies (Lei et al., 2017; Lei et al., 2019; **Chapter 5**) observed no (*quasi*-) menstrual phase-by-ambient profile interaction effect for work completed or mean power output, we hypothesized that measurement error would be unaffected by these factors.

6.2 Methods

6.2.1 Ethical Approval

All previous studies (Lei et al., 2017; Lei et al., 2019; **Chapter 5**) had received approval by the Massey University Human Ethics Committee (Southern A) and were performed in accordance with the latest revision of the *Declaration of Helsinki*, except for registration in a database. Informed, written consent was obtained from all participants prior to participation.

6.2.2 Participants

Thirty-three aerobically trained females participated in this study that yielded 130 separate trials, with physical characteristics displayed in Table 10. All were healthy, non-smokers and free from cardiovascular, metabolic, neurological, and respiratory diseases and were not taking any regular medication apart from those using the OCP. Some of the data herein have been

reported previously in separate studies (Lei et al., 2017; Lei et al., 2019; **Chapter 5**). All eumenorrheic females self-reported a regular menstrual cycle 21-35 days in length (≥3 mo) with no use of hormonal contraception (≥6 mo). All OCP females were taking a monophasic combination OCP (≥1 y) with experimental visits completed during the three weeks of active pill use (see Lei et al., 2019 for further details).

Table 10: Participant characteristics for ovulatory (OVU), anovulatory and/or luteal phase-deficient (ANO) and oral contraceptive pill (OCP) groups.

Characteristic	**OVU (*n*=19)**	**ANO (*n*=4)**	**OCP (*n*=10)**	**Mean (*n*=33)**	***p*-value**
Age (y)	34 (9)	36 (8)	25 (5)*	31 (9)	0.02
Mass (kg)	63 (6)	65 (3)	68 (10)	65 (7)	0.28
A_D (m^2)	1.70 (0.11)	1.69 (0.03)	1.76 (0.13)	1.72 (0.11)	0.45
A_D : mass	0.027 (0.001)	0.026 (0.001)	0.026 (0.002)	0.027 (0.001)	0.30
% fat	23 (5)	22 (6)	24 (5)	23 (5)	0.70
$\dot{V}O_2$max (L min^{-1})	3.3 (0.6)	3.8 (1.0)	3.7 (0.5)	3.5 (0.6)	0.11
$\dot{V}O_2$max (mL min^{-1} kg^{-1})	52 (9)	58 (15)	55 (9)	54 (10)	0.40
Wmax (W)	270 (40)	292 (39)	283 (29)	276 (37)	0.46
Training history (y)	7.3 (3.3)	9.3 (5.3)	3.7 (2.5)*	6.4 (3.8)	0.01
Progesterone (ng mL^{-1})					
Follicular	0.6 (0.4)*	0.2 (0.1)	0.1 (0.1)		<0.01
Luteal	16.1 (12.2)*	1.2 (1.5)	0.2 (0.1)		<0.01
Estrogen (pg mL^{-1})					
Follicular	63.6 (53.1)	42.5 (15.0)	18.3 (23.0)†		<0.01
Luteal	105.2 (77.2)	90.6 (71.2)	20.5 (28.7)*		<0.01

A_D, Dubois body surface area; $\dot{V}O_2$max, maximal rate of O_2 consumption; Wmax, peak aerobic power. Values are Mean (SD). * significantly different to both other groups. † significantly different to OVU.

6.2.3 Experimental Overview

Data collection weas conducted excluding the Southern Hemisphere summer (March-November) where the average daily temperature did not exceed 22 °C, nor had participants spent any time in a warmer climate for at least one month prior to the study. All participants attended the laboratory on six occasions: (1) preliminary submaximal and maximal aerobic capacity test, (2) experimental familiarization, (3-6) experimental trials. For a diagrammatic representation of the experimental overview, see Figure 9. The four experimental trials were a full crossover of (*quasi*-) menstrual phase (early follicular [EF, *65 trials*] and mid-luteal [ML, *65 trials*]) and ambient profile (moderate [MOD, *19 trials*], warm-humid [HUM, *65 trials*], or warm-dry [DRY, *46 trials*]). Grouping for the ambient conditions was based on vapor pressure, such that the following characterized each environment: MOD (1.3±0.1 kPa, 20.5±0.5 °C, 53.1±5.5 relative humidity [rh]), DRY (2.2±0.2 kPa, 34.1±0.2 °C, 41.4±3.4 rh) and HUM (3.4±0.1 kPa, 30.2±1.1 °C, 80.0±3.7 rh). The order of the trials was randomized and counterbalanced except the order of the ambient profile was consistent in different (*quasi*-) phases within participants. Experimental trials were conducted at the same time of day (±1 h) and following >24 h of dietary and exercise control. Each trial consisted of either 12 (*92 trials*) or 20 (*38 trials*) min of fixed-intensity pre-load immediately followed by a 30 min self-paced work trial where only percentage of time elapsed (every 20% or 6 min) was provided to the participant. All exercise was performed on an electronically braked cycle ergometer (Lode Excalibur, Groningen, The Netherlands), with handlebars, seat height and pedal preference standardized according to individual preference. The typical timeline for a participant to complete this study resulted in preliminary testing and familiarization separated by 3-7 days during the (*quasi*-) follicular phase, with half of the participants starting their experimental trials the following (*quasi*-) luteal phase (14 days later) and the other half the following (*quasi*-

) follicular phase (28 days later), with within-phase experimental trials differing by ambient profile separated by three days.

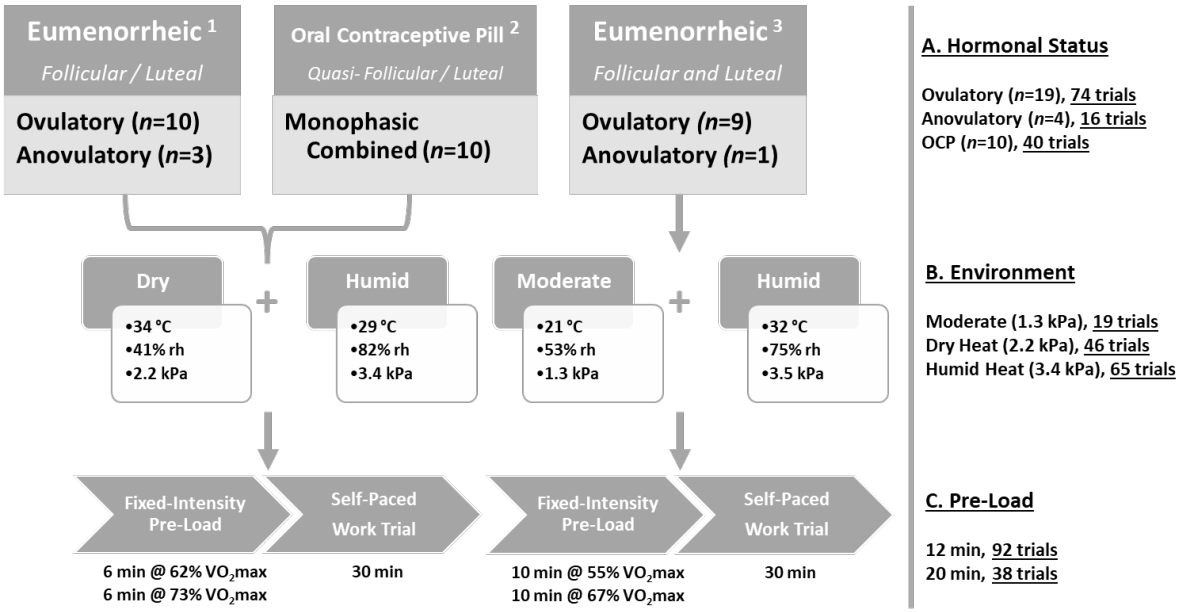

Figure 9. Diagram of experimental overview. Three distinct groups were determined according to their hormonal status (A), with each participant performing trials under two distinct ambient environments (B) and repeated 2 weeks later according to their (*quasi*-) follicular and luteal phases (A). Each 30- min self-paced work trial was preloaded with either a 12 or 20 min fixed-intensity period (C). 1 participants for Lei et al. (2017); 2 participants for Lei et al. (2019); 3 participants for **Chapter 5**). DRY, warm-dry; HUM, warm-humid; MOD, moderate; OCP, oral contraceptive pill; $\dot{V}O_2$max, maximal oxygen consumption**.**

6.2.4 Preliminary Testing and Familiarization

All preliminary testing was conducted in the (*quasi*-) EF phase of each participant's menstrual cycle to minimize the potential effects of menstrual/OCP cycle on their physiological and performance responses during the tests (Sims & Heather, 2018). Following anthropometric measurements (height, weight, body composition), a 24-min steady-state submaximal exercise test was conducted in a temperate laboratory environment (18-22 °C). The submaximal exercise test consisted of four consecutive six-minute stages with power outputs of 100 W, 125 W, 150 W and 175 W at comfortable but constant cadence. O_2 consumption was measured

during the last two minutes of each stage. Following 10 min rest from the submaximal test, a $\dot{V}O_2$max test was performed. The initial workload began at 100 W and increased by 25 W every minute, until volitional exhaustion. The exercise "gear" (linear factor, see Experimental Procedure) during the self-paced exercise was based on 75% of an individual's $\dot{V}O_2$max, which was derived from the linear relationship between the power output and the O_2 consumption during both the steady-state submaximal exercise test and maximal aerobic capacity test. Following at least 24 hours rest from the preliminary session, a familiarization trial was conducted to ensure all participants were familiar with the testing procedures and to minimize the learning effect during trials. This trial replicated entirely the experimental trials outlined below.

6.2.5 Dietary and Exercise Control

Diet and physical activity during the 48 hours prior to the first experimental trial were recorded and participants were instructed to repeat these for the following trials. The day of and prior to any experimental trial was marked by abstinence from alcohol, exercise, and only habitual caffeine use (as abstinence would confound results from withdrawal effects). This dietary and exercise control minimized variation in pre-trial metabolic state. Fluid intake was encouraged to ensure a euhydrated state.

6.2.6 Ovulatory Status and Ambient Conditions

Eumenorrheic females were tested on days 3-6 (EF) and 18-21 (ML) following *start of menses*, whilst OCP females were tested on days 3-6 and 18-21 following *start of OCP use*. Testing for eumenorrheic females was scheduled using the three-step method (Allen et al., 2016) whereby self-reported menses onset and urinary luteinizing hormone testing (EasyCheck® Ovulation Test, Phoenix Medcare Ltd, Auckland, New Zealand) prospectively identified EF and ML, whilst measurement of serum 17β-estradiol and progesterone retrospectively confirmed ML.

A progesterone level of >5 ng·ml^{-1} is good evidence that ovulation has occurred (Schaumberg et al., 2017; Leiva et al., 2015; Janse de Jonge et al., 2019). Therefore, participants were deemed as ovulatory (OVU, >5 ng·ml^{-1}) or anovulatory (ANO, <5 ng·ml^{-1}).

6.2.7 Experimental Procedure

These four trials were conducted in the same environmental chamber with a fan-generated airflow of 19 km·h^{-1}. Upon their arrival at the laboratory, participants voided, and a blood sample was obtained from an antecubital vein after participants had rested seated for 15 min. Participants entered the environmental chamber wearing only cycling shorts and top, shoes and socks. Participants rested seated on the ergometer for 20 min before completing either i) 6 min of cycling at each of 125 and 150 W (62±9 and 73±10% $\dot{V}O_2$max, respectively), or ii) 10 min of cycling at each of 100 and 125 W (55±8 and 67±9% $\dot{V}O_2$max, respectively). Immediately on completion of the second fixed-intensity bout, the ergometer was set to linear mode based on the formula of Jeukendrup et al. (1996), where participants were instructed to perform as much work as possible over 30 min. During this 30 min self-paced period, work completed (kJ) was recorded every 6 min and tap water at 20 °C was provided to drink *ad libitum* throughout to minimize dehydration. Total work completed (kJ) was used as the criterion measure for reliability metrics (see *Statistical Analysis* below).

