IMPACT OF MENSTRUAL CYCLE MEDIATED AND TRADITIONAL HIGH-INTENSTITY INTERVAL TRAINING ON MAXIMAL OXYGEN UPTAKE, ENDOGENOUS ANDROGENS, AND FAT MASS IN NATURALLY MENSTRUATING FEMALES

Paula Havukainen

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University of Jyväskylä
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Research supervisors: Sheila Gagnon and Ritva Mikkonen

TIIVISTELMÄ

Havukainen, P. 2023. Kuukautiskierron mukaan jaksotetun ja perinteisen korkean intensiteetin intervalliharjoittelun vaikutukset maksimaaliseen hapenottokykyyn, endogeenisiin androgeeneihin ja rasvamassaan luonnollisen kuukautiskierron omaavilla naisilla. Liikuntatieteellinen tiedekunta, Jyväskylän yliopisto. Valmennus- ja testausopin pro gradu -tutkielma. 60 s. 1 liite.

Kuukautiskierron aikaansaamien hormonaalisten muutosten on tunnistettu vaikuttavan fysiologisiin toimintoihin niin levossa kuin fyysisen kuormituksen aikana. Nämä muutokset ovat herättäneet mielenkiinnon selvittää kuukautiskierron mukaan jaksotetun harjoittelun hyötyjä suorituskykyyn. Poikittaistutkimuksissa on havaittu korkeamman rasvamassaan sekä metabolisen oireyhtymän olevan yhteydessä androgeenien korkeampaan pitoisuuteen. On viitteitä, että kestävyysharjoittelulla voi saada aikaan myönteisiä muutoksia sekä androgeeneissa että rasvamassassa. Korkeaintensiteettisen intervalliharjoittelun (HIIT) on havaittu parantavan sekä kestävyyssuorituskykyä että vähentävän rasvamassaa. Tämän opinnäytetyön tarkoituksena oli vertailla kuukautiskierron mukaan jaksotetun intervalliharjoittelun sekä tasaisesti jaksotetun intervalliharjoittelun vaikutuksia maksimaalisen hapenottokykyyn (VO_2max). Lisäksi intervalliharjoittelun vaikutuksia androgeeneihin sekä kehonkoostumukseen tarkastellaan.

Luonnollisen kuukautiskierron omaavat fyysisesti aktiiviset naiset (n=36, ikä=29,7 ±4,3 vuotta, BMI 24,3±3,4 kg/m^2) jaettiin satunnaisesti kolmeen harjoitteluryhmään: luteaalivaiheessa painotettuun (LP), follikulaärivaiheessa painotettuun (FP) ja kontrolliryhmään. Koehenkilöt suorittivat 8 viikon HIIT-jakson, jota edelsi 8-viikon kohtuukuormitteinen (MIET) jakso. Kestävyyssuorituskyvyn kehittymistä mitattiin juoksumatolla suoritetulla VO_2max-testillä. Kehonkoostumuksen muutoksia mitattiin bioimpedanssilla ja androgeenien muutokset määritettiin immunomääritysmenetelmällä. Tilastollisina menetelminä ryhmien väliseen vertailuun käytettiin toistettavien mittausten varianssianalyysiä (ANOVA) sekä ryhmien sisäiseen vertailuun parametritonta Friedmanin varianssianalyysiä. Kehonkoostumuksen ja androgeenien yhteyksiä määritettiin Spearmanin korrelaatiokertoimella.

Ryhmien välillä ei havaittu tilastollisesti merkitseviä eroja VO_2max-tuloksissa. Ryhmien sisäisessä vertailussa kontrolliryhmä paransi tilastollisesti merkitsevästi VO_2max-tulostaan koko harjoitusintervention aikana (40,68±5,30<43,14±5,61). LP-ryhmä paransi VO_2max tulosta tilastollisesti merkitsevästi (38,97±4,32<40,40±4,40) MIET jakson aikana. FP-ryhmällä ei nähty tilastollisesti merkitseviä muutoksia intervention aikana. Androgeeneista vapaa testosteroni väheni merkittävästi HIIT-jakson aikana (-14,7±34,9 %) sekä koko harjoitusintervention aikana (7,1±44,2 %). Kehonkoostumuksessa ei havaittu tilastollisesti merkitseviä eroja harjoitusintervention aikana, eikä kehonkoostumusmuutosten ja androgeenimuutosten välillä havaittu tilastollisesti merkitsevää korrelaatiota. Tutkimuksen mukaan kuukautiskierron mukaan jaksotettu HIIT ei ole tehokkaampaa kuin tasaisesti jaksotettu HIIT. LP-painotteinen MIET saattaa olla suositeltavaa, mutta aiheesta tarvitaan lisää tutkimusta.

Asiasanat: Kuukautiskierto, intervalliharjoittelu, androgeenit, kehonkoostumus, rasvamassa

ABSTRACT

Havukainen. P. 2023. Impact of menstrual cycle mediated and traditional high-intensity interval training on maximal oxygen uptake, endogenous androgens, and fat mass in naturally menstruating females. Faculty of Sport and Health Sciences, University of Jyväskylä. Science of Sport Coaching and Fitness Testing, master's thesis, 60 pp, 1 appendix.

Fluctuation of hormones impacts on physiological functions during exercise and rest across the menstrual cycle. This has increased interest to investigate whether menstrual cycle meditated training provokes beneficial adaptations in physical performance. Many cross-sectional studies have observed that a high concentration of endogenous androgens in females is associated with metabolic syndrome and higher body fat mass. Furthermore, both fat mass and endogenous androgens have been observed to change in response to endurance training. High-intensity interval training (HIIT) is an effective training method to improve endurance performance and reduce body fat mass. The objective of this master's thesis was to compare the impact of menstrual cycle mediated training and traditional continuously periodized HIIT on maximal oxygen uptake (VO_2max). Additionally, the impact of HIIT training on endogenous androgens and body composition will be investigated.

Naturally menstruating recreationally active females (n=36, age=29.7 ±4.3 years, BMI 24.3±3.4 kg/m^2) were divided into three training groups: luteal phase emphasized (LP), follicular phase emphasized (FP), and control. The participants executed a 8-week HIIT period, prior to which a 8-week moderate intensity endurance (MIET) period was accomplished. Change in endurance performance was measured with an incremental treadmill running test to determine VO_2max. Changes in body composition were determined using bioimpedance measurement. Changes in endogenous androgens were determined with immunoassay kits. Statistical analysis for between group comparisons were analysed using repeated measures of variance (ANOVA) and for within group comparisons a non-parametric related samples Friedman's two-way analysis of variance test were used. Spearman's correlation was used to determine the relationship between endogenous androgens and body composition variables

Statistically significant differences in VO_2max between groups or during the HIIT groups were not observed. In within group comparisons, it was observed that VO_2max improved in the control groups significantly across the whole training intervention (40.68±5.30<43.14±5.61). The LP group significantly increased VO_2max during the MIET intervention (38.97±4.32<40.40±4.40). The FP group did not experience significant changes across the training intervention. When comparing all participants as a one group, significant reduction in free testosterone was observed across the HIIT-intervention (-14.7±34.9 %) and the whole training intervention (7.1±44.2 %). In body composition variables significant changes were not observed. Hence, no concurrent changes in in the androgen and body composition variables were observed. According to the present study, menstrual cycle mediated HIIT is not superior compared to the traditional continuously periodized HIIT. However, there are indications that LP emphasized MIET training might be beneficial, but more studies considering the topic is needed.

Key words: Menstrual cycle, interval training, endogenous androgens, body composition, fat mass.

ABBREVIATIONS

A4	androstenedione
ACTH	adrenocorticotrophic hormone
ATP	adenosine triphosphate
BMI	body mass index (kg/m^2)
DHEA	dehydroepiandrosterone
DHEA-S	dehydroepiandrosterone-sulphate
DHT	dihydrotestosterone
FP	follicular phase
FSH	follicle stimulating hormone
GnRH	gonadotropin releasing hormones
HIIT	high-intensity interval training
HPO-axis	hypothalamus-pituitary-ovarian axis
LH	luteinizing hormone
LP	luteal phase
MIET	moderate intensity endurance training
SHBG	sex hormone binding globulin
VO_2max	maximal oxygen uptake

CONTENTS

1 INTRODUCTION

In recent decades, more and more women have participated in sports and various forms of physical activity. When looking at the studies considering sport and physical performance, it is noticed that a vast majority of the studies are completed with males as participants. Consequently, the research findings from physical performance and sport with male participants are transferred to females (Kissow et al. 2022; McNulty et al. 2020.) In most premenopausal female, the menstrual cycle causes cyclical fluctuations in endogenous hormones (Davis & Hackney 2017, 2) which adds a new aspect when studying female physiology and physical performance.

Only recently the amount of studies investigating female physiology and menstrual cycle has increased (Elliott-Sale et al. 2021). Similarly, the interest on impact of menstrual cycle on sport performance has also grown. Some studies have suggested that physiological responses are altered along with menstrual cycle: changes in energy metabolism (D'Eon et al. 2002; Hackney et al. 2022) exercise economy (Thompson et al. 2012; Rael et al. 2021) and lactate levels (Zderic et al. 2001) are reported in different menstrual cycle phases. This brings into question whether a menstrual cycle mediated training method would have superior effect compared to traditional training on sport performance. Currently, the scientific research considering endurance training with menstrual mediated protocol is scarce, so the need for good quality research is evident.

Another topic spotlighting females is increased sedentary lifestyle. It is well recognized that overweight among adults has increased remarkably during the past few decades around the world. Of concern, is that prevalence of obesity has risen more with females than males. In the 2000, obesity levels were 6.7% males and 10.6% in females, whereas the numbers in the 2016 were higher at 11.1% in males and 15.1%. (World Health Organization 2019) Endurance training is a frequently used method to reduce fat mass and enhance other health related factors, such as blood pressure, total cholesterol, and fasting glucose (Gripp et al. 2021; Scharhag-Rosenberger et al. 2010; Martins et al. 2016). It is also used to improve maximal oxygen uptake (VO_2max) (Helgerud et al. 2007). Traditionally, endurance training can be effectively completed using a continuous and moderate intensity training method (Murias et al. 2010; Helgerud et al. 2007). Besides the traditional moderate intensity continuous training method, an another regularly used method is high-intensity interval training (HIIT). HIIT is time-

efficient method which provokes at least similar physiological adaptations with reduced time (Martins et al. 2016; Helgerud et al. 2007).

There are indications that excess of endogenous androgens would be associated with obesity and different lifestyle related illnesses in females (Korhonen et al. 2003; Fenske et al. 2015). Androgens are part of the sex-hormone group that are essential for normal and healthy living. Although produced in small amounts in females, androgens have direct and crucial effects on female physiology, e.g., bone density, muscle growth, and erythrocyte production (Slemenda et al. 1996; Karunasena et al. 2017). It is proposed that physical activity could be an approach to lower the concentration of excess endogenous androgens in females, but the knowledge of the topic is far from comprehensive. Some cross-sectional studies have found positive correlations between obesity and increased androgens (Torchen et al. 2020; Korhonen et al. 2003) and longitudinal studies have observed concurrent reductions in both androgens and fat mass in response to endurance training (Keizer et al. 1987; McTiernan et al. 2004). However, this is not always the case as Kumru et al. (2005) and Eklund et al. (2017) have demonstrated that well-trained populations and athletes tend to have lower BMI and higher amount of endogenous androgens.

Studying impact of the menstrual cycle on physical performance gives valuable information for approximately half of the population worldwide. It can provide new insights for athletes and coaches considering every day on-field and help female athletes to reach their full potential. Therefore, this master thesis investigated whether menstrual mediated HIIT and traditional continuously periodized HIIT provoke different adaptations to aerobic capacity, and whether changes in endogenous androgens and body compositions variables were connected recreationally active naturally menstruating females. This may lead to re-examination of modalities used for training physical training and maintenance of an active and healthy lifestyle using evidence-based recommendations in female population.

2 MENSTRUAL CYCLE AND ENDOGENOUS HORMONES

The reproductive system of female is a complex physiological system with many hormonal and regulatory components (Davis & Hackney 2017, 1; Oostjuyse & Bosch 2010). Probably the most specific physiological and hormonal function of the female reproductive system is menstrual cycle. Menstrual cycle is approximately 28 (21-35) days long and is characterized by production and release of different hormones: gonadotropin-releasing hormone (GnRH), follicular stimulating hormone (FSH), luteinizing hormone (LH), estradiol, and progesterone. These hormones fluctuate in a cyclic manner and are regulated by feedback mechanisms. (Messinis et al. 2014; Davis & Hackney 2017, 2) In this master's thesis, females who experience naturally this cyclical fluctuation in the endogenous hormones, are referred as naturally menstruating females.

Generally, the menstrual cycle is divided in two phases, follicular phase (FP) and luteal phase (LP). Usually, these phases are separated with ovulation. The first day of menstrual bleeding is marked as the start of the FP and the first day post-ovulation as the start of the LP. (Davis & Hackney 2017, 6; Constantini et al. 2005) In addition to this commonly used approach, it is important to acknowledge that these phases can be further divided into shorter periods, such as early, mid, and late FP and early, mid, and late LP. According to this, the phases would be then divided into even seven phases. (Elliott-sale et al. 2021) In this chapter, the menstrual cycle and the hormones associated with it are reviewed, and the impact of menstrual cycle on physiological functions and physical performance are discussed

2.1 Hormonal fluctuation of the menstrual cycle

In each menstrual cycle a few follicles start to grow independently under the influence of FSH and one of the follicles becomes a dominant follicle, which starts to grow and mature (Constantini 2005; Davis & Hackney 2017, 6-7). During the FP, the concentration of FSH increases gradually reaching the peak at ovulation after which its' concentration starts to diminish (Dighe et al. 2005; Stricker et al. 2006). As the follicle grows, it starts to secrete estrogen, mainly estradiol. Estradiol is the most responsible estrogen to induce primary and secondary characteristics in the female body (Constantini 2005; Davis & Hackney 2017, 6-7), so this is the main estrogen referred in this master thesis as well. Concentration of estradiol is at the lowest in the early FP and starts to gradually increase until it reaches its' peak

approximately one day before ovulation. This increased amount of estradiol causes a positive feedback loop inducing a surge in LH. (Dighe et al. 2005; Stricker et al. 2006) Approximately 10-12 hours after the LH surge ovulation occurs and the egg is released from the follicle (Reed & Carr 2015). After ovulation LH remains slightly elevated during the early days of the LP. Similarly, the concentration of estradiol drops following ovulation but starts to increase again at mid LP. (Dighe et al. 2005; Stricker et al. 2006) Around one day after ovulation, the ovulated follicle is transformed as a progesterone secreting corpus luteum. Consequently, the concentration of progesterone starts to increase at the LP. (Davis & Hackney 2017, 6-7) Concentration of progesterone is at the highest at the mid LP and starts to decrease at the latter part of the LP phase (Stricker et al. 2006). Since progesterone secretion has decreased, a new cycle begins with menstrual bleeding (Constantini 2005; Davis & Hackney 2017, 6-7). The fluctuation of the hormones and development of the follicle is illustrated in the figure 1.

