SEX DIFFERENCES, HORMONAL CONTRACEPTIVES, AND MENSTRUAL CYCLE EFFECTS ON EXERCISE METABOLIC FLEXIBILITY

by

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(UNDER THE DIRECTION OF NATHAN JENKINS)

ABSTRACT

Metabolic flexibility is the body's ability to switch between fat and carbohydrates in response to exercise or eating. Historically this has been researched in response to meal challenges. However, exercise is an equally robust stressor on the body, which challenges underlying metabolic processes both at the muscular and whole body level. Exercise metabolism has been frequently assessed during steady state or ramped intensity exercise. High intensity interval exercise may, however, act as a test of exercise metabolic flexibility. Additionally, metabolism may be impacted by hormonal shifts, such as those seen in the menstrual cycle. The menstrual cycle can be separated in the low hormone follicular phase (days 1-14), and the high hormone luteal phases (days 16-28) separated by a ovulation, a one day estrogen surge (~day 15). These changes in hormones may impact exercise performance and how the body uses fats and carbohydrates. Estrogens positively influence metabolism in women by improving fat metabolism and performance in the first half of the menstrual cycle. However,

these effects may be negatively impacted by high progesterone in the luteal phase or by hormonal oral contraceptive use. There also appears to be a need for additional research on sex differences in metabolism, as well as on the impact of hormonal contraceptives across on metabolic flexibility and its underlying mechanisms. The proposed project aims to draw connections between postprandial and exercise metabolism, and to investigate the impacts of sex and mitochondrial capacity on metabolic flexibility.

INDEX WORDS: METABOLIC FLEXIBLITY. FAT OXIDATION. CARBOHYDRATE OXIDATION. SUBSTRATE METABOLISM. SEX DIFFERENCES. HIGH INTESNITY INTERVAL TRAINING. EXERCISE METABOLISM. POSTPRANDIAL METABOLISM.

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DEDICATION

To the girls that do not see themselves in sports.

To the girls that do not see themselves in science.

To the girls that do not see themselves in the research.

To my 13-year-old self who first fell in love with sports.

To the 19-year-old me who then fell in love with science.

And to the me today who fell in love with research.

We are smarter than we know.

We are more capable than we know.

We can make a more positive impact than we know.

This is for you.

This is for all of us.

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“Something so hard can be so easy if you just have a little help. In the right place, under the right conditions, you can finally stretch out into what you’re supposed to be.” – Hope Jaren, Lab Girl

To my family for their never-ending support and love during this long journey. To my friends for always supporting me and my big goals and for loving me through every “I’m sorry I can’t” over the last 11 years. For Dr. Nathan Jenkins for his mentorship, but most of all – friendship. To all my mentors who supported me, believed in me and showed me this path was possible.

To the mountains and the miles who continually showed me I could do hard things, lean in, and for the head clearing hours that turned into my best ideas.

To Regis. I love you. I couldn’t have done any of this without you. You are the best lab mate and partner I could have asked for on this journey.

Most of all. To me.

We did it.

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CHAPTER 1

INTRODUCTION

Human metabolism is an integrated communication process between organ systems such as the liver, adipose, pancreas, and muscle tissue and may be impacted by hormonal shifts [1, 2]. Together, these systems regulate nutrient partitioning and fuel metabolism in response to eating and physical activity [1, 2]. Metabolic flexibility is traditionally examined during the transition between fasted and feeding, which requires a cellular cascade dependent on endocrine and mitochondrial shifts [1] and is commonly assessed via meal challenges to elicit metabolic stress on the body. However, another robust test of metabolic switching is the onset of exercise, requiring appropriate shifting of skeletal muscle metabolic pathways and mitochondria to support activity demands [1, 3]. As has previously been established, type I muscle fibers have a robust ability to oxidize lipid and favor oxidative metabolism due to increased mitochondrial density and lipid storage and are well-suited for low-intensity work, with type II fiber supporting rapid ATP generation via phosphocreatine and glycolytic energy pathways [1]. However, substrate overload of the oxidative pathways overfeeding, or metabolic demand high-intensity exercise is only sustainable for a short period. Overtime increased rate of glucose demands will begin to exceed the oxidative system's

capacity, leading to cellular inhibition of fatty-acid pathways [1]. During high-intensity interval exercise (HIIT), an individual's capacity for metabolic shifting from glycolytic to oxidative energy supply systems during recovery periods between intervals may provide an alternative assessment of metabolic flexibility compared to the standard approach using meal challenges. Furthermore, muscle capacity may play an essential role in one's capacity to do so.

The mechanisms contributing to increased fat oxidation and metabolic flexibility during exercise are involved in regulating body mass, metabolic health, and exercise performance [4]. It has also been established that aerobic fitness status plays a significant role in metabolic flexibility during feeding [2, 5-8] and fat oxidation during HIIT [9, 10], with higher fitness status being related to increased fat oxidation capacity. Furthermore, women appear to have a higher capacity to oxidize fat during exercise [11]. However, most previous literature assessing metabolic differences in sex is done during steady-state or often submaximal exercise intensities $\leq$75%VO_{2max} [12-14]. With recent work showing differences in fat oxidation during HIIT according to fitness status [9, 10], it seems possible that HIIT could also detect differences in fuel utilization between sexes and if these previously observed differences are still present during high-intensity exercise.

In the first study of this dissertation project, we investigated the assessment of metabolic flexibility during a high-fat meal and HIIT and the difference in fitness status and sex differences play within this. The results revealed for the first time that the capacity to oxidize fat during HIIT and in response to a high-fat meal challenge is related. Furthermore, our HIIT protocol was more sensitive than the

high-fat meal challenge in detecting metabolic flux between fitness status groups and carbohydrate metabolism between sex. While there were no sex differences in HIIT or high high-fat challenge lipid oxidation, our data suggest that low fitness status may be more detrimental to metabolic flexibility in women than men. Overall, our first study in this area suggests that substrate oxidation during HIIT may be a novel and sensitive test of metabolic flexibility. Furthermore, investigating sex differences and in women may warrant further research. These differences in substrate metabolism between sex or fitness status may also oscillate across the menstrual cycle, where no current studies have investigated differences in exercise substrate utilization during HIIT. The second study in the proposed dissertation will address this knowledge gap.

The menstrual cycle comprises the low hormone follicular phase (days 1-14) and the high hormone luteal phases (days 16-28) separated by ovulation, a one-day estrogen surge (~day 15) [15, 16]. Shifts in ovarian hormones can influence the cardiovascular system, aerobic capacity, thermoregulation, and metabolism [15, 16]. Estrogens appear to positively influence substrate metabolism, including enhanced muscle glycogen storage and fatty-acid availability and oxidation during exercise [17, 18]. These estrogen-associated impacts on metabolism lead to reduced reliance on anaerobic metabolism, lower carbohydrate reliance, sparing glycogen depletion, and lower blood lactate levels [4, 15, 19]. However, due to the cyclic nature of the menstrual phases, women spend half of their cycle in a high hormone estrogen and high progesterone dominant state. Progesterone has been shown to have anti-estrogen effects such

as insulin resistance, decreased glycogen storage, glucose availability, and impaired fat metabolism [19]. Historically women have been underrepresented in exercise metabolism research due to the difficulty in controlling for hormone fluctuations of the menstrual cycle [15]. These fluctuations in hormones and their impacts on metabolism could lead to downstream enhancements or impairments in metabolic flexibility.

Furthermore, these processes may be altered by hormonal oral contraceptive use. Hormonal contraceptives fall into one of two categories 1) combined estrogen and progestin pill or patch or 2) a progestin-only pill, intrauterine device, or implant, all of which work to suppress the regular ovarian release of estradiol and progression across the menstrual cycle by replacing it with synthetic hormonal agonist [15]. In oral contraceptive pills, one of the most common forms of birth control is a 21-day hormone pill phase, followed by a 7-day placebo or withal phase (sugar pills) to mimic the low hormone menstrual cycle phase and suppress ovulation. This suppression of ovarian hormones by oral hormonal contraceptives may impact exercise metabolic flexibility [20].

Metabolic flexibility has historically been assessed in response to high-fat meal challenges or similar. However, as established, exercise is also a robust metabolic stressor and may be an equally demanding test of our metabolism, specifically, the use of HIIT as a measure of metabolic switching during exercise. Furthermore, fitness status and sex may impact substrate oxidation during exercise, specifically HIIT. Our first study set out to investigate the relationship between HIIT and a high-fat meal challenge and differences between fitness status

and sex during this, revealing HIIT as a more sensitive measure of exercise substrate metabolism. From our data, we propose that this protocol can be used as a novel assessment of metabolic flexibility, and offers unique advantages over steady-state substrate protocols and high-fat meal challenges primarily used in the literature.

Furthermore, traditionally, studies on sex differences in exercise metabolism, such as our first study, are carried out with female subjects in the follicular phase, particularly days 2-9 of menses, when female hormones are the lowest and most similar to their male counterparts [15]. However, there is a need to investigate cycle variation, and sex differences should be assessed with female subjects tested in both phases. The impact of hormonal contraceptives on exercise metabolism also needs to be assessed. There has been especially little research looking at females' ability to fuel metabolism during high-intensity interval exercise (HIIT) and recovery between bouts across the menstrual cycle phases. The proposed project will build on our first project's finding on HIIT as a measure of metabolic flexibility, and address these current gaps in the literature on sex differences and the impacts of menstrual cycle hormonal variation on exercise metabolism.

Specific Aims:

Specific Aim 1 was to determine if there was a relationship between high-fat meal challenges and high-intensity interval training and how this differs by fitness status and sex. Hypothesis 1a is that ability to oxidize fat during a high-fat meal challenge

and HIIT will be related. Hypothesis 1b is that substrate metabolism will be impacted by fitness status and gender. Results confirm hypotheses 1a and 1b and support our that fat oxidation in response to a high-fat meal and HIIT is related. Furthermore, fitness status and sex play a role in exercise metabolic flexibility, enhancing the current literature on metabolic flexibility and the role of fitness status and sex on exercise substrate oxidation.

Specific Aim 2 is to determine if exercise metabolic flexibility varies across the menstrual cycle in women and is affected by contraceptive use and fitness status. Hypothesis 2a is that higher fit females will have greater metabolic flexibility indicated by increased fat oxidation and decreased lactate accumulation and glucose reliance during HIIT. Hypothesis 2b is that women will have grater exercise metabolic flexibility during the follicular phase and impaired metabolic flexibility during the luteal phase. Hypothesis 2c is that metabolic flexibility will be negatively impacted by hormonal contraceptive use. Results confirming Hypotheses 2a and 2b will support our overall hypothesis that fitness status and ovarian hormones play a role in exercise metabolic flexibility and will enhance the current literature on the impacts of the menstrual cycle and hormonal contraceptives on exercise metabolism.

CHAPTER 2

Literature Review

Substrate Metabolism

Metabolic Flexibility and Glucose Fatty-Acid Cycle

Glucose, lipids, and amino acids and their storage forms are the primary fuel sources that drive and are used in metabolism [21]. Competition between metabolic substrates and one another's inhibition was demonstrated in vivo and in vitro levels [21]. The reciprocal inhibition of dietary substrates in human tissue and metabolism was initially classified in 1963 within rat heart and diaphragm as the Randle Cycle, otherwise known as the Glucose fatty-acid cycle [21]. This cycle described the competition between glucose and fatty-acids for oxidation within the body, capturing the impacts of high or low glucagon/insulin states on fatty-acid and carbohydrate oxidation. The discovery of the Randle cycle led to the thought that increase fatty-acid oxidation led to the development of insulin resistance, diabetes, and obesity in humans [22]. It was suggested that increased fatty-acid oxidation resulted in a rise of acetyl-CoA, NADH/NAD+, inhibiting PDH, causing a rise in extracellular citrate, inhibiting glucose-6-phosphate, and hexokinase, and decreasing glucose uptake and inhibiting glucose oxidation [23]. The inverse of this occurring during a low carbohydrate state, preserving glucose supply and increasing fatty acid oxidation[24]. This relationship between carbs and fats was

shown to act independently of other endocrine signals or metabolic pathways on one another and appeared to be entirely dependent on nutrient availability and sensing [21].

For years investigators tried to replicate the glucose fatty-acid cycle to classify the extent to which this may exist within the body, liver, and muscle of humans and specifically, in the context of insulin resistance, diabetes, and obesity [22]. Felber and Vannotti, among the earliest, assessed response to a glucose tolerance test with and without fatty-acid infusion [25], finding that infusion of fatty-acids decreased glucose tolerance, despite an elevated insulin response compared to glucose alone. Felber and Vannotti thus proposing that the presence of elevated blood fatty-acids along with glucose resulted in impaired glucose tolerance [25]. Furthermore, this occurred despite adequate insulin, resulting in an increased reliance on fatty-acids and impaired glycolytic flux [25]. Other earlier work using a euglycemic-hyperinsulinemic clamp in the thigh or forearm muscle further clarifies this relationship, finding that there appears to be an inverse relationship between glucose and fatty-acids; it was not as straightforward in humans as previous in vivo data suggested [26, 27]. These data together revealing that while the Randall cycle may predict an increase in glucose uptake following fatty-acid infusion, insulin stimulation in the presence of plasma blood fatty-acids inhibits this effect. Additionally that while insulin impact lipid oxidation, that fatty-acids effect on glucose metabolism may be more dependent on the timing of exposure [23, 28-31]. Furthermore, the glucose fatty-acid cycle proposed that fatty-acids inhibit glucose metabolism by inhibition of PDH; however, studies

suggested that FFAs may directly inhibit together glucose transport, oxidization, and glycogen synthesis, resulting in insulin resistance [23]. These effects glucose being linearly related to the plasma level of FFAs [32] and the use of whole-body respiratory exchange ratio (RER) measurements alongside clamp data show similar suppression of glucose metabolism during an intralipid infusion [33].

Skeletal muscle's role in regulating metabolism, glucose disposal, and insulin resistance was then investigated in a series of studies by Kelley and colleagues. Kelley and Mandarino set out to investigate the mechanism responsible for skeletal muscle insulin resistance in diabetic and healthy controls [34], asking the question: does impaired muscle glucose storage and oxidation occur independently of defects in glucose uptake? Kelly's seminal studies revealed that diabetic individuals had increased basal muscle glucose oxidation, despite elevated plasma FFA levels. Furthermore, this study captured a lower basal leg respiratory quotient (RQ) and insulin-stimulation increase in RQ in the control group, paralleling a significant decrease in basal leg lipid oxidation. Lastly, during hyperglycemia, when glucose uptake and oxidation in diabetic subjects were normalized, glycogen synthesis was normal [34].

Building on nearly three decades of research, the concept of metabolic flexibly emerged in two papers. First, Kelley et al. set out to investigate the mechanism by which plasma FFA metabolism inhibits intracellular insulin mediated glucose metabolism in the skeletal muscle of healthy subjects [35]. This first study finding that FFA infusion suppressed muscle glucose uptake, oxygen consumption, local RQ, and pyruvate dehydrogenase (PDH) activity, displaying a

competition between glucose and FFA in human skeletal muscle. Then, Kelley et al., in a follow-up experiment and foundational paper, assessed skeletal muscle FFA uptake and oxidation of the vastus lateralis of both lean and obese individuals using isotope tracer in fasted and insulin-stimulated states [36]. Obese participants then engaged in a 4-month weight loss intervention. These data were able to capture two critical findings. First, fasting fatty-acid oxidation and storage and insulin stimulate substrate flux towards glucose metabolism were blunted in the skeletal muscle of obese individuals, indicated by similar FFA uptake. However, lower oxidation and increased storage in obese compared to lean participants and blunted insulin-stimulated shift of RER towards carbohydrate oxidation. Second, following weight loss in obese participants, fasting and insulin-stimulated RQ and fatty-acid oxidation were improved. Indicating improved insulin clearance and more significant fatty-acid suppression in the insulin-stimulated state, similar to that of lean subjects, thus restoring metabolic flexibility within skeletal muscle [36]. Glucose and fatty-acids did have a reciprocal relationship in human skeletal muscle. However, insulin, adiposity, and later fitness status were all factors that needed to be accounted for when assessing skeletal muscle and whole-body metabolism.

Since the work of Kelley et al. [36], the concept of metabolic flexibility has gained popularity as a measure of metabolic health due to its tight relationship with glucose and lipid homeostasis. Metabolic flexibility was further defined as responding to physiological availability or demand by appropriately shuttling, storing, and utilizing dietary carbohydrate, lipid, and protein [1, 3]. The inverse,

metabolic inflexibility, displaying components of metabolic syndrome which is captured by and has been further defined as a 1) failure of skeletal muscle to flux between fatty-acid during fasting to carbohydrate in an insulin-stimulated state 2) impaired transition between fatty-acid use and storage following feeding and 3) a reduced capacity for substrate use being reflected by low mitochondrial density, size, and capacity [37]. With mitochondria being one of the major regulatory mechanisms that control the flux of carbons from the metabolic breakdown of glucose, fatty acids, and BCAA's at the cellular level to be oxidized into ATP to support energy production [3]. An overload of this process represents metabolic inflexibility, back pressure on the mitochondria, impaired lipid oxidation, and a predictor and symptom of metabolic diseases such as type two diabetes and a predictor of long term weight gain [5, 38-40].

While this can be assessed via indirect calorimetry at the whole-body level via whole-body RER, it is the regulation of these macronutrients at the cellular level of mitochondria that ultimately control oxidation via the sensing of Acetyl-CoA, NADH, FADH, and ATP production and energy metabolism. Therefore, we can address metabolic flexibility as the mitochondrial ability to shift substrates freely based on physiological cues through the appropriate upregulation or inhibition of specific enzymes at the mitochondrial level such as PDH, carnitine palmitoyltransferase-1 (CPT1), and Branch Chain Ketoacid Dehydrogenase (BCKD) [1, 3, 41]. Metabolic inflexibility can be classified as an overload of the carbons products of carbohydrate fat and protein competing for oxidation. Metabolic inflexibility is detected during chronic overfeeding when these pathways

become dysregulated, leading to poor glucose uptake and oxidation and impaired fatty-acid clearance due to the blunting full switching.