6.2.8 Measurements

For interested readers, other physiological measurements (i.e., thermoregulatory, cardiovascular, inflammatory) were performed during these trials, of which the results can be found in separate studies (Lei et al., 2017; Lei et al., 2019; **Chapter 5**).

6.2.8.1 Anthropometric

Participant height and weight were measured using a stadiometer (Seca, Germany; accurate to 0.1cm) and scale (Jadever, Taiwan; accurate to 0.01 kg), from which surface area was estimated (Dubois & Dobuis, 1916). Body composition was measured using multi-frequency bioelectrical impedance analysis (InBody 230, Korea) using a standard procedure (Kyle et al., 2004).

6.2.8.2 Respiratory

Expired respiratory gases were collected from a mixing chamber and analyzed for O_2 consumption using an online, breath-by-breath system (VacuMed Vista, Turbofit, Ventura, CA, USA) using a 30s average. This system was calibrated before each trial using a zero and *β*-standard gas concentrations, and volume (VacuMed 3L Calibration Syringe).

6.2.8.3 Hormones

Venous blood was collected by venipuncture into a vacutainer (Becton-Dickinson, Oxford, UK) containing clot activator and once clotted (>30 min) the whole blood was centrifuged at 4 °C and 805 *g* for 15 min and aliquots of serum were transferred into Eppendorf tubes (Genuine Axygen Quality, USA) and stored at −80 °C until further analysis. For further detail, please see previous studies (Lei et al., 2017; Lei et al., 2019; **Chapter 5**).

6.2.8.4 Statistical Analysis

All statistical analyses were performed with SPSS software for Windows (IBM SPSS Statistics 20, NY, USA). Descriptive values were obtained and reported as means and standard deviation (SD) or using 95% confidence interval (CI) unless stated otherwise. Homogeneity of variance was examined by Levene's test whilst the normality of the data was examined by the Shapiro-Wilk test, with no significant effects. Participant characteristics were analyzed using a one-way analysis of variance. In order to assess test-retest reliability in work trial performance (work

completed in kJ), several commonly reported measures were calculated (Atkinson et al., 1998). Limits of agreement (LoA; Bland & Altman, 1999) are reported as bias±1.96 SD. Giavarina (Giavarina, 2015) proposed that if the line of equality (x=0) lies within the 95% CI (±1.96 SE) of the mean of the differences, the bias is not significant, and the measurement is reliable. The ICC was calculated based on an absolute agreement, 2-way mixed-effects model with high and clinically valid reliability denoted as >0.9 (Portney & Watkins, 2020; Weir & Vincent, 2021). The SEM (a.k.a typical error) was calculated as $SD\sqrt{(1 - ICC)}$ (Hopkins, 2000). The within-subject CV was calculated as the SD divided by the mean of two repeated trials performed under the same ambient conditions, then multiplied by 100%; we used <5% as acceptable reliability (Currell & Jeukendrup, 2008). Finally, Pearson's correlation coefficient was used to examine the direction and strength of relationships between the independent (participant characteristics) and dependent (work completed in kJ) variables; this was deemed appropriate as data were continuous, related pairs, normally distributed, linear and homoscedastic with minimal outliers. Statistical significance was set at $p<0.05$.

6.3 Results

Although the OCP group was significantly younger with less training history, all three groups were not different on most physical characteristics (Table 10). A higher concentration of progesterone characterized the OVU group, whilst estrogen was suppressed in the OCP group.

With regards to repeated performance of the work trials, individual results can be seen as Brinley and Bland-Altman plots (Figure 10). Results presented as homoscedastic, although two clear outliers were identified *a posteriori* using Tukey's method (interquartile range·1.5); where data were identified as outliers, these points (i.e., reliability between two trials) were removed. Therefore, as all outliers belonged to the same two participants, all of their data (4

trials) were removed. Overall bias was -2.1±54.6 kJ with this bias seemingly not significant and the measurement reliable (Giavarina, 2015).

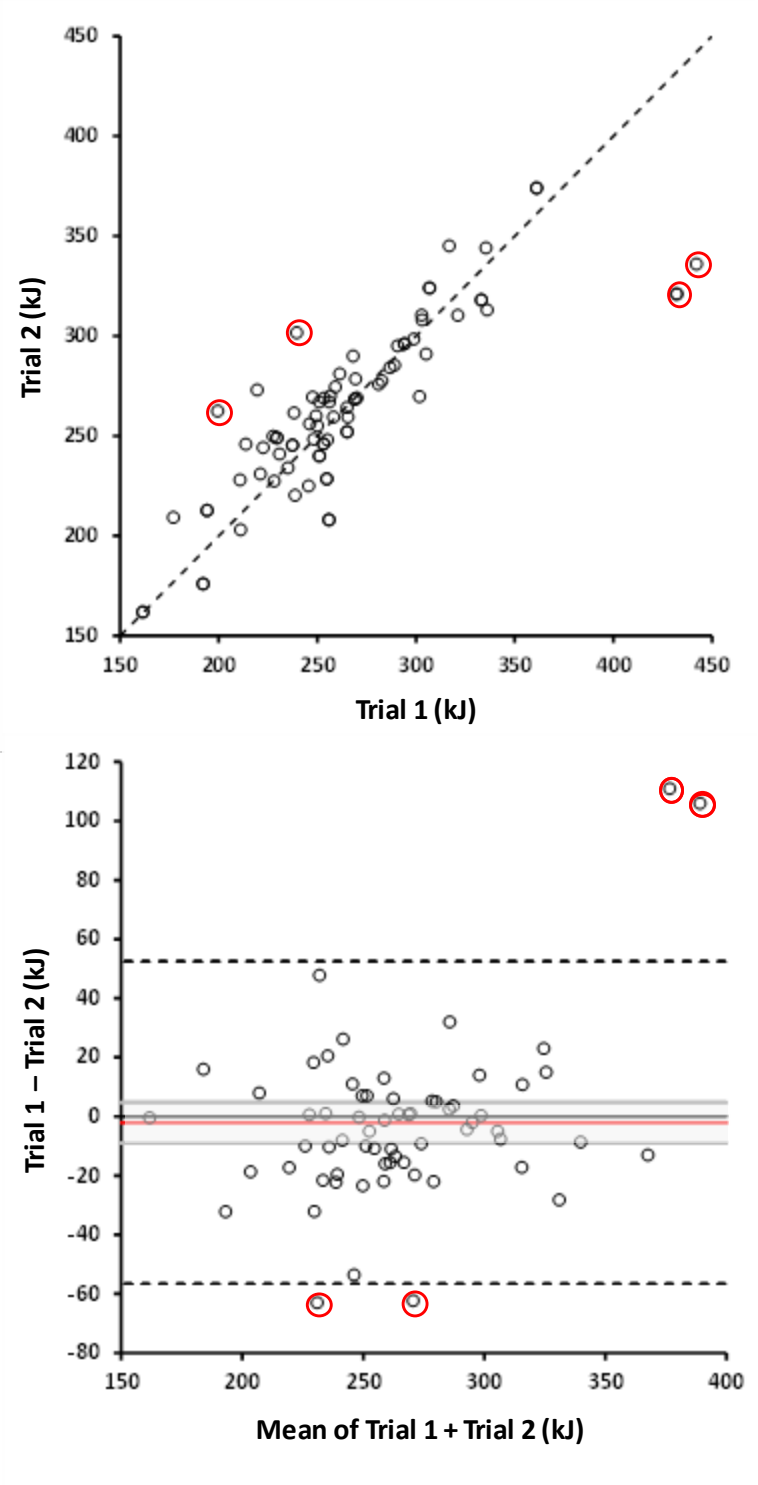

Figure 10. Brinley (top) and Bland–Altman (bottom) plots displaying individual work trial results. Two identified outliers circled red. For Bland–Altman plot, solid black line, line of equality; red line, bias; dashed black lines, limits of agreement; gray lines, confidence limits; shaded area, confidence limits for mean.

Table 11 displays results for the different ways of assessing reliability between repeated work trials, with these results displayed graphically in Figures 11 and 12. Overall, reliability was high, clinically valid (ICC=0.90, $p<0.01$) and acceptable with a mean CV of 4.7%, SEM of 3.8 kJ (2.1 W) and reliable bias of -2.1 kJ (-1.2 W). The different duration of pre-load and varying ambient conditions had no appreciable effect on reliability, whereas it could be argued that hormonal status (ANO≠OVU/OCP) affected reliability due to i) the ICC ($p=0.05$) not being different to 0, ii) having the largest CI for all metrics, and iii) the largest SEM (7.7 kJ or 4.3 W). However, on closer inspection this is likely a reflection of sample size as demonstrated best by removal of the two identified outliers (circled red, Figure 10). Following the removal of the two outliers the change in values were greatest for ANO for ICC (OVU: 0.96 [0.91-0.98], ANO: 0.98 [0.88-1.00], Overall: 0.95 [0.91-0.97], all $p<0.01$), % CV (OVU: 4.1 [2.7-5.4], ANO: 1.7 [0.2-3.1], Overall: 3.8 [2.9-4.7]) and SEM (OVU: 2.0 kJ, ANO: 0.7 kJ, Overall: 2.1 kJ).

In order to determine the potential factors contributing to the variance in work trial performance, bivariate correlations were performed between the within-participant % CV and physical characteristics in Table 10. In absolute terms, only peak aerobic power (Wmax, W) correlated with % CV ($r=0.37$, $p<0.01$); all other correlations $r<0.20$ and $p>0.10$.

Table 11: Measures of reliability for work completed (kJ) during the 30-min self-paced cycling work trial.

	Work (kJ, 95% CI)	**ICC** (95% CI)	**CV** (%, 95% CI)	**SEM** (kJ)
Hormonal Status				
Ovulatory	**265** (253-277)	**0.91*** (0.82-0.95)	**4.9** (3.1-6.7)	**4.1**
Anovulatory	**265** (249-281)	**0.72** (-0.15-0.94)	**5.6** (-0.6-11.9)	**7.7**
OCP	**265** (256-275)	**0.91*** (0.77-0.97)	**4.0** (2.7-5.2)	**3.1**
Environment				
Moderate	**277** (250-303)	**0.86*** (0.35-0.97)	**5.9** (0.7-11.0)	**6.7**
Dry	**269** (259-279)	**0.91*** (0.78-0.96)	**4.1** (2.3-5.8)	**3.2**
Humid	**260** (248-271)	**0.91*** (0.81-0.95)	**4.9** (3.1-6.6)	**3.8**
Pre-Load				
12 min	**260** (253-268)	**0.91*** (0.82-0.95)	**4.5** (3.2-5.8)	**3.4**
20 min	**277** (258-296)	**0.88*** (0.70-0.96)	**5.3** (2.3-8.2)	**5.6**
Overall	***265*** *(257-273)*	***0.90**** *(0.83-0.94)*	***4.7*** *(3.5-5.9)*	***3.8***

ICC: intraclass correlation coefficient; **CV**: coefficient of variation; **SEM**: standard error of measurement. **CI**: confidence interval. * significant at $p<0.01$ following analysis of variance.