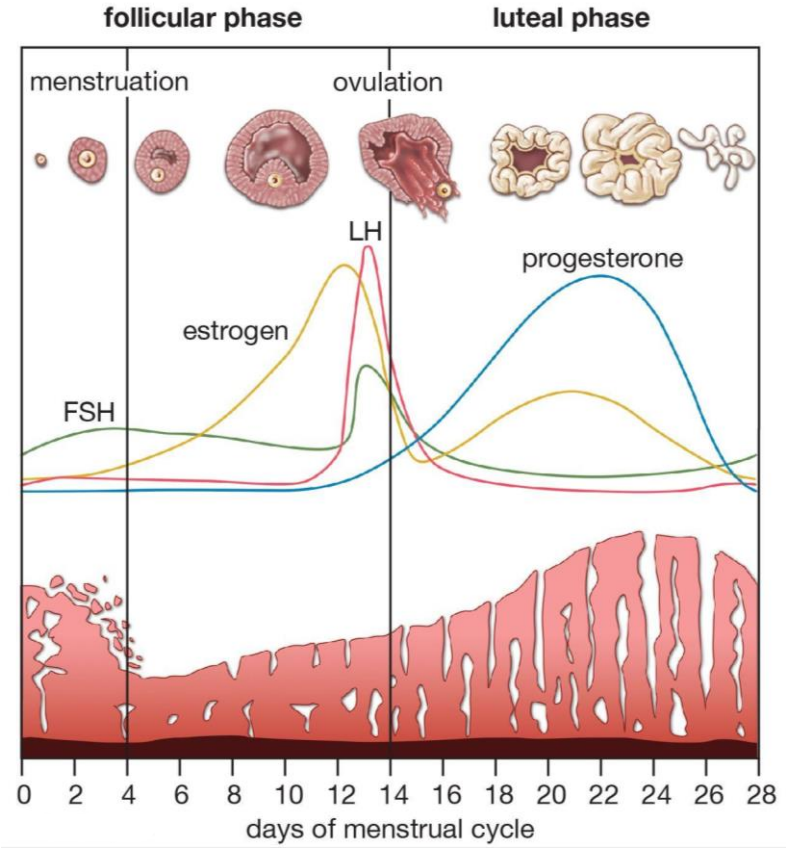


FIGURE 1. Fluctuation of endogenous hormones, follicular development, and development of endometrial lining during the menstrual cycle (Encyclopædia Britannica 2022).

2.2 Hypothalamus-pituitary-ovarian axis

Regulation of the female reproductive system and the menstrual cycle consists of a complex including the hypothalamus, pituitary, and ovaries, collectively referred to as the hypothalamic-pituitary-ovarian axis (HPO-axis) (Davis & Hackney 2017, 2). The control of the hypothalamic, hypophyseal, and ovarian hormones happens via several positive and negative feedback loops. The key hormones of this system are GnRH, LH, FSH, estradiol, and progesterone. (Messinis et al. 2014) The HPO-axis and its regulative feedback loops are presented in the figure 2.

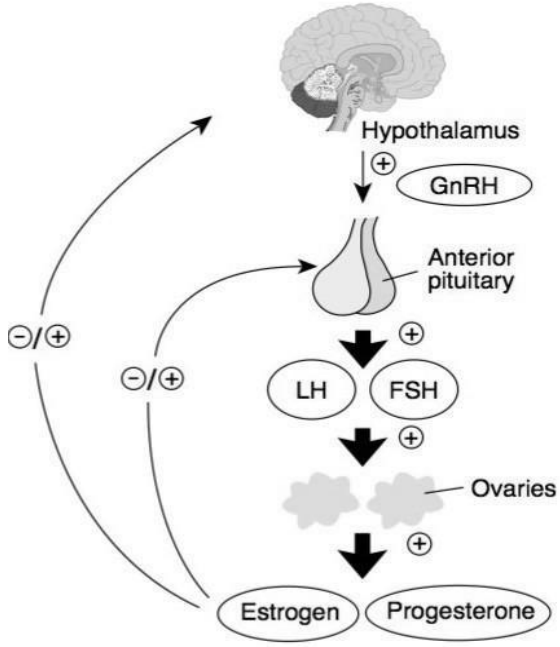


FIGURE 2. Control of the hypothalamus-pituitary-ovarian axis. The figure represents the positive and negative feedback loops which regulate the secretion of GnRH, LH, FSH, estrogen, and progesterone. (Kong et al. 2014)

2.2.1 Gonadotropin-releasing hormone

Gonadotropin-releasing hormone is a hypothalamic peptide which has a significant and direct control of female reproductive system (Thompson & Kaiser 2014; Wu et al. 2021). GnRH is produced in the neurosecretory cells in the hypothalamus where it is released in a pulsatile manner into hypothalamic circulation. Thereafter GnRH is transported to the gonadotropic cell, which is located in the anterior pituitary gland, where it binds to a seven-transmembrane

receptor (GnRHR) on the cell surface. After binding in pituitary gonadotropins, biosynthesis, and secretion of LH and FSH starts. (Leung & Chen 2005) GnRH implements its reproductive functions indirectly in the ovaries, but GnRH receptors have been identified in other reproductive tissues too, such as endometrium and myometrium (Thompson & Kaiser 2014; Wu et al. 2021).

The upstream regulators of GnRH release include different neurotransmitters and neuropeptides, which impact on GnRH neurons causing the GnRH secretion. The ovarian steroid hormones, estradiol and progesterone, are the principal mediators of the negative feedback loop for the GnRH secretion. (Wu et al. 2021)

2.2.2 Luteinizing hormone and follicle stimulating hormone

The gonadotropin hormones, LH and FSH, respond to hypothalamic GnRH. LH and FSH hormones are synthetized and released in different ways from the gonadotropic cells in the anterior pituitary. As GnRH is secreted in a pulsatile manner, the secretion of LH and FSH follow a similar pattern. (Thompson & Kaiser 2014; Davis & Hackney 2017, 2-3) GnRH stimulates synthesis and release of FSH directly, whereas LH is secreted via regulated signalling pathway, where it is stored in secretory granules, until it is released under the influence of GnRH (Thompson & Kaiser 2014).

The main function of LH and FSH is the regulation of reproductive hormones (steroidogenesis and gametogenesis) (Thompson & Kaiser 2014). In the ovaries, LH binds to LH receptors in the theca cells and starts to convert available cholesterol into androgens. After the conversion, androgens are transported to the granulosa cells where FSH binds to FSH receptor which stimulates conversion of androgens into estradiol. Similarly, progesterone production is modulated by LH; LH stimulates LH receptors in the granulosa cells, which leads to conversion of cholesterol to progesterone. (David & Hackney 2016, 3-5)

2.2.3 Estradiol and progesterone

The main regulators of the HPO-axis are ovarian steroid hormones: estradiol and progesterone. As the concentrations of estradiol and progesterone increases, they assert negative feedback to the hypothalamus and the pituitary (Messinis et al. 2014). Estradiol and progesterone belong to steroid hormone group and are produced via steroidogenesis (Kalogera et al. 2013). In addition to estradiol, estrogens refer also to estrone, estriol, and estetrol. Estrone and estriol are less abundant than estradiol. Estetrol is produced only during pregnancy. (Davis & Hackney 2017, 2-3; Iorga et al. 2017) In premenopausal females, most of the estradiol is produced and secreted by the ovaries, but some of it is produced in other tissues as well, such as adipose, brain, bone, and vascular endothelium (Iorga et al. 2017). Progesterone belongs to progestogen steroid hormone group and is produced predominantly in the ovaries, but some other tissues produce it locally (Graham et al. 1997).

To express their functions in the body, these hormones bind to the specific receptors to induce estrogenic or progesteronic functions. Estrogens bind to the estrogen receptors (ER) (Iorga et al. 2017). Similarly, progesterone binds to the progesterone receptor (Graham et al. 1997). ERs are expressed in several tissues and organs which advocates that estrogen has various functions in human body. ERs are located in the female reproductive systems, such as in the uterus, ovaries, breasts, but ERs are also evident in the nervous and the male reproductive systems (Tang et al. 2019). In addition, ERs are discovered in the skeletal muscles (Wiik et al. 2009) and the cardiovascular systems (Tang et al. 2019) too, which reinforces that estrogens, especially estradiol, play a significant role in vascular health. Estradiol increases vasodilation, participates in angiogenesis, and decreases oxidative stress (Iorga et al. 2017). In a reproductive purpose, progesterone prepares endometrial lining for fertilization and pregnancy (Davis & Hackney 2017, 5). Some studies have reported that elevated concentration of progesterone increases body temperature, therefore, it is indicated that progesterone has thermoregulative effects (Goldsmith & Glaister 2020; Thompson et al. 2012).

2.3 Effect of estrogen and progesterone on physical performance

As estrogen and progesterone have various impacts on physiological functions, the impact of these hormones on physical performance has also been examined (Hackney et al. 2022; Oosthuyse & Bosch 2005; de Jonge et al. 2003). Studies have indicated that fluctuation of

estrogen and progesterone might enhance or decrease specific metabolic pathways, ventilatory parameters, or heart rate during the exercise (D'eon et al. 2002; Thompson et al. 2012).

2.3.1 Metabolic pathways

It has been suggested that the ovarian hormones may influence the metabolic pathways utilized during physical exercise. For example, a study by D'Eon et al. (2002) observed that exogenously elevated estrogen increased lipolysis and fatty acid availability during submaximal bicycle exercise at the intensity of 60% VO_2max. Elevated estrogen spared the utilization of carbohydrates by decreasing muscle glycogenolysis. They also observed, that when elevated estrogen was combined with elevated progesterone, the sparing impact of estrogen on carbohydrates utilization of fatty acids was attenuated. (D'Eon et al. 2002) Similar observations have been reported, when the impact of endogenous estrogen and progesterone on fat oxidation during submaximal exercise has been examined. In the study of Hackney et al. (2022) it was reported that females rely more on fat metabolism during exercise when the menstrual cycle sifts from the FP to the LP. The study suggested that the interaction of changes in progesterone and estradiol influences substrate metabolism, and progesterone's increase relative to estradiol might attenuate the extent of the fat oxidation shifts between the FP to the LP.

Better utilization of lipids and the sparing effect of glycogen during exercise during the LP is also supported by Zderic et al. (2001) who observed that lactate levels were lower during the LP phase compared to the FP when the intensity of cycling exercise was 90% of the lactate threshold. This indicated that oxygen was utilized more efficiently in the LP compared to the FP. They did not see a similar effect when the training was executed at lower intensity (70% of the lactate threshold). A possible explanation for this observation can be that the impact of estrogen on metabolic pathways may depend on the intensity of exercise, where the sparing effect is noticeable only when the intensity is high enough to utilize glucose (Oosthuyse & Bosch 2010).

2.3.2 Ventilation, heart rate, and temperature

Besides the potential impact of the menstrual cycle phases on metabolic pathways, it is observed that menstrual cycle might affect physiological functions in other ways as well. It is reported that resting body temperature is significantly elevated during the LP compared to early and late

FP. (Goldsmith & Glaister et al. 2020) This observation is supported by Thompson et al. (2012) who reported that resting body temperature, rating of perceived exertion (RPE), resting heart rate, and heart rate during submaximal exercise were increased during the LP compared to FP when environmental conditions were hot and humid (32°C, 60% relative humidity), but not when environmental conditions were temperate (20°C, 45% relative humidity).

Changes in the ventilatory response and exercise economy across the menstrual cycle have been reported in various studies: minute ventilation (Ve) is reported to be higher during the LP with submaximal and HIIT exercises (William & Krahenbuhl 1997; Thompson et al. 2012; Rael et al. 2021) Thompson et al. (2012) reported Ve being increased in the submaximal exercise in the LP compared to the FP when conditions were hot and humid. A study of Williams and Krahenbuhl (1997) observed that Ve is highest at mid LP, hence resting VO_2 was lower during the early FP than in mid LP. The oxygen demand was also higher in mid LP than in the early FP when the exercise intensity was 80% of the VO_2max, which suggests that running economy (RE) is decreased during mid LP if the intensity is high enough (Williams & Krahenbuhl 1997). Similar findings were reported from Goldsmith & Glaister (2020) who recognized RE to be decreased in mid LP compared to other phases. It is suggested that the increase in Ve in the LP is observed due to the elevation of progesterone concentration. It has been recognized that progesterone increases Ve by increasing the sensitivity of the medulla's respiratory centre, which in turn increases VO_2 at the submaximal loads (Barba-Moreno et al. 2022; Williams & Krahenbuhl 1997). Table 1 summarises how different studies have observed fluctuation of physical performance in relation to menstrual cycle phase.

TABLE 1. Impact of hormone concentration and/or the menstrual cycle phase on physical performance.

Reference	Hormone concentration or the menstrual cycle phase	Exercise	Impact
D'Eon et al. (2002)	High estradiol*	60 min submaximal bicycle ergometer at 60% VO_2max	RER ↓ Fat utilization↑ Glucose utilization ↓
	High estradiol *+ and high progesterone*		RER ↔ Fat utilization ↔ Glucose utilization ↔
Goldsmith & Glaister (2020)	High progesterone/mid LP	Submaximal incremental running test until lactate ≥ 4 mmol/l	RE↓ Ve ↑
Hackney et al. (2022)	High Estradiol + high progesterone/mid LP	60 min submaximal treadmill run at 65% VO_2max	Fat utilization ↑
Williams & Krahenbuhl (1997)	High Estradiol + high progesterone/mid LP	12 min (6 min + 6 min) submaximal running test at 55% VO_2max and 80% VO_2max	Ve ↑ at 55% and 80% VO_2max RE ↓ at 80% VO_2max
Zderic et al. (2001)	High estradiol/LP	50 min (25 min + 25 min) submaximal bicycle ergometer exercise at 70% LT and 90% LT	Glucose utilization ↓ at 90% LT Fat utilization ↑ at 90% LT Lactate ↓ at 90% at LT

*Exogenous stimulation, ↓ = reduced, ↑=increased, ↔=not significant difference, RE=running economy, Ve=minute ventilation, RER=respiratory exchange ratio. All results represented as $p < 0.05$ and compared to other menstrual cycle phases.

2.3.3 Menstrual cycle mediated training

As studies have advocated, fluctuation of hormones impact on physiological functions and possibly on physical performance as well. Some studies have found changes in physical performance in naturally menstruating females related to menstrual cycle (Oosthuyse & Bosch 2005; de Jonge et al. 2003; McNulty et al. 2020) which has increased the interest to examine if the body's ability to respond to training is altered by the menstrual cycle and should be considered in training periodization. The literature is inconsistent around the topic. Even if some studies have found changes in physical performance related to the menstrual cycle (Oosthuyse & Bosch 2005; de Jonge et al. 2003), some studies have not found any significant changes (Bailey et al. 1985; McLay et al. 2007; Vaiksaar et al. 2011). As lipid metabolic pathway seems to be enhanced in relation to estrogen and the LP (Zderic et al. 2001; D'eon et al. 2002; Hackney et al. 2022) it would advocate that endurance performance might be better in the LP compared to the FP. This assumption is supported by a review and meta-analysis conducted by McNulty et al. (2020) who concluded that existing research indicates that exercise performance might be reduced trivially during early FP compared to other phases. In contradiction, it has been shown that ventilation and HR parameters increase, and exercise economy decrease during the LP (William & Krahenbuhl 1997; Thompson et al. 2012; Rael et al. 2021) suggesting that there is a slight decrement in aerobic performance in the LP compared to the FP. These inconsistent findings emphasizes that deeper knowledge of the role of the menstrual cycle phase on physical performance is needed.