Exercise Metabolism

Early exercise physiologists began to find a clear relationship of metabolism in response to exercise and during exercise itself [42]. Exercise can be considered a robust stressor on the body's metabolic systems promoting or shifting metabolism away from lipid or glucose storage toward energy production [42]. Furthermore, while this can be looked at during acute exercise on metabolism, the long term effect of physical activity and fitness status also play an important role in modulating this via shifts in mitochondrial and skeletal muscle proteins, eliciting long term impacts on whole-body metabolism [42, 43]. Investigators began to understand the relationship between lipid and carbohydrate during exercise and how factors such as training status, sex, and intensity influenced those.

Early studies set out to mimic early studies on the Randal cycle and investigate how elevated FFA impacted glucose and exercise metabolism. First, Costill et al. assessed seven men undergoing 30 minutes of treadmill exercise at 70% VO_{2max} with heparin (lipid) infusion with and without prior glucose (75g) ingestion [44], revealing elevated plasma FFA and subsequent decrease muscle glycogen depletion under heparin infusion. Furthermore, following prior glucose consumption, noting increased plasma insulin, glucosemand carbohydrate oxidation even in the presence of elevated fatty-acids. Similarly, Ravussin et al. investigated five males during 2.5 hours of exercise at 44% VO_{2max} in the presence

of elevated FFA levels via intralipid infusion, with and without glucose ingestion [45]. Under heparin without glucose, higher FFA concentrations were present across exercise, and carbohydrate or lipid oxidation only different for the first 30 minutes. The ingestion of glucose did not significantly alter the contribution of carbohydrate or lipid oxidation to total energy expenditure. However, while the effect of glucose ingestion was not significant, it should be noted that lipid oxidation did decrease from 36±4 to 29±2% following carbohydrate ingestion [45]. Furthermore, Coyle at al. assessed six trained men undergoing a constant infusion of a long and medium-chain FFAs while cycling for 40 minutes at 50% VO_{2max} after both an overnight fast and after the ingestion of 1.4g/kg of glucose at 60 minutes and ten minutes before exercise [46], finding an elevation in insulin and blood glucose following ingestion of carbohydrate paralleled with a suppressed oxidation of fatty-acids. More specifically, the inhibition of long-chain fatty-acid oxidation. These studies together suggest carbohydrate feeding alone or in the presence of FFAs may impact exercise metabolism.

Furthermore, Wolfe et al. assessed FFA turnover and fat metabolism in five normal subjects during 4 hours of treadmill exercise at 40% VO_{2max} and 2 hours of recovery [47]. Wolf et al. finding FFA appearance, lipolysis, and fat oxidation increased across exercise, with recovery FFA and lipolysis rapidly dropping down but remaining higher than baseline. Lastly, a decline in fatty-acid reesterification during exercise accounted for over half the increase in fat oxidation during exercise [47]. Following this, Klein et al. investigated whole-body lipid kinetics via glycerol and FFA rate of appearance in five untrained and five endurance-trained men

during 4 hours of treadmill exercise at 20 ml/kg/min and 1 hour of recovery after [48]. Glycerol and FFA rate of appearance did not differ between groups. However, the trained subject's triglyceride oxidation was significantly higher, and post-exercise recovery kinetics were significantly faster, indicating oxidation of non-plasma oxidation sources likely plays a role in improved fat oxidation seen here [48]. Other work on seven men during 2 hours of cycling at 60% VO2peak before and after eleven weeks of endurance training (three days/week running and three days/week cycling) found similar effects. Following training, these men had decreased glucose oxidation and turnover and a lower RER, indicating significantly higher whole-body fat oxidation [49]. Lastly, Romijn et al. investigated basal whole-body lipid kinetics via the rate of appearance of glycerol and palmitate in untrained vs. endurance-trained individuals [50], reporting a 2-3x higher lipid turn over shifted towards FFA mobilization in trained individuals. Romijn et al. proposing greater basal lipid metabolism and availability during the onset of exercise as a possible mechanism behind elevated FOX during the onset of exercise. Together, these studies demonstrated that training status plays a role in increasing fat metabolism [47, 48] and possibly carbohydrate sparing during exercise [49].

A series of studies assessed fat and carbohydrate metabolism during cycling at 25%, 65,% and 85% VO_{2max} in five trained men [51] and eight women [52]. These studies found that plasma glucose uptake and glycogen oxidation increased linearly with exercise intensity, paralleling findings of Ravussin et al. [45], with slightly lower glucose metabolism at lower intensities in women. Contrastingly, whole-body fatty-acid rate of appearance gradually increased during

25% and 65% VO_{2max} but decreased during 85%. With fat oxidation highest at 65% VO_{2max} and no difference between 25% and 85% VO_{2max}, despite significantly lower FFA uptake and three-fold higher energy expenditure at 85% VO_{2max} [52]. Additionally, as measured by the glycerol rate of appearance, muscle lipolysis was only stimulated at 65 and 85% VO_{2max}. Similar results were observed in women aside from slightly lower glucose disappearance rates at low exercise intensities [52]. Furthermore, across 120 minutes of exercise at 65% VO_{2max}, there was a gradually greater reliance on muscle triglyceride and decrease glycogen utilization. These studies suggest that while carbohydrate metabolism was straightforward and linear during exercise of increasing intensity, lipid metabolism was not [51].

Building on their previous work, Romijn et al. assessed the role of plasma FFAs during exercise at 85% VO_{2max} in trained six cyclists with and without a fatty-acid infusion [53]. Exercise fat oxidation was increased 27% by lipid infusion and was only partially restored compared to rates of fat oxidation at 65% VO_{2max} in previous work by Romjin discussed above [51]. These findings suggest that the limits of fat oxidation at higher intensities are partially due to decreased fatty-acid mobilization rather than oxidative capacity alone [53]. Similarly, Martin et al. found a 41% increase in fat oxidation following eleven weeks of endurance training in thirteen males during 90-120 minutes of cycling and a shift in lipid utilization to non-plasma sources of FFA's [54]. To further investigate differences at higher intensity efforts, Klein et al. assessed glycerol rate of appearance in five trained and five untrained men during 60 minutes of cycle exercise at 70% VO2peak [55], finding whole-body glycerol rate of appearance and lipid response to exercise was

significantly greater in trained men across the full exercise session. All these data are together supporting that fitness status, exercise intensity, and duration all play roles in fatty-acid mobilization [54] and glucose oxidation during exercise [51, 56].

During this time, other work began to show that exercise training improved skeletal muscle mitochondrial content and enzymatic activity associated with fatty-acid metabolism, citrate cycle, and electron transport chain [57, 58], reflecting this enhanced metabolic efficiency to utilize fatty-acids at the same or similar levels than the untrained state [57]. Furthermore, that metabolic response to a given exercise bout was determined by intensity, fitness, nutrition, and environment. Lastly, studies such as Romijn et al. [53] were able to capture the limits of fatty-acid oxidation as possibly being due to FFA mobilization rather than oxidation capacity alone. More data began to emerge that suggested that limits of fat oxidation were 1) due to decreased fatty-acid transport into the mitochondria via CPT-1 which 2) may in part be suppressed by an increase in malonyl CoA by glucose and 3) skeletal muscle played a large role in this [58].

The Crossover Concept and Impact of Exercise Intensity on Metabolism

With these studies finding fat oxidation during exercise would increase, then drop after specific intensities, the foundational crossover concept was finally proposed by Brooks and Mercier [59]. The crossover concept thus capturing the preceding exercise metabolism research leading up to this time suggesting 1) lipid is the primary substrate for muscle and the body at rest; 2) flux between lipid and carbohydrate metabolism is primarily determined by exercise intensity; 3) glycogen

and glucose use increase exponentially, with lipid oxidation peaking then diminishing, with increasing intensity; 4) and endurance training and dietary intake impact substrate metabolism during exercise [60]. More simply, the power output of exercise in which energy dependence rapidly increases towards carbohydrates reliance parallels muscle tissue's increased reliance on glycolytic enzymes, glycolysis, fast glycolytic muscle fibers; with decreased mitochondrial fatty-acid uptake [59, 60]. Lastly, that over time, exercise training shifts this point to higher power outputs, compared to untrained, reflecting the increased efficiency of lipid oxidation in trained individuals, primarily due to improvements in mitochondrial mass, reticulum, and resulting increase in capacity oxidize lipids [59].

A series of studies in the Brooks lab assessed the effects of 9-12 weeks of cycle training (60 min, five times/week at 75% VO2peak) in healthy males on intramuscular lipid metabolism [61], lactate kinetics [62], glucose kinetics [63], the contribution of muscle to whole-body lipid metabolism [64] and fatty-acid turnover and oxidation in men [65] and women [66]. Participants were tested pre and post at their relative 45% and 65% VO2peak and post-training at the same absolute intensity of their 65% VO2peak baseline. This series of studies finding increased working muscle FFA uptake at 65%VO2 peak, but not higher RER or RQ values [61]. Furthermore, increased fatty-acid turnover but not oxidation following training in men [65] and training increase in fatty-acid kinetics and utilization in women [66]. Additionally, this series of studies found that muscle lipid oxidation represented ~62% of whole-body oxidation at 45% and absolute 65% VO2peak following training. However, muscle lipid oxidation dropped to ~30% of whole-body lipid

oxidation at higher intensities (relative 65% VO2peak) even after training [64], with carbohydrates still supplying the majority of muscle energy [64]. This is supported by data on glucose kinetics, which appear to increase following training [63], and blood glucose uptake increasing from 61% to 81% at the same relative intensity (65% VO2peak) and 38% at the same absolute pre-training intensity [63]. Moreover, training decreased muscle glucose uptake during moderate-intensity exercise and increased shunting during high-intensity exercise towards active tissue [63], suggesting at the muscular level preference of carbohydrate sources at higher intensities is prominent despite training [61, 64, 65] with training increasing women's fat oxidation at higher intensities than males [66].

Other data support these findings of lower glucose disappearance despite a similar rate of appearance in trained compared to untrained males during 30 minutes of cycling at 80% VO_{2max} [67]. These noted glucose kinetics likely a result of increased muscle PDH, hexokinase, and GLUT-4 [68] and as suggested in the crossover theory, blood glucose and muscle glycogen utilization increase with intensity regardless of training status [69]. Additionally, other investigations found that these changes in glucose and lipid metabolism were impacted by dietary status at lower to moderate exercise intensities [70] but at higher intensities ($\geq$60% VO2peak). Together these data implying carbohydrate oxidation remains the preferred substrate at higher intensities regardless of feeding or training status [70] and training decreases carbohydrate reliance at lower to moderate intensities but improves efficiency in carbohydrate metabolism at higher intensities [63, 67, 68].

While carbohydrate appears to be the favored substrate during moderate to high-intensity exercise, postexercise substrate oxidation during recovery was assessed in moderately trained healthy men and women after 89 minutes at 45% and 60 minutes at 65% VO2 peak, compared to a controlled trial [71]. These data finding postexercise RER values lower than both rest and control, suggesting a significant postexercise contribution to lipid oxidation and possible shifting of metabolism towards fat oxidation following exercise [71]. Other data looking at RER the day after high-intensity submaximal interval exercise (1min @ 80% max aerobic power output followed by 1 min recovery at 40% max aerobic power output), compared to 60 minutes continuous exercise at 45% VO_{2max}, vs. control found a decreased RER and a corresponding increase in lipid oxidation following both protocols [72]. These data reveal that while working muscle appears to prefer carbohydrates during high intensity exercise may, lipid oxidation seems to be favored in the recovery period.

Lactate Metabolism During Exercise

While fat and carbohydrate metabolism got much attention, blood lactate also became an important measure of metabolism. Lactate metabolism was investigated before and after 9-12 weeks of cycle training (60 min, five times/week at 75% VO2peak) by formerly mentioned Bergman et al. [61]. These data revealing similar lactate turnover after training at the same relative intensity (65% VO2peak), but an increase in leg lactate uptake and oxidation and decreased appearance and disappearance at the same absolute workload after training [61]. Dubouchaud et

al. performed an investigation following the same training protocol and found lactate dehydrogenase localization to the sarcolemma following training [73] and monocarboxylate transporter-1 (MCT1), a major lactate shuttle, increasing significantly after training in muscle and mitochondria [73]. This finding was further supported by other studies finding higher metabolic clearance at the same relative intensity between trained and untrained males [74] and increased lactate oxidation in trained individuals [75]. Together, these data suggest that endurance training decreases blood lactate concentrations by increasing lactate clearance at the same relative intensities [62, 73].

Furthermore, other evidence has shown that lactate is also important during post-meal carbohydrate metabolism and sustaining exercise, playing a role as an intermediate between glucose, glycogen storage, and energy production; lactate began to be viewed as a critical metabolic intermediate rather than a dead-end of metabolism [68, 76, 77]. From these studies, the lactate shuttle hypothesis emerged, suggesting that lactate forms as a product during both rest and exercise to shuttle from active tissue to the heart or liver via the Cori cycle to be metabolized into glucose or glycogen storage, or even metabolized directly in the muscle cell [68, 76, 77]. Lastly, this increase in lactate metabolism is tightly tied to mitochondrial increases seen with exercise training, favoring increased lactate clearance alongside improved aerobic respiration [77].

Since then, lactate has been suggested to be a “fulcrum of metabolism” [78] and, more recently, a marker of metabolic health [79, 80]. The role of lactate has

been defined as 1) a major energy source for mitochondrial respiration, 2) a major gluconeogenic precursor, and 3) a signaling molecule [78]. Lactate, acting as a metabolic intermediate between lipid and glucose oxidation and regulation of mitochondrial regulation. With new thinking about lactate viewing it more so as a balance between clearance and production, rather than production alone [79]. Moreover, lactate dynamics play a major role in both mitochondrial redox status and whole-body metabolism, capable of inhibiting lipolysis and mitochondrial transport proteins such as CPT via malonyl-CoA, inhibiting mitochondrial fatty-acid uptake [78]. These mitochondrial proteins all also being factors that are closely related to the mechanisms that regulate maximal fat oxidation during exercise and metabolic substrate switching [78, 81, 82]. Furthermore, with improved training status being shown to improve substrate use via mitochondrial biogenesis [78] and higher mitochondrial capacity being associated with improved metabolic flexibility [79] as mitochondria have been described as the “gatekeepers of fuel metabolism” [3, 79] and metabolic flexibility. This relationship of mitochondria and substrate oxidation suggests these organelles’ ability to flux to substrate demand lactate, and lipid and carbohydrate make sense as is a pivotal role in human metabolism.

It became evident that there was a robust relationship between exercise and metabolic flexibility. However, while many studies classified exercise metabolism, few had used exercise to classify metabolic health itself with exercise alone. San Millan and Brooks [83] set out to investigate lactate as a measure of metabolic flexibility during incremental cycling exercise in male professional athletes, moderately active and with metabolic syndrome. Understanding the role

of substrate metabolism and during exercise, metabolic flexibility, fitness status, and metabolic health, this study captured the original crossover concept proposed by Brooks two decades prior [59] with an inverse relationship between lactate and fat oxidation. Furthermore, higher fitness status was associated with sustaining higher power outputs, having superior lipid-derived fuel metabolism, and lower lactate accumulation [83]. Previous studies supported their findings in men with high and low insulin sensitivity, demonstrating more efficient exercise metabolic flexibility in the high insulin-sensitive group [84] and other data in 33 endurance athletes showing a relationship between elevated lactate accumulation and the point of maximal fat oxidation and lactate threshold and fat minimum [85]. These studies are together highlighting both a maximal inflection point of fat oxidation during exercise and its inverse relationship to exercise intensity and lactate accumulation [83]. Their data insinuating lactate may act as a non-invasive mechanism of assessing both metabolic flexibility during exercise and mitochondrial function [83].

Impacts of Exercise and Fitness Status on Metabolism

It also emerged that regular exercise performance improved metabolic capacity during exercise and postprandially as well [5, 43, 86]. Increased postprandial metabolic capacity was shown in work such as Batttaglia et al., who assessed fatty-acid oxidation in lean and obese subjects in skeletal muscle in response to a three-day high-fat diet before and after ten days aerobic exercise training [8]. Lean subjects increased fatty-acid oxidation, with obese showing no

changes in response to the high-fat day. However, ten days of aerobic training increased muscle fatty-acid oxidation in response to the high-fat diet in both groups, revealing recovered metabolic flexibility in the obese subjects [8]. Other studies revealed that two months of bed rest alone were enough to decrease fatty-acid transport and oxidative enzymes such as CD36, CPT1, and COX4, increase intramuscular lipid and impair metabolic flexibility in women and men [5, 6]. Furthermore, Hansen et al. investigated metabolism of ten women before and after transitioning into a four-day high-fat diet while following three different, incremental activity levels. Finding that higher activity levels were significantly associated with a compensatory decrease in RER and an increase in fat oxidation [87]. Other data compiled from bed rest and training studies revealed that higher physical activity levels were associated with superior metabolic flexibility as indicated by a higher flux of postprandial RER, and decreased insulin response was positively correlated with activity level [5].

Data revealing that larger fat oxidation rates improved metabolic disease symptoms, as well as exercise performance, led to lines of work assessing both exercise and nutritional strategies to maximize exercise fat oxidation [88, 89]. This data also revealed that fat oxidation increased with exercise duration [88, 89] and with intensity from 25% to 65% VO_{2max} but decreased by 85% [51]. Due to this, series of studies assessed when fat oxidation peaked and ended [90] by examining graded intensity vs. fat oxidation [88, 90, 91], finding maximal fat oxidation rates to be around ~63% of VO_{2max} in moderately and ~86% VO_{2max} in highly trained individuals [88, 91]. A fat max at ~63% VO_{2max} was also found in 33 endurance

athletes [85], with moderately trained individuals having lower absolute fat oxidation rates than highly trained individuals at the same relative intensity and considerable variation within fitness status [88, 91]. Differences may be due to total energy expenditure and impacted by more than training status itself [88, 91]. Furthermore, building on this, other work looking at 300 healthy men and women finding maximal fat oxidation to occur at ~48% VO_{2max}, with women having both greater total fat oxidation and maximal fat oxidation [92] with a large variance in maximal fat oxidation remaining unexplained [92]. Inversely, Peric et al. looked for the point of minimum fat oxidation in 30 male athletes and 17 non-athletes, finding it to occur at ~87% and ~85% VO_{2max}, respectively [93]. Confirming other data showing that trained individuals have a higher capacity to oxidize fat [10]. However, it is worth noting that the VO2 max difference between these groups was 56.17 ± 4.95 and 46.04 ± 3.25 ml/kg/min, with the non-athlete group still reflecting moderately trained individuals [93].