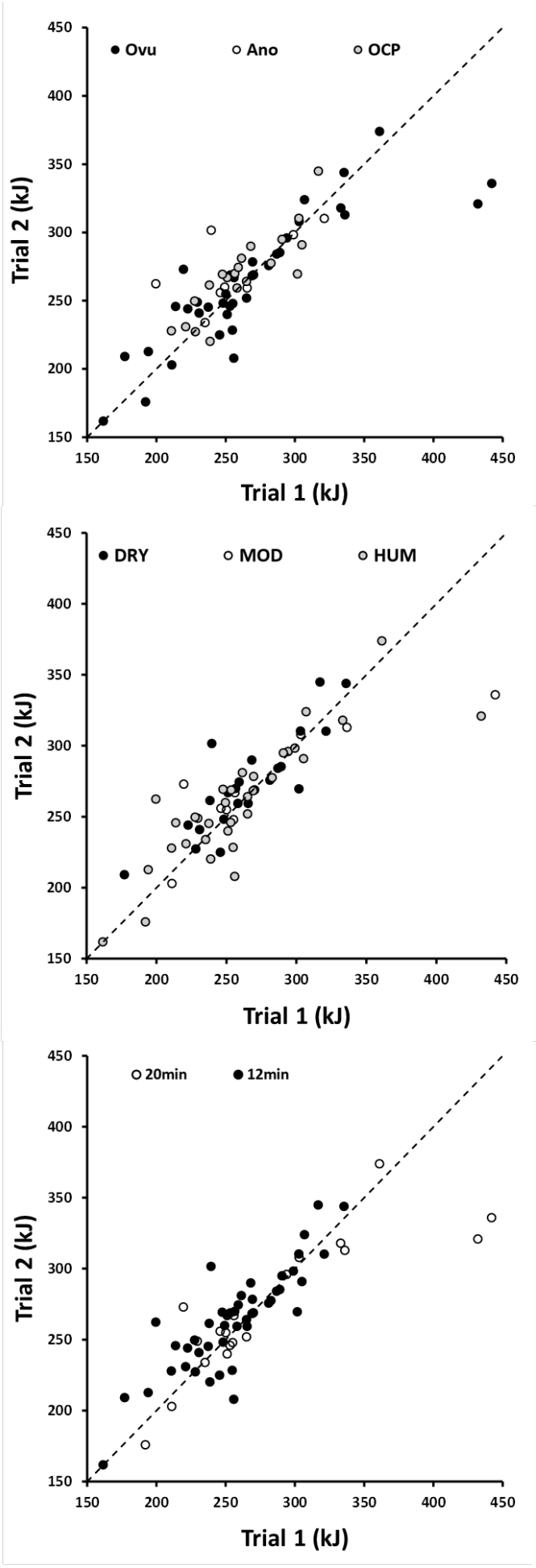

Figure 11. Brinley plots displaying individual work trial results and grouped by hormonal status (top, OVU, ovulatory; ANO, anovulatory; OCP, oral contraceptive pill), ambient condition (middle, MOD, moderate; DRY, warm-dry; HUM, warm-humid), and preload duration (bottom).

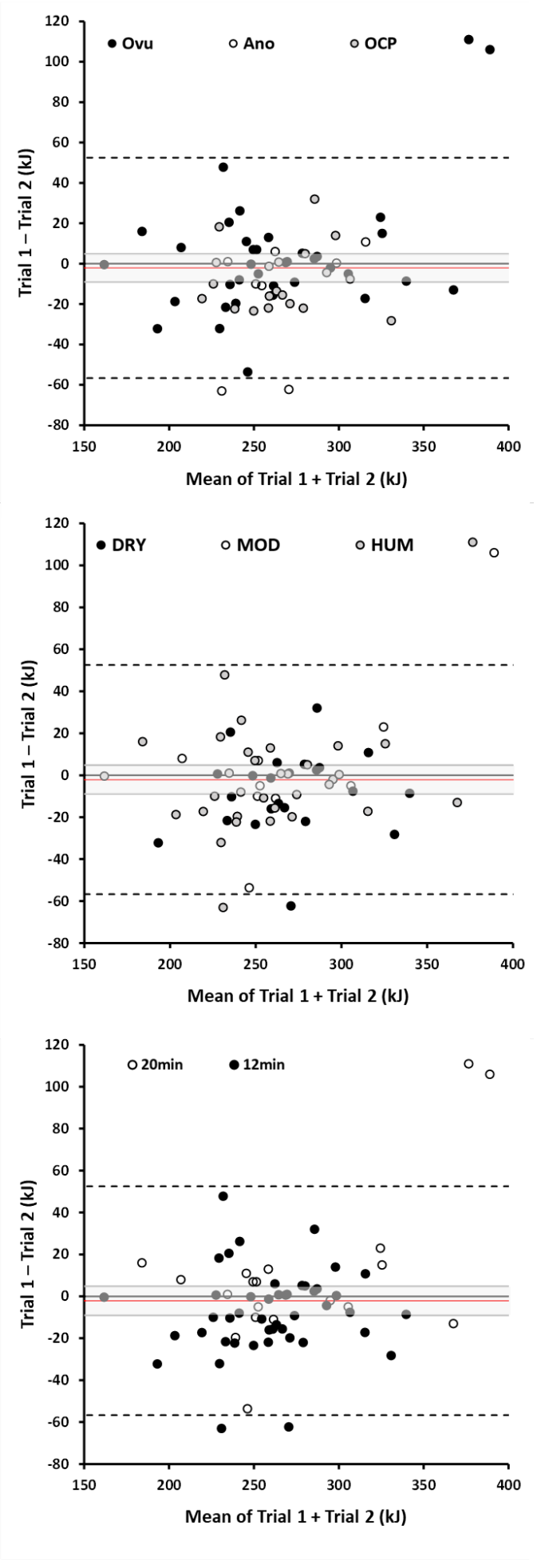

Figure 12. Bland–Altman plots displaying individual work trial results and grouped by hormonal status (top, OVU, ovulatory; ANO, anovulatory; OCP, oral contraceptive pill), ambient condition (middle, MOD, moderate; DRY, warm-dry; HUM, warm-humid), and preload duration (bottom).

6.4 Discussion

The important results from this chapter were that: 1) hormonal/ovulatory status and the introduction of greater ambient heat and humidity did not moderate measurement error of 30-min self-paced work trials that were completed ~two weeks apart, and 2) aerobically trained women repeatedly performed this test with acceptably low variability.

Previously, Bishop (1997) observed a CV of 2.7%, SEM of 3.4 W and ICC of 0.97 for average power output in female cyclists and triathletes completing two 60-min cycling work trials separated by a week. Our results are comparable, observing an overall CV of 4.7%, SEM of 3.8 W and ICC of 0.90. Taken together, the results of these two studies support the use of a self-paced work trial for the assessment of aerobic performance (or behavior) to determine intervention success in trained women, and indicate little difference to trained males (CV <5% as acceptable reliability, Currell & Jeukendrup, 2008) despite early concerns (Hopkins et al., 2001). Moreover, it has been speculated that increased ambient temperatures could introduce further variance to self-paced (constant work and duration) trials (Salgado et al., 2020), although the evidence from studies in trained men is to the contrary, i.e., a CV ≤ 3.6% irrespective of ambient temperature and humidity (Jeukendrup et al., 1996; Marino et al., 2002; Che Jusoh et al., 2015). The current results (Table 11, Figures 11 and 12) support the latter and corroborate these findings to trained women, thereby refuting the original supposition (Salgado et al., 2020).

Recent meta-analyses of the literature concluded trivial effects (Cohen's $d < 0.2$) of OCP use and menstrual cycle phase on exercise performance (Elliott-Sale et al., 2020; McNulty et al., 2020), indicating little meaningful effect from the variance in endogenous or exogenous ovarian hormone concentrations. The current results are the first to determine performance reproducibility in relation to trained but hormonally distinct women, with an important finding

that women often excluded *a posteriori* from physiological investigations due to subtle menstrual disturbances, display similar reliability to eumenorrheic athletes (Table 11, Figures 11 and 12). This is likely reflective of or consequent to similar body composition, functional capacity and training history (Table 10), as supported by these characteristics not being correlated with percent CV. However, Wmax did correlate positively with % CV, and the two identified outliers (Figure 10) were two of the 'best' performers – both in terms of Wmax (95th percentile) and race performance/results: one a former professional and continental road cycling champion whilst the other a national masters road cycling champion.

Total measurement error is composed of both systematic bias (e.g., learning or fatigue effects) and random error (e.g., biological or mechanical variation), with both components ideally quantified (Atkinson & Nevill, 1998). The most common methods for assessing relative reliability (*r* and ICC) are highly influenced by the range of values in the sample and cannot by themselves assess systematic bias, therefore, should not be used to extrapolate results to new individuals or compare between different measurement tools (Atkinson & Nevill, 1998). The most common methods for assessing absolute reliability overcome some of these issues and are expressed in the actual unit of the measurement (SEM) or dimensionless (CV), although represent (only) ~68% of the error present for an average individual (Atkinson & Nevill, 1998). The LoA quantifies systematic bias (-2.1 kJ or -1.2 W) and random error (±54.6 kJ or 30.3 W), such that for any new female athlete the difference between her two work trials should lie within these limits with a ~95% probability. It is also possible to determine a signal-to-noise ratio (sensitivity index) knowing total measurement error, such that one can be confident of the true effect of an intervention on performance. See APPENDIX 1 for a worked example.

6.5 Considerations

The observations herein are valid only for the current sample(s), protocol(s), and condition(s). Although the time between trials (~two weeks) in the current study is both longer (weekly trials for males) and shorter (four weeks +, training interventions) than might be required, Hopkins et al. (2001) concluded that time-between-trials is likely to have a smaller effect than the type of test and measure used, athletic status, and duration of test (Hopkins et al., 2001). We were unable to distinguish between anovulatory or luteal phase-deficient cycles as detection of a urinary luteinizing hormone surge (alone) cannot confirm luteal phase sufficiency without a reduction in estrogen concentrations (Elliott-Sale et al., 2020; Scheid & De Souza, 2010), which warrants further investigation. It is noteworthy that (sub-) sample size appeared to affect our results, although impossible to improve/increase in such a retrospective analysis. The two smallest data samples (<40, Figure 9), the anovulatory group and moderate ambient condition, displayed considerably lower reliability than the other two groups/conditions (Table 11). However, as demonstrated in the *Results*, omission of only one of the identified outliers improved reliability disproportionately compared to the larger data set. This is a well-known phenomenon of small data sets as they are more sensitive to heterogeneity, and why only larger samples (>40) are recommended to be examined by limits of agreement (Atkinson & Nevill, 1998). Thus, we caution interpretation of the current results, especially for these two sub-samples, with further confirmatory research required with *a priori* data samples >40.

6.6 Perspectives and Significance

Global warming and urbanization present a current and increasing threat to human health and performance/productivity, with nearly one-third of the global population regularly exposed to

extreme heat events, and a pertinent risk factor being increased physical activity for sport, exercise, or occupation (Luber & McGeehin, 2008; Mora et al., 2017). Concurrently, the number of women working in physically demanding occupations (e.g., mining, logging, construction, firefighting, military etc.) continues to increase (*"Women at Work"*, 2017), alongside the rising number of sports open to, events for, and number of competitive female athletes (*"Factsheet - Women in the Olympic Movement"*, 2021). Thus, mitigation strategies (or ergogenic aids) for active women encountering this combined heat load (metabolic and environmental) are warranted. The current study should provide the impetus for being able to correctly identify the true effects of, for example, exercise training, heat acclimation, hydration, cooling and dietary interventions (Grahn et al., 2005; Szymanski et al., 2018; Ravanelli et al., 2019; Chapman et al., 2020) in the athletic female population.

Chapter Seven

7.0 Do E_2 and P_4 Contribute to the Explained Variance in Core Temperature Response for Trained Women During Exertional Heat Stress When Metabolic Rates are Very High?

The formatting of this chapter is consistent with the accepted publication: Zheng, H., Badenhorst, C. E., Lei, T. H., Che Muhamed, A. M., Liao, Y. H., Fujii, N., Kondo, N., & Mündel, T. (2022). Do E_2 and P_4 contribute to the explained variance in core temperature response for trained women during exertional heat stress when metabolic rates are very high?. *Eur J Appl Physiol*. 122(10), 2201-2212.