To the best of our current knowledge, there are no existing studies which have examined periodizing endurance training according to the menstrual cycle (e.g. menstrual cycle mediated training). Kissow et al. (2022) discuss in their review article that the current evidence considering menstrual cycle mediated training research is limited and focusing mostly only on resistance training. In a study of Sung et al. (2014) a menstrual cycle mediated resistance training intervention was executed with eumenorrheic females during three menstrual cycles. In this study, menstrual cycle mediated training was executed by emphasizing training volume remarkably either in the FP or the LP: one leg had eight training sessions in the FP and only two training sessions in the LP, and the other leg had only two training sessions in the FP and eight training sessions in the LP. After the intervention, it was observed that maximum isometric force improved more with the leg which was trained more in the FP compared the LP. Also, the muscle diameter was improved more in the FP trained leg compared the LP trained

leg. Possible mechanisms behind this difference may be the higher concentrations of total testosterone and free testosterone in the FP compared to the LP. (Sung et al. 2015) Kissow et al. (2022) deduced that menstrual cycle mediated resistance training emphasizing the FP may be better compared to the LP for strength gains, but further studies are needed. Figure 5 illustrates how menstrual cycle mediated training was executed in the study of Sung et al. (2015).

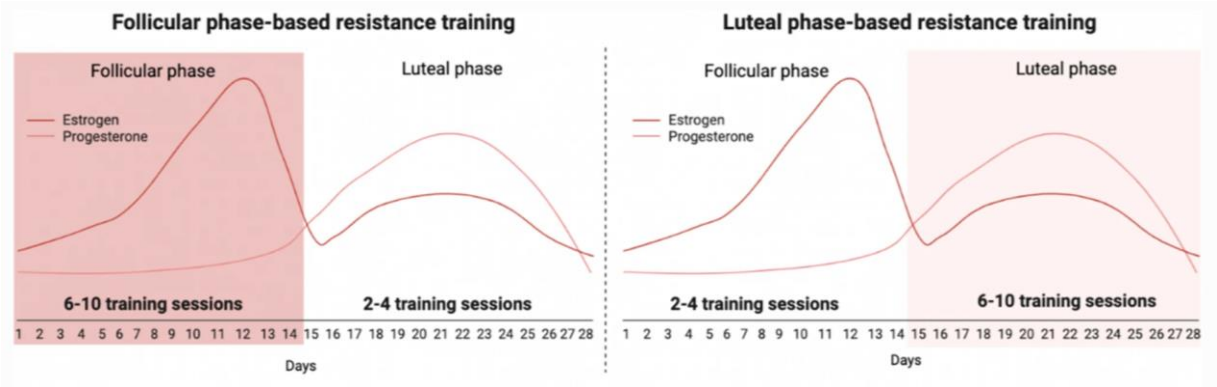


FIGURE 3. Typical fluctuation of estrogen and progesterone across the menstrual cycle and organization of menstrual mediated resistance training in the study by Sung et al. (2015).

3 ENDURANCE TRAINING

Endurance can be defined as the capacity to sustain given velocity or power output at the longest possible time. With prolonged exercising, the endurance performance is improved due to improved physiological functions which enhance the delivery of oxygen to the mitochondria and enable better regulation of muscle metabolism. (Jones & Carter 2000) Endurance training impacts on several physiological functions, such as oxidative capacity of the muscle, haematological, and cardiorespiratory factors (Murias et al. 2010; Montero et al. 2015; Jacobs et al. 2013). Improved cardiorespiratory fitness has been seen to be connected to other health related factors, such as improved blood pressure, total cholesterol, fasting glucose and triglyceride, and body composition (Gripp et al. 2021; Scharhag-Rosenberger et al. 2010; Martins et al. 2016).

An important key factor for aerobic fitness is maximal oxygen uptake (VO_2max). Other factors for aerobic fitness are exercise economy, lactate and ventilatory thresholds and oxygen uptake kinetics. The other factors that are determinant for the endurance performance are related to these factors as well. (Jones & Carter 2000) In this this chapter factors related to endurance performance are shortly introduced and two different training methods are reviewed.

3.1 Maximal oxygen uptake

It is widely accepted that VO_2max is probably the most important factor determining endurance performance (Jones & Carter 2000). For example, in elite female cross-country skiers it is established that VO_2max is the most significant indicator of performance in distance and sprint competitions (Carlsson et al. 2016). Maximal oxygen uptake is usually reported as an absolute value (l/min) in sports where bodyweight is not needed to be supported, while in sports, where bodyweight is supported, like in running, VO_2max is reported as a relative value e.g., as millilitres of oxygen used in one minute per kilogram of bodyweight (ml/kg/min). (Coulson & Archer 2011, 187)

Endurance performance relies highly on the aerobic resynthesis of adenosine triphosphate (ATP) which increases the demand to transport oxygen in the mitochondrial electron transport chain in efficient way. Higher VO_2max seems to reflect the ability to oxygenate the exercising muscles and generate ATP via aerobic resynthesis. (Jones & Carter 2000; Carlsson et al. 2016)

Therefore, improved peripheral oxidation capacity and skeletal muscle oxygenation, elevated mitochondrial density, and increased cytochrome c oxidase activity are paramount to improve VO_2max (Jacobs et al. 2013). Several other factors influence on VO_2max as well. For instance, hemodynamical factors, such as increases in cardiac output and in arterio-venous-O_2 difference (De Revere et al. 2021), elevated blood volume, plasma volume, red blood cell volume, and haemoglobin mass (Montero et al. 2015) have been associated with increased VO_2max.

3.1.1 Aerobic and anaerobic thresholds

Generally, adaptation to endurance training is investigated during a graded exercise test by interpreting ventilatory and lactate thresholds in addition to VO_2max. These thresholds are called as aerobic and anaerobic thresholds. (Faude 2009; Cerezuela-Espejo et al. 2018) The ventilatory thresholds (VT1 and VT2) are determined by measuring gas exchange parameters via indirect calorimetry (Cerezuela-Espenjo et al. 2018). The blood lactate thresholds (LT1 and LT2) are commonly measured during a graded exercise test, which provides an exponential curve of blood lactate responding to exercise intensity (Faude 2009). The aerobic and anaerobic threshold are usually determined by interpreting both the lactate and ventilatory curves. It is well documented, that VT1 and LT1 and VT2 and LT2, correspond with each other. (Cerezuela-Espejo et al. 2018; Pallarés et al. 2016)

Aerobic threshold is determined at LT1 and VT1. In VT1 the oxygen consumption (VO_2) and carbon dioxide production (VCO_2) are increased from baseline, but bicarbonates in blood manages to buffer the acidosis. (Cerezuela-Espenjo et al. 2018) In LT1 the intensity of the exercise is still rather low, but there is a point when blood lactate starts to increase above the baseline levels. It is suggested that this would be the upper limit for the exclusive aerobic energy production. (Faude 2009)

Anaerobic threshold is determined when a more drastic increase occurs in LT and VT. Anaerobic threshold has been reported as the highest steady state condition when the energy is supplied by oxidative phosphorylation. After this turn point, the buffering capacity of blood is not able to maintain homeostasis which causes a radical increase in blood lactate and in ventilatory parameters. (Faude 2009; Cerezuela-Espejo et al. 2018) In VT2 the blood lactate accumulates and increases considerably compared to baseline and aerobic threshold. One frequently used determination for anaerobic lactate threshold is the intensity when the blood

lactate concentration raises to 4 mmol/l (OBLA 4) (Faude 2009). A higher anaerobic threshold seems to be favourable in endurance sports, as higher intensity where OBLA 4 occurs has been positively correlated with better long-distance performance (Carlsson et al. 2016).

3.1.2 Exercise economy and oxygen uptake kinetics

Exercise economy is defined as a relationship between required oxygen uptake and given intensity. The lower the VO_2 at a given submaximal intensity, the better the exercise economy is. (Jones & Carter 2000; Williams & Krahenbuhl 1997) Muscle fibre type distribution influences on an exercise economy, hence it has been reported that fast type of muscle fibres are less economical than slow type muscle fibres (Krustrup et al. 2008). There are some indications that running economy is impaired in some menstrual cycle phases. The studies have reported that exercise economy would be decreased in the LP due to increased body temperature and higher minute ventilation (Williams & Krahenbuhl 1997; Goldsmith & Glaister 2020).

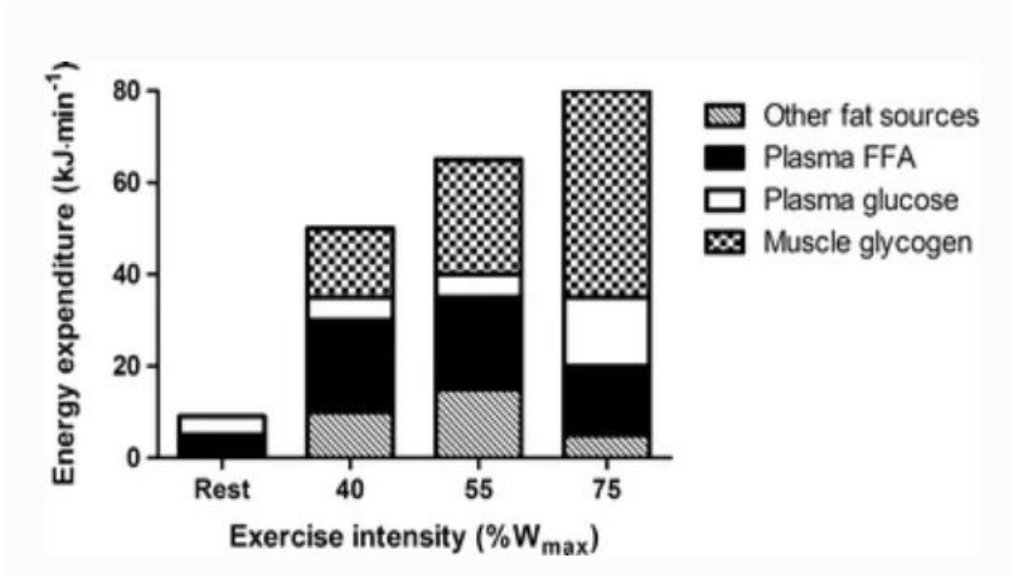
Oxygen uptake kinetics (VO_2 kinetics) reflect to how fast pulmonary oxygen uptake achieves a steady state at a given intensity. During a constant exercise intensity, oxygen deficit might increase the blood lactate level momentarily in the beginning of exercise until it attains a steady state. (Jones & Carter 2000) Endurance training has indicated to be an effective way to improve and to speed up VO_2 kinetics in older and younger females (Murias et al. 2011; Murias et al. 2010).

3.2 Energy metabolism in endurance performance

To produce any kind of physical activity, formation of ATP is required. Roughly, ATP can be produced in two routes, anaerobically in the cytosol or aerobically in the mitochondria via the citric acid cycle and respiratory chain during several oxidation-reduction (redox) reactions. Anaerobically ATP is produced via carbohydrate metabolism in anaerobic glycolysis where glucose is transformed to lactate under the influence of several enzymes. Aerobically, instead of lactate, a pyruvate molecule is formed. This molecule is transported to the mitochondria, where it is degraded as acetyl-CoA when it can be carried to the citric acid cycle and to electron transport chain. When producing energy aerobically, oxygen is always needed to form ATP. This way to synthetize energy is slower than anaerobic glycolysis but produces more ATP.

(McArdle et al. 2015, 136-151) One goal of endurance training is to enhance this utilization of oxygen so the energy can be produced more efficiently via aerobic metabolism.

Fat and carbohydrates are two primary fuel sources in prolonged endurance exercise. In low or moderate-intensity exercise, fat is the dominant energy source (Cermak & van Loon 2013). Fat is stored in the body as triacylglycerols mostly in adipose tissue but also in muscles and plasma. Energy from triacylglycerol can be yielded via lipolysis where fatty acids are released and delivered to the skeletal muscle mitochondria for oxidation. During exercise the blood flow through the adipose tissue increases significantly which increases fatty acid removal from the adipose tissue when those can be utilized as an energy source more efficiently. Lipolysis during exercise permits sustained physical activity and delays the onset of glycogen depletion. (Horowitz & Klein 2000) Estradiol has lipolysis enhancing effects and it has been suggested that females rely more on fat metabolism during exercise. It is also proposed that energy metabolism during an exercise could be altered depending on the concentration of estrogen across the menstrual cycle. (Oosthuyse & Bosch 2012; Hackney et al. 2022; D'Eon et al. 2002) When the intensity of exercise increases, metabolism starts to rely more on carbohydrate and the glucose becomes more important source of energy (Cermak & van Loon 2013). Figure 4 presents the relative energy sources in different exercise intensities.



FIQURE 4. The distribution of energy sources at different exercise intensities. FFA=plasma free fatty acids. $\%W_{max}$=percentage of maximal workload capacity. (Cermak & van Loon 2013)

3.3 MIET and HIIT as training methods for endurance capacity

There are many different training methods implemented with the aim of increasing endurance capacity. Probably the most used and traditional training method is moderate intensity continuous training referred also as MICT or MIET. MIET occurs generally at low intensities, which enables training in high volume (Stöggl & Sperlich 2015; Norton et al. 2010). A training method to provide similar physiological adaptations with reduced time is high-intensity interval training (HIIT) (Wood et al 2019). It is well documented, that both MIET and HIIT methods lead to positive adaptations in endurance capacity. Increases in VO_2max are reported after MIET (Scharhag-Rosenberg et al. 2010; Murias et al. 2010; Helgerud et al. 2007) and after HIIT (Martins et al. 2016; Jacobs et al. 2013; Helgerud et al. 2007).

Even though the endurance performance improvement might be similar with different training methods, it is important to note that physiological adaptations behind improved performance might be different. For example, Montero et al. (2015) suggested that haematological and cardiovascular factors were more crucial in improved endurance performance with MIET. They reported that increases in cardiac output, blood volume, plasma volume, red blood cell volume, and haemoglobin mass were crucial factors for endurance performance improvement. Similar observations have been seen after a 12-week cycling MIET intervention where Murias et al. (2010) reported premenopausal females improved VO_2max, with concurrent increased maximal cardiac output and improved maximal arterial-venous O_2 difference (a-vO_2 diff). In particular, increased maximal cardiac output was a crucial factor for improved VO_2max (Murias et al. 2010). Besides these factors, MIET seems to improve peripheral factors, such as mitochondrial volume density and capillary content as well, but the authors suggested that haematological changes are more crucial (Montero et al. 2015).

High-intensity interval training. High-intensity interval training has been seen to be an effective training method considering the improvements in physiological performance with reduced time (Wood et al. 2019; Helgerud et al. 2007). The interval training is usually executed with lower volume since the intensity is higher compared to MIET. Usually, the anaerobic threshold is considered as the critical point above which the interval training should be executed. (Stöggl & Sperlich 2015) While haematological and cardiovascular factors are the key factors in MIET, there are indications that peripheral factors determine the improvements with HIIT (Jacobs et al. 2013). Jacobs et al. (2013) observed enhanced peripheral oxidation capacity and skeletal

muscle oxygenation, such as increased mitochondrial density and increased cytochrome c oxidase activity were the determinant factors for the increase of VO_2max. In that study, the cardiovascular factors did not change during the HIIT intervention.