Many studies to this point have described fat oxidation changes at low, moderate, and high intensities [50-52, 59]; however, these were primarily done during steady-state or graded exercise intensities, with few capturing metabolic flux of exercise between high and low intervals [50-52, 59]. However, high-intensity interval training (HIIT) may be a robust metabolic stress on both whole-body substrate oxidation and muscle substrate flux, challenging the body to rapidly adapt to higher work bouts but giving time to recover between may also capture differences in exercise fat oxidation. HIIT style training has been shown to improve mitochondrial biogenesis, aerobic fitness stats, and fat oxidation [7, 94, 95].

However, a few studies have investigated the changes in substrate oxidation during high-intensity interval training (HIIT) [10, 96]. Hetlelid et al. examined substrate oxidation during HIIT consisting of 4-minute work bouts separated by 2 minutes of recovery in well-trained (VO_{2max}: 71±5 mL/min/kg) and recreationally (VO_{2max}: 55±5 mL/min/kg) trained males [10]. Work bouts were set at RPE ~18 between groups. Hetlelid et al. found that while RPE, blood lactate, and carbohydrate oxidation were the same, fat oxidation was significantly higher in the well-trained males, accounting for 33% of total energy expenditure, compared to just 16% in the well trained [10]. Furthermore, finding a strong significant correlation between VO_{2max} and fat oxidation rate during high-intensity intervals. Similarly, Aslankeser et al. [9] assessed substrate oxidation in 18 well trained (VO_{2max}: 60±8 mL/min/kg) and untrained males (VO_{2max}: 47±7 mL/min/kg) during HIIT of 4 minute hard bouts at ~80% VO_{2max}, with 2 minutes of recovery between bouts. Lastly, finding elevated fat oxidation in the highly trained group, with similar carbohydrate oxidation and fat oxidation, made up ~25% of total substrate oxidation in the trained group and just ~2% in the untrained. However, they found significantly greater lactate concentrations in the untrained group across the workout [9]. Together, these two studies reflect that fat oxidation can contribute significantly to substrate oxidation in highly trained individuals during short higher intensity work, with blunted substrate flux in untrained individuals [9].

NIRS and Muscle Oxygen Kinetics

Another novel line of work that has evolved in assessing muscle metabolism and mitochondrial capacity has the application of been near-infrared spectroscopy (NIRS) [97-100]. NIRS is assessed using a device that uses near-infrared spectrum light (700-850 nm) to evaluate muscle oxygenation and oxidative metabolism via light scattering and absorption in tissue [97]. This is accomplished through calculating relative changes in oxygenated, deoxygenated, and total hemoglobin and myoglobin using the beer-lambert law [97]. Since energy metabolism is driven by ATP synthesis, the demand for oxygen in the skeletal muscle (mVO2) during the onset exercise can increase upward of 50 fold above baseline [97]. Additionally, NIRS has been widely used also to test mitochondrial capacity non-invasively [99-101]. This is important since the strong reliance of skeletal muscle mitochondria and metabolism on oxidative pathways is reflective of exercise performance [97, 101] and clinical outcomes [102]. Lagerwaard et al. found post-exercise muscle oxygen consumption and mitochondrial capacity to be significantly greater in healthy males with high aerobic fitness (VO2peak $\geq$57 ml/kg/min) vs. low aerobic fitness (VO2peak $\leq$47 ml/kg/min) [100]. Furthermore, Achten et al. [90] assessed O2 uptake kinetics on six healthy women during a transition exercise protocol of 6 minutes at 20w, then three transitions between 6 minutes at 90% lactate threshold and 8 minutes at 20 watts [90]. Women were tested during the menstrual cycle's follicular and luteal phases using indirect calorimetry and deoxygenated hemoglobin/myoglobin via near-infrared spectroscopy (ΔHHb) of the vastus lateralis, finding no differences in VO2 kinetics

or ΔHHb response, or RER or lactate threshold. However, it is worth noting that plasma hormone concentrations were lower than average, possibly resulting in a smaller difference in RER or VO2 kinetics due to smaller differences in hormonal profile across the phases [90].

Menstrual Cycle Physiology

The menstrual cycle is split into the low hormone follicular phase (days 1-14) and the high hormone luteal phases (days 16-28) separated by ovulation, a one-day estrogen surge (~day 15) [15, 16]. Shifts in ovarian hormones can influence the cardiovascular system, aerobic capacity, thermoregulation, and metabolism [15, 16]. Estrogens appear to positively influence substrate metabolism, including enhanced muscle glycogen storage and fatty-acid availability and oxidation during exercise in women [17, 18] and possibly improved metabolic flexibility of skeletal muscle [103]. These estrogen-associated impacts on metabolism may lead to reduced reliance on anaerobic metabolism, lower carbohydrate reliance, sparing glycogen depletion, and lower blood lactate levels [4, 15, 19, 104, 105] with data in men receiving estrogen supplementation also reflecting increased fatty-acid oxidation and glucose sparing [4, 106, 107]. These mechanisms are thought to be due to women having more type one oxidative muscle fibers and store more intramuscular triglycerides than men [104, 108]. Additionally, recent reports suggest that the mitochondria themselves are responsible for sex differences in metabolism [109] and estrogen receptors, ERα. These receptors are predominant in type one muscle fibers and are associated

with increased fat oxidation, glucose tolerance, and insulin sensitivity in both men and women [4, 110], which may act on downstream targets associated with fatty-acid metabolism such as PPARα, PPARδ, PGCα, fatty-acid transport proteins such as CD36 or CPT1 and mitochondrial biogenesis [4, 110]. Lastly, when matched for fitness status, women have been shown to have a higher percentage of the total substrate and lower RER than males at 75 and 85% VO_{2max}, matched with increased mitochondrial volume density and mitochondrial fatty-acid respiration [111].

However, due to the menstrual cycles cyclic nature, women spend half of their cycle in a progesterone dominant state, which may have anti-estrogen effects such as insulin resistance, decreased glycogen storage and glucose availability, and impaired fat metabolism [19]. Progesterone's effects may inhibit estrogen's positive effect on skeletal muscle and metabolic flexibility [103] and increase core temperature, impairing thermoregulation, and fluid regulation [15, 112]. Women have been underrepresented in exercise metabolism research due to the difficulty in controlling for hormone fluctuations [15], leading to incongruent findings across studies expanded on below [113, 114]. Additional push for nutritional control, proper menstrual cycle phase, and hormonal confirmation have made these studies difficult to compare [15, 115]. Furthermore, recent reviews suggest possible low-level evidence for slightly impaired performance in the first half of the menstrual cycle [114] or oral contraceptive use [113], possibly due to lower circulating levels of sex hormones such as estrogen. With hormonal fluctuations, impacting metabolism [116], exercise performance, cardiovascular, respiratory

systems, and thermoregulatory systems, female sex hormones' impacts on metabolism and exercise became increasingly important [15, 16].

Impact of Menstrual Cycle on Exercise and Substrate Metabolism

While substrate metabolism variations across the menstrual cycle during exercise became apparent, a few studies assessed diet effects on exercise metabolism as well. Kanaley et al. assessed substrate response of seven trained endurance women during the early follicular, late follicular, and mid-luteal phases while running for 90 minutes at 60% VO_{2max} [117], finding no significant influence of cycle phase on exercise carbohydrate or fat oxidation, nor blood glucose or lactate associated with cycle phase. Furthermore, other early work by Bonen et al. assessing glucose, lactate, FFAs, glycerol between follicular and luteal phases of the menstrual cycle during fasted, fed, or glucose fed exercise [118]. Six women underwent treadmill exercise of 30 min at 40% and 80% VO_{2max}, noting no significant differences between menstrual cycle phases during fasted exercise. However, FFAs during the luteal phase were found to be lower in a glucose loaded state, revealing an important role of pre-exercise nutrition on menstrual cycle exercise metabolism [118]. Similarly, Wentz et al. assessed nine women during the mid-follicular and mid-luteal phases during rest and exercise at 30, 50, and 75% VO_{2max} following either a three-day low or high carbohydrate diet [119]. Their data revealing a significant interaction between the menstrual cycle phase and diet condition during 30% and 50% VO_{2max}, with carbohydrate oxidation being the

lowest and lipid oxidation highest during the luteal phases and low carbohydrate condition [119].

Building on the importance of role nutrition intake plays, work by Campbell et al. examined eight moderately trained women while cycling at 70% VO2peak for two hours followed by a four kJ/kg time trial [120]. Each woman completed a control fasted trial and a glucose fed trial in the follicular and luteal phases. During the control trials, glucose rates of appearance and disappearance were higher during the 2nd hour of exercise, with participants exhibiting a higher total percent carbohydrate oxidation (F: 80.1±1.2%, L: 77.0±1.0%), total percent fat oxidation (F: 19.9±0.%9, L: 23.0±1.0%) and total fat oxidized (L: 30±1, F: 35.3±3 g) and increased time trial performance (F: 24:30±2:07, L: 28.17±3:13 min:sec) in the follicular compared to luteal phases. However, during the glucose fed trial, performance improved from control in both phases (F: 19:53±0:52, L: 20:55±0.56 min:sec), with carbohydrate ingestion increasing percent carbohydrate oxidation and decreasing fat oxidation from control, with no differences between phases [120].

Furthermore, Suh et al. looked at glucose kinetics, and metabolism in eight moderately trained women at rest and during 60 minutes of exercise at 45 and 65%VO2peak during the follicular and luteal phases [14]. All trials were completed 3 hours after a standardized meal (308 kcal: 75% CHO (55 g), 16% protein, 9% fat), with RER, lipid, and carbohydrate oxidation significantly different between exercise intensities (45%: LP: 0.90±0.01, FP: 0.90±0.01, 65%: LP: 0.93±0.01, FP: LP: 0.93±0.02), but not between phases of the menstrual cycle. Additionally, lipid

oxidation was not different between phase or intensity (45%: LP: 2.0±0.1, FP: 2.2±0.4, 65%: LP: 2.2±0.2, FP: LP: 1.8±0.5 kcal/min) with an increase in carbohydrate oxidation only across intensities (45%: LP: 4.1.1±0.3, FP: 3.7±0.2, 65%: LP: 6.7±0.2, FP: LP: 6.7±0.7 kcal/min). Lastly, finding the rate of glucose appearance and disappearance (metabolic clearance rate) being significantly impacted by intensity ($p<0.05$), but not the menstrual phase. These data together reflecting that glucose metabolism is directly related to intensity and that pre-exercise feeding may play a significant role in exercise metabolism in women than the menstrual cycle phase itself [14].

Hackney et al. assessed the effects of sex hormones and the menstrual cycle in a series of studies. The first study assessed carbohydrate and lipid oxidation rates of nine women during a treadmill run of ten-minute increments of 35%, 60%, and 75% VO_{2max} during the mid-luteal and mid-follicular phases [121]. Significantly lower carbohydrate and higher lipid oxidation rates were found during 35% and 60% VO_{2max} during the mid-luteal phases, and no difference between carbohydrate and lipid oxidation 75%. Follow this, Hackney et al. examined six women's metabolic responses to 60 minutes of exercise at 70% VO_{2max} during the mid-follicular, ovulatory and mid-luteal phases [122]. RER of these women during the ovulatory phases (0.86±0.02) was significantly lower than mid-follicular(0.94±0.02) but not mid-luteal (0.89±0.01), with data on fat oxidation reflecting the same results. Inversely, there was higher carbohydrate oxidation during mid-follicular than ovulatory. RPE was also higher in the ovulatory trials at 30-60 min of exercise [122]. These studies are together reflecting greater lipid

oxidation during the mid-luteal phases and carbohydrate oxidation during the mid-follicular submaximal intensities [121], but no major differences in these phases at higher intensities (>70%VO2) [122] and greater fat oxidating matching the rise in estrogen that occurs with ovulation [122]. These findings are supported by Hackney et al. in six women during the low hormone early follicular phase and then again under pharmaceutically manipulated sex hormone (estrogen and progesterone) conditions during 60 minutes of exercise at 65% VO_{2max} [123]. This study revealing significantly elevated fat oxidation and lower carbohydrate oxidation rates under high hormone conditions, further supporting the impact of elevated sex hormones on exercise substrate metabolism [122], specifically lipid oxidation [123].

Similarly, D'Eon et al. looked at endogenous estrogen and progesterone's effects in regulating exercise substrate oxidation [124]. Women underwent three conditions, baseline (low estrogen and low progesterone), estrogen-only (estrogen high and progesterone low), and estrogen + progesterone (estrogen and progesterone both high). During each condition, women cycled for 60 minutes at ~60% VO_{2max}, blood glucose disappearance, total carbohydrate oxidation, and estimated muscle glycogen utilization was assessed. Carbohydrate oxidation was significantly lower with estrogen than baseline and the estrogen + progesterone conditions, with glucose rate of disappearing trending lower with estrogen alone and estrogen + progesterone relative to baseline. Estimated muscle glycogen utilization was 25% lower with estrogen alone than baseline or estrogen + progesterone. FFAs use was inversely related to estimated muscle glycogen use

(r^2=0.49). Together, these data suggest that estrogen lowers carbohydrate oxidation, leading to reduced muscle glycogen use and glucose uptake. Additionally, progesterone increases muscle glycogen use but not glucose uptake, supporting previously observed opposing actions of estrogen and progesterone on glycogen use and FFA availability [123].

More recent work by Frandsen et al. looked at the peak rate of fat oxidation and maximal fat oxidation intensity across the menstrual cycle in the mid-luteal, mid-follicular and late follicular phase in 29 healthy women [125]. Participants first underwent a fat max test starting 60 watts for 3 minutes, increasing 25 watts every 3rd minute till an RER $\geq$1.0 was achieved for 30 seconds. After resting for 10-15 min, a VO2peak test of 100 watts for 5 minutes followed by an increase of 25 watts/min till volitional exhaustion. However, there were no differences in peak fat oxidation (MF: 0.379 g/min; LF: 0.375 g/min; ML: 0.382 g/min), VO2 peak, lactate, glucose, or FFAs across the menstrual cycle. Furthermore, this data found no relationship between peak fat oxidation with plasma estradiol or FFAs. Nor Estradiol and FFAs or glycerol. However, it is worth noting that this study's protocol was shorter (<30 min) and more aggressive in progressing through exercise phases, unlike many other studies previously mentioned using longer (>30) steady-state intensities. This protocol's short duration and more progressive nature may miss differences that occur with longer duration exercise, whereas menstrual cycle differences in substrate oxidation may be less different as intensity increases and time decreases [125].

Similarly, Redman et al. looked at the exercise status of fourteen sedentary women in both the follicular and luteal phases during incremental cycling exercise to exhaustion and steady-state submaximal exercise at 25% and 75% VO2peak [126]. During the incremental test time to exhaustion, maximal power output, total work done, VO2peak, ventilation, heart rate, or lactate were not different between phases. However, overall VCO2 (F: 3.04±0.15, L: 2.93±0.14) and RER from 50-60% VO2peak (F: 1.16±0.04, L: 1.05±0.03) were lower during the luteal phase. VO2 was higher at 80-100%VO2 peak, despite being non significantly different overall (F: 2.64±0.15 l/min, L: 2.73±0.15 l/min). Furthermore, while there were no differences between lactate threshold or blood lactate values, there was higher lactate in the follicular phase. During a steady-state submaximal exercise session, VO2, RER, and lactate at both 25% and 75% were all greater in the follicular phase. Similarly, Lebrun et al., who looked at aerobic capacity, anaerobic capacity, and high-intensity endurance in sixteen trained women during the early follicular and mid-luteal phases [127], yielded no significant differences between phases in heart rate, ventilation, RER at max, anaerobic performance, time to fatigue (at 90%VOmax). However, both absolute (EF: 3.19±0.009, ML: 3.13±0.03 l/min, P=0.04) and relative (EF: 53.7±0.09 ml/kg/min, ML: 52.8±0.8 ml/kg/min, P=0.06) VO_{2max} were slightly lower in the luteal than the follicular phase. Taken together, these studies suggest that maximal exercise metrics may not be impacted by the cycle phase [127] and metabolism may not be altered during harder increases in intensity [125] but differences may be more apparent during lower-intensity steady-state exercise [126].

Impacts of The Menstrual Cycle on Glucose and Glycogen Metabolism

While previous research revealed differences in carbohydrate and lipid metabolism, glucose metabolism alone began to emerge not only as a sex difference but a menstrual cycle difference. First, Nicklas et al. looked at exercise performance, muscle glycogen, and substrate response during exhaustive exercise of 70% VO_{2max} during the mid-luteal and mid follicular phases in six eumenorrheic women [18]. Each exhaustive exercise test was preceded by a depletion bout (90 min at 60% VO_{2max} and 4x1 min at 100% VO_{2max}) and three days of dietary control. Time to exhaustion trended higher ($p<0.07$) during the mid-luteal (139.2±14.9 min) than in the mid-follicular(126±17.5 min) phase [18]. Muscle baseline glycogen (ML: (15.5±27, MF: 20.2±3.2 umol/g) was not different at baseline but was lower in the luteal phase. Nor was muscle glycogen depletion different during exhaustive exercise. However, the repletion of glycogen following the depletion trial (before exhaustive exercise) was significantly greater during the luteal phase (ML: 103.7±5.5 umol/g vs. MF 93.0±5.9 umol/g), revealing possible enhancements of the luteal phase on exercise performance and glycogen resynthesis. Furthermore, finding no differences in blood glucose, lactate, or RER between phases, but overall elevated in the luteal phases. However, a sample size of just six women may have reduced the authors' ability to detect these differences. Following this work, Hackney et al. looked at ten women's muscle biopsies during the mid-follicular and mid-luteal phases of their cycle [17]. Biopsies were randomized, with diet and physical activity being controlled for 36 hours before

each one. These data finding mid-follicular (90.76±3.56 umol/g) muscle glycogen content to be significantly less than mid-luteal (102.05±497 umol/g), matching that of Nicklas et al. [18]. In another study, Hackney et al. assessed muscle glycogen at rest and in response to 60 minutes of cycling at 70% VO_{2max} exercise in eight women during the mid-follicular and mid-luteal phases [128]. This study finding no differences in resting glycogen at rest, but slightly higher in the luteal phase, with post-exercise glycogen being reduced significantly more in the follicular phase and a significant negative correlation in the luteal phase (r=-0.71) between estrogen (E2) levels and muscle glycogen utilized [128], indicating as estrogen is lower more glycogen is utilized. These data suggesting enhanced lipid metabolism and a glycogen sparing effect, supported by a lower RER and higher lipid oxidation during the luteal phase (0.31±0.06 g/min) than the mid-follicular phase (0.17±0.05 g/min) [128]. Furthermore, proposing differences between phases may exist in glycogen storage and repletion, favoring the luteal phase [17, 18]. However, while the diet was controlled, it is worth noting these differences may also be due to favoring of lipid oxidation, as supported by Hackney et al. [128] and possible sparing of glucose and glycogen noted in the luteal phase [4, 108, 116].