Abstract

Women remain underrepresented in the exercise thermoregulation literature despite their participation in leisure-time and occupational physical activity in heat-stressful environments continuing to increase. In this chapter we determined the relative contribution of the primary ovarian hormones (estrogen [E_2] and progesterone [P_4]) alongside other morphological (e.g., body mass), physiological (e.g., sweat rates), functional (e.g., aerobic fitness) and environmental (e.g., vapor pressure) factors in explaining the individual variation in core temperature responses for trained women working at very high metabolic rates, specifically peak core temperature (T_{peak}) and work output (mean power output). Thirty-six trained women (32±9 y, 53±9 $ml \cdot kg^{-1} \cdot min^{-1}$), distinguished by intra-participant (early follicular and mid-luteal phases) or inter-participant (ovulatory vs. anovulatory vs. oral contraceptive pill user) differences in their endogenous E_2 and P_4 concentrations, completed a self-paced 30-min

cycling work trial in warm-dry (2.2±0.2 kPa, 34.1±0.2 °C, 41.4±3.4% RH) and/or warm-humid (3.4±0.1 kPa, 30.2±1.2 °C, 79.8±3.7% RH) conditions that yielded 115 separate trials. Stepwise linear regression was used to explain the variance of the dependent variables. Models were able to account for 60% of the variance in T_{peak} ($\bar{R}^2$: 41% core temperature at the start of work trial, $\bar{R}^2$: 15% power output, $\bar{R}^2$: 4% [E_2]) and 44% of the variance in mean power output ($\bar{R}^2$: 35% peak aerobic power, $\bar{R}^2$: 9% perceived exertion). E_2 contributes a small amount towards the core temperature response in trained women, whereby starting core temperature and peak aerobic power explain the greatest variance in T_{peak} and work output, respectively.

7.1 Introduction

Determining the factors that influence the female response to exertional heat stress is not new (Nunneley, 1978; Stephenson and Kolka, 1993), although different research approaches have been employed. One approach compares *differences in the group mean* with that of an intervention or other matched group when all characteristics apart from the one under investigation are standardized (Gagnon and Kenny, 2012; Charkoudian and Stachenfeld, 2014). Another approach considers the *relative contribution of independent variables* in explaining a dependent variable from individual responses of a (usually larger) heterogenous sample, seen as a better representation of the population distribution (Foster et al., 2020). Concerning the latter, previous studies (Havenith et al., 1998; Notley et al., 2019b) with the largest number of recreationally active women (*n*=36 and 43, respectively), have sought to determine thermoregulatory responses to low-moderate fixed-intensity cycle ergometry for 30-60 min bouts measured in a range of ambient conditions (from temperate to warm-humid and hot-dry). Both studies used regression analysis to determine which morphological (body mass, surface area and % fat etc.), physiological (metabolic rate or heat production, whole-body or

local sweat rates etc.), functional (aerobic fitness and power) and environmental (ambient temperature and absolute humidity) factors explained the variance in the women's' core temperature (T_{core}) response. Results indicated that the strength of the relationships and variance explained (10-59%) was dependent on the heat load i.e., combined exercise intensity and ambient thermal profile of the trials (Havenith et al., 1998; Notley et al., 2019b). Whilst these important results are valid for occupational and leisure-time physical activity completed at a low-moderate intensity (or metabolic rates), they are unlikely to be representative of or applicable to aerobically trained women undertaking such activities at higher intensities for a number of reasons.

Firstly, metabolic heat production in trained women at these higher intensities is likely double the values previously examined in the literature i.e., metabolic rates of 148-389 vs. 464-716 $W \cdot m^{-2}$ (Lei et al., 2019; Notley et al., 2019b), whilst trained women have a greater capacity to deal with a heat load on account of their enhanced heat loss effectors (Kuwahara et al., 2005). Next, these previous studies have not reported or accounted for differences in thermoregulation secondary to fluctuations in the primary ovarian steroids (E_2 and P_4), whereby generally speaking E_2 promotes heat dissipation and lowers T_{core} whilst P_4 has the opposite effect (Charkoudian and Stachenfeld, 2014). This is important to consider as this may differ to less trained counterparts (Kuwahara et al., 2005) and has been shown to contribute to the variance in T_{core} at rest (Lei et al., 2017). Finally, the nature of a fixed-intensity protocol denies the user of behavioral thermoregulation (Schlader et al., 2011a), thereby ignoring the fundamental premise that heat loss needs only to equal heat production (Nielsen, 1938), and is considered to be less ecologically valid (than self-pacing) for most leisure-time and occupational physical activity apart from few i.e., forced marching.

The purpose of the current paper was to determine the relative contribution of the E_2 and P_4 alongside other morphological, physiological, functional and environmental factors in

explaining the individual variation in trained women when considering the core temperature response (peak T_{core}, [T_{peak}]) and work output (mean power output) with very high metabolic rates. To achieve this, we retrospectively analyzed results from thirty-six trained women completing a self-paced 30-min work trial that has been shown to be unaffected by ovulatory status, ambient environment and pre-load/warm-up duration (Zheng et al., 2021b). Participants were distinguished by intra-participant (i.e., early follicular and mid-luteal phases) or inter-participant (i.e., ovulatory vs. anovulatory vs. OCP user) differences in their endogenous E_2 and P_4 concentrations. We hypothesized that in addition to previously identified factors such as body mass, aerobic fitness and metabolic heat production (Havenith et al., 1998; Notley et al., 2019b) the ovarian hormones would contribute significantly towards the variance explained in T_{core} during exercise.

7.2 Methods

This chapter combines data from three separate experiments (Lei et al., 2017, 2019; **Chapter 5**), which included *n*=28 ovulatory and OCP-user female cyclists/triathletes and adds to this new data the *n*=8 participants that did not complete all trials or were excluded from the final analyses on account of being deemed anovulatory (Lei et al., 2017; **Chapter 5**). Interested readers are directed to these studies for further methodological details and results.

7.2.1 Ethical Approval

All original studies (Lei et al. 2017, 2019; **Chapter 5**) had received approval by the Massey University Human Ethics Committee (Southern A) and were performed in accordance with the

latest revision of the *Declaration of Helsinki*, except for registration in a database. Informed, written consent was obtained from all participants prior to their participation.

7.2.2 Participants

Thirty-six aerobically-trained women participated, yielding 115 separate trials (*n*=23 completed 4 trials, *n*=10 completed 2 trials, *n*=3 completed 1 trial, see Figure 13). Their physical characteristics are displayed in Table 12. Inclusion criteria were that participants were healthy non-smokers not taking any regular medication (apart from those using the OCP), cycling regularly (≥3 days per week) with a maximal aerobic capacity ($\dot{V}O_2max$) ≥40 $ml \cdot kg^{-1} \cdot min^{-1}$. Exclusion criteria included any cardiovascular, metabolic, neurological, and respiratory diseases. All eumenorrheic women self-reported a regular menstrual cycle 21-35 days in length (≥ 3 mo) with no use of hormonal contraception (≥ 6 mo). All OCP women were taking a monophasic combination OCP (≥ 1 y) with experimental visits completed during the three weeks of active pill use (see Lei et al., 2019 for further details).

Table 12: Participant characteristics for ovulatory (OVU), anovulatory (ANO) and oral contraceptive pill (OCP) groups.

Characteristic	**OVU (*n*=19)**	**ANO (*n*=7)**	**OCP (*n*=10)**	**Mean (*n*=36)**	***p*-value**
Age (y)	34±9 (19-46)	37±10 (22-51)	25±5 (20-36)*	32±9 (19-51)	0.01
Mass (kg)	63±6 (46-69)	60±7 (46-69)	68±10 (58-82)	64±8 (46-82)	0.13
A_D (m^2)	1.70±0.11 (1.49-1.94)	1.63±0.12 (1.37-1.72)	1.76±0.13 (1.60-1.98)	1.70±0.12 (1.37-1.98)	0.12
A_D:mass	0.027±0.001 (0.024-0.029)	0.027±0.002 (0.025-0.030)	0.026±0.002 (0.023-0.028)	0.027±0.002 (0.023-0.030)	0.37
% fat	23±5 (15-37)	20±5 (13-29)	24±5 (16-32)	23±6 (13-37)	0.19
$\dot{V}O_2max$ ($L \cdot min^{-1}$)	3.3±0.6 (2.3-4.6)	3.4±0.9 (2.7-5.0)	3.7±0.5 (3.0-4.8)	3.4±0.6 (2.3-5.0)	0.22
PPO (W)	270±40 (225-392)	283±31 (250-325)	283±29 (248-325)	276±35 (225-392)	0.56
Training history (y)	7.1±3.5 (4-16)	8.1±5.1 (1-15)	3.7±2.5 (2-10)*	6.3±3.9 (1-16)	0.03

A_D, Du Bois body surface area; **PPO**, peak aerobic power; **$\dot{V}O_2max$**, maximal rate of O_2 consumption. Values are mean±SD (range). * significantly different to both other groups.

7.2.3 Ovulatory Status and Ambient Conditions

Eumenorrheic women were tested on days 3-6 (EF) and 18-21 (ML) following the *start of menses*, whilst OCP women were tested on days 3-6 and 18-21 following the *start of active OCP use*. Our rationale for comparing EF and ML was based on maximizing the differences in E_2 and P_4 occurring naturally, permitting comparison with/expansion beyond previous results, and that ovulatory women are in EF and ML for ~50% of their reproductive lives. Although this approach represents the phases of lowest hormone exposure and peak P_4, it does not include for comparison the late-follicular/pre-ovulatory phase. Although the late-follicular/pre-ovulatory phase captures when E_2 peaks, the duration of <72h makes it difficult to perform repeated tests (such as this study) and comprises a much smaller proportion (~10%) of the reproductive life for these women. Testing for eumenorrheic women was scheduled using the three-step method (Allen et al., 2016) whereby self-reported menses onset and urinary luteinizing hormone testing (EasyCheck® Ovulation Test, Phoenix Medcare Ltd, Auckland, New Zealand) prospectively identified EF and ML, whilst measurement of serum 17β-estradiol (E_2) and P_4 retrospectively confirmed ML. A P_4 level of >5 $ng \cdot ml^{-1}$ is good evidence that ovulation has occurred (Leiva et al., 2015; Schaumberg et al., 2017; Scheid et al., 2010). Therefore, participants were deemed as ovulatory (OVU, >5 $ng \cdot ml^{-1}$) or anovulatory (ANO, <5 $ng \cdot ml^{-1}$) as detection of a urinary luteinizing hormone surge (alone) cannot confirm luteal phase sufficiency (Scheid et al., 2010). Ambient conditions were distinguished by vapor pressure, such that the following characterized each environment: DRY (2.2±0.2 kPa, 34.1±0.2 °C, 41.4±3.4% RH, wet-bulb globe temperature: 27.0±0.5 °C) and HUM (3.4±0.1 kPa, 30.2±1.2 °C, 79.8±3.7% RH, wet-bulb globe temperature: 28.2±0.8 °C).

7.2.4 Experimental Overview

Data collections were conducted excluding the Southern Hemisphere summer (i.e., March-November) where the average daily temperature did not exceed 22 °C, nor had participants spent any time in a warmer climate for at least one month prior to the study. All participants attended the laboratory on the following occasions: (1) preliminary submaximal and maximal aerobic capacity test, (2) experimental familiarization, (3) experimental trials. For an overview of the experimental design see Figure 12. The experimental trials consisted of the following factors: (*quasi*-) menstrual phase (early follicular [EF, *56 trials*] and mid-luteal [ML, *59 trials*]) and ambient profile (warm-humid [HUM, *69 trials*] and warm-dry [DRY, *46 trials*]). The order of the trials was randomized and counterbalanced except the order of the ambient profile was consistent in different (*quasi*-) phases within participants. Experimental trials were conducted at the same time of the morning (±1 h) and following >24 h of dietary and exercise control. Each trial consisted of either 12 or 20 min of fixed-intensity pre-load that was kept consistent within participants, immediately followed by a 30 min self-paced work trial where only percentage of time elapsed (every 20% or 6 min) was provided to the participant. All exercise was performed on an electronically braked cycle ergometer (Lode Excalibur, Groningen, The Netherlands), with handlebars, seat height and pedal preference standardized according to individual preference. The typical timeline for a participant to complete this study resulted in preliminary testing and familiarization separated by 3-7 days during the (*quasi*-) follicular phase, with half of the participants starting their experimental trials the following (*quasi*-) luteal phase (i.e., 14 days later) and the other half the following (*quasi*-) follicular phase (i.e., 28 days later), with within-phase experimental trials differing by ambient profile separated by three days.