HIIT has been reported to induce good or even better adaptations compared to MIET and with less time (Martins et al. 2016; Helgerud et al. 2007). One example of this is from the study of Martins et al. (2016) who investigated the differences between two different HIIT protocols (1/2 HIIT = 10 min; HIIT = 20 min) and MIET (32 min) programs on body composition and cardiovascular fitness in sedentary population. They observed that all three different 12-week training programs led to increases in fat free mass, reductions in fat mass, and improvements in VO_2max, but no significant differences were observed between the training groups, yet the exercise duration was remarkably shorter in HIIT programs. Helgerud et al. (2007) compared four different training programs, including two longer continuous intensity programs and two HIIT programs. One HIIT program was 47 repetitions of 15-seconds intervals at 90-95% of maximal heart rate (HR_{max}) with 15-seconds active recovery and the other was 4 x 4-minutes at 90-95% of HR_{max} with active resting recovery. One continuous training group did long slow distance running at 70% HR_{max} for 45-minutes, and the other continuous run at lactate threshold 85% HR_{max} for 24- minutes. They observed that the HIIT groups increased the VO_2max significantly compared to the other programs.

Despite the smaller volume of HIIT compared to MIET, it has been reported that HIIT is also as effective to reduce fat mass and increase lean mass compared to MIET (Martins et al. 2016). Some studies have reported HIIT to be even better to cause favourable adaptations in the body composition and other health related factors. A study by Gripp et al. (2021) observed that HIIT was more effective to reduce fat mass, visceral mass, BMI, and to improve systolic blood pressure, VO_2max, total cholesterol, fasting glucose and triglyceride profile compared to MIET. These observations indicate, that HIIT would be more effective training method to improve health related factors compared to MIET.

4 ANDROGENS AND FEMALES

Steroid hormones are hormones converted from cholesterol and are of great importance for specific physiological functions, such as the body's growth, metabolism, and sexual development. Steroid hormones are transported in the circulation, and are generally carried bound to specific carrier proteins, such as sex-hormone binding globulin (SHBG). (He et al. 2017, 26) Steroid hormones induce their effect on the target tissues by binding in their specific receptors. In order at interact with their receptors, the hormones need to be converted into their active forms in the presence of specific enzymes. According to their binding receptor the steroid hormones are classified, in addition to estrogens and progestogens, into glucocorticoids, mineralocorticoids, and androgens. (Kalogera et al. 2013)

Androgens bind to the androgen receptor, through which they embody several androgenic functions in the human body (Heinlein & Chang 2002; Kalogera et al. 2013). Even though the production of androgens is small in females, androgens have several physiological functions (Enea et al. 2011). The androgens have a crucial role in muscle growth, reproductive system and maturation, and the bone health as well (Enea et al. 2011; Heinlein & Chang 2002). For example, a loss in the concentration of total testosterone is related to bone mineral density loss in premenopausal females (Slemenda et al. 1996). The number of circulating androgens is also directly proportional to the erythropoiesis, haemoglobin, and haematocrit (Karunasena et al. 2017). The concentration of circulating androgens has been reported be to impact on body composition, but with contradictory findings (Eklund et al. 2017; McTiernan et al. 2004). In this chapter the endogenous androgen production in females is shortly introduced and the relationship between endogenous androgens, exercise, and body composition is reviewed.

4.1 Androgen synthesis

In humans, the production of steroid hormones, or steroidogenesis, occurs mainly in the adrenal glands, gonads (ovaries and testicles) and placenta. In addition to the gonads and the adrenal glands, steroid hormones are synthetized in peripheral tissues such as in the adipose tissue, brain, and skin. (Kalogera et al. 2013; Bianchi et al. 2021) Especially in females, adrenal production of endogenous androgens is in a significant role (Rege et al. 2013). Endogenous androgens include dehydroepiandrosterone (DHEA) and its sulphate (DHEA-S), androstenedione (A4), testosterone, and dihydrotestosterone (DHT). DHEA, DHEA-S, and

androstenedione are considered as precursor androgens since they can be converted to more active androgens. Testosterone and DHT have the most androgenic activity and bind actively to the androgen receptors. (Bianchi et al. 2021) Androgen synthesis is catalysed by several enzymes, but the two critical enzymes for the biosynthesis of androgens are p450 side-chain cleavage (P450scc) and P450c17 (Enea et al. 2011).

Adrenal production of androgens. Androgens are part of the adrenocortical hormones that are secreted i.e., by the adrenal cortex, part of the adrenal gland. The adrenal cortex has three different layers, in each which different hormones are secreted; zone glomerulosa, zona fasciculata, and zone reticularis. Androgens are secreted from the zone fasciculata and zona reticularis. (Guyton & Hall 2011, 921-922) The synthesis of these adrenal androgens is mainly regulated by adrenocorticotrophic hormone (ACTH) secreted by the anterior pituitary gland (Rege et al. 2013; Enea et al. 2011).

Ovarian production of androgens. Androgen production in the ovaries is mainly regulated by LH. Androgen synthesis in the ovaries is predominantly located in the vascularised theca cell layer of the follicle. The receptors that bind LH are luteinizing hormone/human chorionic gonadotropin receptors (LHCGRs) which are located exclusively in the theca cells in the growing follicle. In the late follicular phase, LHCGRs are expressed in the granulosa cells of the preovulatory follicle, and after ovulation in the corpus luteum as well. Further, FSH enhances the androgen conversion into estrogens. Ovarian synthesis of androgens is needed to provide enough precursors for conversion of estrogen and progesterone, and for the growth and maturation of ovarian follicle. (Franks 2021)

Peripheral production of androgens. Peripheral tissues are at great importance of androgen production. For example, testosterone can be converted from androstenedione and DHEA in peripheral tissues (Bianchi et al. 2021). Also, DHEA is considered to be the most important precursor androgen since all active androgens produced in peripheral tissues via enzymatic conversion are originated from DHEA (Labrie et al. 2017). Especially adipose tissue is recognized to be an important source of androgens (Puche et al. 2002). Physiologically, adipose tissue has several androgen receptors via androgens can express their androgenic functions directly on the tissue. Androgens effect on adipose tissue by regulating fatty acid uptake, lipid metabolism, insulin signalling, and adipokine production. (O´Reilly et al. 2014)

4.2 Precursor androgens

Precursor androgens, androstenedione, DHEA, and DHEA-S, have only little androgenic activity but provide a pool for peripheral conversion to more active androgens. Approximately half of the circulating androgens are converted from precursor androgens and estradiol. (Turcu et al. 2014; Burger 2002; Bianchi et al. 2021) Most of the precursor androgens are of adrenal origin, and consequently these hormones are sometimes referred as adrenal androgens (Turcu 2014). In addition to adrenal production of the androgens, DHEA, DHEA-S, and androstenedione can be produced also by the ovarian theca cells and peripheral enzymatic conversion (Bianchi et al. 2021; Burger 2002). It is recommended, that to measure androgenic activity in females, precursor androgens should be measured in addition to active androgens because testosterone and DHT circulate in very low concentrations (Labrie et al. 2006). Besides androgenic functions of the precursor androgens, these hormones are important for production of other sex hormones, estrogens and progesterone (Kalogera et al. 2013). Figure 5 represents conversion of precursor androgens and androgens, and other sex hormones.

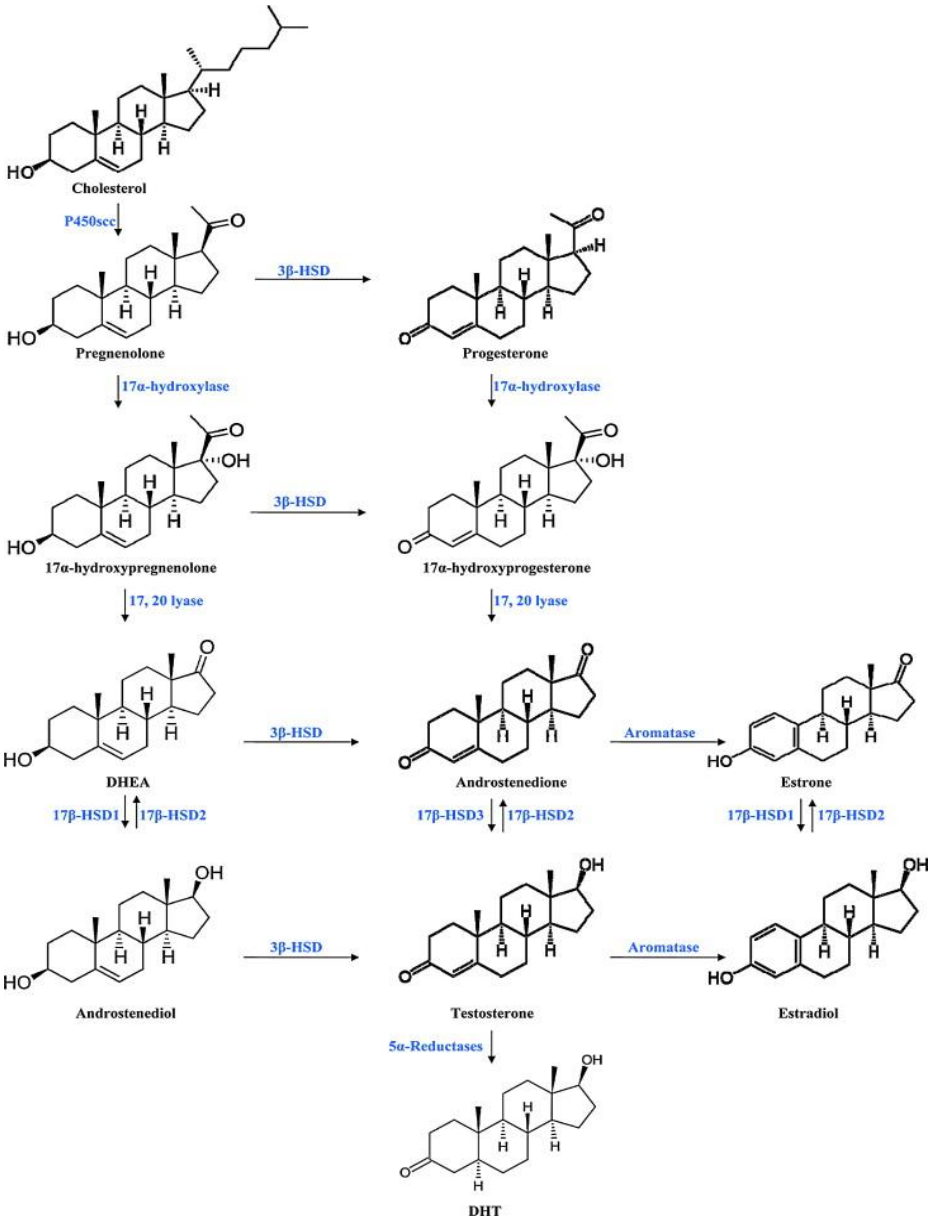


FIGURE 5. Conversion of sex hormones (Kalogera et al. 2013).

4.3 Active androgens

Testosterone and DHT are referred as active androgens. These hormones can bound to androgen receptor to induce the androgenic functions to the target cell. (Bianchi et al. 2021) As illustrated earlier in figure 5, testosterone and DHT are produced by the enzymatic conversion of precursor androgens and estradiol; approximately 30-50 % of testosterone and DHT are produced by enzymatic conversion of these precursor androgens. In addition to this form or production, ovaries, and adrenal glands are able to produce active forms of androgens too. (Bianchi et al.

2021) Testosterone can be converted to DHT and back to the precursor androgen androstenedione or estradiol (Kalogera et al. 2013). DHT is the most potent androgen and binds to the androgen receptor with the greatest affinity, so it is unable to be converted further to other forms of androgens or estrogens. This enhances its half-life. (Bianchi et al. 2021) In females, testosterone and DHT circulate in very low concentrations, and menstrual cycle makes the hormones fluctuate across the menstrual phases in premenopausal healthy females. For example, the concentration of testosterone is at lowest in the early FP, peaks at ovulation, and remains a bit elevated at the mid LP. Free testosterone and DHT remains rather stable during all these phases. (Rothman et al. 2011) Table 2 represents the fluctuation of active androgens and SHBG across menstrual cycle.

TABLE 2. Fluctuation of testosterone, DHT, SHBG, and free testosterone across the menstrual cycle. Adapted from Rothman et al. (2011).

Hormone	Early FP	Midcycle	Mid LP
Testosterone (ng/dl)	15.4 (1.6)	22.7 (1.7)	18.7 (1.5)
DHT (ng/dl)	8.1 (0.85)	8.4 (0.75)	8.5 (0.88)
SHBG (nmol/l)	85.3 (9.2)	99.0 (8.8)	103.4 (10.7)
FreeT (ng/dl)	0.15 (0.013)	0.20 (0.017)	0.16 (0.021)

FP=follicular phase, midcycle=within 48 hours after a surge in LH. LP=luteal phase. FreeT= free testosterone. The results are represented as mean and standard error of the mean. Adapted from Rothman et al. (2011).

Androgens are transported in the circulation bound to carrier proteins. The most abundant carrier protein is albumin, which binds all kinds of steroid hormones with low affinity, thought the most important carrier proteins for sex steroid hormones is SHBG. SHBG is a plasma glycoprotein that binds active forms of androgens and estrogens with a great affinity. SHBG is mainly produced in the liver and its main purpose is to control the transportation, metabolic clearance, and bioavailability of sex hormone steroids. (Hammond 2011) Only a small proportion (1-3%) of the total pool of androgens is free and bioavailable, and a major part of androgens are bound to carrier proteins. There is an inverse relationship between SHBG and free androgens; increased amount of SHBG decreases amount of free and bioavailable androgens and vice versa. (Bianchi et al. 2021; Enea et al. 2011) According to the free hormone hypothesis, free forms of androgens are the forms of hormones, which can bind to an androgen receptor and thus cause the androgenic actions in the target cell (Enea et al. 2011; Rosner 2006).

This suggests that to measure androgenic activity it is recommended to measure the total serum androgens and the free fraction of the androgens.

4.4 Body composition, exercise, and androgens

As lifestyle related sicknesses, such as metabolic syndrome and type 2 diabetes mellitus, have been increasing remarkably (Prasad et al. 2012), many cross-sectional studies have indicated that higher concentration of androgens and low concentration of SHBG may be related to these illnesses and obesity in premenopausal females (Fenske et al. 2015; Korhonen et al. 2003). Fenske et al. (2015) observed that higher levels of free testosterone and low amount of SHBG were risk factors for metabolic syndrome with pre- and postmenopausal females. Similarly, Korhonen et al. (2003) observed same patterns in the Finnish population as they reported that premenopausal females with metabolic syndrome had significantly higher levels of free testosterone, free androgen index, and lower levels of SHBG.