Furthermore, Zderic et al. assessed glucose kinetics and substrate oxidation in the follicular and luteal phase in 6 moderately trained women during cycling for 25 minutes at the intensity of 70% then 90% of their lactate threshold [129]. There were no differences in glucose rate of appearance or disappearance or fat oxidation at rest or at 70% lactate threshold between phases. However, the rates of glucose appearance (28.8±4.8 vs. 33.7±4.5 μmol/min/kg; $P< 0.05$) and

disappearance (28.4±4.8 vs. 34.0±4.1 μmol/min/kg; *P*< 0.05), and carbohydrate (82.0±12.3 vs. 93.8±9.7 umol/min/kg P <0.05) oxidation at 90% of the lactate threshold were significantly lower in the luteal phase [129] and fat oxidation greater (7.46±1.01 vs. 6.05±0.89 umol/min/kg, P <0.05). Similarly, Horton et al. looked at substrate metabolism during the early follicular, mid-follicular, and mid-luteal phases of the menstrual cycle of thirteen menstruating women during resting and 90 minutes of exercise at 50% VO_{2max} [130]. This study finding no differences in substrate oxidating (EF 0.84±0.01, MF 0.85±0.01, ML 0.85±0.01) or glucose rate of appearance or disappearance in any of the phases. However, they found significantly elevated glucose concentrations during the first 45 minutes of exercise in the mid-luteal vs. earl or mid follicular phases, indicating that greater glucose may be present despite a similar substrate use pattern, which may be due to elevated sex hormones [130]. Furthermore, glucose rate of appearance and disappearance may also be impacted by exercise intensity. Zderic et al. data reflected this, whose 70% Lactate threshold was likely more similar to the 50% VO_{2max} used by Horton [129]. Matching the previous findings that glucose rate of appearance and disappearance, glycogen utilization, and carbohydrate oxidation appear to be greater in the follicular phase, with elevated fat reliance in the luteal phase [4, 108, 116].

Impacts of the Menstrual Cycle on Lactate Metabolism

With differences in substrate metabolism becoming apparent during exercise, investigations of other performance metrics and lactate metabolism

across the menstrual cycle were undertaken. Early work by Jurkowski et al. examined nine women during the mid-follicular and mid-luteal phases during cycling for 20 minutes at light 33%, heavy 66%, and exhaustive 90% Maximal power output (Wmax) [131]. Finding ventilation was significantly higher in the luteal (88±5.6 l/min) than the follicular phase (80±5.4 l/min). Time to exhaustion was more significant in the follicular (2.97±0.63 min) than the luteal phase (1.57±0.32), similar to Nicklas et al. [18]. Additionally, blood lactate was significantly elevated during the follicular phase at rest, at the end of heavy (66% Wmax) exercise, and at exhaustion (90% Wmax), but not light exercise (33% Wmax), reflecting improved performance with decreased lactate in the luteal phase. A follow-up experiment with a subset of five women undergoing 45 minutes of cycling at 50% Wmax found no effect of the cycle phase on lactate disappearance during exercise following an infusion of 2-mmol/kg bolus of lactate [131]. The lack of lactate metabolism changes here, reflecting increased steady-state lactate production rather than disappearance as a possible mechanism for lactate differences across the cycle. However, findings appeared to be inconsistent and only slightly reflective of exercise at 66% Wmax, suggesting hormonal differences likely played a role in elevated lactate and appearance rates seen during the follicular phase [131].

Contrastingly, McCracken et al. found no time to exhaustion differences in nine women between the mid-follicular (32.5±0.5 min) and mid-luteal (32.4±0.5 min) phase during exercise of ten minutes increments at 35%, 60%, and 75% VO_{2max} followed by 90% VO_{2max} to exhaustion [132]. However, similarly to Jurkowski et al. [131], recovery lactate taken at 3 (5.4±1.2 v s 8.7±1.8 mmol/L) and

30 (2.4±0.4 vs. 4.0±1.3 mmol/L) minutes post recovery was significantly higher during the mid-follicular phase, possibly a mechanism of estrogens effect on lipid oxidation, sparing carbohydrate, and decreasing lactate in the luteal phase [4, 108, 116]. Furthermore, Dean et al. [133] assessed the lactate threshold in 8 moderately active women during the early follicular, mid follicular, and mid-luteal phases; finding moderate significance (p=0.09) of the lactate threshold between the early follicular (52.1±1.7 % VO_{2max}) and mid-luteal (55.7±1.5 % VO_{2max}) phases. Work by Smekal et al. in 19 women undergoing incremental cycle test to voluntary exhaustion during the follicular phase and luteal phase [134] yielded no differences in power output, VO2, lactate, heart rate, or RER. However, Smekal et al. found higher VE/VCO2 and VE/VCO2 and VE at rest and exhaustion during the luteal phase, reflecting an enhanced ventilatory drive in the luteal phase, possibly due to thermoregulation changes during the luteal phase [134]. Together, all of these data that the menstrual cycle may play a role beyond metabolism by impacting exercise performance ventilation and lactate metabolism.

Sex Differences in Exercise Substrate Metabolism

While data on the impacts of the menstrual cycle on metabolism were not as clear, hormones in women alone compared to males were. First, Horton et al. looked at substrate metabolism in trained and untrained men (n=14) and women (n=14) before, during, and 2 hours after 2 hours of cycling at 40% VO_{2max} [135]. Subjects were matched for training status, and women were studied during the follicular phase of their menstrual cycle. During the exercise trials, women used

significantly more total energy from fat (W: 50.9±1.8 and M: 43.7±2.1%, p< 0.02), with men oxidizing significantly more energy from carbohydrate (53.1±2.1 and 45.7±1.8% for men and women, respectively, p< 0.01). Mittendorfer et al. similarly tested substrate oxidation and lipid kinetics in five men and five women exercising for 90 minutes at 50% VO_{2max}, matched for training status [13]; all women were tested in their follicular phase. A ~65% greater total increase in glycerol and FFA rate of appearance was seen here during exercise in women than men, but similar whole-body fat oxidation and RER (M: 0.87±0.02, W: 0.82±0.02) in both sexes. However, the contribution of FFAs to whole-body fat oxidation was significantly greater in women (76±5%) than in men (46±5%, p<0.05). The authors interpreted this to be due to adipose lipolysis during exercise being greater in women than men, leading to greater plasma FFAs and greater uptake for oxidation. Lastly, there were no differences in whole-body fat oxidation, likely resulting from decrease use of non-plasma FFA sources, such as intramuscular, in women [13].

Furthermore, Roepstorff et al. looked at substrate utilization in fourteen endurance-trained men (n=7) and women (n=7) during cycling at 57% VO2peak for 90 minutes [136]. Female participants were tested in the mid-follicular phase. During exercise, substrate oxidation measured by RER at the whole-body level (F: 0.89±0.02, M: 0.91±0.01) or leg respiratory quotient was not different between sex. However, intramuscular triacylglycerol degradation was negligible in males but was significantly decreased in women. This use of intramuscular triacylglycerol was representative of 25.0±6.0% of total oxygen uptake in females and just 5.0±7.3% in males (p<0.05), and plasma FFAs, blood glucose, and glycogen use

not being significantly different between sex. This data reflecting that in females, substrate oxidation accounted for 99% of leg oxygen uptake and only 28% in males, suggesting that males and females use different lipid sources during exercise, despite no differences in whole-body substrate oxidation during submaximal exercise.

Building on this work by Roepstroff et al., similar differences across the menstrual cycle were seen with training by Carter et al. who looked at the effects of seven weeks of endurance cycle training of 5 days/week for 60 min at 60%VO2 max in eight men and eight women on substrate metabolism during exercise [137]. Subjects were assessed during a 90-minute cycle test at 60% of their VO2 max at baseline and twice at post both at the absolute intensity of their baseline and new relative post-training intensity of 60% VO_{2max}. Female subjects were tested in the early to mid-follicular phase. Endurance training significantly increased VO_{2max} and fat oxidation, inversely with carbohydrate oxidation at the same absolute intensity. Lactate was significantly decreased by training in the absolute trial and 30 minutes of exercise in the relative trial. After training, glucose rates of appearance and disappearance were significantly lower during absolute and relative trials. Furthermore, glycerol rate of appearance or disappearance was significantly lower at 75 and 90 minutes following training during the absolute trial, with FFA concentrations being lower following training, matching previous work by Romijn finding increased lipid kinetics in trained vs. untrained subjects [50]. Furthermore, Females oxidized a significantly greater percentage of total energy from fat and less carbohydrate than males across all trials [137], with no differences in lactate

or glucose kinetics between males and females before or after training. However, glucose's metabolic clearance rate was significantly lower in females at 75 and 90 minutes of exercise, which is matched with a trend for women to have higher glucose concentrations across exercise (p=0.056) and possibly resulting in greater lipid utilization, matched with a greater glycerol rate of appearance higher plasma FFAs in females across all time points. Furthermore, men had a higher RER and a greater glucose appearance, disappearance, metabolic clearance rate, and higher glycogen during exercise than women in both phases. Proglycogen utilization was also greater than women in the luteal phase compared to men, but not follicular phase. All these data suggest women oxidize more fat than males, with a lower metabolic clearance rate of glucose regardless of training status. Moreover, endurance training alone appears to decrease carbohydrate use during exercise, regardless of sex [137]. However, Romijn et al. found no differences in substrate oxidation across 25%, 65%, or 85% VO_{2max} between men and women but did find a similar decrease in glucose disappearance at 25% VO_{2max} [52].

These findings are similar to that of Derives et al., who looked at glucose turnover and muscle glycogen utilization during 90 minutes of cycling at 65% VO2peak in men and women [12]. Women in the luteal phase had lower glucose rates of appearance and disappearance and a lowered metabolic clearance rate, with a greater decrease in proglycogen, macroglycogen, and total glycogen during the follicular phase. These findings further supporting the data of Nicklas et al. [18] and Hackney et al. [17, 128] together suggesting sex may have a greater influence on glycogen and glucose metabolism perse than the menstrual cycle itself [12,

137] and no lactate differences across the menstrual cycle or between sexes during exercise [12, 137]. Furthermore, Steffensen et al. looked at myocellular triacylglycerol (MCTG) during 90 minutes of submaximal exercise at 60%VO2 max in 42 untrained (UT), moderately trained (MT) or endurance-trained (END) females (n=21) and males (n=21) [138]. All subjects' diets were controlled eight days prior, and women underwent testing in the mid-follicular phase. Women had significantly greater resting MCTG than men across all fitness status (W: UT: 48.4±4.2, MT: 48.5±8.4 and END: 52.2±5.8 mmol/kg dry, M: UT: 34.1±4.9, MT: 31.6±3.3 and END: 38.4±3.0 mmol/kg, $p<0.05$). Exercise decreased MCTG by 25% in women regardless of training status, with males being unaffected by exercise. RER was similar across all groups (F: UT: 0.80±0.02, MT: 0.81±0.02, END: 0.79±0.02, M: UT: 0.85±0.05, MT: 0.80±0.03, and END: 0.79±0.01), decreasing only after 60 minutes of exercise only in the endurance-trained groups. There were no sex differences at any time points, but a significant correlation between baseline MCTG and use during exercise ($r=0.61$, $p<0001$). These data suggesting elevated baseline and intramuscular triglyceride use in women, supporting data previous of greater fat oxidation in women than males [137].

As previously discussed, Friedlander et al. looked at the effects of exercise intensity and training on lipid metabolism [66] and carbohydrate metabolism [139] in women and men [140] following an eleven-week exercise program (5x/week, 60 min at 75%VO2peak). Participants underwent two pre-training trials at 45 and 65% VO2peak, followed by two post-training trials at the same absolute and relative 65% VO2 peak workload. Exercise sessions were conducted in the mid-luteal

phase for female participants. Pre-training rates of FFAs appearance, disappearance, and oxidation were found to be similar between 45% and 65% VO2peak in women [66]. With, post-training absolute and relative 65%VO2peak having increased FFA rate of appearance and disappearance and whole-body fat oxidation (Pre 45%: 67.61±6.03%, Pre 65%: 51.33±4.43%, Absolute 65%: 61.22±5.51%, 65% Relative: 71.76±3.91% %Energy). When looking at the change in carbohydrate metabolism in women [139], at baseline, there was a significant increase in glucose rate of appearance, disappearance, and oxidation from 45 to 65% intensities ($p<0.05$). Training decreased glucose rate of appearance and disappearance at the same absolute workload ($p<0.05$), but not at the same relative workloads [139]. However, after training at both relative and absolute 65%, there was significantly lower carbohydrate oxidation (Pre 45%: 63.1±3.51%, Pre 65%: 72.6±7.46%, Absolute 65%: 52.7±3.52%, Relative 65%: 56.7±3.34 %Energy). Compared to previous work done in men using the same protocol [140], women showed similar glucose flux compared to men [139]. However, women tending to have lower RERs than men at similar relative intensities, despite similar glucose appearance and disappearance and lower blood lactate than males at both baseline 65% and absolute and relative intensities. These findings on RER, glucose, and lactate support that men and women may have differences in substrate oxidation, even when women are tested in the lowest hormone phase (mid-follicular), and that remains with exercise training [61, 66, 140].

Lastly, work by Henderson et al. looked at glucose kinetics in ten men and eight women under three different exercise conditions [141]. Women were tested

in their earlier follicular phase. Glucose rate of appearance, disappearance, and metabolic clearance rate were assessed before, during, and after 90 min of exercise at 45% VO2peak, 60 min at 65% VO2peak, and a time-matched sedentary control trial. Glucose kinetics were significantly elevated in 65% than 45% VO2 peak conditions in men and women, with average rate of appearance, disappearance, and metabolic clearance rate remained significantly elevated in men over control during 3 hours of recovery after 45% and 65% exercise and blood glucose remaining lower. Women experienced no depression in glucose during recovery or elevated glucose rate of appearance or disappearance during recovery. Only metabolic clearance rate remaining elevated after exercise at 65% VO_{2max} in women, suggesting that women may be better at maintaining glucose levels during the post-exercise period, and men expressing an increased need in counter-regulation of glucose production [141]. Overall, these data indicating better glucoregulation in women may be the cause of sex differences in post-exercise substrate metabolism (i.e., greater fat oxidation in women) [141].

Impacts of Oral Contraceptives on Exercise Performance and Metabolism

The natural menstrual cycle's impact on substrate metabolism and exercise performance is important; however, nearly half of women participating in sport report hormonal contraceptive use [142]. Leaving the question, how do these hormonal agonists impact exercise and fuel metabolism? First, Bonen et al. looked at substrate metabolism in seven women on oral contraceptive and eight menstruating women while walking for 30 minutes at 40% VO_{2max} and 85%VO2

max [143]. The OC group underwent sessions during the active pill and inactive pill phases. Menstruating women underwent testing in the follicular and luteal phases. Blood draws taken at rest, 15, 30, 45, and 60 minutes of exercise and 30 min after were analyzed for glucose, lactate, and FFAs, finding no differences during exercise between OC phase or menstrual cycle phase. However, FFA concentrations were significantly higher during 40% VO_{2max} exercise, and glucose concentrations were significantly lower at rest and across exercise in the OC group and no differences in lactate at rest or exercise between groups.

A series of studies out of the Brooks lab looked at the effect of oral contraceptives on VO_{2max} and maximal exercise kinetics and substrate oxidation [144], triglyceride mobilization [145] and fatty-acid kinetics and fat oxidation [146] and glucose flux [147]. Normally menstruating women were tested in the follicular and luteal phases and again following four months of oral contraceptives in the active and inactive pill phases. All participants consumed a standardized breakfast (308 kcal: 75% CHO, 16% protein, 9%) before each session. Casazza et al., finding no differences between phases of the cycle on RER (Baseline: FP: 1.18±0.01, LP: 1.17±0.01 vs. OC: Inactive: 1.16±0.03, Active: 1.16±0.02) or performance. However, oral contraceptive use slightly decreased VO2peak (-11%), likely due to increases in body size [144]. Casazza et al. also looked at women during 90 minutes of rest and 60 minutes of cycle exercise at 45 and 65% VO_{2max}. Finding no difference in RER at 45% VO2 peak (FP: 0.90±0.01, LP: 0.90±0.01, IP: 0.90±0.01, HP: 0.89±0.01) or 65% $\dot{V}O_2$ peak (FP: 0.93±0.01, LP: 0.93±0.02, IP: 0.94±0.01, HP: 0.93±0.01). Furthermore, whole-body fat oxidation

was similar between phases at 45% VO2 peak (FP: 2.0 ± 0.1, LP: 2.0 ± 0.4, IP: 2.0 ± 0.2, HP: 2.2 ± 0.3 kcal/min) and 65% VO2 peak (FP: 2.2 ± 0.2, LP: 1.8 ± 0.5, IP: 1.6 ± 0.3, HP: 1.9 ± 0.3 kcal/min). Conversely, oral contraceptive use significantly increased the glycerol rate of appearance during the high hormone pill phase at 45% VO2peak and at 65% in the inactive pill phase. Jacobs et al., using the same exercise protocol, found no significant difference in plasma FFA on appearance or disappearance between menstrual cycle or oral contraceptive phases at either exercise intensities. However, compared with both menstrual cycle phases, oral contraceptive use increased plasma FFA reesterification and decreased the rate of disappearance that was oxidized during rest and exercise. Suggesting the noted increase in FFAs during exercise with oral contraceptive use was not matched with an increase in fat oxidation [146]. Building on this, Suh et al. found a significant reduction in glucose rates of appearance and disappearance during both exercise intensities with oral contraceptives, but not at rest. Furthermore, Suh et al. found no phase effects or effect of oral contracept use on carbohydrate, lipid, RER, lactate at rest, or exercise intensity [147]. These data together inferring that the order of importance for the impact of ovarian hormones and steroids on triglyceride mobilization in women during exercise is 1) energy flux, 2) oral contraceptive use, then 3) nutritional intake [145].