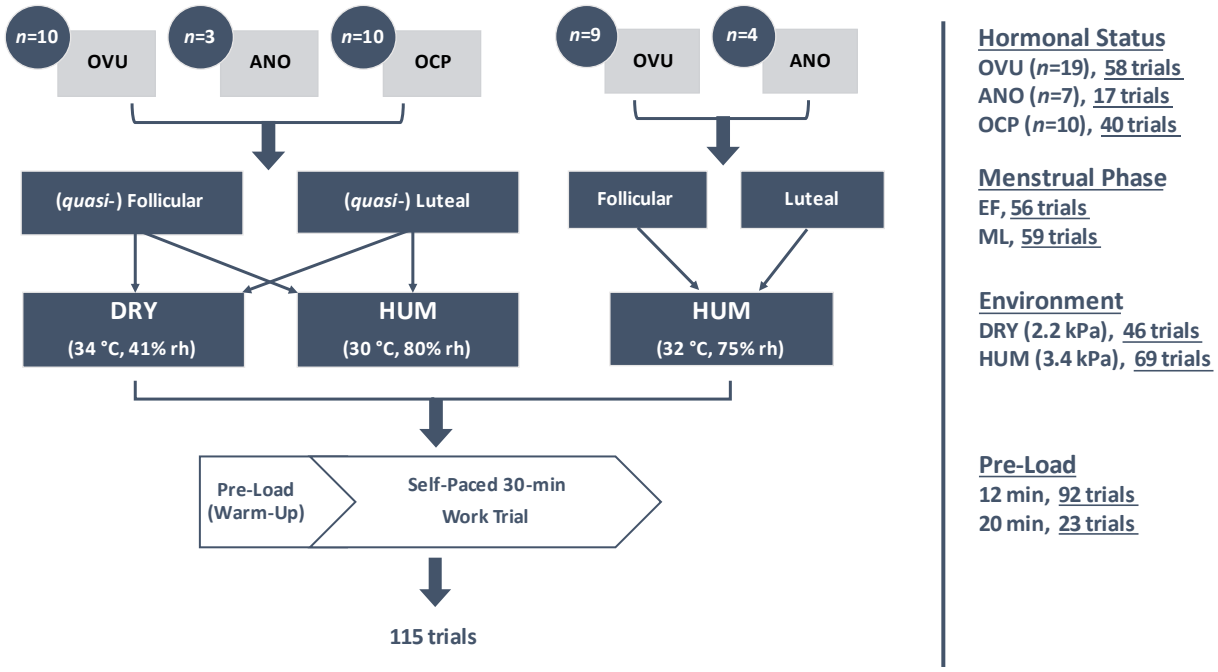

Figure 13. Diagram of experimental overview. Ovulatory (OVU), anovulatory (ANO) and oral contraceptive pill (OCP) users performed trials in their (*quasi*-) early follicular (EF) and/or mid-luteal (ML) phases in warm-dry (DRY) and/or warm-humid (HUM) environmental heat. *n*=23 completed four trials and *n*=10 completed two trials; whereas *n*=3 completed only one trial due to scheduling difficulties and drop-out.

7.2.5 Preliminary Testing and Familiarization

All preliminary testing was conducted in the (*quasi*-) EF phase of each participant's menstrual cycle to minimize the potential effects of menstrual / OCP cycle on their physiological and performance responses during the tests (Sims and Heather, 2018). Following anthropometric measurements (height, weight, body composition), a 24-min steady-state submaximal cycle ergometer test was conducted in a temperate laboratory environment (18-22 °C) with a fan-generated airflow of 19 $km \cdot h^{-1}$ facing participants. The submaximal cycle test consisted of four consecutive six-minute stages with power outputs of 100 W, 125 W, 150 W and 175 W at comfortable but constant cadence. O_2 consumption was measured during the last two minutes of each stage. Following 10 min rest from the submaximal test, a $\dot{V}O_2max$ cycle ergometry test was performed. The initial workload began at 100 W and increased by 25 W every minute,

until volitional exhaustion. The exercise intensity during the self-paced exercise was based on 75% of an individual's $\dot{V}O_2max$, which was derived from the linear relationship between the power output and the O_2 consumption during both the steady-state submaximal exercise test and maximal aerobic capacity test. Following at least 24 hours rest from the preliminary session, a familiarization trial was conducted to ensure all participants were familiar with the testing procedures and to minimize the learning effect during trials. This trial was replicated entirely during the experimental trials outlined below.

7.2.6 Dietary and Exercise Control

Diet and physical activity during the 48 hours prior to the first experimental trial were recorded and participants were instructed to repeat these for the following experimental trials. The day of and prior to any experimental trial was marked by abstinence from alcohol, exercise, and only habitual caffeine use (as abstinence would confound results from withdrawal effects). This dietary and exercise control minimized variation in pre-trial metabolic state. Fluid intake was encouraged to ensure a euhydrated state.

7.2.7 Experimental Procedure

These trials were conducted in the same environmental chamber with a fan-generated airflow of 19 $km \cdot h^{-1}$. Upon their arrival at the laboratory, participants voided, producing a urine sample to confirm a urine specific gravity <1.020 to ensure adequate hydration (Sawka et al., 2007). Following this, nude body weight was recorded and participants self-inserted a rectal thermistor 12 cm beyond their anal sphincter. A blood sample was obtained from an antecubital vein after participants had rested seated for 15 min. Participants entered the environmental chamber wearing only cycling shorts and top, shoes and socks. Participants rested seated on the ergometer for 20 min during which they were instrumented, and baseline measurements were recorded. They then completed either i) 6 min of cycling at each of 125 and 150 W (62±9 and

73±10% $\dot{V}O_2$max, respectively, *92 trials*), or ii) 10 min of cycling at each of 100 and 125 W (56±8 and 68±10% $\dot{V}O_2$max, respectively, *23 trials*); notably, where participants completed multiple trials the warm-up duration was kept constant. Physiological measurements taken during the final 2 min of each intensity included expired gas, and rating of perceived exertion RPE, whilst rectal temperature (T_{rec}) was measured continuously. Immediately on completion of the second fixed-intensity bout, the ergometer was set to linear mode based on the formula of Jeukendrup et al. (1996), where participants were instructed to perform as much work as possible over 30 min. During this 30 min self-paced period, work completed (kJ), and RPE were recorded every 6 min, whilst T_{rec} was measured continuously and tap water at 20 °C was provided to drink *ad libitum* throughout to minimize dehydration. Total work completed (kJ) was used as criterion measure for performance although this was expressed as mean power output for the trial to allow wider application. After the completion of the 30 min self-paced exercise, the participant towel-dried and recorded nude body weight.

7.2.8 Measurements

Results reported in the current study were those for which a maximal number of measures were recorded for the *n*=36. For interested readers, other physiological (i.e., thermoregulatory, cardiovascular, inflammatory) and reliability measurements were performed during these trials and can be found in separate studies (Lei et al., 2017, 2019; **Chapter 5 and 6**).

7.2.8.1 Anthropometric

Participant height and weight were measured using a stadiometer (Seca, Germany; accurate to 0.1 cm) and scale (Jadever, Taiwan; accurate to 0.01 kg), from which surface area (A_D) was estimated (Du Bois and Du Bois 1916). Body composition was measured using multi-frequency bioelectrical impedance analysis (InBody 230, Korea) using a standard procedure (Kyle et al., 2004).

7.2.8.2 Respiratory

Expired respiratory gases were collected from a mixing chamber and analyzed for O_2 consumption using an online, breath-by-breath system (VacuMed Vista,Turbofit, Ventura, CA, USA) using a 30-s average. This system was calibrated before each trial using a zero and *β*-standard gas concentrations, and volume (VacuMed 3L Calibration Syringe).

7.2.8.3 Body Temperature and Sweat Loss

T_{core} was indexed from T_{rec} measured with a rectal thermistor (Covidien Mon-a-Therm, USA; accurate to 0.1 °C) and recorded continuously using TracerDAQ software (Measurement Computing Corporation, Norton, MA, USA). Whole-body sweat rate (WBSR) was estimated from nude body mass loss, corrected for fluid consumed and time.

7.2.8.4 Hormones

Venous blood was collected by venipuncture into a vacutainer (Becton-Dickinson, Oxford, UK) containing clot activator and once clotted (>30 min) the whole blood was centrifuged at 4 °C and 805 *g* for 15 min and aliquots of serum were transferred into Eppendorf tubes (Genuine Axygen Quality, USA) and stored at −80 °C until further analysis. Serum samples were analyzed using enzyme-linked immune assays for E_2 (Demeditec Diagnostics, Kiel, Germany) and P_4 (IBL International, Hamburg, Germany) with a sensitivity of 6.2 $pg \cdot ml^{-1}$ and 0.045 $ng \cdot ml^{-1}$, respectively, and an intra-assay variation of <6 and <7%, respectively.

7.2.8.5 Perceived Exertion

RPE was measured using the 15-grade scale, from 6 to 20 (Borg 1970).

7.2.8.6 Data and Statistical Analyses

The dependent variables were mean power output and T_{peak}. The independent variables included: age, mass, A_D, A_D:mass, % body fat, aerobic fitness, peak aerobic power, training

history, E_2, and P_4, P_4:E_2, T_{core} at baseline (T_{base}), T_{core} at start of work trial (T_0), WBSR, vapor pressure and power output.

All statistical analyses were performed with SPSS software for Windows (IBM SPSS Statistics 25, NY, USA). Descriptive values were obtained and reported as means and standard deviation (±SD). Data were checked for normality by calculating skewness and kurtosis, whereby values within ±2 were deemed to be acceptable (Weir and Vincent, 2021). Participant characteristics were analyzed using one-way ANOVA and Student *t* Test. Correlation coefficients were calculated to reveal the direction and strength of any potential relationships between variables; Pearson's correlation coefficient and Spearman's Rho were determined for data that did or did not (E_2, P_4, P_4:E_2) follow a normal distribution, respectively. Finally, in line with and to allow comparison to previous research (Havenith et al., 1998; Notley et al., 2019b) stepwise linear regression was used to explain the variance of the dependent variables. A total of 104 (T_{peak}) and 103 (power output) cases were included for the regression (not 115, due to missing E_2, P_4 and sweat rate data), where data that did not follow a normal distribution (E_2, P_4, P_4:E_2) were log-transformed before entering. Independent variables were only included in final models if their tolerance value was >0.5 in order to avoid unacceptable collinearity between predictors. Data were screened for influential cases using Cook's distances, leverage values and standardized residuals. Test assumptions for normality, linearity and homoscedasticity were determined by scatter and residual plots. Since some participants completed repeated trials, residuals from each final regression model were tested for serial correlation using the Durbin-Watson test, whereby a value between 1.5 and 2.5 was deemed acceptable (Durbin and Watson, 1950). Statistical significance was set at $p \leq 0.05$.

7.3 Results

As can be seen from Table 13, a wide range of intra- and inter-participant endogenous concentrations in E_2 and P_4 was evident. By contrast, other dependent and independent variables displayed far less variability between participants, (*quasi*-) menstrual phases and ambient environments (Table 14).

Table 13. Participant hormone concentrations.

	(*quasi*-) Follicular						**(*quasi*-) Luteal**					
	Warm-Humid			**Warm-Dry**			**Warm-Humid**			**Warm-Dry**		
	OVU	**ANO**	**OCP**	**OVU**	**ANO**	**OCP**	**OVU**	**ANO**	**OCP**	**OVU**	**ANO**	**OCP**
E_2 (pg·ml^{-1})	63±60	46±14	19±23	55±43	24±n.d.	17±25	108±70	156±128	21±31	86±76	44±7	20±28
	(2-255)	(36-56)	(1-75)	(9-137)	(-)	(0.2-79)	(45-297)	(42-386)	(0-102)	(15-288)	(39-48)	(0-89)
P_4 (ng·ml^{-1})	0.6±0.4	0.2±0.1	0.2±0.2	0.5±0.4	0.2±0.1	0.1±0.1	16.4±10.0	1.8±2.9	0.2±0.2	17.1±18.8	0.6±0.6	0.1±0.1
	(0.1-1.2)	(0.1-0.3)	(0.0-0.4)	(0.1-1.1)	(0.1-0.2)	(0.01-0.4)	(6.5-52.7)	(0.1-7.9)	(0.02-0.5)	(5.5-69)	(0.2-1.3)	(0.01-0.5)
P_4:E_2	47±150	5±2	28±42	15±14	5.5±n.d.	31±59	184±98	23±45	16±11	276±300	16±15	12±13
	(1-667)	(4-6)	(2-130)	(2-36)	(-)	(3-185)	(44-415)	(1-114)	(4-34)	(88-1048)	(5-26)	(5-44)

n.d., SD was not determined due to missing data. **E_2**, 17β-estradiol; **P_4**, progesterone; **P_4:E_2**, ratio of progesterone to 17β-estradiol. **OVU**, ovulatory group; **ANO**, anovulatory group; **OCP**, oral contraceptive user group. Values are mean±SD (range).