High concentration of androgens is associated also with high BMI. This is supported by the finding with a study by Torchen et al. (2020) noted that premenopausal females without any illnesses who have elevated free testosterone, total testosterone, and DHEA-S tend to have higher BMI. Hyperandrogenism is also connected to polycystic ovary syndrome (PCOS), which is a metabolic syndrome of reproductive age females (Coviello et al. 2006; Apridonidze et al. 2005). It is indicated that the body fat distribution might play a role in endogenous hormone, as concentration and reduction of fat mass in associated with decreased androgen concentrations. A longitudinal study by Wabitsch et al. (1995) observed that in obese adolescent girls with the abdominal obesity was associated with higher levels of total and free testosterone than girls with gluteal-femoral obesity. After a 6-week weight loss program the participants with abdominal obesity experienced greater reductions in free and total testosterone than girls with gluteal-femoral obesity. The study indicated that the reduction in fat mass is related with improvements in steroid hormone abnormalities.

Studies suggests that exercise might be an effective way to impact on blood androgen levels. It is reported that a single exercise bout increases acutely on circulating androgens (Enea et al. 2011). Increased endogenous androgens, such as DHEA, DHEA-S, and testosterone, have been observed immediately after resistance and endurance exercise (Enea et al. 2009; Copeland et al. 2002).

Asides from acute outcomes, there are indications that exercise would influence androgens also chronically. In well-trained populations basal levels of circulating androgens tend to be lower (Enea et al. 2009; Enea et al. 2011) suggesting that there is a chronic adaptation in androgens from exercise. Basal levels of androgens might decrease after endurance training intervention. Keizer et al. (1987) reported decreases in circulating androgens after 3-months of endurance training in previously untrained premenopausal females. They examined the subjects in the FP and LP of the menstrual cycle. They reported decreases in ACTH and DHEA-S in the FP and in the LP together with decreases in testosterone in the LP after the training intervention. McTiernan et al. (2004) observed declines in androgens after 12-months moderate intensity endurance training in sedentary postmenopausal females. The study reported significant decreases in circulating testosterone and free testosterone in exercising group who did experience loss in body fat mass compared to control group.

Possible mechanisms for how association of fat mass and androgens might be related to peripheral production of androgens. Adipose tissue has a lot of androgen receptors so the androgens can augment their androgenic actions directly on the adipose tissues. As peripheral tissues can produce active androgens via enzymatic conversion, can excess of adipose tissue increase the androgen production. (O´Reilly et al. 2014) In addition to impact of fat mass on endogenous androgens, another possible factor inducing concentration of circulating androgens may be connected to ACTH secretion. Exercise is a form of stress which can augment the secretion of ACTH, which in sequentially increases androgen secretion from adrenal cortex. It is also proposed, that to provoke ACTH secretion, the intensity and duration needs to be great enough. (Enea et al 2011) For example, elevated endogenous androgens were not observing after 30-seconds Wingate test (Enea et al. 2009). Keizer et al. (1987) proposed that prolonged endurance training would reduce ACTH secretion and Duclos et al. (2001) indicated that endurance training inhibits the adrenal sensitivity to ACTH secretion.

Interestingly, even though it has been observed that exercise reduces total amount of androgens, there are also contradictory findings how exercise and body composition are connected to the concentration of the endogenous androgens in females. Kumru et al. (2005) reported that regular exercise (> 10 h per week ≥ 5 years) was related to lower BMI and higher levels of serum testosterone compared to sedentary controls in age-matched premenopausal females. Both Eklund et al. (2017) and Cook et al. (2018) have observed higher levels of androgens in female athletes compared to non-athletes. Eklund et al. (2017) reported higher levels of DHEA

and androgen metabolites 5-androstene-3β, 17β-diol (5-Diol) and etiocholanolone glucuronide (Etio-G) compared to non-trained subjects. The difference in androgen profile was not explained with differences in BMI, since all subjects were homogenous when comparing the BMI. The study suggested that with elite athletes the endogenous androgens are associated with more anabolic body composition. These inconsistent findings indicate that there is a gap in literature how body composition, mainly fat mass and endogenous androgens are connected and modified with training in naturally menstruating females.

5 RESEARCH QUESTIONS AND HYPOTHESIS

The aim of this master thesis was to examine the impact of menstrual mediated and traditional HIIT on VO_2max, endogenous androgens, and fat mass. The primary focus of this study was to investigate: 1) if LP-focused HIIT is better than FP-focused and traditional HIIT to gain improvements in aerobic capacity (VO_2max) and; 2) to examine the effect of HIIT on the basal endogenous androgen profile and fat mass in premenopausal females.

RQ 1: Does LP-focused HIIT induce significant improvements in VO_2max compared to FP-focused or traditional HIIT in naturally menstruating females?

H 1: **No.** Although the 4 x 4 min HIIT protocol has been shown to induce significant increases in VO_2max (Helgerud et al. 2007), and endurance performance at the LP might be improved due to enhanced fat utilization and glucose-sparing effect (Hackney et al. 2022; D'Eon et al. 2022; Zderic et al. 2001) it is unlikely that significant changes between the groups will be seen due to volume matched training protocols and a relatively short training period.

RQ 2: Does 8-weeks of HIIT promote reductions in body fat mass, concurrent with a reduction in basal endogenous androgen profile in naturally menstruating females?

H 2: Yes. There are indications that HIIT is effective for reducing fat mass (Gripp et al. 2021) and endurance training might reduce concentration of DHEA and testosterone (Keizet et al. 1987) and free testosterone (McTiernan et al. 2004) and probably in other endogenous androgens in naturally menstruating females. Concurrent reductions in fat mass and endogenous androgens in females has been reported as well (McTiernan et al. 2004; Wabitsch et al. 1995).

6 MATERIAL AND METHODS

The study was a longitudinal observational study part of a bigger research project. The project examined the impact of menstrual cycle mediated endurance training on physical performance and body composition together with energy availability, metabolism, the risk factors of cardiovascular illnesses, quality of life and body image. The study design was approved by the ethics committee. The participants were informed about the voluntary participation in the research, and they were allowed to withdraw from the study at any time.

6.1 Participants and training groups

The inclusion criteria were that the participants were 18-35 years old naturally menstruating females with BMI between 19.5-35 kg/m^2. They were not allowed to be using any kinds of hormonal contraceptives for the past 12-months. The participants were required to be healthy without any medication, chronic or musculoskeletal illnesses which would prevent performing endurance training or tests involved in the research project. Participants were considered recreationally active, and prior the inclusion, it was ensured that the intervention would not decrease their training load. They were required to be non-smokers, not training competitively, not pregnant or breastfeeding. Their menstrual cycle was considered normal, and the criteria for the menstrual cycle length was 28-35 days. However, some participants reported about slightly shorter or longer menstrual cycles, but they were still included, if the other inclusion criteria were fulfilled. Prior the research project, the participants filled a health questionnaire, and if there something which could prevent the participating, a doctor contacted the participant, and the issue was resolved.

Participants meeting inclusion criteria were randomized into one of three groups: control group, follicular phase (FP group), or luteal phase (LP group) emphasized training group. All groups had the same number of participants (n=13) at the beginning of the intervention. The control group's training volume was continuously periodized across the training and two experimental groups had a greater training volume in one menstrual cycle phase: the FP emphasized training group had higher training volume at the follicular phase and lower training volume at the luteal phase, and the LP emphasized group had higher training volume at the luteal phase, and the lower training at the follicular phase. Descriptive characteristics of the study participants are

presented in the table 3. However, three of the participants dropped out before the first measurements, so their descriptive characteristics are not included in the table 3.

TABLE 3. General and anthropometric data of the participants divided by group

	LP group	FP group	Control group	All
n	12	13	11	36
Age (years)	29.8 ± 5.4	29.5 ± 3.2	29.8 ± 4.6	29.7 ±4.3
Height (cm)	164.5 ± 4.9	165.8 ± 4.4	165.7 ± 5.9	165.3 ± 4.9
Weight (kg)	66.2± 10.1	67.3 ± 9.1	65.6 ± 11.5	66.4 ± 9.8
BMI (kg/m^2)	24.5 ± 4.1	24.5 ± 3.1	23.8 ± 3.2	24.3 ± 3.4
Fat mass (kg)	26.9 ± 7.8	26.5 ± 8.1	26.2 ± 6.6	26.3 ± 7.4
MC length (days)	29.4 ± 7.9	28.2 ± 4.5	28.9 ± 4.4	28.8 ± 5.6
VO_2max (ml/kg/min)	38.1 ± 4.8	39.2 ± 5.3	39.5 ± 4.2	39.1 ± 4.8

LP group=Luteal phase emphasized group, FP group=follicular phase emphasized group. MC= Menstrual cycle. Results represented as means ± standard deviation.

6.2 Study design

The research project lasted approximately six months. First, the participants completed a 4–8-week (1 menstrual cycle) control period followed by 8-weeks (2 menstrual cycles) moderate intensity endurance training period (MIET) and 8-weeks (2 menstrual cycles) HIIT-period. Before the control period, the participants had a short familiarization visit when they were informed about the procedures, risks, and benefits of the participation and asked for written consent. In addition, they were familiarized with performance test measurements and equipment used during the study. The participants monitored their training and heart rate data continuously across the project so the sport watch (Garmin® Venu Series, Garmin Ltd., Taiwan) and heart rate monitor (Garmin® HRM-Dual, Garmin Ltd., Taiwan) were given for a participant at the familiarization visit.

Each participant had six measurement points during the study. Each measurement point included two days of subsequent measurements (measurement day 1 and measurement day 2). The participants were measured after the control period, after MIET period, and after HIIT period. After each period, the participants were measured two times, at mid-LP and early-FP according to their menstrual cycle. To minimize the possible impacts of hormones across the

menstrual cycle phases on measured variables, the LP timepoints were analysed in the present thesis. Based on their baseline incremental treadmill running test heart rates, individualized training intensities and heart rate zones were determined for each training block. During the control period the participants continued their normal daily activity without a specific training program and were educated to monitor their menstrual cycle. During the training interventions the participants were instructed to continue their normal daily activities. Figure 6 illustrates the study design.

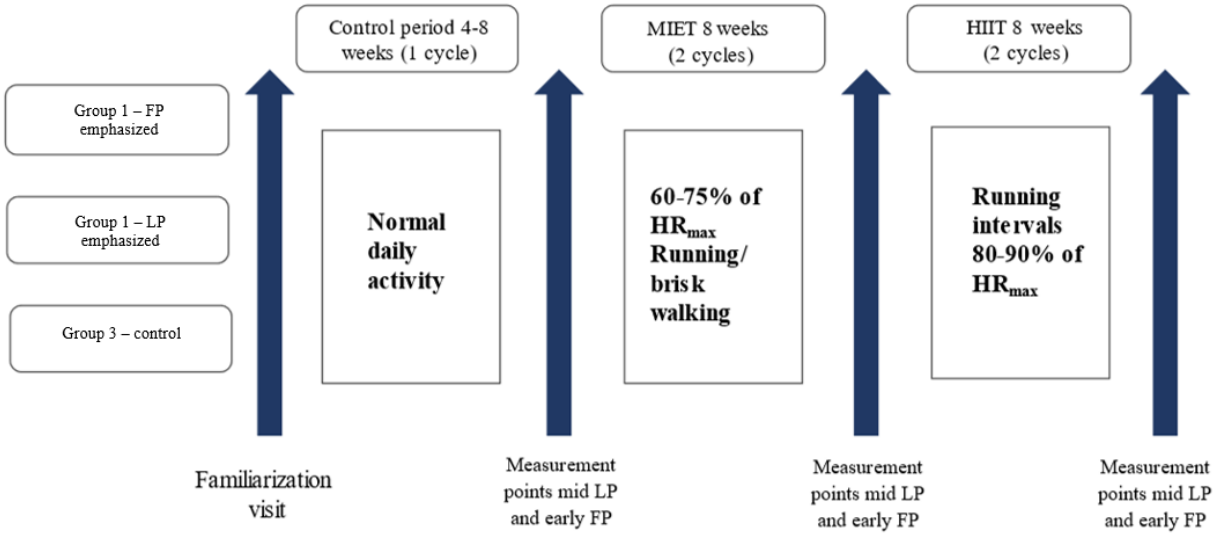


FIGURE 6. Study design and measurements time points. FP=follicular phase, LP= luteal phase, MIET=moderate intensity endurance training, HIIT=high-intensity interval training, HR_{max}=Maximal heart rate.

6.3 Menstrual cycle monitoring

Participants were instructed to monitor their menstrual cycle throughout the study. The menstrual cycle phase was verified by using urinary ovulation test (Clearblue® Advanced), which measures LH and estrogen, in each menstrual cycle during the study, including the control cycle. The ovulation test was started a few days prior the estimated ovulation based on the menstrual cycle history and was done according to the manufacturer's guidelines (Appendix 1). In addition to ovulation test, the menstrual cycle phase was verified from fasting blood sample hormones taken on the measurement day 1. Measurements days were scheduled on specific days according to each participant's individual menstrual cycle. The measurement

days were fixed for mid luteal phase (4-8 days after positive ovulation test) and for early follicular phase (1-3 days after the start of the menstrual bleeding).

6.4 Measurements

Measurement days 1 and 2 were executed either on two subsequent days or a maximum of one day in between. Before measurement day 1 the participants were informed to be fasting 12 h and refrain from strenuous activity for 24 h prior the measurements. Before measurement day 2 participants were instructed to refrain from other strenuous activities than the peak fat oxidation (PFO) test from the measurements of the previous day. Height was measured once on the first visit and weight was measured each visit. On measurement day 1, weight was measured in a fasted state wearing underwear and on day 2 weight was measured before the tests without shoes in the clothing they performed the tests. To avoid possible confounding effects of circadian rhythm, the tests were completed at the same time of day (±2 hours) for each measurement timepoint. Figure 7 shows the tests that were completed on each measurement day.

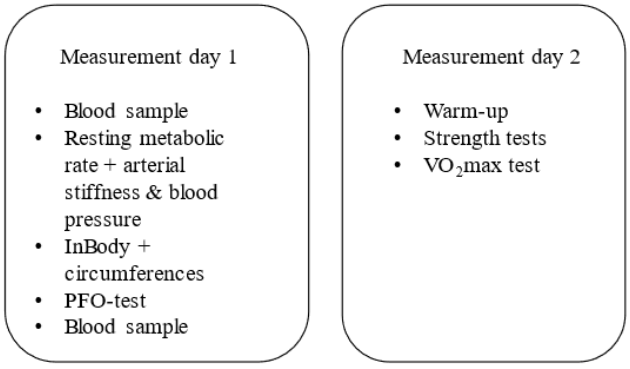


FIGURE 7. Measurement days and the executed tests at the approximate order. PFO=Peak Fat Oxidation test.

On measurement day 2, participants performed a standardized warm-up which included 3 minutes of brisk walking or light jogging on a treadmill after they performed 10 squats, 10 lunges, 10 side lunges, and 10 calf raises. After the warm-up, participants performed strength tests including countermovement jump (CMJ) and maximal isometric bilateral strength test on a leg press. The participants performed 2 to 3 warm-up CMJ with 1 minute rest after they performed 3 to 5 maximal CMJ until the result didn´t improve. After CMJ the participants

performed isometric warm-ups in leg press at 50%, 75%, and 90% of their self-estimated maximum. Warm-ups were separated with 1 minute rest. After warm-up participants performed 3 to 5 maximal performances with 2 minutes recovery until the result didn´t improve.