Likewise, Issaco et al. looked at the effects of energy intake and oral contraceptives on exercise substrate oxidation [148] in ten regularly menstruating women in their luteal phase and eleven using oral contraceptives during their active pill phase. Testing was done during 45 min of exercise, 65% VO_{2max} in the

fasted and fed state (50% carbohydrate, 35% fat, 15% protein, 9.5kcal/kg). At rest, the oral contraceptive group had higher triglyceride concentrations but no influence on substrate oxidation or metabolic response to exercise. In the fasting state, regardless of oral contraceptive status, women exhibited a greater reliance on fat oxidation than in a postprandial state, matched with higher FFAs and glycerol levels, further suggesting oral contraceptives do not modify exercise whole-body substrate oxidation [143, 149]. It is also worth noting all these trials were performed with participants in the fed state, reflecting the impact of pre-exercise meal on exercise substrate oxidation, supporting the idea that nutritional status affects exercise substrate use more than hormonal status alone [120, 147, 148].

Further work by Issaco et al. looked at maximal fat oxidation and anaerobic exercise parameters in healthy untrained women, with eleven women on oral contraceptives and ten healthy menstruating women [150]. Women on oral contraceptives were tested during their pill phase and menstruating women during the luteal phase of their cycle in a fasted state. Participants underwent two sessions: first, a maximal incremental cycle exercise test to exhaustion; second, a maximal lipid oxidation test consisting of a 6-minute stage graded cycling exercise starting at 20% of their Wmax, increasing by 10% each stage till 60% of Wmax. There were no differences in cardiorespiratory parameters at the anaerobic threshold (VO2, VCO2, VE, RER, or power output) or maximal aerobic capacity between groups. However, women on oral contraceptives had a greater maximal lipid oxidation rate (7.6±1.9 vs. 4.6±1.0 mg min/kg FFM; $p<0.01$). Additionally, they had a higher point of maximal lipid oxidation (45.2±5.2 vs. 36.2±4.1 % of VO_{2max};

p<0.001) compared to menstruating women. Suggesting that while oral contraceptives may not impact parameters at the anaerobic threshold, the hormonal status of oral contraceptive uses appears to increase lipid oxidation. It is worth noting that there were no differences in absolute VO_{2max} (OC: 35.1±7.0, LF: 39.2±4.1, p=0.12) or at the anaerobic threshold (OC: 27.5±4.9, LF: 26.4±5.9, p=0.65) between groups, suggesting this is not the result of differences in fitness status on lipid oxidation.

Building on this, Mattu et al. looked at the impact of the menstrual cycle or oral contraceptive phases on submaximal exercise oxygen uptake kinetics, maximal lactate steady-state, and VO_{2max} during the time to exhaustion test in healthy active women [151]. Fifteen women on oral contraceptives and fifteen normally menstruating women were tested twice in the mid-follicular phase and again in the mid-luteal phases or active and inactive pill phases. Women underwent a VO2 kinetics test, one incremental ramp test, a 2-3 constant load cycling trial to determine maximal lactate steady-state, and a time to exhaustion trial. Neither the menstrual cycle phase nor oral contraceptive use affected the time to constant VO2 response, VO2 max, maximal lactate steady-state, or time to exhaustion. RPE during the maximal lactate steady-state trial was significantly greater in the mid-follicular vs. mid-luteal phase. Together, these data suggest hormonal fluctuations between the menstrual cycle and oral contraceptive use had no significant effects on submaximal or maximal exercise performance [151]. Furthermore, work by Lynch and Nimmo also found no differences in performance between cycle phase (mid-luteal vs. late follicular) or pill use (week one vs. week

2) in five women [152]. Women underwent an intermittent running protocol of repeated 20-second sprints with 100 seconds of passive recovery. Sprints started at a speed of 14.3 km/h and 10.5% incline, with each sprint increasing by 1.2 km/h, until exhaustion. In the oral contraceptive group, mean blood lactate during exercise was significantly different from week 1 to week 2 testing cycle, but there were no other phase differences in menstruating women [152].

Furthermore, Gordon et al. examined VO_{2max} and cardiorespiratory dynamics in ten normally menstruating women and six on oral contraceptives [153]. All women were tested in the menstrual, mid-follicular, mid-luteal, and premenstrual phases, or each pill week. Results indicated no significant difference between groups or across phases in VO_{2max}, heart rate max, stroke volume max, or cardiac output max. However, for the change in VO_{2max} during the last 60 seconds of the trial, there were significant differences between oral contraceptive and non-oral contraceptive users, with women taking oral contraceptives showing no plateau in VO2 in 3 of the 4 phases tested. These data suggest that oral contraceptives may affect the VO2plateau but that VO_{2max} performance is independent of the menstrual cycle phase.

Vaiksaar et al. looked at the effects of menstrual cycle phase and oral contracept use on endurance performance in rowers [149] and endurance capacity [154] in two separate studies. First, looking at twenty-four women divided into competitive and normal menstruating (n=7), recreationally and normal menstruating and recreationally trained on oral contraceptives (n=9). All participants performed two voluntary exhaustion rowing tests starting at 40W,

increasing by 15 W every minute till exhaustion. Participants were tested during the follicular and luteal phase in the post-absorptive state, with no significant differences in power output, heart rate, RER, VO_{2max}, or lactate between cycle phases or oral contraceptive use, only higher VE/VCO2 values in the luteal phase in oral contraceptive users. Similar results were observed in their study of eight rowers using oral contraceptive pills. Rowers underwent 1 hour of rowing at 70% VO_{2max} [154], during the inactive and active pill phases, showing no differences in energy expenditure, carbohydrate or lipid oxidation, blood lactate between oral contraceptive pill phases. These together suggest no differences in cycle phase or pill phases, at least in a postprandial state.

Lastly, Barba-Moreno et al. looked at the cardiorespiratory response to endurance exercise in menstruating women (n=15) and those taking oral contraceptives (n=8) [155]. Menstruating women were tested in the early follicular, mid-follicular, and luteal phases, with oral contraceptive users being tested during active and inactive pill phases. All participants underwent 40 minutes of running at 75% of their speed at their VO_{2max}. Data in menstruating women found oxygen uptake was significantly greater and percent maximal oxygen uptake during the mid-follicular phase vs. the early follicular phase ($p<0.05$), with heart rate, ventilatory equivalent for oxygen, ventilatory equivalent for carbon dioxide, and tidal volume all increased in the luteal phase compared to the mid-follicular phase. With data in oral contraceptive users' showing breathing frequency, ventilatory equivalent for oxygen, and ventilatory equivalent for carbon dioxide are elevated during the active pill phase, resulting in possible lower cardiorespiratory efficiency

during higher hormonal phases (luteal, active pill). Nevertheless, as supported by earlier studies by Vaikssar et al., [149, 154] these variations are minor, but that the menstrual cycle itself does have small impacts on submaximal exercise performance. These studies are together suggesting greater FFA or lipid metabolism despite no differences in whole-body substrate oxidation [150], with possibly minor lower cardiorespiratory performance [149, 154] and likely minimal if any differences in performance [149].

CHAPTER 3

HIGH INTENSITY INTERVAL EXERCISE VS HIGH FAT MEAL CHALLENGE AS TESTS OF METABOLIC FLEXIBILITY: THE ROLE OF FITNESS, SEX AND LEAN MASS IN YOUNG ADULTS.[1]

[1] Olenick et. al, 2022. To be submitted to Experimental Physiology.

Abstract

We explored whether high intensity interval exercise (HIIE) could provide a novel means of testing metabolic flexibility. Fat and carbohydrate oxidation were assessed during HIIE (n=21) of 4x4:3 minute work:recovery (0W) intervals on a cycle ergometer at a power output of halfway between ventilatory threshold and $\dot{V}O_{2peak}$. A high fat meal challenge (~81% fat) (n=19) included fasted and postprandial resting metabolic rate. Participants were split by sex and fitness status ($\dot{V}O_{2peak}$: High$\geq$40 ml/kg/min, low<40 ml/kg/min). HIIE fat oxidation (g/kg) was greater in high fitness (AUC: High: 7.10±0.04 vs. Low: 7.06±0.01, p=0.017). HIIE carbohydrate oxidation (g/kg) was higher in men (AUC: Men: 8.27±0.14 vs. Women: 7.87±0.15, $p\leq0.001$). The high fat meal challenge did not capture fitness or sex differences. Fat oxidation (g/kg) was lower in low fit women during HIIE (AUC: high fit men (n=6): 7.09±0.02, low fit men (n=5): 7.07±0.01, high fit women (n=5): 7.10±0.05, low fit women (n=5): 7.04±0.01, p≤0.05), and high fat meal challenge (AUC: high fit men: 0.58±0.15, low fit men: 0.55±0.13, high fit women: 0.59±0.14, low fit women: 0.33±0.12, p≤0.05). Our data suggest that HIIE may detect greater metabolic differences associated with training not captured by high fat meal challenge.

Abbreviations

COX Carbohydrate Oxidation

FOX Fat Oxidation

HIIE High Intensity Interval Exercise

HFM High Fit Men

HFW High Fit Women

LFM Low Fit Men

LFW Low Fit Women

NIRS Near-infrared spectroscopy

REE Resting Energy Expenditure

RMR Resting Metabolic Rate

RPE Rating of Perceived Exertion

Introduction

Metabolic flexibility is the ability to efficiently adapt metabolism by substrate sensing, trafficking, storage, and utilization, dependent on availability and requirement [1]. Metabolic flexibility is essential for maintaining energy homeostasis in nutrient excess or energy demand and relies on a whole body control between systems such as the endocrine system, muscle tissue and mitochondria [1, 3]. Communication between these systems is vital in maintaining energy balance, and disruptions in metabolic flexibility can have implications for exercise performance and development of metabolic disease [1, 3, 5, 40, 156]. Metabolic flexibility is traditionally examined during the transition between fasted and feeding, which challenges whole body endocrine and mitochondrial shifts [1]. Another robust test of metabolic flexibility is the onset of exercise, as it requires

appropriate shifting of skeletal muscle metabolic pathways to support activity demands [1, 3].

High intensity interval exercise is a unique style of exercise that temporarily stresses whole body, muscle and mitochondrial to match energy demands, and recover between bouts. Therefore, body's ability to switch between carbohydrate and fat metabolism during HIIE and recovery periods may provide an alternative, perhaps a less-appreciated assessment of metabolic flexibility. However, historically, high intensity exercise as a means to assess substrate oxidiation has been avoided due to the increased anerobic demands, elevated blood lactate levels, and subsequent accumulation of hydrogen ions [H^+] in excess of what can be buffered [157]. These together, resulting in an elevate CO_2 response, inflating carbohydrate oxidation measures. While these are valid concerns, previous work shows that the assessment of substrate oxidation via indirect calorimetry yield similar results to isotope infusion during high intensity exercise [157]. Due to these limits only a few studies have investigated exercise metabolic differences in well trained and recreationally trained males suggesting the use of fat during exercise upwards of 85% VO2max [9, 10, 158]. With no reports comparing the metabolic response during HIIE between sexes, in women, or as a measure of metabolic flexibility.

Mechanisms that contribute to increased fat oxidation and metabolic flexibility during exercise are involved in managing body mass, metabolic health, and

exercise performance [4]. Aerobic fitness status also plays an important role in metabolic flexibility during feeding [5, 6, 8] and fat oxidation during incremental and HIIE is increased with fitness status [8-10, 83]. Despite the known connection of fitness status and metabolic flexibility, it is unknown if the ability to oxidize fat during the postprandial period and during exercise are related. Furthermore, no one has yet classified the metabolic differences in response to high intensity interval exercise as an assessment of metabolic flexibility, or it's relationship with the post prandial period.

Additionally, women have reported higher fat oxidation during exercise [11, 104, 111]. However, most previous studies assessing metabolic differences in sex use steady state, submaximal exercise protocols [12-14]. With recent work showing differences in fat oxidation during HIIE according to fitness status in men [9, 10], it seems plausible that HIIE could also detect differences in fuel utilization between or within sexes. Current gaps in the literature on women in sport science research leave unclear gaps in knowledge, and warrant further comparisions between sexes and in women [159]. Therefore, assessing the metabolic response to high fat meal and HIIE compared to men as well as the impact of fitness status in women is merited.

Assessment of substrate oxidation in response to feeding captures only one dimension of metabolic flexibility. Furthermore, the relationship between exercise metabolism and postprandial metabolic response has not been established.

Therefore, we aimed to assess substrate oxidation during HIIE and in response to a high-fat meal challenge and determine if HIIE and high-fat meal challenge-based assessments of metabolic flexibility are influenced by fitness and sex. We hypothesized that (i) female sex and a higher fitness status would be associated with higher fat oxidation during the high-fat meal challenge and HIIE and (ii) capacity to oxidize fat during exercise and postprandial feeding would be related. We also explored whether HIIE could uncover unique differences in metabolic flexibility among study groups not captured by a traditional high-fat meal challenge.

Methods

Ethical Approval

The study was approved by the University of Georgia Institutional Review Board (study no. 286), with written informed consent obtained prior to any experimental procedures. The study conformed to the standards set by the *Declaration of Helsinki*, except for registration in a database.

Participant Characteristics

Twenty-Two healthy, non-smoking, recreationally active (cardiovascular exercise ≥150 min/wk and resistance exercise ≥2 h/wk) men and women (23.00 ± 0.86 yrs; 172.41 ± 1.78 cm; 76.24 ± 2.94 kg; 40.92 ± 1.49 mL/kg/min) were recruited to the Integrative Cardiovascular Physiology Laboratory for three testing sessions. Participants were free of any history of cardiovascular, metabolic, or musculoskeletal disease. Participants were not taking medications that affect

metabolism, including hormonal or non-hormonal birth control or any supplements or vitamins during the study period. For purposes of this study, we operationally categorized fitness and defined “high fitness status” as having a $\dot{V}O_{2peak}$ of ≧40 mL/kg/min, and low fitness status as <40 mL/kg/min. Participants were then further split by both fitness status and sex.

Design

Participants completed three trials consisting of a baseline testing session followed by counter balanced high-fat meal challenge and HIIE sessions. Baseline testing consisted of anthropometrics, body composition, skeletal muscle mitochondrial oxidative capacity test and $\dot{V}O_{2peak}$ testing. The second session included high intensity interval exercise, and third session high-fat meal challenge and resting muscle oxygen uptake assessment. Session two and three were randomized. All sessions were separated by >48h , and all data collection was completed within 2 months. Participants refrained from exercise and alcohol ingestion for 24 h before each trial and were fasted overnight for 10-12 hours prior to high-fat meal challenge and HIIE. Subjects were instructed to maintain current dietary habits. Dietary records were collected for 3 days prior to each session and assessed using the United States Department of Agriculture National Nutrient Database for Standard Reference (http://www.nal.usda.gov/fnic/foodcomp/search/) to ensure that quantity of macronutrients and total energy consumed did not vary significantly between visits. Participants were instructed to consume a standard pre-fasting meal, consisting of 30% predicted resting energy expenditure (50% carbohydrate,

30% fat and 20% protein) [160]. All female participants completed HIIE and high-fat meal challenge sessions within days 2-10 following self-reported onset of their menstrual cycle following baseline testing [15]. See Figure 1.1 for an overview of experimental procedures.

Baseline

Participants' height, weight, body composition (via dual-energy X-ray absorptiometry, Horizon® DXA System, Hologic, Inc., Marlborough, MA, USA), blood pressure and resting heart were assessed. Skeletal muscle mitochondrial oxidative capacity test and $\dot{V}O_{2peak}$ were performed.

Skeletal muscle mitochondrial oxidative capacity test

Mitochondrial oxidative capacity was determined by measuring changes in continuous wave near-infrared spectroscopy (NIRS) (CW-NIRS; PortaMon, Artinis Medical Systems, Einsteinweg, The Netherlands) signals during periods of ischemia, as previously described [101]. To measure the rate of recovery of muscle oxygen uptake ($m\dot{V}O2$) back to resting levels, four mitochondrial oxidative metabolism tests were performed consisting of a series of six brief occlusions (5s on/5s off of 250-300 mmHg) following 30s of muscular twitch by stimulation. Briefly, each participant laid supine on a table with a CW-NIRS optode fixed to their Vastus Lateralis and secured using elastic tape. The muscle was percutaneously stimulated proximal and distal to the CW-NIRS optode with a rapid inflating blood pressure cuff (Hokanson SC12; D.E. Hokanson Inc., Bellevue, WA) placed

proximal to the CW-NIRS optode. CW-NIRS signals were analyzed using Matlab-based analysis software MATLAB® R2018b (MathWorks Inc., Natick, MA), and a rate constant for the return of muscle oxygen uptake to resting levels was calculated as previously described [101].

Peak oxygen uptake ($\dot{V}O_{2peak}$) Test

Participants were fitted with a mask to collect respiratory gasses via indirect calorimetry (TrueOne 2400, Parvo Medics, Sandy, UT, USA). $\dot{V}O_{2peak}$ measured via a ramp protocol on an electromagnetically braked cycle ergometer (Lode Excalibur Sport, Groningen, Netherlands). Participants completed a warmup by cycling for 3-minutes at 20 watts, after which work rate increased 20 W/min until volitional fatigue or participants could no longer sustain a cadence of $\geq$60 RPM.