Table 14. Descriptive statistics for dependent and independent variables.

	(*quasi*-) Follicular		**(*quasi*-) Luteal**	
	Warm-Humid	**Warm-Dry**	**Warm-Humid**	**Warm-Dry**
Independent variables				
T_{base} (°C)	37.2±0.3 (36.6-37.8)	37.3±0.3 (36.8-37.8)	37.4±0.3 (36.6-37.8)	37.4±0.2 (36.9-37.7)
T_0 (°C)	37.7±0.3 (37.1-38.2)	37.8±0.3 (37.4-38.4)	37.9±0.3 (37.1-38.3)	37.8±0.2 (37.6-38.3)
Sweat rate ($kg \cdot h^{-1}$)	0.8±0.3 (0.4-1.6)	0.9±0.2 (0.4-1.3)	0.8±0.3 (0.4-1.9)	0.9±0.4 (0.2-1.8)
RPE	15.1±1.5 (12.6-17.6)	15.5±1.7 (12.3-18.2)	15.2±1.7 (11.8-19.8)	15.5±1.7 (12.4-18.2)
Absolute humidity (kPa)	3.4±0.1 (3.2-3.6)	2.2±0.2 (1.8-2.6)	3.4±0.1 (3.2-3.6)	2.2±0.2 (1.9-2.6)
Dependent variables				
Power output (Watt)	147±29 (90-240)	149±19 (116-192)	144±24 (90-208)	150±20 (98-191)
T_{peak} (°C)	38.6±0.3 (37.6-39.2)	38.7±0.3 (38.0-39.4)	38.7±0.4 (37.9-39.6)	38.7±0.3 (38.1-39.3)

RPE, rating of perceived exertion; T_0, T_{core} at start of work trial; T_{base}, T_{core} at baseline; T_{peak}, peak T_{core}. Values are mean±SD (range).

7.3.1 T_{peak}

Correlation coefficients between the independent variables and T_{peak} measured during the 30-min work trial can be seen in Figure 14 (left panel). Factors included in the regression analysis to explain the variance in T_{peak} were A_D:mass, log(E_2), T_0, and power output. The decision to enter A_D:mass was made as it is a function of both individual factors and that it provided the strongest correlation to T_{peak}, whilst T_0 (but not T_{base}) was entered to reduce collinearity and because it provided far stronger correlation to T_{peak}. The resulting model can be seen in Table 15, with no evidence of serial correlation in the model (2.15), and very high tolerance values indicating acceptable collinearity and model stability. Variables that were excluded from the models were A_D:mass (β=0.08, p=0.26). Overall, the model was able to account for 60% of the variance in T_{peak}, with T_0 the largest contributing variable (Figure 14, right panel). It is noteworthy that the resulting model remained unchanged even when the omitted variables (A_D, mass and T_{base}) were included *a posteriori*, supporting the decision process.

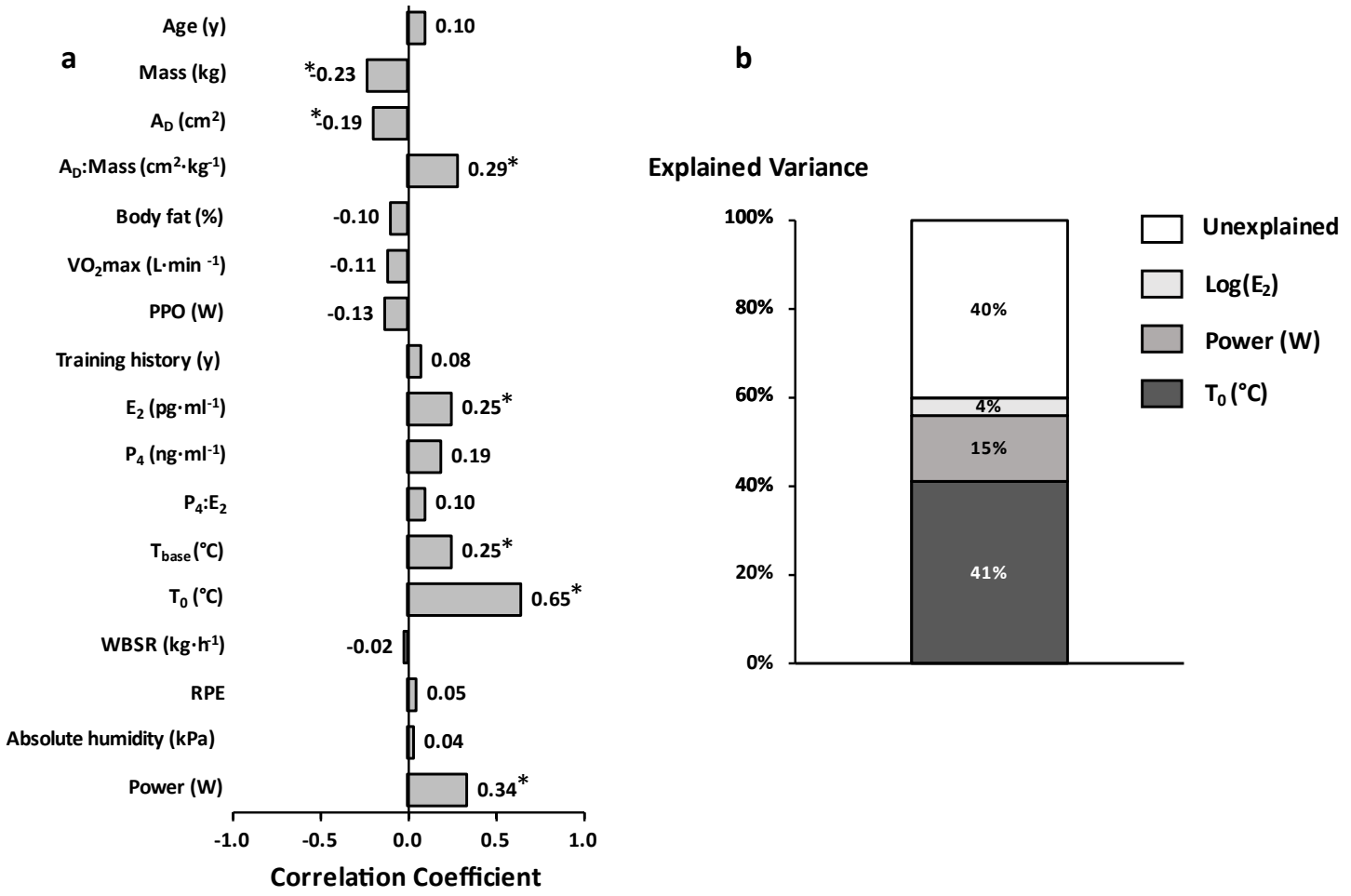

Figure 14. a: Bivariate associations between independent variables and peak T_{core} (T_{peak}) on all common data points. *p<0.05. **b**: The percentage of explained and unexplained (residual) variance ($\bar{R}^2$) for explaining T_{peak}.

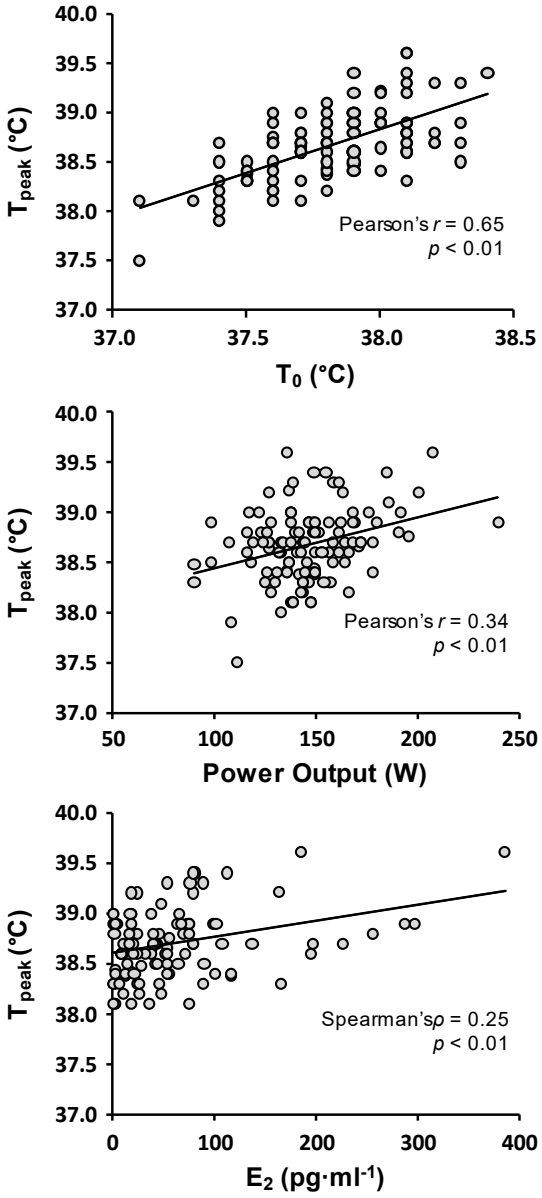

Figure 15. Bivariate associations between peak core temperature (T_{peak}) during exercise and core temperature at the start of the work trial (T_0; top row, n=115); between T_{peak} and mean power output during the work trial (middle row, n=115); between T_{peak} and E_2 concentration measured before exercise (bottom row, n=104). Values are all common individual data points, analyzed using Pearson's correlation coefficient and Spearman's Rho, respectively.

7.3.2 Power Output

Correlation coefficients between the independent variables and mean power output achieved during the 30-min work trial can be seen in Figure 16 (left panel). Factors included in the regression analysis to explain the variance in power output were A_D, $\dot{V}O_2$max, PPO, training history, WBSR, and RPE. The resulting model can be seen in Table 15, with no evidence of serial correlation in the model (1.86), and very high tolerance values indicating acceptable collinearity and model stability. Variables that were excluded from the models were A_D (β=-0.03, p=0.72), $\dot{V}O_2$max (β=0.16, p=0.11), training history (β=0.09, p=0.22), and WBSR (β=0.10, p=0.24). Overall, the model was able to account for 44% of the variance in power output, with peak aerobic power the largest contributing variable (Figure 16, right panel).