6.4.1 Incremental treadmill running test

Following the strength tests incremental treadmill running test was executed to determine aerobic capacity, VO_2max, and lactate thresholds. Prior the test, a dampened heart rate monitor (Garmin® HRM-Dual, Garmin Ltd., Taiwan) was placed on a bare skin close to participant's sternum (xiphoid process) to measure the heart rate. A reusable mask was placed on participant's face and adjusted with a head strap and a blood sample was taken from the fingertip for further lactate analyses. The respiratory gas analyzer (Vyntus CPX, Vyaure Medical GmbH, Hoechber Germany) was calibrated according to manufacturer instructions before each test and connected to the mask. The participant was informed about the test protocol and ending of the test. The participants were encouraged verbally to continue the test until maximal exhaustion, but they were allowed to stop the tests between the grades or at any time during the stage. For safety reasons, participant wore a harness during the test.

Treadmill incline remained constant (0.6°) and speed increased by 1 km/h for each stage. speed was 6 km/h and participants were allowed to either walk or jog the first stages. All tests were standardized how the participant completed the first stage (ie. walking or jogging). Each stage lasted 3 minutes and the stages were continued until volitional exhaustion. Respiratory gases and VO_2 were measured using breath-by-breath throughout the entire test. Heart rate was monitored continuously during the test with a sport watch (Garmin® Venu Series, Garmin Ltd., Taiwan). After each stage the treadmill was stopped and a fingertip blood sample was taken for the analysis of lactate (Biosen C-line Clinic, EKF Diagnoctics GmbH, Barleben Germany). If the treadmill was stopped over 45-seconds for blood sample, one minute was added on the next stage. Rate of perceived exhaustion (RPE) was assessed using Borg´s scale (6-20) and was recorded 30-seconds prior to the end of each stage. Each stage's heart rate was written down 15-seconds prior the stage end. After the test was ended, fingertip blood sample was taken immediately after the termination and at 1 and 3 minutes after the termination.

VO_2max was measured and analysed automatically with K-lab 3.1.25 software (2121.10.01 Aino Health Management Oy, Helsinki). If the automatic analysis failed to describe the

thresholds, manual corrections were made according to general guidelines. K-lab analysed VO_2max as 60 second time-interval average oxygen consumption. The results are presented as absolute (l/min) and relative (ml/kg/min) values.

6.4.2 Body composition measurements with bioimpedance

Participants underwent body composition measurements every measurement day 1. They were measured in a 12 h fasted state to minimize possible confounding effects of hydration or nutrition status on variables. Subjects wore their underwear and were asked to remove all excess shoes, watch, and jewellery prior the measurement. Subjects wiped their palms and soles with damp towel before they stepped on the scale. The body composition measurements were done with Bioimpedance (Inbody 770 body composition analyzer, Biospace Co. Ltd, Seoul, South Korea). Participants were instructed to stay still and avoid speaking during the measurement. To avoid possible intentional or unintentional behaviours affecting the body composition subjects did not see the bioimpedance result by themselves. The body composition variables chosen for the further analysis were visceral fat area (VFA), body fat percentage (BF %), body fat mass (BFM), and fat free mass (FFM).

6.4.3 Hormone measurements

For hormone analyses blood samples were collected from the antecubital vein using sterile needles into serum tubes (Vacuette® TUBE, Greiner Bio-One, Austria) by a qualified laboratory technician. Blood samples were collected between 6:30 and 8:30 on measurement day 1. After 10 ml of blood was collected, the sample was centrifuged at 2245 g (Megafuge 1.0R, Heraeus, Germany) for 15 minutes after which the serum was removed from the blood sample and pre-frozen at -20°C after which the samples were stored at -80°C until further analysis. From the serum, concentrations of estradiol, progesterone, androstenedione (A4), free testosterone (freeT), total testosterone (Tt), DHT, SHBG, DHEA, and DHEA-s were determined with chemical lumiscence techniques by using Immulite®2000 XPi-analyzer (Siemens, New York, NY, USA) or with ELISA-kits (Biovendor) tests by using Dynex DS2® (Dynex ® technologies, Cantilly, USA).

6.5 Training program interventions

The training groups had same number of trainings across the whole training intervention. The experimental groups emphasized the training volume in one menstrual cycle phase: the FP emphasized group had higher training volume in the FP and lower volume at the LP. The LP emphasized group had lower volume at the FP and higher volume at the LP. The control group consistently trained the same volume each week, 2 trainings each week, despite during the weeks 7 and 8, when the amount of the trainings was 3 and 1.

All groups executed 8-week MIET before the HIIT block. MIET intensity was 60-75% of the HR_{max}. Depending on the individual, it was light running or brisk walking. MIET training served as a familiarization period for the HIIT-program. The complete MIET program consisted of 21 separate training sessions. If the menstrual cycle was unexpectedly long or short the participant was instructed to do 30-minutes training sessions for maintenance or to move on to the next cycles program.

Each HIIT session included a 10-minute warm-up (brisk walking or light running) at 60-65% of HR_{max}, after which participants executed 4 x 4 min running intervals at 80-90% of HR_{max}. The HIIT-program was modified according to Hellerud et al. (2007) HIIT program. Between each interval there was a 3-minute recovery at 60-65% of HR_{max}. After the running intervals, a 10-minute running cool-down at 60-65% HR_{max} was executed. The 8-week training program for each group is presented in the table 4.

TABLE 4. HIIT program with all training groups.

	Control	FP group	LP group
Week 1	**TESTING**	**TESTING**	**TESTING**
	2 HIIT sessions = 58 min	2 HIIT sessions = 58 min	1 HIIT session = 29 min
Week 2	2 HIIT sessions = 58 min	3 HIIT session = 87 min	2 HIIT sessions = 58 min
Week 3	2 HIIT sessions = 58 min	2 HIIT sessions = 58 min	3 HIIT session = 87 min
Week 4	2 HIIT sessions = 58 min	1 HIIT session = 29 min	2 HIIT sessions = 58 min
Week 5	2 HIIT sessions = 58 min	3 HIIT session = 87 min	2 HIIT sessions = 58 min
Week 6	2 HIIT sessions = 58 min	2 HIIT sessions = 58 min	1 HIIT session = 29 min
Week 7	3 HIIT session = 87 min	2 HIIT sessions = 58 min	4 HIIT session = 116 min
Week 8	**TESTING**	**TESTING**	**TESTING**
	1 HIIT session = 29 min	1 HIIT session = 29 min	1 HIIT session = 29 min

The table presents total amount and total length of the HIIT-sessions executed per each HIIT-intervention week. Testing=Weeks when the fasting and performance tests were done.

6.6 Statistical analysis

Statistical analyses were done with IBM SPSS statistics 26 (IBM Corporation, US). Prior to statistical analysis the normality of the data was tested using the Shapiro-Wilk test and possible differences in VO_2max, body composition variables (VFA, BFM, BF%, FFM) and basal levels of endogenous hormones (A4, FreeT, tT, DHT, SHBG, DHEA, and DHEA-S) between groups was tested with One-Way ANOVA with a level of significance set $p<0.05$. Percentual changes of body composition variables and endogenous androgens were calculated Microsoft Excel (Microsoft Corporation, US) software.

A repeated measures ANOVA was performed to compare the effect of the training program on VO_2max with a level of significance set $p<0.05$. Due to not normally distributed data, within group changes were tested with non-parametric Related-Samples Friedman's Two-Way analysis of Variance. To identify which groups differed from each other a Post Hoc test was done, and significant values were adjusted by the Bonferroni correction for multiple tests with a level of significance set $p<0.05$. Changes in body composition variables and endogenous androgens and estradiol and progesterone for all participants as one group across the whole intervention were measured with non-parametric Related-Samples Friedman's Two-Way analysis of Variance with a level of significance set $p<0.05$ was computed to assess the correlation between percentual changes of body composition variables and endogenous androgens.

7 RESULTS

No statistically significant differences were observed between groups at the baseline measurements in absolute or relative VO_2max values, body composition variables, or endogenous androgens between the groups. For the final VO_2max results, the number of the participants in FP group was 4, in LP group was 7, and control group was 5. When using repeated measures ANOVA, a statistically significant effect of time was observed for absolute ($F(2,12)=0.5039$ and $p=0.026$) and relative ($F(2,12)=10.202$ and $p=0.003$) but not for group. In within group comparisons, statistically significant changes were not observed across the HIIT intervention in any of the groups or when comparing all groups as a one group. Within group changes in VO_2max are represented in tables 5 and 6.

TABLE 5. Absolute VO_2max across the training intervention between the groups.

		VO_2max (l/min)		
	n	Baseline	Pre HIIT	Post HIIT
FP Group	4	2.72±0.23	2.70±0.27	2.71±0.31
LP Group	7	2.50±0.23	2.59±0.23	2.57±0.26
Control	5	2.55±0.27	2.66±0.30	2.69±0.28*
All	16	2.57±0.25	2.64±0.25**	2.64±0.27*

Results as l/min presented as means±SD. *=Statistically significant difference of $p<0.05$ between baseline and post HIIT. **=Statistically significance difference of $p<0.05$ between baseline and pre HIIT.

TABLE 6. Relative VO_2max across the training intervention between the groups.

		VO_2max (ml/kg/min)		
	n	Baseline	Pre HIIT	Post HIIT
FP Group	4	39.13±7.50	38.70±7.27	39.25±8.31
LP Group	7	38.97±4.32	40.40±4.40**	39.69±5.70
Control	5	40.68±5.30	42.64±6.12	43.14±5.61*
All	16	39.54±5.18	40.68±5.54**	40.66±9.18*

Results as means±SD. *=Statistically significant difference of $p<0.05$ between baseline and post HIIT. **=Statistically significance of $p<0.05$ between baseline and pre HIIT.

The tables 5 and 6 present that the FP group did not experience any statistically significant difference in absolute or relative VO_2max values across the whole training intervention, but the control group experienced a statistically significant increase between baseline and post HIIT timepoints and almost statistically significant increase between timepoints baseline and pre HIIT ($p=0.058$) as in absolute and relative VO_2max values. For the LP group there was a statistically significant increase in relative VO_2max between baseline and pre HIIT as relative and almost statistically significant increase between baseline and post HIIT ($p=0.061$). The increase was not observed in absolute VO_2max values. When comparing all participants as one group, a statistically significant difference between baseline and pre HIIT and baseline and post HIIT was observed. Figure 8 illustrates the relative VO_2max changes between groups across the whole training intervention and figure 9 illustrates the relative VO_2max changes between groups across the HIIT intervention.

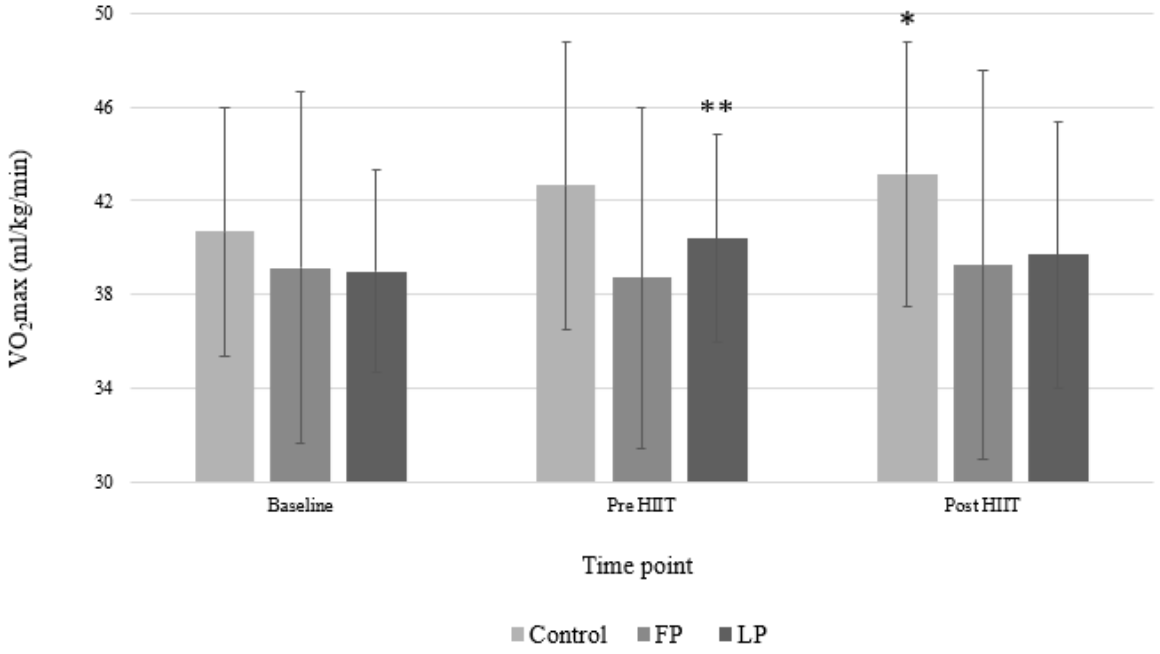


FIGURE 8. relative VO_2max changes across the training intervention between the groups. Results represented as means±SD. **=statistically significant difference of $p<0.05$ between baseline and pre HIIT. *=statistically significant change of $p<0.05$ between baseline and post HIIT.

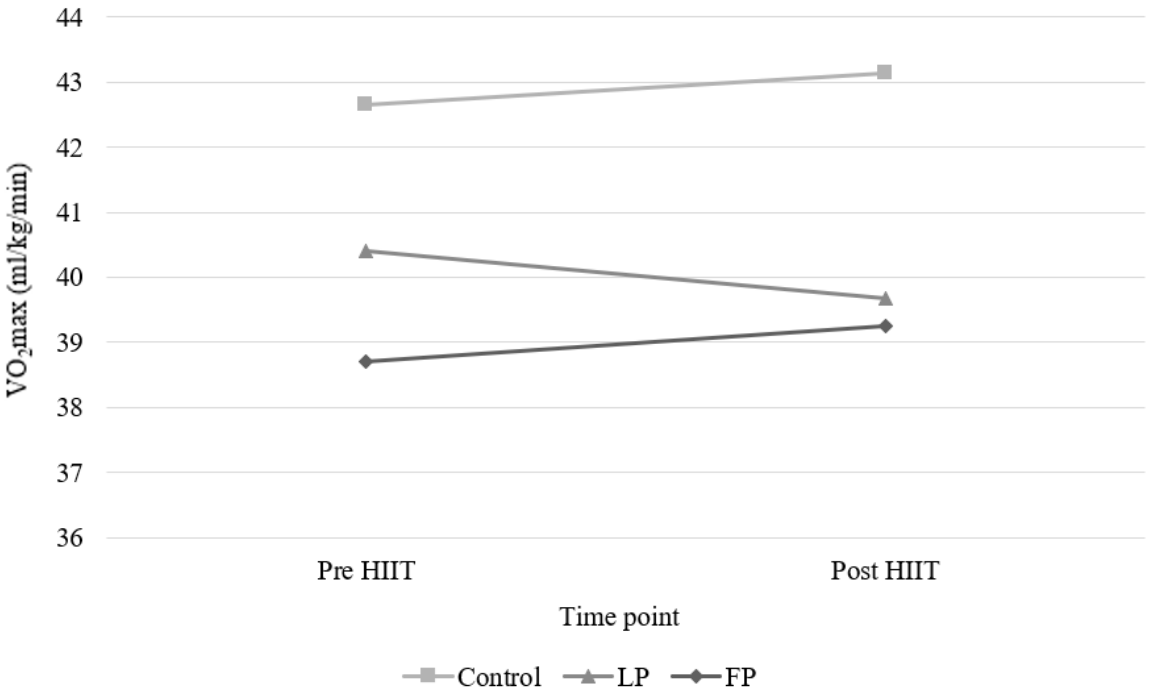


FIGURE 9. Relative VO_2max before and after the HIIT program. Values represented as means.