High-Fat Meal Challenge

Participants arrived at the laboratory the morning, following an overnight fast (10-12 h) to undergo a high-fat meal challenge as previously described [161]. Upon arrival a fasting resting metabolic rate (RMR,kcal day^{-1}) assessment was performed. Subjects then ingested a high-fat test meal consisting of 0.5 g carbohydrate, 1.3 g fat and 0.2 g protein standardized per kg of fat free mass (~13.79% carbohydrate, 80.69% fat and 5.52% protein) [161]. Postprandial RMR was measured for 20 minutes immediately (0 m) and every 30 m during the 4 h postprandial period (TrueOne 2400, Parvo Medics, Sandy, UT, USA). Participants

rested in a supine position throughout the 4 h postprandial period. Blood glucose was measured at baseline (-30), 30, 60, 120, 180, and 240 minutes.

Resting Muscle Oxygen Uptake Assessments

Muscle oxygen uptake ($m\dot{V}O_2$) was assessed during the high-fat meal session in a subset of 12 participants (M9/F3; 22 years, 176.08±0.28 cm, 78.5±0.28 kg). Muscle oxygen uptake of the vastus lateralis was determined by measuring changes in continuous-wave near-infrared spectroscopy (NIRS) (CW-NIRS; PortaMon, Artinis Medical Systems, Einsteinweg, Netherlands) signals during 30 second periods of ischemia from a rapid inflating blood pressure cuff (Hokanson SC12; D.E. Hokanson Inc., Bellevue, WA). Between each RMR test, participants laid supine with a CW-NIRS optode fixed to their Vastus Lateralis. Three ischemic cuffs were completed (30 seconds on, 90 seconds off with an occlusion pressure of 250-300 mmHg). The slope of CW-NIRS signals calculated resting metabolism following occlusion [98]. Data were analyzed using Matlab-based analysis software MATLAB® R2018b (MathWorks Inc., Natick, MA) as previously described [101]. The average slope (1/sec) of the three cuffs was recorded.

High-Intensity Interval Exercise Session

Participants arrived at the laboratory in the morning after an overnight fast (10-12 h) to undergo a HIIE exercise session. Participants were fitted with a mask to collect respiratory gasses for analysis via indirect calorimetry (TrueOne 2400, Parvo Medics, Sandy, UT, USA). 5 minutes of seated resting metabolism were

recorded. High-intensity intervals were four minutes long and was determined by identifying the halfway point between gas exchange threshold and $\dot{V}O_{2peak}$. V-slope method was used to determine gas exchange threshold and independently verified by two study team members, as previously described [162]. Low intervals were three minutes of unloaded cycling. Warm up and cool down were 3 min long at a self-selected intensity of RPE 8/20. Exercise was performed on an electromagnetically braked cycle ergometer pre-programmed to the previously determined power output (Lode Excalibur Sport, Groningen, the Netherlands). Participants were asked to maintain a cadence of ≥60 RPM across all intervals. If a participant could not sustain ≥60 RPM power output, it was decreased in 5W increments till the participant could complete the work (n=4). Seated recovery metabolism was assessed for 20 minutes post-exercise. Blood glucose and lactate via finger stick and RPE were collected following each interval and every five minutes during recovery.

Lipid and Carbohydrate Oxidation Assessment

Macronutrient oxidation rate was assessed for HIIE and high-fat meal challenge using equations developed by Frayn [163]. The first 5 min of each 20 min test was discarded for the high-fat meal challenge to ensure steady state metabolic data analysis. For HIIE sessions, macronutrient oxidation rate was averaged for the duration of each interval. Total grams of substrate oxidized was calculated by multiplying the rate of substrate oxidation by time duration and summed for total exercise (high interval 1, 2, 3 and 4), recovery (low 1, 2, 3, 4, and recover 5-20 min), and full session. Rate of substrate oxidation was multiplied by time during

individual high intervals and recovery to estimate total amount of respective substrate oxidized. Due to the contribution of bicarbonate and hydrogen buffering that occurs during exercise and may impact $\dot{V}CO_2$ values during exercise, a small subset of data was analyzed using equations developed by Jeukendrup for exercise intensities between 50-75% $\dot{V}O_{2peak}$ [164]. Owing to the lack of equations for very high intensity (85–95% VO2max), Fran questions slightly inflate carbohydrate oxidation, thowever his difference is not apparent when controlling for body weight or fat free mass. Therefore all data analysis was conducted using Frayn equations, which has been used elsewhere during HIIE exercise as well [9].

Statistics

A two-way (condition × time) repeated-measures ANCOVA was conducted to assess statistical significance of overall effects and effects of each group (*fitness* and *sex)* on fat (FOX) and carbohydrate oxidation (COX) rates. To further assess impacts of fitness and sex (*fitness x sex*), a third analysis was conducted to assess impact of fitness (high vs. low) stratified by sex (male vs. female) on study outcomes. This stratification produced four groups: high fit men (HFM), low fit men (LFM), high fit women (HFW), low fit women (LFW). Postprandial fat and carbohydrate oxidation curves (AUC) were calculated using the trapezoid rule [165]. Student's paired t-test was used to compare total fat and carbohydrate oxidized calculations, total fat and carbohydrate oxidation AUC, skeletal muscle mitochondrial oxidative capacity, and all group characteristics. Substrate oxidation analyses were analyzed with data expressed relative to total body weight (kg/BW)

and fat free mass (kg/FFM). Pearson's correlation coefficient was used to determine correlations between $\dot{V}O_{2peak}$, skeletal muscle mitochondrial oxidative capacity, postprandial fat oxidation and exercise fat oxidation. Assumptions of normality were verified for all outcome measures. Statistical significance was accepted at $P \leq 0.05$. Data are presented as means ± SD. All statistical analyses were performed with SPSS Statistics version 25.0 (IBM Corp., Armonk, NY, USA).

Results

Participant Characteristics

Twenty-two participants were recruited and completed the study. Twenty-one participants completed the HIIE session, nineteen the high-fat meal challenge and thirteen the resting muscle metabolism assessment. Two participants did not complete all sessions due to personal reasons. Two were unable to complete testing due to the University's cessation of all non-essential research activities during the COVID-19 pandemic. All data was included in the final analysis. Participant characteristics are presented in Figure 1.1. Participants all consumed a similar diet across all trials (Carbohydrate: 237.36±95.36 g, Protein: 119.78±51.60 g, Fat: 74.76±34.46 g; calories: 2101.40±898.10).

Baseline Measures

Men were taller, weighted more and had more muscle, had lower body fat, had more visceral adipose tissue and a smaller waist to hip ratio than our women (Figure 1.1, $p \leq 0.001$). There were no sex differences in $\dot{V}O_{2peak}$ (Figure 1.1,

p>0.050). High fit individuals had less fat mass and less visceral adipose tissue, but a greater $\dot{V}O_{2peak}$ and mitochondrial oxidative capacity than low fit individuals (Figure 1.1, *p*>0.050). High fit men had less fat mass, less visceral adipoise tissue and a greater $\dot{V}O_{2peak}$ than low fit men (Figure 1.1, *p*≤0.050). High fit women had lower body fat and a greater $\dot{V}O_{2peak}$ than low fit women (Figure 1.1, *p*≤0.050). There were no other noted differences between groups.

High Intensity Interval Exercise Session

HIIE had a significant effect on fat *(*ANOVA*, time; p*≤0.001*)* and carbohydrate oxidation *(*ANOVA*, time; p*≤0.001) for all participants, and conditions.

Fitness Status

With participants classified according to fitness status (high n=11 vs. low n=10), there were between subjects effect of fat oxidation (ANOVA: *group*; *p*=0.035). Significant differences in exercise metabolism were noted when controlled for total body weight (Figure 1.2) and fat free mass (Supplemental Figure 1.1). Results indicated higher fat oxidation during HIIE in high vs. low fitness individuals at High intensity exercise intervals 1 (*p*=0.005) and 2 (*p*=0.039) (Figures 2a & Supplemental 1a). Moreover, high fitness individuals had higher fat oxidation AUC (Figure 1.2c *p*=0.017, Supplemental Figure 1.1c *p*=0.047), and total grams of fat oxidized across the full session (Figure 1.2e *p*=0.019, Supplemental 1e *p*=0.029) and within exercise intervals (Figure 1.2e *p*=0.019, Supplemental Figure 1.1e *p*=0.025). No differences in carbohydrate oxidation were observed between high

and low fitness groups when controlled for total body weight and fat free mass (ANOVA, *group*; *p*=0.939; AUC *p*=0.563, Figures 2b & Supplemental Figure 1.1b).

Sex

With participants classified by sex (men n=11 vs. women n=10), there was a significant within group effect for carbohydrate oxidation when controlled for total body weight (ANOVA, *group*; *p*≤0.001). When controlled for total body weight and fat free mass, we observed higher rates of fat oxidation in men vs. women at baseline (*p*=0.041), warmup (*p*=0.029) and recovery 15 minutes (p=0.038) for kg body weight (Figure 1.3a). When controlled for total body weight, carbohydrate oxidation was higher in men than women across all exercise and recovery time points (Figure 1.3b, *p*≤0.050) and AUC (Figure 1.3d *p*≤0.001), with no differences between men and women observed when controlled for fat free mass (Supplemental Figure 1.2b and 2c, AUC *p*=0.371).

Fitness Within Sex

When participants were controlled for fitness within sex (HFM=6, LFM=5, HFW=5, LFW=5) there was no effect of fitness status on fat (ANOVA, *group*, p=0.420) or carbohydrate oxidation (ANOVA, *group*, p=0.900) in men, or carbohydrate oxidation in women (ANOVA, *group*, p=0.363). However, their was a moderat effect of fitness status on fat oxidation in women (ANOVA, *group*, p=0.069). When grouped by fitness within sex, we found lower fat oxidation in low fit women compared to high fit women, and men at various time points. Furthermore, we

found lower carbohydrate oxidation in women than men across various timepoints (Figure 1.4a-d, Supplemental Figure 1.3a-d $p \leq 0.050$). When controlled for total body weight and fat free mass, fat oxidation AUC during exercise was lower in LFW compared to HFM (p=0.004), HFW (p=0.050) and LFM (p=0.010) (Figure 1.4e & Supplemental Figure 1.3e). Carbohydrate oxidation as indicated by AUC and at various time points during HIIE were lower in HFW (HFM, p=0002; LFM, p=0.017) and LFW (HFM, p≤0.001; LFM, p=0.004) compared to HFM and LFM when controlled for body weight (Figures 4f, $p \leq 0.050$). No differences were observed when expressed relative to fat free mass (Supplemental Figure 1.3b-c, 3f). Total grams of fat oxidized during the full HIIE session was significantly lower in LFW than HFW (p=0.009) and HFM (p=0.015) (Figure 1.4g and Supplemental Figure 1.3g $p \leq 0.050$). Total grams of fat oxidized during exercise intervals was significantly lower in LFW than HFW (p=0.009), HFM (p=0.016) and LFM (p=0.006) during exercise intervals alone (Figure 1.4g and Supplemental Figure 1.3g $p \leq 0.050$).

Blood Lactate

There was a significant effect of HIIE on blood lactate (ANOVA, *time*; $p \leq 0.001$). There were no significant differences in blood lactate across HIIE between high (n=11) and low (n=10) fitness status, men (n=11) and women (n=10), HFM (n=6), and LFM (n=5). There was a significantly higher blood lactate value in LFW (n=5) than HFW (n=5) at 10 min post HIIE Recovery ($p \leq 0.048$) (Figure 1.5d).

High-fat Meal Challenge

There was a significant effect of the high fat meal on fat (ANOVA, *time*; $p \leq 0.001$) and carbohydrate oxidation (ANOVA, *time*; $p \leq 0.001$) for all participants, and conditions.

Fitness

When participants were classified based on their fitness status (high n=9 vs. low n=10), we observed no effect of fitness status on postprandial fat (ANOVA, *group*; $p=0.388$) or carbohydrate oxidation (ANOVA, *group*; $p=0.700$) relative to total body weight and fat free mass (Figure 1.6a-d and Supplemental Figure 1.4a-d). Furthermore, high-fat meal challenge-evoked increases in fat oxidation and decreases in carbohydrate oxidation were significantly different from baseline in both groups (Figure 1.6a-b and Supplemental Figure 1.4a-b, $p \leq 0.05$). There were no differences in fat AUC (p=0.415) or carbohydrate oxidation AUC (p=0.690) between groups (Figure 1.6c-d and Supplemental Figure 1.4c-d).

Sex

When participants were classified by sex (men n=11 vs. women n=8), we observed no effect of sex on postprandial fat (ANOVA, *group*; $p=0.151$) or carbohydrate oxidation (ANOVA, *group*; $p=0.238$) relative to total body weight (Figure 1.7a-d) and fat free mass (Supplemental Figure 1.5a-d). However, men appeared to increase fat oxidation or decrease carbohydrate oxidation compared to baseline across more time points compared to women when controlled for body weight and

fat free mass (Figures 7a and Supplemental Figure 1.5a $p \leq 0.050$). No differences between groups were detected in carbohydrate AUC (p=0.194) or fat oxidation AUC (p=0.127) (Figures 7c-d and Supplemental Figure 1.5c-d).

Fitness Within Sex

When participants were controlled for fitness within sex (HFM=6, LFM=5, HFW=5, LFW=4) there was no effect of fitness status on fat (ANOVA, *group*, p=0.697) or carbohydrate oxidation (ANOVA, *group*, p=0.678) in men, or carbohydrate oxidation in women (ANOVA, *group*, p=0.145). However, their was a significant effect of fitness status on fat oxidation in women (ANOVA, *group*, p=0.028). Fat oxidation was lower in LFW (n=4) than HFM (n=5), LFM (n=6) and HFW (n=4) at various time points when controlled for body weight (Figures 8a $p \leq 0.050$) and fat free mass (Supplemental Figure 1.6a, $p \leq 0.050$). Fat oxidation AUC was lower in LFW compared to HFM (p=0.030), LFM (p=0.036), and HFW (p=0.030) only when expressed relative to body weight (Figure 1.8e). Carbohydrate AUC (g/kg/FFM) was significantly higher in LFW than HFM (p=0.048).

Correlations

Multiple correlations were observed in relation to fitness status, including: $\dot{V}O_{2peak}$ (mL/kg/min) and skeletal muscle mitochondrial oxidative capacity (1/sec) (Supplemental Figure 1.7a, r=0.500; p=0.018, n=21), $\dot{V}O_{2peak}$ (mL/kg/min) and fat oxidized (g) during HIIE exercise intervals (H1-H4) (Supplemental Figure 1.7b, r=0.631; p=0.004, n=21), and $\dot{V}O_{2peak}$ (mL/kg/min) and total fat oxidized (g) during

the entire HIIE session (Supplemental Figure 1.7c, r=0.554; p=0.009, n=21). Additionally, a positive relationship was observed between total fat oxidized (g) during the high-fat meal challenge and HIIE sessions (Supplemental Figure 1.7d, r=0.631; p=0.005, n=18).

Skeletal Muscle Metabolism and Whole Body Metabolism

When examining the relationship between resting energy expenditure and muscle metabolic rate, there were positive relationships between skeletal muscle oxygen uptake (1/sec) and REE for the full high-fat meal challenge (Figure 1.9a, r =0.347; p≤0.001, n =13) and at each time point up to 120 min postprandial (Figure 1.9, p≤0.050, n=13). Correlations at time points beyond 120 minute postprandial were not statistically significant.

Discussion

There are four main findings of the current study: 1) HIIE may be a more sensitive measure of detecting metabolic flexiblity between fitness status in healthy young individuals, as indicated by higher differences in fat oxidation between groups during HIIE compared to the high-fat meal challenge, 2) There are no sex differences in fat oxidation during HIIE, but when stratified by fitness status and sex, low fitness women appear to be less metabolically flexible during feeding and exercise, 3) There is a positive relationship between the ability to oxidize fat during HIIE and following a high-fat meal, and 4) Quadriceps muscle oxygen uptake is correlated with whole body REE for 2 hrs during the postprandial period.

Fitness

We found higher fat oxidation in high fitness individuals as indicated by fat oxidation AUC and total grams of fat oxidized during HIIE, with similar carbohydrate oxidation rates between high and low fitness participants (Figure 1.2a-d). These results were similar to fat and carbohydrate oxidation data expressed relative to body weight and fat free mass (Supplemental Figure 1.1a-d). While ability to oxidize fat is thought to be decreased above 65-70% $\dot{V}O_{2peak}$ [88], current data challenges this by displaying an increase capacity to oxidize fat across the entire exercise period in high fitness individuals (Figure 1.2a, 2c Supplemental Figure 1.1a, 1c). Furthermore, we found that aerobic fitness status as measured by $\dot{V}O_{2peak}$ was strongly correlated with total fat oxidized during the entire HIIE session and each high intensity exercise interval (Supplemental Figure 1.7c-d). Our data are consistent with two previous studies utilizing similar high intensity interval exercise in males, indicating that despite similar carbohydrate oxidation rates (g/min) in high- and low-fitness groups, a high level of aerobic fitness is associated with a significantly higher utilization of fat oxidation [9, 10]. Our present findings with these previous data support the notion that trained individuals have a greater ability to oxidize fat during HIIE while maintaining similar energy supply from anaerobic energy systems than their lower fitness counterparts (Figure 1.2 and Supplemental Figure 1.1). This is likely due to training-induced adaptations such as improved mitochondrial content, oxidative capacity, and delivery, as reflected in significantly greater mitochondrial oxidative capacity in the high fitness group (Figure 1.1) and positive relationship between mitochondrial

oxidative capacity and $\dot{V}O_{2max}$ (Supplemental Figure 1.7a). These adaptations result in improved fuel utilization at whole body, muscle, and mitochondrial level [166]. Notably, lactate concentrations increased significantly throughout HIIE to a similar extent in high and low fitness groups (Figure 1.5a). These data, taken with higher work output, increased $\dot{V}O_{2peak}$, and higher fat oxidation in high compared to low fitness, collectively suggest that the increased capacity for work during HIIE in high fitness is associated with increased oxidative capacity, but not necessarily a concomitant reduction of the glycolytic system's contribution to ATP supply.