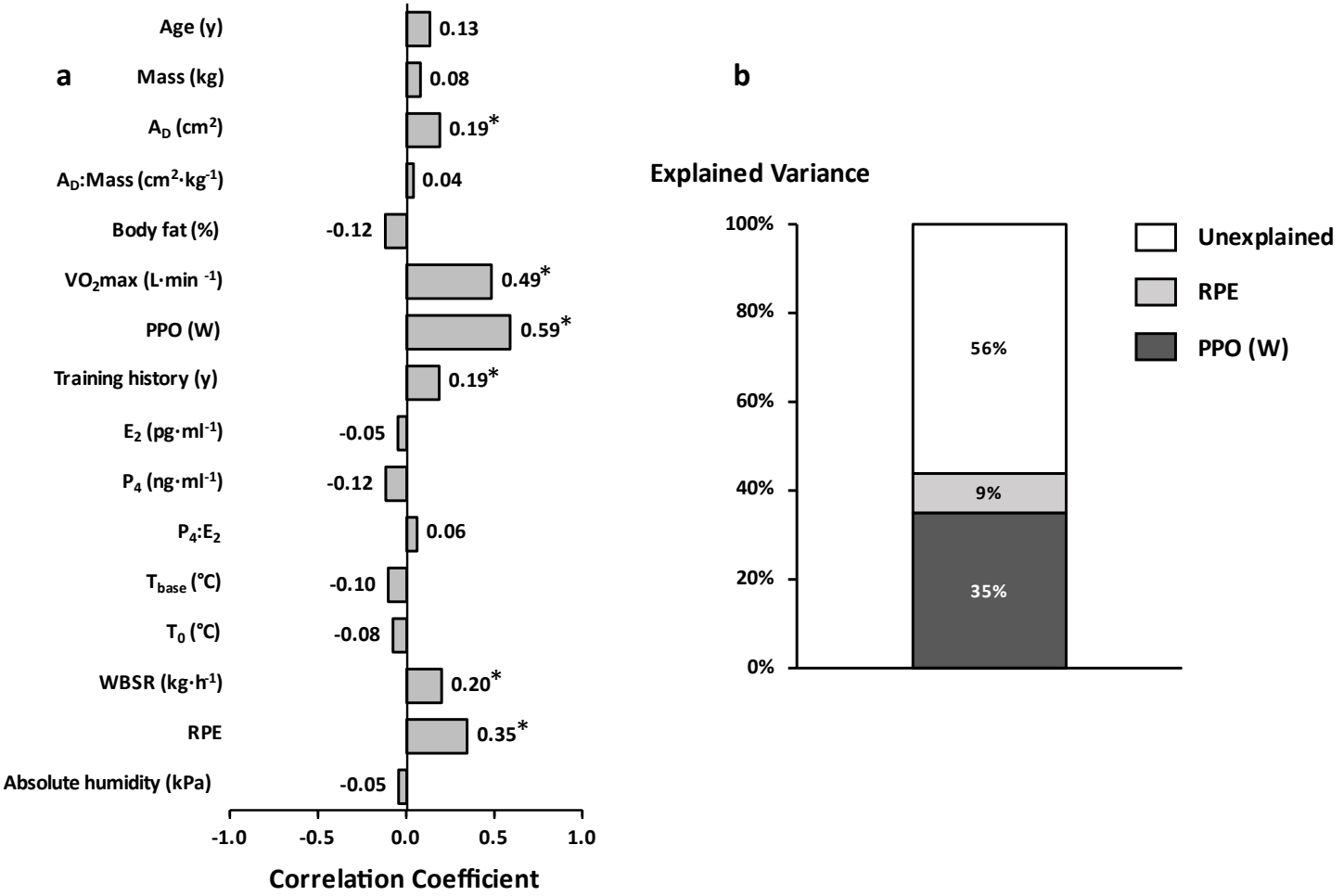

Figure 16. a: Bivariate associations between independent variables and mean power output on all common data points. *p<0.05. **b**: The percentage of explained and unexplained (residual) variance ($\bar{R}^2$) for explaining mean power output.

Table 15. Multiple regression models for explaining the core temperature response (T_{peak}) and performance (mean power output).

	B	**95% CI**	**β**	***p***	**Tolerance**	$\overline{R}^2$
T_{peak}						
Explaining						
Constant	4.10	-2.49-10.68		0.22		
T_0, °C	0.89	0.71-1.06	0.65	<0.01	0.98	41.1%
Power, W	0.01	0.00-0.01	0.41	<0.01	0.99	14.9%
log(E_2)	0.12	0.04-0.19	0.20	<0.01	0.98	3.5%
Power output						
Explaining						
Constant	-34.20	-77.99-7.24		0.11		
PPO, W	0.40	0.30-0.51	0.58	<0.01	1.00	34.7%
RPE	4.60	2.43-6.77	0.31	<0.01	1.00	9.2%

B, unstandardised regression coefficient; **95% CI**, confidence intervals of the slope coefficient or intercept; **β**, standardised regression coefficient; $\overline{R}^2$, adjusted partial contribution to total variance.

7.4 Discussion

The current investigation fills an important gap in the literature that describes a woman's vulnerability to exertional heat stress in this literature. Namely, it is the first study to determine the relative contribution of independent variables (individual factors) in explaining the core temperature response to exertional heat stress in women *at very high metabolic rates*, and *when accounting for the inter- and intra- variation in ovarian hormone concentrations* (cf. Havenith et al., 1998; Notley et al., 2019b). In partial support of our hypothesis, we observed that E_2 contributes a small amount towards the core temperature response (T_{peak}), whereby starting core temperature and power output (≈metabolic heat production) explained the greatest variance.

In the current study E_2 was positively associated with T_{peak}, although was only able to explain ≤4% of its variance (Figure 14, Table 15). This seemingly contradicts other research (Charkoudian and Stachenfeld, 2014) and is inconsistent with the previous study by Lei et al. (2019). A subset of these results (Lei et al., 2019) showed that the OCP group had attenuated heat loss mechanisms (↑ forearm vascular resistance, ↓ forearm blood flow, local and whole body sweat rates) compared to their matched eumenorrheic counterparts, concurrent with lower concentrations of E_2 (19±26 vs. 78±65 pg·ml^{-1}; p<0.01; Cohen's d=1.2), although these differences were insufficient to change T_{core}. Furthermore, despite no change in endogenous E_2 and P_4, the OCP group still demonstrated a consistent and significant increase in resting and exercising T_{core} during their *quasi*-ML compared to EF (Lei et al., 2019). Using the current analysis (and design), it is difficult to determine whether it is the intra-participant or inter-participant E_2 driving this relation (or both, Table 13, Figure 15). Similarly, what modulating effect P_4 might be contributing is unclear and is probably best explored using different methods e.g., use of progestin-only OCP or temporary suppression of the menstrual cycle with a

gonadotropin releasing hormone (ant)agonist (Charkoudian and Stachenfeld, 2014). A confounding factor in this analysis may be that the group with lowest concentrations of E_2 was younger and had a lower training history (Table 12). Aerobic training, independent of aerobic fitness ($\dot{V}O_2max$), has been shown to improve T_{core} and heat loss responses in both men (Ravanelli et al., 2021) and women (Ichinose et al., 2009) synonymous with phenotypic heat adaptation. Clearly, further research on this topic is necessary in additional cohorts (e.g., ages and training status), nevertheless the effect of E_2 on T_{peak} was still considerably less than that of starting T_{core} and power output.

That T_0 was able to explain ~40% of the T_{core} response should reinforce for women what is already known and practiced for men with regards to heat-specific interventions; namely, trained women should focus and prioritize interventions (e.g., aerobic training, active heat adaptation, pre-exercise cooling, fluid ingestion etc.) that effectively lower T_{core} before competition, attenuate the rise in T_{core} during or (perhaps) extend T_{core} at the end of exercise in order to improve work output (Alhadad et al., 2019). Moreover, power output explained ~15% of the T_{core} response, which reaffirms the contribution of metabolic heat production (Nielsen, 1938; Notley et al., 2019b). This highlights the role that behavioral thermoregulation (self-pacing) plays during exercise in the heat by being able to reduce metabolic heat production, thereby improving heat exchange with the environment to decrease thermoregulatory strain; something that a fixed-intensity protocol does not permit (Schlader et al., 2011a, 2011b, 2011c).

Few studies have previously quantified contributors to aerobic performance during self-paced exercise in the heat; to the authors' knowledge, this is the first study to do so using women. The single greatest contributor towards work output (performance) was a participant's peak aerobic power (Figure 16, Table 15). These results support those of James et al. (2017) who demonstrated that velocity at $\dot{V}O_2max$ (i.e., PPO) was the strongest predictor of 5-km running

performance in the heat in men. Thus, the results of the current study and James et al. (2017) concur with a recent meta-analysis (Alhadad et al., 2017) that placed aerobic training as the single greatest factor for determining endurance performance in the heat, above heat acclimation, pre-exercise cooling and fluid ingestion; something that athletes and practitioners should consider.

Notable differences between our results and those previously (Havenith et al., 1998; Notley et al., 2019b) include: i) anthropometric factors such as body mass and A_D (or composite, A_D:mass) did not contribute towards variance explained in T_{peak} despite significant correlations (Figure 14); ii) the functional factor of $\dot{V}O_2$max did not contribute towards variance explained in T_{peak} (Figure 14), and although it correlated with power output, it did not contribute towards variance explained (Figure 16); iii) the environmental factor of vapor pressure did not contribute towards variances explained (Figures 14 and 16). As already mentioned, we believe these differences to be likely a function of the different sample training status and protocol used (intensity and self-pacing). However, it is also acknowledged that like other retrospective analyses of existing datasets (Havenith et al., 1998; Notley et al., 2019b) the current analysis has certain limits. Our primary focus was whether and by how much the T_{core} response to exertional heat stress in women can be explained by accounting for the variation in ovarian hormone concentrations. To maximize predictive/explanatory power we chose to include all factors into one model each for power output and T_{peak} i.e., by not separately grouping by vapor pressure, pre-load duration etc. Thus, due to our partially-nested design we cannot be certain of the independent effect of these variables. Nevertheless, if we were to take by example the dependent and independent variables with greatest explanatory power (T_{peak}, power output, T_0, RPE) and compare between vapor pressures and pre-load duration no differences are found (all p>0.21). Furthermore, were the factor of vapor pressure to exert an effect then this should be evident as a positive (T_{peak}) or negative (power output) correlation, which is not evident in our

results (Figures 14 and 16). Moreover, it is noteworthy that the resulting models (±1-6%) and predictors remain largely unchanged if vapor pressure and pre-load were separated.

7.5 Considerations

The observations herein are valid only for the current sample(s), protocol(s), and condition(s), and inference of association does not imply causation. It is regrettable that measurement of autonomic thermoeffectors and thermodynamic data were not collected in ~40% of the sample, which may have strengthened the results. Our decision to use T_{peak} as our primary dependent variable was guided by the fact that i) ethics committees and professional bodies use absolute, not relative, thresholds for T_{core} in their guidelines and policies; ii) not all participants reached their highest T_{core} at the end of exercise due to the self-paced nature of the protocol. However, *a posteriori* re-analysis of our data for ΔT_{core} did not change any of the significant independent variables. Whilst it may be tempting to interpret the results as E_2 having a negligible influence on T_{core}/T_{peak}, it is worthwhile considering that as an individual factor E_2 did contribute a small amount towards the variance explained for T_{peak} whereas A_D:mass did not, a variable that has previously been shown to have one of the largest effects (Havenith et al., 1998). Finally, our data should not be generalized to other OCP formulations (e.g., triphasic combination and progestin-only) or to the late-follicular/pre-ovulatory phase of a menstrual cycle.

7.6 Perspectives and Significance

Women remain underrepresented in the exercise thermoregulation literature and >70% of studies still *do not* report ovulatory status or menstrual phase (Hutchins et al., 2021). Ovulatory status should not inhibit inclusion into this research topic (Schaumberg et al., 2017; **Chapter**

6) although, importantly, the current results support calls for future measurement and consideration of ovarian hormone concentrations being standard (Elliott-Sale et al., 2021). Individualization of human thermoregulation models improves the prediction of heat strain, largely through an increase in the number of input parameters (Havenith, 2001). The current results suggest an additional factor (E_2) might be considered in future work, although data saturation has not been reached. Similarly, Flouris et al. (2018) have identified simple metrics that can successfully be used as screening criteria to prospectively identify individuals at greater risk of acute exertional heat stress. Flouris et al. (2018) argue health professionals and occupational management to (re)consider whether different criteria for women should be utilized on account of their unique body morphology/physiology, something the current results support.

Chapter Eight

8.0 General Discussion

This thesis sought to provide insights on improving female athletes' well-being when experiencing heat exposures from two different perspectives. Firstly, **Chapter Five** clarified whether environmental heat stress is detrimental to female athletes' iron metabolism. Based on the data collected for **Chapter Five** along with previously reported data in our lab, **Chapter Seven** elucidated the contributions made by a series of potentially relevant factors when considering the risk for heat injury during exercise-heat exposures. In addition, **Chapter Six** assessed the reliability of the exercise protocol used in **Chapters Five** and **Seven**, which provides future researchers with a reliable protocol to evaluate the effect of interventions on female athletes' performance.