Endogenous androgens, estradiol, progesterone, and body composition were analysed as all participants as a one group. Concentration of estradiol and progesterone across the measurement points are presented in the table 7. For the final body composition analysis, the amount of the participants was 17. No change in body composition variables were observed in VFA, BF %, BFM, or in FFM over time (table 8)

TABLE 7. Concentration of progesterone and estradiol across the measurement points.

	n	Baseline	N	Pre HIIT	n	Post HIIT
Estradiol (pmol/L)	33	550.42 (276.00-1523.00)	15	543.93 (184.00-892.00)	11	673.81 (337.00-1553.00)
Progesterone (nmol/L)	33	23.19 (1.01-49.90)	15	30.24 (1.45-63.60)	11	25.51 (1.78-49.60)

Results presented as mean and minimum and maximum values.

TABLE 8. The VFA, BF %, BFM, and FFM across the measurement points.

	N	Baseline	Pre HIIT	Post HIIT
VFA (cm^2)	17	82.44±42.69	80.52±42.44	78.98±42.96
BF %	17	27.59±7.41	27.38±7.51	26.95±7.52
BFM (kg)	17	18.71±7.93	18.45±7.97	18.19±8.10
FFM (kg)	17	46.85±3.97	46.64±3.87	46.89±4.09

VFA=Visceral fat area, BF %=Body fat percentage, BFM=Body fat mass, FFM=Fat free mass. Results presented as mean±SD.

For endogenous androgens, the number of the participants for the final analysis was 5 for A4, SHBG, DHEA-S, and tT, 9 for DHEA and DHT, and 15 for FreeT. No change over time was observed for androgens, expect for FreeT (table 9). Free testosterone decreased statistically significantly across the HIIT intervention (-14.7±34.9 %) and from baseline to post HIIT (-7.1±44.2 %) The change in DHEA concentration was increasing between timepoints baseline and post HIIT, and the change was almost statistically significant (p=0.059). The changes in the endogenous androgens across the whole training intervention are represented in the table 9.

TABLE 9. The endogenous androgens across the timepoints.

	N	Baseline	Pre HIIT	Post HIIT
A4 (nmol/L)	5	12.81±8.24	10.17±5.46	12.64±8.15
SHBG (nmol/L)	5	65.12±6.50	54.40±20.39	65.64±14.90
tT (nmol/L)	5	0.98±0.77	0.69±0.33	0.92±0.45
DHEA-s (umol/L)	5	5.53±1.72	4.98±2.27	4.97±1.12
DHT (pg/ml)	9	671.44±407.73	670.89±286.74	712.22±398.75
DHEA (ng/ml)	9	11.89±4.90	13.96±6.27	16.54±6.79
FreeT (pg/ml)	15	2.67±1.50	2.83±1.85 *	2.17±1.18 **

A4=androstenedione, SHBG=sex hormone binding globulin, tT=total testosterone, DHEA-S=dehydroepiandrosterone sulphate, DHT=dihydrotestosterone, DHEA=dehydroepiandrosterone, FreeT=free testosterone. The results represented as mean±SD. * =statistically significant difference of $p<0.05$ between pre HIIT and post HIIT, **=statistically significant difference of $p<0.05$ between baseline and post HIIT.

Spearman correlations for association of percent change in body composition and androgenic hormones are presented in the tables 10 and 11. Some variables had weak to moderate correlations, but those did not reach statistical significance. Because the n of A4, SHBG, tT, and DHEA-s was relatively small across the HIIT intervention (n=5), these variables were not included in pre and post HIIT correlation analysis.

TABLE 10. Spearman correlation coefficient (*r*) between the percent changes of body composition and androgen hormones during the high-intensity interval training intervention.

	DHEA Δ%	DHT Δ%	FreeT Δ%
VFA Δ%	-0.250	0.067	-0.439
BF % Δ%	0.100	0.600	-0.282
BFM Δ%	0.000	0.217	-0.461
FFM Δ%	0.067	-0.517	-0.250

A4=androstenedione, SHBG=sex hormone binding globulin, tT=total testosterone, DHEA-S= dehydroepiandrosterone sulphate, DHT=dihydrotestosterone, DHEA=dehydroepiandrosterone, FreeT=free testosterone. Δ%= Percentual change between pre HIIT and post HIIT timepoints.

TABLE 11. Spearman correlation coefficient (*r*) between the percent changes of body composition and androgen hormones during the whole training intervention.

	A4 Δ%	tTΔ%	DHEA-s Δ%	DHEA Δ%	DHT Δ%	SHBG Δ%	FreeT Δ%
VFA Δ%	0.291	0.045	0.509	0.042	0.152	-0.218	-0.93
BF % Δ%	0.245	-0.109	0.391	0.164	0.345	-0.100	0.068
BFM Δ%	0.218	0.118	0.491	0.139	0.333	-0.164	0.025
FFM Δ%	-0.178	0.542	0.588	0.261	0.345	-0.478	0.352

A4=androstenedione, SHBG=sex hormone binding globulin, tT=total testosterone, DHEA-S= dehydroepiandrosterone sulphate, DHT=dihydrotestosterone, DHEA= dehydroepiandrosterone, FreeT=free testosterone. Δ%= Percentual change between baseline and post HIIT timepoints.

8 DISCUSSION

The main objective of this master thesis was to examine whether menstrual cycle mediated HIIT is better than traditional HIIT to improve endurance capacity measured with VO_2max and whether HIIT is beneficial to promote concurrent reductions in endogenous androgens and fat mass. In accordance with the hypothesis 1, it was observed that there were no significant differences in absolute or relative VO_2max values between groups. Interestingly, no group increased their VO_2max during the HIIT intervention. However, in within group comparisons, it was observed that control group experienced statistically significant changes between baseline and post HIIT (40.68±5.30<43.14±5.61) timepoints and the LP groups between baseline and pre HIIT (38.97±4.32<40,40±4.40) timepoints. Against the hypothesis 2, the HIIT training did not induce concurrent reductions in endogenous androgens and fat mass, some of the correlations were moderate, but those did not reach the level of statistically significance set. The VFA, BF %, BFM and FFM variables did not experience any significant changes across the intervention. There was a significant decrease in FreeT across the HIIT (p=0.018) and the whole training intervention (p=0.045), but for other androgens there was not change.

8.1 Discussion on the HIIT training and VO_2max results

In the present study, there were no statistically significant differences between or within groups in endurance performance measured as VO_2max improvements during the HIIT intervention. The non-appearance of the group differences might be explained by the observation that the direction of the VO_2max change in most of the participants was similar. Despite that, in within group analysis group differences were observed how VO_2max improved between different time points. The control group improved from the baseline to post HIIT, and the LP group improved from the baseline to pre HIIT. The FP group did not experience any statistically significant changes across the intervention.

It was hypothesized that the LP group might benefit during the HIIT intervention due to enhancing effects of estrogen on fat utilization. In line with that, current literature advocates that lipolysis might be enhanced in the LP due to higher concentration of estrogen (Oosthuyse & Bosch 2012; Hackney et al. 2022; D'Eon et al. 2002). It is noteworthy to point out that many of the studies which have observed the effect have used submaximal tests (Hackney et al. 2022; D'Eon et al. 2002; Zderic et al. 2001). In submaximal loads fat is the most important source of

energy and as the intensity increases over the lactate threshold, glucose becomes the most important source of energy (Cermak & van Loon 2013). In the present study, the HIIT training session consisted of 4 x 4 minutes running at 80-90 % of HR_{max}, which makes the training rather anaerobic, and the energy production relies more on glucose. This could mask lipolysis enhancing effects of estrogen and explain why the LP group did not improve their VO_2max during the HIIT intervention. This is supported in the study of Rael et al. (2021) who did not see statistically significant changes between menstrual cycle phases in most of the cardiorespiratory variables during the HIIT exercises, which consisted of 8 x 3 minutes running at 85% of the maximal aerobic. They explained the finding with that a high-intensity exercise is alone the most crucial factor to influence physiological adjustments, and the fluctuation of hormones are not high enough defeat them.

In addition to estrogen, another possible reason why the LP group did not improve their endurance performance during the HIIT might be explained with the role of progesterone. As progesterone has thermoregulatory effects resulting in an increase in body temperature and heart rate (Goldsmith & Glaister 2020; Thompson et al. 2012), combined with chemosensitivity enhancing effects of the hypothalamus chemoreceptors resulting in increases in Ve (Goldsmith & Glaister 2020; Williams & Krahenbuhl 1997), it is noticed that high levels of progesterone might decrease running economy in submaximal loads (Barba-Moreno et al. 2022; Williams & Krahenbuhl 1997). These effects might cause a greater cardiovascular strain in the LP compared to other phases, which might hinder endurance performance enhancing effects of estrogen in the LP. These reasons together might explain why the LP emphasized HIIT might hinder the development of the aerobic capacity.

Even though this study focused on the HIIT intervention, the role of the MIET intervention cannot be disparaged. When all participants were compared as a whole, a statistically significant improvement between baseline and pre HIIT and baseline and post HIIT was observed in both absolute and relative VO_2max values. The MIET consisted of submaximal walking or moderate running exercises at 60-75 % of HR_{max} and the training volume in minutes was higher compared to HIIT. It is possible that the noteworthy decrease in time spent on training from MIET to HIIT caused the plateau in VO_2max development. Interestingly, the LP group was the only group which experienced statistically significant increase in relative VO_2max between baseline and pre HIIT time points. This observation is in line with previous studies (Hackney et al. 2022; D'Eon et al. 2002; Zderic et al. 2001) that have observed enhanced fat oxidation at submaximal

loads in the LP. It is possible, that during the MIET the LP group did benefit from estrogen's lipolysis improving effect and enhanced the VO_2max improvement.

The FP group did not experience a statistically significant changes in absolute or relative VO_2max across the HIIT intervention, nor the whole training intervention. This is in line with the review article of McNulty et al. (2020) which concluded that exercise performance could be trivially reduced in the FP. This assumption might explain why the FP emphasized endurance training might not be the most effective to improve VO_2max. In within group comparisons the control group did improve the most across the whole training intervention. They experienced statistically significant increase in relative VO_2max from baseline to post HIIT and almost statistically significant increase from baseline to pre HIIT (p=0.058). In the sight of this result, a continuously periodized endurance training might be more suitable compared to menstrual cycle mediated endurance training in recreationally active naturally menstruating females.

The participants were guided to monitor their trainings with heart rate monitor and fill a training log. Unfortunately, the specific training log data were not available in this master's thesis due to scheduling constraints. It is possible, that some participants did follow the guided heart rate zones during the training, or they performed less trainings although they were included in the statistical analyses. As many participants could not participate in all measurement days due to illness, we can assume that for the same reason training sessions were not executed at the right time point or perhaps at all. Hence, results need to be interpreted carefully.

8.2 Discussion on the endogenous androgens and body composition

There were no statistically significant changes in body composition variables between HIIT nor over the whole training intervention. The decrease in freeT across the HIIT intervention was statistically significant (-7.1±44.2 %). Other changes in hormone variables did not reach statistical significance. Interestingly, the increase in DHEA concentration between baseline and post HIIT timepoints was near statistical significance (p=0,059). Finally, there were no statistically significant correlations between body composition and endogenous androgen variables across the HIIT or the whole training intervention.

Possible explanation for non-significant changes in body composition variables might be explained with participants training history and baseline fat mass. It has been previously

observed that 12-weeks of HIIT and MIET performed three times a week influenced fat mass loss in sedentary and obese participants (Martins et al. 2016). In the present study the inclusion criteria were that participants were not training competitively, and the training intervention would not decrease their usual training volume. Baseline relative VO_2max values as means in all groups were from 38.1 ml/kg/min to 39.5 ml/kg/min. When these values are compared to international reference values of 30 years old women, these values are considered "good" (Shvartz & Reibold 1990). With this information we can conclude that the studies considering sedentary females won´t apply to our study population. This hypothesis is supported by a study by Helgerud et al. (2007) where recreationally active male subjects executed very alike 8-week 4 x 4 minutes HIIT running intervention, and they did not experience significant reductions in their body weight even thought they increased their VO_2max. Also, the BMI of the participants was 24.3 ± 3.4 kg/m^2 which is considered normal. Hence, we can question whether reduction in fat mass would even be expected in this population.

The statistically significant reduction in freeT across the HIIT was in line with the study of McTiernan et al. (2004) who detected statistically significant reductions in freeT and total testosterone after 12-month of moderate intensity training walking and bicycling intervention. The reductions in freeT and total testosterone were significant with the group which decreased ≥ 2% of body fat. In the present study the reduction in freeT was not associated with reductions in fat mass variables, so this observation does not apply to this study sample. The trend in DHEA was increasing and reached almost a statistically significant change across the HIIT. Increases in DHEA in response to training has been seen in well trained athletes, for instance a study of Filaire et al. (1998) discovered a that 16-week training program, consisted of strength training, endurance, and sprint training, increased DHEA levels in female athletes. In agreement with the present study, they did not find correlations between DHEA variation and body composition variation.

The concurrent reductions and changes in fat mass and endogenous androgens is still rather unknown as a topic. In previous studies, a positive correlation between fat mass and androgens has been observed in females who have metabolic syndrome or type two diabetes (Korhonen et al. 2003; Fenske et al. 2015). The inclusion criteria of the present study were that subjects needed to be healthy without any chronical illnesses. In this regard, these studies might not apply to our study population. Also, concurrent reductions in androgens and fat mass are observed in populations who were obese and sedentary (McTiernan et al. 2004). As discussed

before, the population of this study was not sedentary, we can postulate that the concurrent changes may be observed only in previously untrained or obese females. Further, studies with well-trained populations or with athletes, it has been observed that higher concentration of endogenous androgens is associated with lower fat mass and higher fat free mass or with lower BMI (Eklund et al. 2017; Kumru et al. 2005). Hence, as the Enea et al. (2011) note in their review article, the literature is still inconclusive how endogenous androgens change in response to endurance training or physical activity in recreationally active females with normal BMI. It also raises a question, whether endogenous androgen monitoring is even meaningful for healthy recreationally active females with normal BMI. These observations emphasize that the topic needs to more research.

One limitation of the study was that ACTH was not measured. It is proposed that prolonged exercise might inhibit the adrenal sensitivity to ACTH secretion (Duclos et al. 2001) which could reduce the ACTH secretion that might decrease the endogenous androgen concentration in response to endurance training (Keizer et al. 1987). As the adrenal production of androgens is strongly regulated by ACTH (Rege et al. 2013; Enea et al. 2011), change in ACTH might influence on the endogenous androgens' caused by the training intervention. Albeit some studies have observed positive correlations between androgen and fat mass changes, it seems that the causality of the androgen reduction is still unknown.