Interestingly, we found no notable differences in metabolism during the high-fat meal challenge between fitness status groups when controlled for total body weight or fat free mass (Figure 1.6 and Supplemental Figure 1.4). These data are not consistent with previous findings suggesting that lower fitness status or activity levels are associated with greater metabolic flexibility and lower capacity to oxidize fat in a postprandial state [5, 6, 8]. We speculate this discrepancy is due to participants being young, healthy, and normal weight status [167]. Since our study is the first to our knowledge to compare postprandial and exercise metabolic flexibility, it is worth noting that we found a significant relationship between total grams of postprandial fat oxidation and fat oxidized during HIIE (Supplemental Figure 1.7b). These data suggest that these two measures may be related. We speculate that the exercise test may be a stronger assessment of underlying skeletal muscle mitochondrial oxidative capacity to switch fuel efficiently in response to a metabolic stressor in younger healthy populations. It has been

proposed that mitochondria act as regulators of substrate metabolism and that dysfunction either precedes or parallels impairments whole body and skeletal muscle oxidation [3, 168]. While the larger literature focuses on metabolic flexibility in response to feeding, the concept is less often assessed during exercise [2]. Here we find that there are notable impairments in exercise fat oxidation in low fit individuals compared to their more fit counterparts (Figure 1.2 and Supplemental Figure 1.1), suggesting that an exercise test such as HIIE may be an alternative test for metabolic flexibility.

Sex

Another key finding of our current study is that neither fat nor carbohydrate oxidation were different between men and women during HIIE (Figure 1.3 and Supplemental Figure 1.2) or in response to a high-fat meal challenge (Figure 1.7 and Supplemental Figure 1.5) when controlled for kg FFM or total body weight. This may be explained in part by fact that women were tested during the onset of their menstrual cycle, where estrogen and progesterone levels are at their lowest and more similar to men [15], or the intensity of the exercise [88]. Many studies show increased fat oxidation during exercise in women, opposite of what we see here [12, 13, 169]. However, they are often carried out using moderate intensity (<65-75% $\dot{V}O_{2max}$), continuous or incremental exercise protocols. As fat oxidation appears to diminish at higher relative work rates [81], sexes differences may be minimal at higher power outputs required during HIIE. Additionally, future research is necessary to examine the impact of menstrual cycle status on metabolic

flexibility. All together, it appears that during HIIE sex does not impact exercise fat or carbohydrate oxidation.

Fitness × Sex Interactions

Our current data indicate that a lower fitness status in females may be more detrimental to fat oxidation capacity during HIIE and high-fat meal challenge (Figures 4, 7 and Supplemental figures 3 and 6) than men. More specifically, low fitness status appears to impact metabolic flexibility more negatively in women, with highly trained women showing a more favorable metabolic response comparable to both subsets of men. An impaired ability to oxidize fat at rest, in the postprandial state, or during exercise may result from impaired endocrine and mitochondrial capacity [3]. Prior work has indicated that women exhibit a higher postprandial rate of glucose appearance and higher total glycemic AUC in response to a glucose challenge [170]. While Basu, Dalla Man [170] did not control for fitness, women had a $\dot{V}O_{2peak}$ comparable to LFW in the present report (34.6±11 mL/kg/min). Moreover, $\dot{V}O_{2peak}$ of men (42.9±1.2 mL/kg/min) was more similar to that of HFM and HFW in the present study (Figure 1.1) [170]. Although our study did not assess glucose turnover, these data may in part explain the higher carbohydrate oxidation relative to total lean mass (g/kg FFM) in LFW (Figures 4c-d, 4f and supplemental figures 3c-d, 3f).

A number of physiological characteristics of the LFW group may contextualize our metabolic findings. LFW had a significantly higher body fat coupled with the lowest

lean tissue mass compared to all groups. Due to the collective impact of adiposity, low muscle mass, and poor mitochondrial function on metabolic flexibility [1, 3], these data may partly explain our findings of lower fat oxidation during HIIE in LFW. Additionally, skeletal muscle mitochondrial oxidative capacity was lower in LFW compared to that of HFW (Figure 1.1). While muscle mitochondrial oxidative capacity was not statistically different, it still may be reflective of lower mitochondrial oxidative capacity in LFW and a result of low sample size in our populations (Figure 1.1). Importantly, differences within sex and fitness were largely preserved when normalizing substrate oxidation data to fat free mass compared to when expressed relative to total body mass (Figures 4, 7 and Supplemental figures 3 and 6). Therefore, it appears that impaired metabolic flexibility of the LFW group reflects differences in fitness status and oxidative capacity of muscle, and not simply lower absolute amount of lean tissue observed in LFW relative to other study groups.

An interesting finding of our study was that there was a significantly higher carbohydrate oxidation rate in men during HIIE compared to women controlled for total body weight, but not fat free mass alone (Figures 4c-d, 4f and Supplemental figures 3c-d, 3f). This may be due to higher muscle mass in men, which has been shown to be more glycolytic [12]. Men have a greater reliance on carbohydrate at a whole body level during rest and exercise than women [107, 167, 171]. Furthermore, estrogen (E_2) supplementation has been shown to lower glucose kinetics and preserve muscle glycogen in men [107]. Previous data have found

lower rates of glucose appearance, disappearance, and metabolic clearance rate and higher fat oxidation in women in their follicular phase than men of similar fitness status while cycling for 90 minutes at 65% VO_{2peak} [12]. Additionally, data in untrained men and women have shown that men oxidized a higher percentage of total substrate from carbohydrate while cycling at 50% VO_{2peak} for 90 minutes than their female counterparts [13]. Lastly, men also appear to have a higher sympathetic nervous system response and downstream breakdown of muscle and hepatic glycogen stores during exercise compared to women [11]. These data are consistent with our findings of higher glucose oxidation when control for total body weight in men than women (Figures 4c-d, 4f, and Supplemental figures 3c-d, 3f), supporting higher carbohydrate metabolism (g/kg) in men compared to women.

Resting Muscle Oxygen Uptake Assessments

Lastly, our study is the first to our knowledge to use CW-NIRS to track skeletal muscle oxygen uptake following a high-fat meal challenge to assess localized muscle oxygen uptake (Figure 1.9). We found a significant positive correlation between skeletal muscle oxygen uptake and REE and across the entire high-fat meal challenge (Figure 1.9a). However, it appears this relationship is stronger for baseline and 30 - 120 min postprandially (Figures 10b-f). Our data showing skeletal muscle oxygen uptake and whole body REE are related across during a high-fat meal challenge reflects the important role of skeletal muscle in whole body metabolism and metabolic flexibility [1]. Furthermore, previous data showing skeletal muscle characteristics of the forearm are related to whole body energy

expenditure and substrate oxidation [172, 173], with ours, further suggest that resting muscle metabolism play a role in whole body metabolism during the postprandial period. Low metabolic rate and flux may be risk factors for weight gain and long term detriments to metabolic health [38, 172], reflecting the value of a non-invasive measure to assess muscle metabolism in response to feeding. While further group analysis was limited by our subset of participants (n=12), this approach may be a way to assess differences in muscle metabolism kinetics during the postprandial period.

Limitations

One limitation of our current study is that we had no biomarker measures of circulating estrogen or progesterone in our female participants. Additionally, we only tested women during the first half of the menstrual cycle. While we attempted to control menstrual cycle timing by using the onset of menses as our physiological indicator of time during phase, differences in phase length, ovulation, or irregularities in cycle were not assessed [15]. Furthermore, a notable limitation is the small sample size in the sub-group analysis between fitness within sex groups. While the cessation of research activities limited our testing and recruitment in March 2020 due to the COVID-19 pandemic, a larger sample size may have yielded stronger trends between these groups and clarified our results. Lastly, while previous previous work has shown the use of indirect calorimietry accurately reflects substrat oxidation at higher intensities [157], the authors acknowledge the limits on quantifiying carbohydrate oxidation during high intensity due to excess

CO_2 production. The work by Romijn et al. demonstrated the viability of using indirect calorimetry upward of 80-85% VO_{2max}, with our average %VO2 for each exercise interval being well below this established limit at 69±12%. Lastly, this impact appears to be minimized when controlling data for body weight and does not impact fat oxidation assessments presented here. Future literature should work to develop exercise substrate equations for high intensity exercise that account for these differences.

Further work is needed to determine the differences in substrate metabolism between sex groups and across the menstrual cycle. Additionally, impaired metabolic flexibility in response to high-fat feeding has been associated with future weight gain. Future research is necessary to determine the impacts of an impaired capacity for fat oxidation during exercise on long-term adiposity and insulin resistance [2, 38]. Lastly, our NIRS assessment of muscle metabolism during the postprandial period may be limited by only testing a limited subset of participants. Our goal was to test the feasibility of this method alongside indirect calorimetry and should be replicated before firm conclusions can be drawn about the contribution of skeletal muscle to the postprandial increase in whole body energy expenditure following a high-fat meal challenge.

Conclusions

In summary, we found for the first time that capacity to oxidize fat during HIIE and in response to a high-fat meal challenge are related. Furthermore, our HIIE

protocol was more sensitive than the high-fat meal challenge in detecting metabolic flux between fitness status groups and carbohydrate metabolism between sex. Furthermore, that while there were no sex differences in HIIE or high-fat meal challenge lipid oxidation, it appears that low fitness status may be more detrimental to metabolic flexibly in women than men. Lastly, we demonstrated via non-invasive measures (CW-NIRS) the relationship between skeletal muscle metabolism and whole body resting and postprandial energy expenditure, highlighting the important role muscle metabolism plays on whole body metabolism. Overall, our study suggests that substrate utilization during an HIIE protocol may be an indicator of metabolic flexibility. The authors suggest adapting exercise-based metabolic flexibility testing as an alternate or additional way to assess full body metabolism. Specifically, due to the unique stress exercise presents to the muscle metabolic system as a way to capture the effects of training status on underlying metabolic flexibility, an exercise test may be more useful in detecting poor metabolic flexibility in otherwise healthy populations. Future research should explore the link between exercise metabolic flexibility and long term metabolic health.

COMPETING INTERESTS

N.T.J. reports consultancies with CrossFit, Inc. and Renaissance Periodization, LLC separate from the submitted work.

AUTHOR CONTRIBUTIONS

Experiments were conducted in the Integrative Cardiovascular Physiology Laboratory located in the Department of Kinesiology at the University of Georgia. A.A.O. and N.T.J. conceived and designed the research. A.A.O. collected data. A.A.O. and N.T.J. analyzed the data. All authors contributed to interpretation of the results. A.A.O. and N.T.J. drafted the manuscript. All authors edited and revised manuscript. All authors have read and approved the final version of the manuscript and agree to be accountable for all aspects of the work in ensuring the questions related to the accuracy or integrity of any part of the work are appropriately investigated and resolved. All persons designated as authors qualify for authorship, and all those who qualify for authorship are listed.

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Table 1. Participant Characteristics. Data represented as mean(SD). [a]=Significantly different between groups p≤0.050., [b]=Significantly different than HFM p≤0.050 b , [c]=significantly different than LFM p≤0.050 c, HFM, high fitness men; LFM, low fitness men; HFW, high fitness women; LWF, low fitness women.

Measure	Overall	Sex			Fitness			Fitness within Sex					
		Men	Women	*p*	high	low	*p*	HFM	LFM	*p*	HFW	LFW	*p*
n (M/W)	22	12	10		11 (6/5)	11 (6/5)		6	5		5	5	
Age (years)	23.00 (3.8)	23.17 (4.5)	22.90 (3.3)	*0.879*	23.81 (4.6)	22.26 (3.2)	*0.373*	25.00 (5.7)	21.80 (2.0)	0.271	22.20 (2.5)	23.60 (4.0)	*0.537*
Height (cm)	172.41 (7.8)	177.15 (6.3)[a]	166.73 (6.8)[a]	*0.001*	172.66 (5.9)	172.16 (10.9)	*0.892*	175.9 (6.3)	178.36 (6.8)	0.577	170.6 (3.7)	162.86 (7.3)[bc]	*0.070*
Weight (kg)	76.24 (12.7)	84.43 (11.7)[a]	66.41 (7.4)[a]	*0.001*	74.87 (6.5)	77.61 (5.8)	*0.654*	79.41 (4.9)[b]	92.69 (13.3)[c]	0.081	68.33 (4.9)[bc]	64.49 (8.8)[bc]	*0.444*
Fat Free Mass (kg)	57.92 (11.9)	66.39 (10.0)[a]	47.75 (5.4)[a]	*0.001*	58.41 (8.2)	57.42 (16.9)	*0.858*	63.61 (6.5)[b]	71.66 (12.7)[c]	0.202	50.54 (2.8)[bc]	44.95 (6.2)[bc]	*0.105*
Fat Mass (kg)	18.32 (4.3)	18.04 (4.2)	18.66 (3.8)	*0.728*	16.46 (3.8)a	20.18 (3.3)a	*0.024*	15.80 (4.8)	21.03 (1.9)	0.042	17.78 (3.4)	19.53 (4.4)	*0.510*
Body Fat (%)	23.60 (5.3)	20.89 (3.9)[a]	26.99 (4.2)[a]	*0.001*	21.43 (4.4)	25.93 (5.1)	*0.817*	19.68 (4.6)	22.98 (2.3)	*0.197*	25.92 (3.5)[ab]	30.14 (4.2)[abc]	*0.012*
Visceral Adipose Tissue (g)	242.15 (94.2)	290.40 (87.3)[a]	181.85 (56.8)[a]	*0.002*	227.70 (80.0)[a]	277.00 (94.0)[a]	*0.027*	246.6 (81.3)	353.8 (68.9)[c]	*0.045*	194.2 (64.8)[c]	211.0 (53.6)[c]	*0.667*
Waist-to-Hip Ratio	1.28 (0.1)	1.22 (0.1)[a]	1.34 (0.1)[a]	*0.001*	1.27 (0.1)	1.28 (0.1)	*0.812*	1.24 (0.0)[b]	1.18 (0.1)[c]	*0.258*	1.34 (0.0)[c]	1.33 (0.1)[bc]	*0.999*
$\dot{V}O_{2peak}$*(mL/kg/min)*	40.92 (6.5)	42.79 (7.8)	38.65 (5.4)	*0.173*	46.64 (4.5)[a]	35.19 (2.4)[a]	*0.001*	49.21 (3.9)a[b]	37.90 (4.4)a[c]	0.001	43.10 (3.7)a[bc]	34.20 (1.6)a[bc]	*0.002*
Mitochondria oxidative capacity (1/sec)	1.56 (0.6)	1.66 (0.8)	1.42 (0.4)	*0.485*	1.85 (0.8)[a]	1.20 (0.2)[a]	*0.040*	2.00 (1.00)	1.24 (0.2)	0.115	1.75 (0.6)	1.17 (0.1)	*0.104*

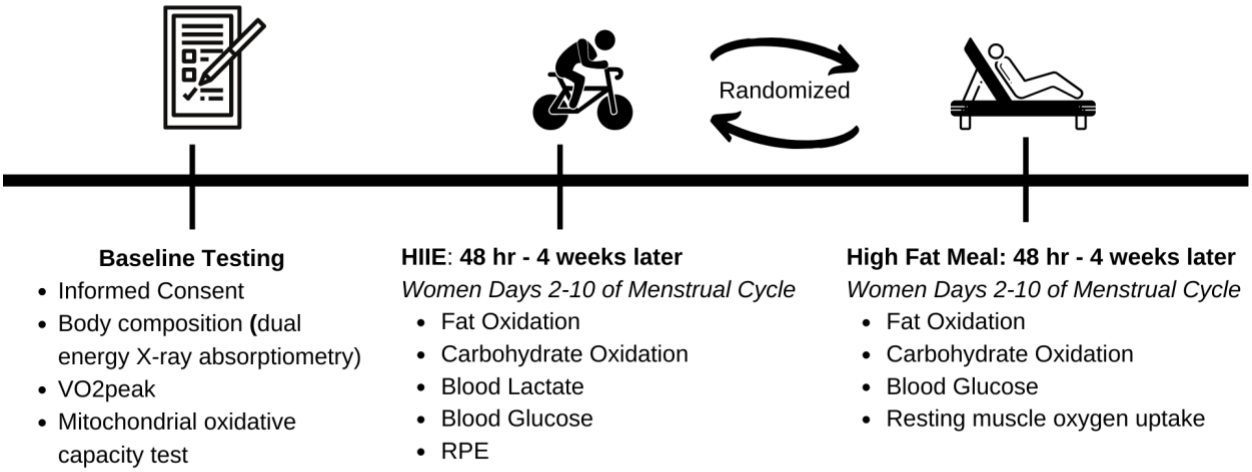


Figure 1.1 Study Timeline

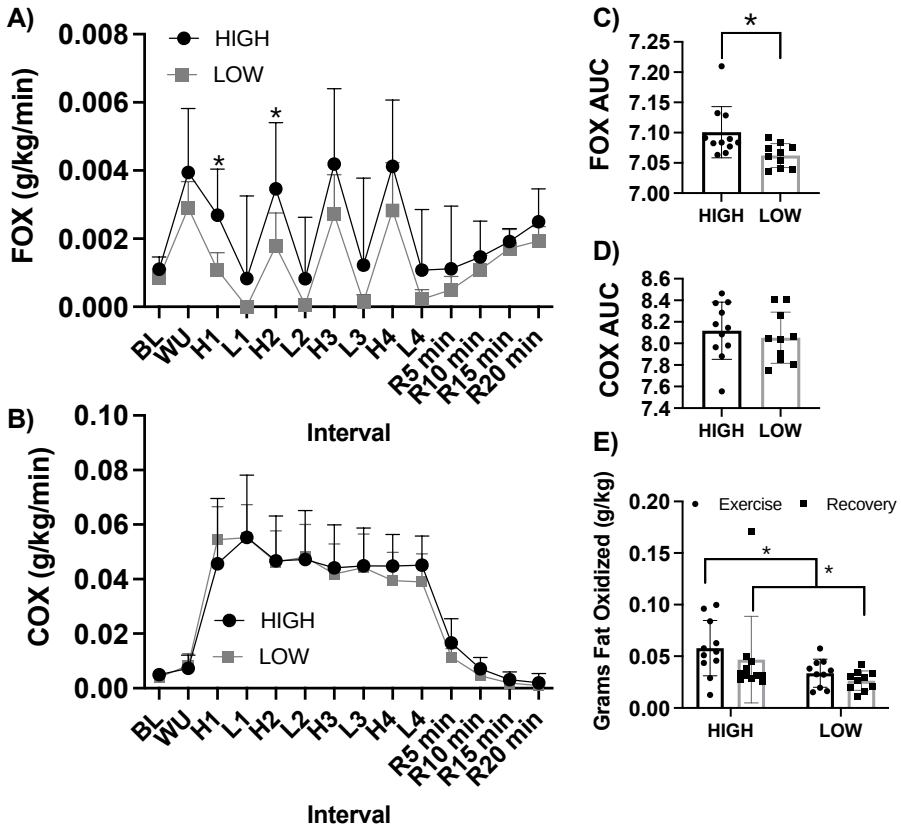


Figure 1.2. Baseline, exercise and recovery fat and carbohydrate oxidation in response to HIIE by fitness status controlled by kg total body weight (high n=11, low n=10) (A) Fat oxidation (g/kg/min) (ANOVA: time, p≤0.001, group, p=0.035) (B) Carbohydrate oxidation (g/kg/min) during HIIE (ANOVA: time, p≤0.001) (C) Fat oxidation AUC during HIIE (Students paired t test: p=0.017) (D) Carbohydrate oxidation (g/kg/min) and AUC during HIIE (ANOVA: time, p≤0.001) (E) Total grams of fat oxidized (g/kg) during HIIE stratified by during exercise alone (Students paired t test, p=0.019) and exercise and recovery (Students paired t test, p=0.019). HIIE, high intensity interval exercise; FOX, fat oxidation; COX, carbohydrate oxidation; BL, baseline, WU, warmup; H1-4, HIIE 1-4; L1-4, low intensity intervals; R5-15, post exercise recovery minutes 5-15. *P≤0.05 high vs. low fitness status.