8.1 General Aim I

Chapter Five firstly elucidated the influence of ambient heat stress on females' peri-exercise iron metabolism, specifically, hepcidin secretion mediated by inflammation. Although the higher increase in leucocyte, neutrophil and platelet counts were observed following exercise under heat stress, it was not enough to trigger a greater increase in IL-6 and subsequently hepcidin. Thereby, for Hypothesis 1 in **Chapter 3**, the null hypothesis was retained.

Furthermore, the comparison between menstrual phases also demonstrated that neither the inflammatory responses nor hepcidin secretion were affected by the fluctuation of ovarian hormones over the menstrual cycle when a self-paced protocol was applied.

8.2 General Aim II

As **Chapter Five** once again demonstrated that well-trained females' performance is not affected by menstrual cycle but ambient conditions, **Chapter Six** confirmed this conclusion by evaluating the test-retest reliability of the protocol being used. The novelty of this experimental chapter is that we included the data not only from eumenorrheic females, but also anovulatory females and females using oral contraceptives. By assessing the relative and absolute reliability of the work output completed during the time trial, it was ascertained that the pre-loaded self-paced work trial is a reliable protocol when measuring aerobically trained females' performance, regardless of the environmental conditions. Thereby, for Hypothesis 2 in **Chapter 3**, the alternative hypothesis was accepted. In addition, this research provides more norms regarding athletic females' performance to the scarce literature in this area.

8.3 General Aim III

For the first time, **Chapter Seven** included ovarian hormones in the analysis when considering female athletes' risk for exertional heat injury during exercise in the heat. Interestingly, E_2 levels were found to positively correlate to peak T_{core} during exercise. We were unable to fully explain this positive correlation as it is well-recognized that E_2 facilitates heat dissipation (Charkoudian & Stachenfeld, 2011). Further research is required to elucidate the role of E_2 in T_{core} during heat exposure(s). When explaining the variance of peak T_{core}, E_2 made a small yet

significant contribution of 4%; whereas, starting core temperature and power output made a much greater contribution. Taken together, for Hypothesis 3 in **Chapter 3**, the alternative hypothesis was accepted. In addition, **Chapter Seven** also indicated that peak aerobic power is the only factor that significantly contributes to female athletes' performance under heat stress.

8.4 Limitations and Future Direction

Like most others, this thesis also has limitations that need to be appraised. Firstly, cycling is the only exercise modality that was investigated in this thesis. This should be noted especially for **Chapter Five**, as different exercise modalities affect iron status to a different extent. Running and resistance exercise have been found to increase hepcidin levels to a greater degree compared to cycling due to their weight-bearing nature (Sim et al., 2014; Goto et al., 2020). As both these modalities are popular in sports, especially as some marathon events are held during warmer/summer seasons, further research specific to these exercise modes is warranted. Secondarily, to improve athletes' thermal tolerance/performance under heat stress, heat acclimation has been the gold standard and widely used. An effective heat acclimation lasts from 5 days to more than 10 days, with ~1 h exercise-heat exposures each day consecutively (Sawka et al., 2011). This was further investigated on females specifically, which demonstrated that it takes longer for females to improve their endurance exercise performance in the heat than males (Kirby et al., 2019). Previous studies have observed diminished iron stores following cumulative training (e.g., Auersperger et al., 2013), with no conclusive findings on hepcidin and IL-6 responses. Thereby, it is important that further research is conducted to address the effect of heat acclimation on females' iron metabolism, as maintenance of a normal iron status is critical for athletes' performance as well as their wellbeing.

Although this thesis had a focus on females, it was not possible to comprehensively investigate females with different hormonal profiles. Such that, in **Chapter Five**, only eumenorrheic females were investigated in order to reveal the influence of menstrual cycle on their iron status and hepcidin levels. However, as mentioned in **Chapter Six**, there are a considerable number of physically active females that suffer from menstrual disturbances, with one potential cause being chronic energy deficit (De Souza and Williams, 2004). This is especially worth noting as energy deficit has been demonstrated to augment the increase in hepcidin following exercise in males (Hennigar et al., 2021). Although it was demonstrated that a pre-exercise breakfast after an overnight fasting makes no difference on post-exercise IL-6 and hepcidin responses in females (Hayashi et al., 2018), more research is still warranted to reveal the relationship between chronic low energy availability, menstrual function and hepcidin levels. Furthermore, although **Chapter Six** included eumenorrheic, anovulatory females as well as females taking OCP, more anovulatory participants should be included in **Chapter Six** to draw a stronger conclusion (please see **Chapter Six** for more detailed discussion). From a similar perspective, following on from the findings in **Chapter Seven**, it is also worth elucidating the factors predicting heat injury risk of peri/postmenopausal women. This population group of females are suspected to be vulnerable to heat stress due to their declining E_2 levels. This is a topic that urgently needs to be investigated, not only for athletes, but also for less trained populations, as there are an enormous number of healthcare workers working under uncompensable heat stress due to the use of personal protect equipment during the COVID outbreak.

8.5 Special considerations of this thesis

Originally, this thesis was designed to investigate both the acute and cumulative effect of exercise-heat exposures on females' iron metabolism over the menstrual cycle. However, just to investigate the acute effect, it took almost two years to collect a complete data set of $n = 13$. It took longer time than the candidate expected to recruit eligible participants and schedule trials in the desired menstrual phases. Participants' dropout and omission of anovulatory participants also made the data collection process longer. Not to mention COVID-19 drastically changed the research situation and environment within New Zealand. Without access to participants and laboratory space as a result of government and university policies, the candidate did two retrospective studies instead (**Chapter Six and Seven**). The candidate took the leading role when switching the research direction, which includes reviewing literature in a brand-new area, designing and conducting the analysis, as well as writing up the manuscripts. Although there are blemishes in terms of study design (small sub-group sample size in **Chapter Six**, lack of relevant data in **Chapter Seven**), this thesis as a whole still provides valuable insights in terms of improving female athletes' performance in hot environments without sacrificing their health.

8.6 Conclusions

The purpose of this thesis was to shed light on female athletes' iron metabolism and their risk of heat injury during acute heat exposure, as well as to evaluate the reliability of the exercise protocol used in this thesis. Corresponding to the Aims and Hypotheses in **Chapter Three**, the following conclusions were made based on the experimental chapters:

1) When aerobically trained females are allowed to pace themselves during exercise in the heat, they voluntary sacrifice their performance. This appears to limit the inflammatory response, so the additional environmental heat stress has no detrimental effect on their iron status.
2) Menstrual phase has no effect on either peri-exercise inflammatory responses or iron metabolism for female athletes under acute heat exposure.
3) A 30 min self-paced work trial with a fixed-intensity preload is reliable in terms of monitoring the performance of aerobically fit females with different hormonal/ovulatory status's, under different environmental conditions.
4) Among ovarian hormones, only E_2 contributes to the risk of exertional heat stress when female athletes exercise under heat stress, yet a small portion (4%). The effect of E_2 is less than the starting core temperature and mean power output. Peak aerobic ability is the only contributor when explaining females exercise performance in hot environmental conditions.

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APPENDIX 1

Calculating a signal-to-noise ratio (sensitivity index).

Currell and Jeukendrup (2008) proposed a quantitative measure of sensitivity expressed as the ratio between an intervention 'signal' and measurement error 'noise', whereby a higher value indicates greater protocol sensitivity. A sensitivity index ≤1 infers that the test completed under those conditions and with that sample is not sufficiently sensitive/reliable, or that the true effect of the intervention is small/negligible. The first step is to identify the change in performance and within-subject CV. From Table 11 we can see that the CV for OCP is 4.0%, whilst the effect of a reduction in ambient thermal stress (lower vapor pressure) results in a change in performance of 5.5% in this cohort (Lei et al., 2019). Therefore, relating the signal (5.5%) to the noise (4.0%) means solving the equation:

sensitivity index*=signal/noise* or ***1.4****=5.5/4.0.*

APPENDIX 2 Publication for Chapter Five

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

Candidate's Signature:	Huixin Zheng Digitally signed by Huixin Zheng Date: 2022.07.13 17:09:23 +12'00'
Date:	13-Jul-2022
Primary Supervisor's Signature:	Toby Mundel Digitally signed by Toby Mundel Date: 2022.07.13 18:51:39 +12'00'
Date:	13-Jul-2022

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.

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APPENDIX 4 Publication for Chapter Seven

DRC 16

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UNIVERSITY OF NEW ZEALAND

GRADUATE RESEARCH SCHOOL

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:	Huixin Zheng
Name/title of Primary Supervisor:	Dr Toby Mündel
In which chapter is the manuscript /published work:	Chapter Seven

Please select one of the following three options:

◉ The manuscript/published work is published or in press

- Please provide the full reference of the Research Output:

Zheng, H., Badenhorst, C. E., Lei, T. H., Che Muhamed, A. M., Liao, Y. H., Fujii, N., Kondo, N., & Mündel, T. (2022). Do E2 and P4 contribute to the explained variance in core temperature response for trained women during exertional heat stress when metabolic rates are very high?. Eur J Appl Physiol. Advance online publication. https://doi.org/10.1007/s00421-022-04996-2.

○ 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:	Huixin Zheng Digitally signed by Huixin Zheng Date: 2022.07.13 17:09:23 +12'00'
Date:	13-Jul-2022
Primary Supervisor's Signature:	Toby Mundel Digitally signed by Toby Mundel Date: 2022.07.13 18:52:07 +12'00'
Date:	13-Jul-2022

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.

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APPENDIX 5 Note for Examiners

MASSEY UNIVERSITY
TE KUNENGA KI PŪREHUROA
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.

September 2021

MASSEY UNIVERSITY
TE KUNENGA KI PŪREHUROA
UNIVERSITY OF NEW ZEALAND

GRADUATE RESEARCH SCHOOL

Note for Examiners
Explanation of COVID-19 Impacts

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, and in particular from 17 August 2021 when the whole of New Zealand again moved to Level 4 lockdown for an extended period. The Auckland region remained in alert level 3 or 4 for a number of months. Please see the NZ Government website for more information on lockdown dates.

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 have been substantial 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.

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

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:	Huixin Zheng	ID Number:	16427969
Supervisor Name:	Toby Mündel	Date:	22-Jul-22
Thesis title:	Factors influencing the exertional heat stress response in athletic females		

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*]

When Lizzie (Huixin) was about to start data collection for her second study as originally planned, New Zealand went into lockdown due to the COVID-19 pandemic. As her research project involved human participants undertaking exercise training sessions in our laboratory and collecting exhaled gas/venous blood samples, we were unable to carry out any participant testing for approximately 3 months (March - May 2020). After this period, there were still health and safety restrictions regarding laboratory testing with human participants, and participant recruitment become even more challenging as most people were wary of their inter-person contact. Meanwhile, as an international student, Lizzie has been away from family, as well as the support network she had built at Massey.

Considering the uncertain situation, what was planed to achieve when Lizzie started her PhD seems to be unachievable within the time frame due to the ongoing pandemic. Thereby we decided to change the research topic and part of the thesis will be two retrospective studies based on the analysis of data previously collected in our lab - which are vastly different from what has been originally planned. Thereby, this thesis is not as coherent as other ones. However, we are still very proud that we managed to publish 3 publications during Lizzie's 4 years of study, when the pandemic jumped in halfway.

Approved by DRC 10/Feb/2021
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Updated September 2021

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	Huixin Zheng	Digitally signed by Huixin Zheng Date: 2022.07.13 17:01:12 +12'00'
Supervisor	Toby Mundel	Digitally signed by Toby Mundel Date: 2022.07.22 12:44:44 +12'00'
Head of Academic Unit (or nominee)	Andrew Foskett	Digitally signed by Andrew Foskett Date: 2022.07.22 12:58:25 +12'00'

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