8.3 Methodological considerations

The current literature is still inconsistent considering the impact of the menstrual cycle on training adaptations, and the impact of physical activity on endogenous androgens´ in naturally menstruating females. Some studies have observed variation in physiological variables across the menstrual cycle, but not in subjective perceived rate of exhaustion, or at actual performance time (Rael et al. 2021; Thompson et al. 2012; Ooshuyse et al. 2005). Similar forms of exercise seem to increase, decrease, or have no effect on endogenous androgens depending on the population (Enea et al. 2011). Major reasons for inconsistent results might be the varying methods and small number of participants in the literature and lack of individual consideration in the studies.

When studying the impact of the menstrual cycle on various factors, one methodological issue considers how the menstrual cycle is determined. The current literature consists of many

different methods used to detect the menstrual cycle, some being more suitable and accurate than others. Non-invasive methods, like calendar-based counting alone, might fail to detect the shift from FP to LP. Consequently, the most reliable methods are combination of different methods, like calendar-based counting combined with ovulation tests and the blood samples which detect the actual number of hormones in circulation (de Jonge et al. 2019). In the present study, the menstrual cycle was determined with this three-step method consisting of calendar-based counting, ovulation tests, and blood samples, which is recommended by de Jonge et al. (2019) and Schaumberg et al. (2017) and should be adapted in the research investigating female reproductive system. According to Verdonk et al. (2019) the estradiol reference values at mid LP are 151-1941 pmol/L. For progesterone, it is recommended to reach the limit of 16 nmol/L at the LP (Thompson et al. 2012; de Jonge et al. 2019) In the present study, the average estradiol values were at the reference intervals and progesterone avarage values were over 16 nmol/L, so the menstrual cycle phase was monitored accurately. It is important to acknowledge, that even if the menstrual cycle was detected with a strong probability in the present study, the comparison to other studies is not trustworthy if the methodology in the other studies is not reliable.

In addition to determination of the menstrual cycle, individuality should be taken into account when studying the impact of the menstrual cycles on physical performance. One study has observed that even 42.5% of naturally menstruating healthy females experience intracycle variability as great as 7 days (Fehring et al. 2006). Besides the variation of the length of menstrual cycle, it is not uncommon that a naturally menstruating woman faces anovulatory menstrual cycle or luteal phase deficiency (LPD) cycle. In this case, the concentration of progesterone will not peak at the luteal phase. (Thompson et al. 2012; de Jonge et al. 2019) In addition to physiological variation between and within individuals, it is common that subjective perceptions of menstrual cycles impact on physical performance might influence on actual performance. This is supported in a study by de Carvalho et al. (2023) who observed no differences in endurance performance between menstrual cycle phases, but in a subgroup of participants, who perceived the interference of the menstrual cycle on physical performance, had a decrease in performance variables.

Moreover, low energy availability or HPA-axis disruptions, such as endometriosis or PCOS might impact on the fluctuation of reproductive hormones and androgens (Elliott-Sale et al. 2021; Enea et al. 2011). For example, PCOS causes a decline in SHBG causing increases in

bioavailability of androgens (Dumitrescu et al. 2015). In the present study, the subjects were required to be healthy without any chronical illnesses, and if they had PCOS, they were excluded, or symptoms related to it, a doctor evaluated whether they were able to participate. In the future studies, similar approach should be used that the homogeneity of the participants can be assured. As in studies considering menstrual cycle, the discrepancies in the studies focusing on androgens and physical activity in females occur due to non-homogenic subject populations and differing experimental designs (Enea et al. 2011).

Probably the main methodological issue considering the studies focusing on menstrual cycle and physical performance is a small number of participants, which is also an evident issue in the present study. For the final VO_2max analysis LP group had 7 participants, control group 5 participants, and the FP group had 4 participants. Also, for the final A4, SHBG, DHEA-S, and tT analyses there was only 5 participants whose androgen measurements were done successfully, because some androgens were not able to include in this study due to scheduling constraints. In the beginning of the intervention the number of the participants was 36. This was the recommended number of participants calculated with Faul's et al. (2009) power analysis with a level of statistical power of 80%. The number of participants decreased during the intervention due to illnesses and other reasons, or they were not able to participate in all measurement timepoints so they could not be included for the final analysis. As the training intervention was long, 5-6 months, illnesses, and possible other reasons for drop-out were expected, and the study was conducted in the middle of the global COVID-19 pandemic, which probably impacts on the overall health conditions. However, the small number of the participants might decrease the reliability of the findings and between group comparisons, because individual variance may impact on the results too much. The small number of the participants has been an issue in previous menstrual cycle studies as well (McNulty et al. 2020).

These observations about individual variability and methods determining the menstrual cycle and androgens in females claim the need for more uniformly recruited participants and more specific inclusion and exclusion criteria to get more comparable results and studies in the future. Consequently, individuality should be in the spotlight when studying menstrual cycles impact on physical performance. (de Jonge et al. 2019; Elliott-Sale et al. 2021)

8.4 Strengths and limitations of the study

One major strength of the study was that it was one of the first to study impacts of menstrual mediated endurance training and of HIIT on endogenous androgens in naturally menstruating recreationally exercising females with normal BMI. The study had homogeneous participants at baseline VO_2max, BMI, fat mass, and endogenous androgens which increases the reliability of the study. The inclusion criteria were strict, and during the recruitment, a doctor did further assessment if anything which could present the inclusion rose from the health questionnaire. Also, the methodological strength was that menstrual cycle phases and ovulation were determined using a reliable method.

One limitation of the study was the small number of the participants, which is the major constrain of the study. The dropout rate was high and some of the participants were not able to take part to all measurement points due to illnesses etc. so they were excluded from the final analysis. Also, even though the method to determine menstrual cycle was pretty reliable, in few LP measurement points the blood samples were collected at the point when progesterone was not peaking yet (<16 nmol/l) which might impact on the measured variables. Finally, there was a missing data for training logs. As the amount of the trainings or the intensities of the trainings cannot be monitored, the VO_2max and changes in androgens needs to be interpreted carefully.

8.5 Conclusions

Based on this study, there are no significant differences in endurance performance improvement in recreationally active naturally menstruating females whether the training is periodized traditionally and continuously or mediated by the menstrual cycle. However, when interpreting the within group results, it appears that traditionally and continuously periodized endurance training might be more effective to improve VO_2max, and the LP emphasized MIET training might provoke better improvements in VO_2max, but more future studies are needed around this topic. Also, HIIT training can induce significant reductions in freeT but not in other androgens or in fat mass variables in recreationally active naturally menstruating females. Concurrent reductions in endogenous androgens and fat mass variables in recreationally active naturally menstruating females are not observed in this population.

8.6 Practical applications

Each individual is different when considering the impact of the menstrual cycle on the physical performance. Hence, formatting general guidelines is difficult and probably impractical. For coaches and athletes who are considering periodizing training according to menstrual cycle, a personalized approach should be taken into account. In the future studies considering menstrual cycle and endogenous androgens, more uniform participants and specific inclusion and exclusion criteria to get more comparable studies are needed.

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APPENDIX 1. OVULATION TEST – CLEARBLUE

ENGLISH

Clearblue

Ovulation Test

ADVANCED DIGITAL

Peak Fertility is displayed for 48 hours

Test using first urine after longest sleep

Never hold test with tip pointing upwards

For self-testing. Read this leaflet carefully before testing

1 When to test

You need to know the length of your cycle to know which day you should start testing. Your cycle length is the total number of days from Day 1 (first day of full bleeding) up to and including the day before your next period starts.

Cycle length (days):	21 or less	22	23	24	25	26	27	28	29	30	31	32	33	34	35	36	37	38	39	40	41 or more
Start testing on cycle day:	5	6	6	6	7	7	7	8	9	10	11	12	13	14	15	16	17	18	19	20	20 days before you expect your next period

If your cycles vary, use the shortest over the last 6.
If you don't know how long your cycle is, wait one cycle and note the length of this.

2 Prepare the test

a Take test stick out of foil and **remove cap**.

b Insert test stick into the holder before testing. Line up arrows.

c Before doing the test wait for the test ready symbol to appear.

New cycle symbols will flash briefly the first time you use the test.
See 'Other symbols'.

3 Choose your testing method

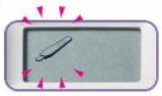
Place the absorbent tip:

in your urine stream

or

in a collected urine sample

4 Wait



Replace the cap and lay the test flat.
Do not eject the test stick.

Within **1 minute** the test ready symbol will flash.

After 5 minutes the display will show your result.

5 Results

Low Fertility: small chance of getting pregnant
Test again tomorrow. Low Fertility results are displayed for 8 minutes. If you miss seeing your result, eject the test stick and it will appear for another 2 minutes.

High Fertility: have intercourse for increased chance of getting pregnant
Test again tomorrow. High Fertility is displayed for 8 minutes and will be displayed after each test until Peak Fertility. If you miss seeing your result, eject the test stick and it will appear for another 2 minutes. High Fertility smiley face **flashes** on the display.

Peak Fertility: have intercourse for the best chance of getting pregnant
Stop testing. Peak Fertility is displayed constantly for 48 hours.
You don't need to test again during this cycle. If you try, the holder will not be able to read the test.

Your cycle and hormone pattern is unique. The number of fertile days you see and when you see them each cycle is personal to you so it's not unusual to see different results each cycle. See 'Questions & answers'.

6 Eject the test stick

Throw the test stick away with normal household waste.
When you are ready to test again follow the instructions using the holder and a new test stick.

Symbols

Error symbols

If something has gone wrong during testing or with the holder a symbol will be displayed within 10 minutes.

A 

Re-insert the test stick quickly – it was ejected too soon.

B 

Test again with a new test stick. If you have collected urine use this once the display is blank. Otherwise test in your urine stream but drink normally and test again in 4 hours.
When testing again remember the following:
- The test stick must be inserted into the holder before you test. Check step 2.
- Don't use too much or too little urine. Check step 3.
- Keep the test pointing downwards or flat after sampling.
- Don't eject the test stick too soon. Check step 4.

C 

You cannot use this holder again.

D 

- If the display remains blank when you insert the test stick try ejecting and re-inserting it.
- If it's inserted correctly and the display is blank the holder is no longer working.
- If the display is blank after testing the holder may have gotten too wet.
- If your holder is no longer working you can use any unused test sticks but you will need a new holder.

Other symbols

E 

This appears when you use a test stick for the first time.
It also appears if you are testing after you have seen Peak Fertility, or if you have not done a test for 3 or more days in a row.

Tips for trying to get pregnant

It's a good idea to consider a few changes before you start trying for a baby and during pregnancy, for example it's best to avoid smoking, recreational drugs and alcohol. Start taking folic acid if you're planning to get pregnant and then continue for the first 12 weeks of your pregnancy. Stay fit and maintain a healthy weight, and don't forget to involve your partner. Becoming parents is an exciting time for you both.

For more information on Clearblue Advanced Ovulation Test and top tips on getting pregnant visit www.clearblue.com.

Questions & answers

1 How can Clearblue Advanced Ovulation Test help you get pregnant?
There are only a few days each cycle when you can get pregnant – the day of ovulation and the days leading up to it. Knowing your fertile days can help you get pregnant faster. Clearblue Advanced Ovulation Test detects your fertile days by tracking 2 key fertility hormones – estrogen and luteinizing hormone (LH). When a rise in estrogen is detected you have reached High Fertility, and when a surge in LH is detected you have reached Peak Fertility. Having intercourse on High and Peak Fertility days maximizes your chances of getting pregnant. Clearblue Advanced Ovulation Test is over 99% accurate at detecting the LH surge.

2 What if you don't see High Fertility?
If you don't see any High Fertility days or you see fewer than expected, it may be that in this cycle your estrogen level is not high enough to be detected. Alternatively, it may be your hormone changes occur close together or you started testing too late. Even if you don't see High Fertility you may still see Peak Fertility.

3 What if you don't see Peak Fertility in a cycle?
If you don't see Peak Fertility, it may be that in this cycle your LH surge was too low to be detected or you may have missed a test around your LH surge. It is also possible that you have not ovulated this cycle. This isn't unusual but we would recommend you see your doctor if this happens for 3 cycles in a row.

4 What if you started testing on the right day and saw 9 or more High Fertility days?
You may wish to stop testing this cycle.

Further information

Medical conditions

You may get misleading results if you are pregnant, have recently been pregnant, have reached menopause, have impaired liver or kidney function, have polycystic ovarian syndrome (PCOS).

Medication

Always read the manufacturer's instructions for any medication you are taking before testing.

You may get misleading results if you are taking fertility drugs containing luteinizing hormone (LH) or human Chorionic Gonadotrophin, or are taking antibiotics containing tetracyclines. Some fertility treatments, such as clomiphene citrate, may give misleading High Fertility results but Peak Fertility results should not be affected.

If you have recently stopped using hormonal contraception your cycles may be irregular so you may wish to wait until you have had 2 cycles before testing.

Checking with your doctor

As prenatal care is very important for a baby's health, we recommend that you consult your doctor before you try to conceive.

Check with your doctor if you are taking any medication or have any medical condition before planning a pregnancy.
If you have a medically diagnosed fertility problem, ask your doctor if this test is suitable for you and if you do get unexpected results discuss them with your doctor.

If you are under 35 years and haven't become pregnant, we recommend you see your doctor after 12 months.
If you're over 35 years see your doctor if you haven't become pregnant after 6 months, and straight away if you are over 40.

Disposing of your holder

When you have finished using the holder, separate the upper and lower halves starting at the end nearest the display. Remove the batteries from under the central metal cover and dispose of them according to the appropriate recycling protocol. **Caution:** Do not take apart, recharge, or dispose of the batteries in fire. Do not swallow. Keep away from children. Dispose of the rest of the holder according to the appropriate recycling protocol for electrical equipment. Do not dispose of electrical equipment in fire.

Please note test sticks are not available separately.

www.clearblue.com
Clearblue Helpline
Monday-Friday 8:30 a.m. - 5:00 p.m. Eastern Time.
Toll-free. **1-800-321-3279**
Please have the product and packaging, including any foil pouches, with you when you call. Calls are recorded for training and quality control.

The sensitivity of the LH detection in Clearblue Advanced Digital Ovulation Test is 40mIU/ml measured against the Third International Standard for urinary LH and FSH for Bioassay (71/264).

Holder made in China. Packaged in the U.S.A.
Distributed by Procter & Gamble, Cincinnati, OH 45202.

Home ovulation test. For self-testing at home. For *in vitro* diagnostic use only. Not for internal use. Do not reuse test sticks. Keep out of reach of children. Store at 36° - 86°F (2°-30°C). Bring to room temperature for 30 minutes if refrigerated. Do not use if the foil wrapper containing the test stick is damaged. Do not use a test stick after the expiration date.

Only use test sticks for Clearblue Advanced Digital Ovulation Test with the holder. Not for contraceptive use.

Results must be read on the display and not by any lines you might see on the test stick.

This device complies with part 15 of the FCC Rules. Operation is subject to the following two conditions: (1) This device may not cause harmful interference, and (2) this device must accept any interference received, including interference that may cause undesired operation.

The EMC countermeasures employed within the electronic instrument will provide reasonable protection against electromagnetic interference effects likely to be encountered in the home environment. Do not use this instrument in close proximity to sources of strong electromagnetic radiation (e.g. mobile phones), as these may interfere with the proper operation.

Changes or modifications not expressly approved by the manufacturer will void the authority to operate this equipment.

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