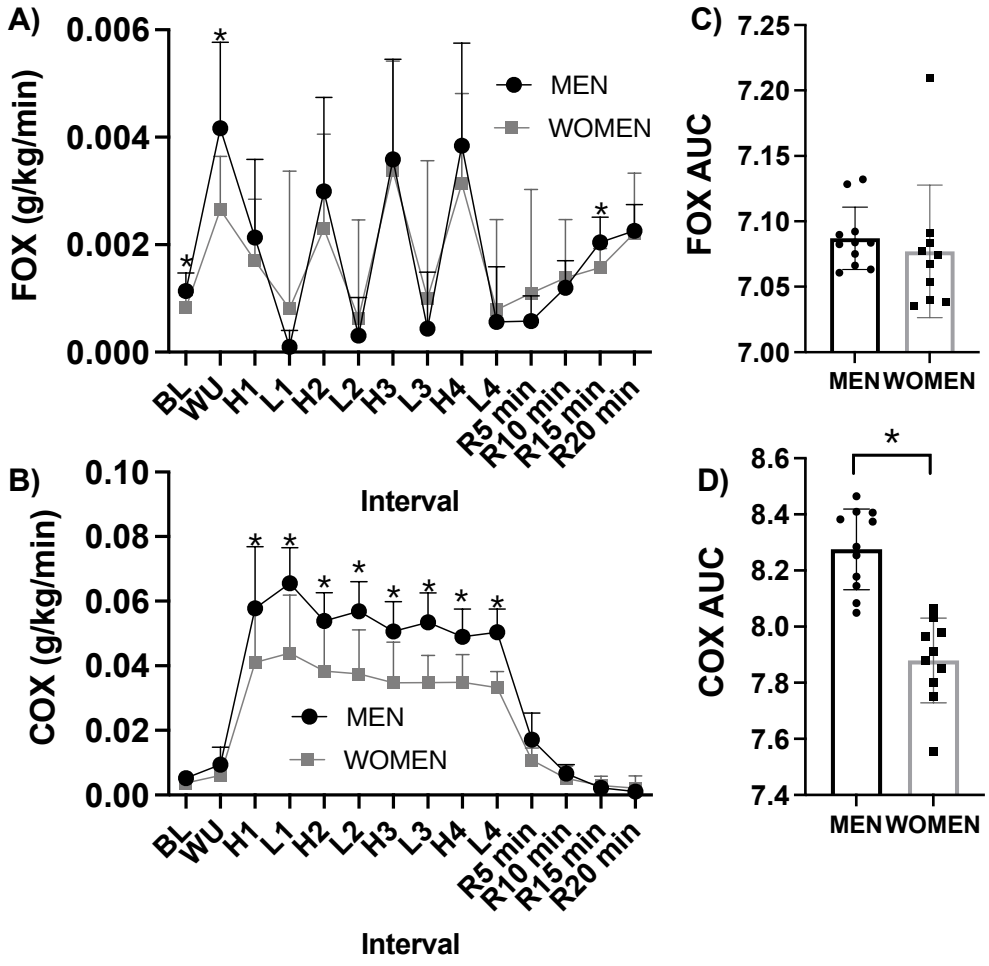


Figure 1.3. Baseline, exercise and recovery fat and carbohydrate oxidation in response to HIIE by sex controlled for by kg total body weight (men n=11, women n=10) (A) Fat oxidation (g/kg/min) during HIIE (ANOVA: *time*, $p \leq 0.001$) (B) Carbohydrate oxidation (g/kg/min) (ANOVA: *time*, $p \leq 0.001$) (C) Fat oxidation AUC during HIIE (D) Carbohydrate AUC during HIIE (Students paired *t* test: $p \leq 0.001$) HIIE, high intensity interval exercise; FOX, fat oxidation; COX, carbohydrate oxidation; BL, baseline, WU, warmup; H1-4, HIIE 1-4; L1-4, low intensity intervals; R5-15, post exercise recovery minutes 5-15. *$p \leq 0.05$ Men vs. Women

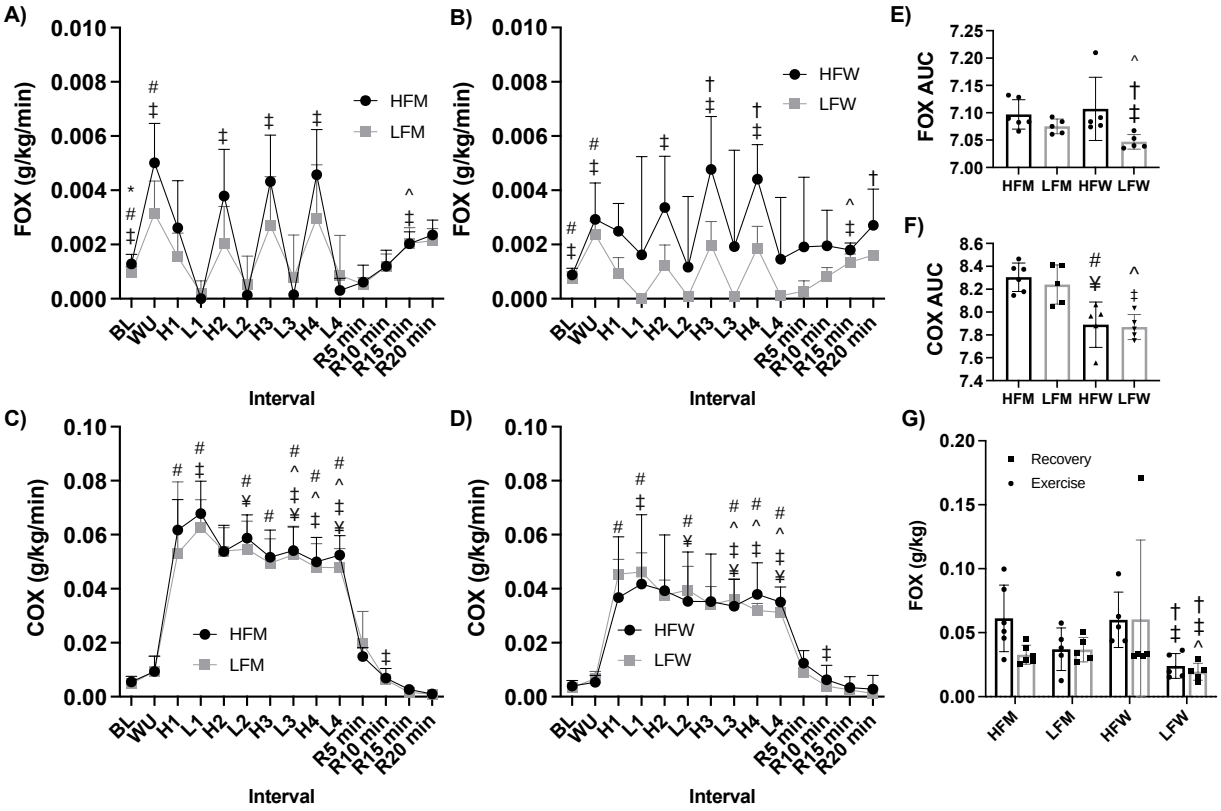


Figure 1.4. Baseline, exercise and recovery fat and carbohydrate oxidation in response to HIIE by fitness status controlled for by kg body weight (HFM n=6, LFM n=5, HFW n=5, LFW n=5) (ANOVA; *time, p*≤0.001, n=21) (A) Fat oxidation (g/kg/min) during HIIE for men (ANOVA: *time*, *p*≤0.001) and (B) Fat oxidation (g/kg/min) during HIIE for women (ANOVA: *time*, *p*≤0.001; *group, p*=0.069) (C) Carbohydrate oxidation (g/kg/min) during HIIE for men (ANOVA: *time*, *p*≤0.001) and (D) Carbohydrate oxidation (g/kg/min) during HIIE for women (ANOVA: *time*, *p*≤0.001) (E) Fat oxidation AUC (Students paired *t* test: *p*≤0.05) (F) Carbohydrate oxidation AUC (Students paired *t* test: *p*≤0.05) (G) Total grams of fat oxidized (g/kg) during HIIE stratified by during exercise alone (Students paired *t* test: *p*≤0.05) and exercise and recovery (Students paired *t* test: *p*=0.050). HIIE, high

intensity interval exercise; FOX, fat oxidation; COX, carbohydrate oxidation; HFM, high fitness men; LFM, low fitness men; HFW, high fitness women; LFW, low fitness women; BL, baseline, WU, warmup; H1-4, HIIE 1-4; L1-4, low intensity intervals; R5-15, post exercise recovery minutes 5-15. *$p \leq 0.05$ *HFM VS. LFM,* #$p \leq 0.05$ *HFM VS. HFW*, ^$p \leq 0.05$ *LFM VS. LFW*, †$p \leq 0.05$ *HFW VS. LFW*, ‡$p \leq 0.05$ *HFM VS. LFW*, ¥$p \leq 0.05$ *LFM VS. HFW*

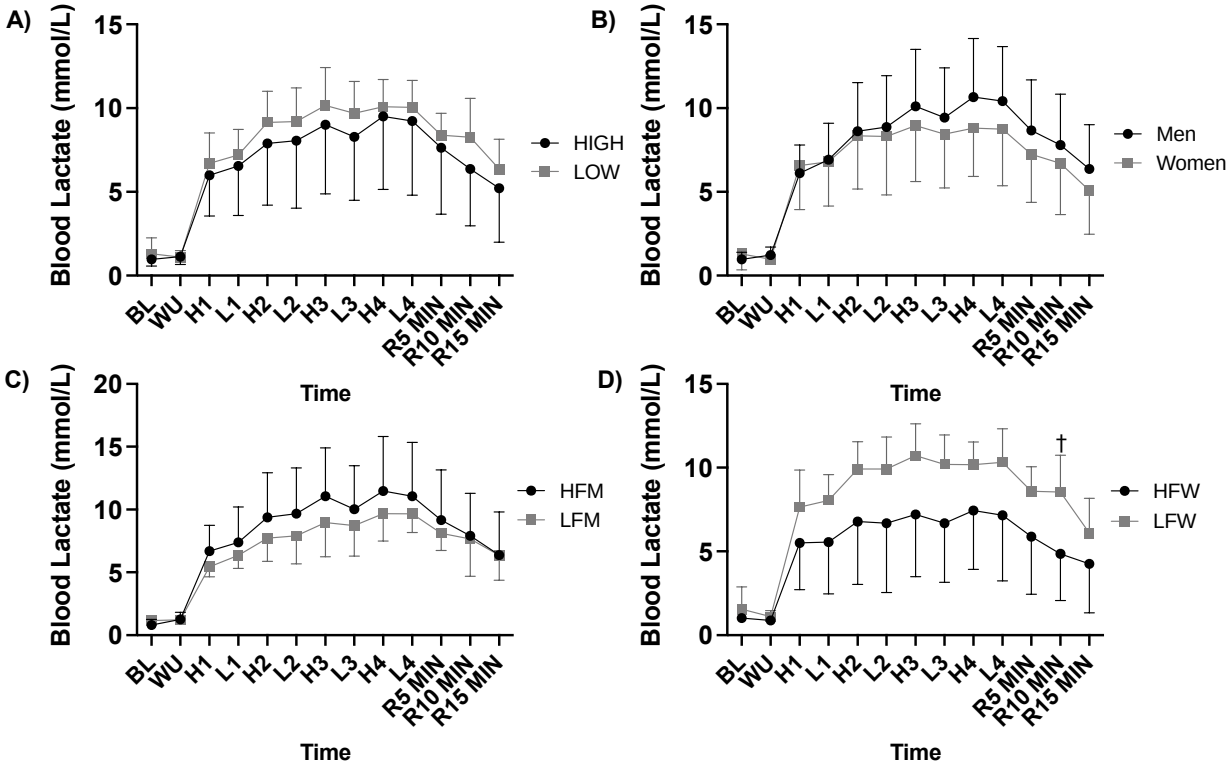


Figure 1.5. Baseline, exercise and recovery blood lactate in response to HIIE (ANOVA; *time* p≤0.001) by (A) fitness status (high n=11, low n=10) (B) Sex (men n=11, women n=10) and fitness within sex for (C-D) men and women (HFM n=6, LFM n=5, HFW n=5, LFW n=5). HIIE, high intensity interval exercise; HFM, high

fitness men; LFM, low fitness men; HFW, high fitness women; LFW, low fitness women; BL, baseline, WU, warmup; H1-4, HIIE 1-4; L1-4, low intensity intervals; R5-15, post exercise recovery minutes 5-15. † $p \leq 0.05$ HFW VS. LFW

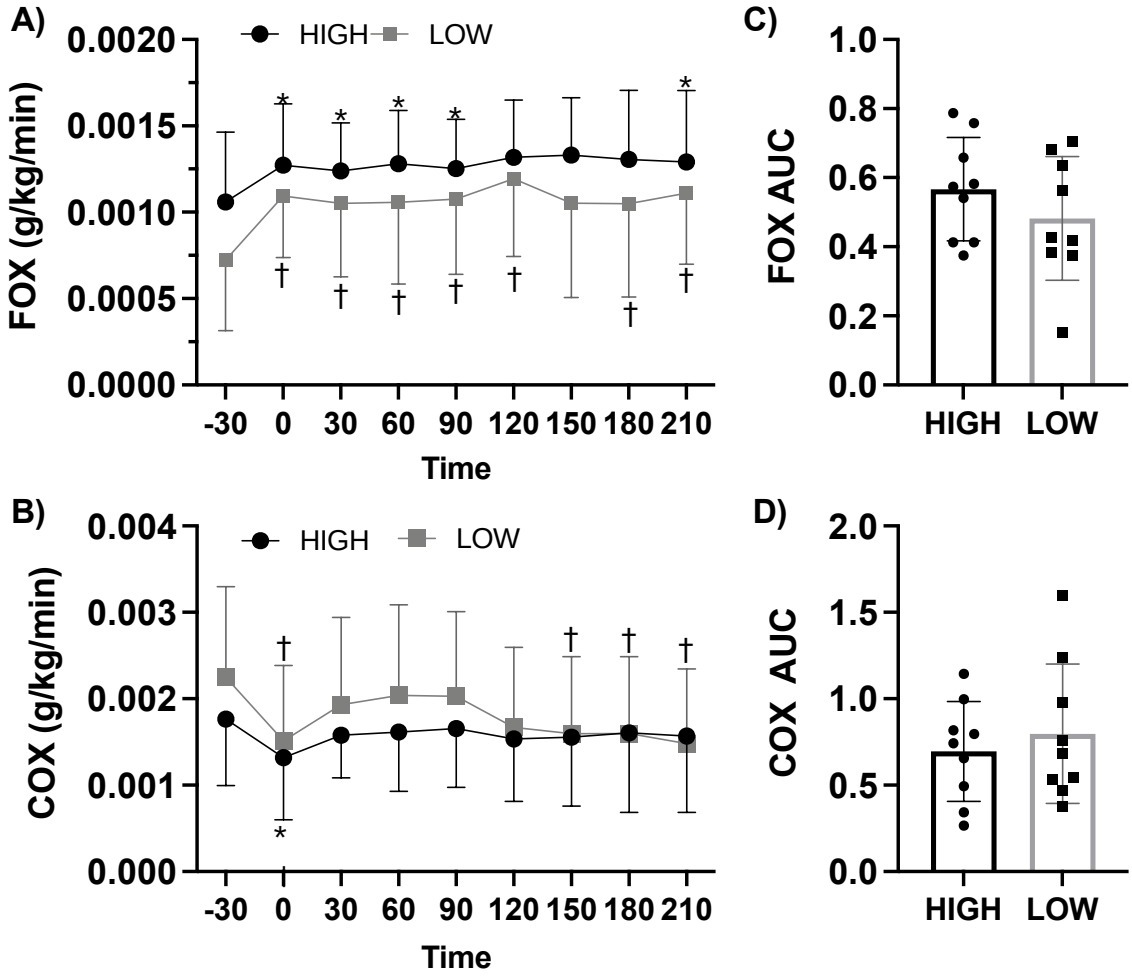


Figure 1.6. Baseline and postprandial response to a high-fat meal challenge by Fitness Status controlled for total kg body weight (high n=9, low n=10) (ANOVA; time p≤0.001, n=19) (A) Fat oxidation (g/kg/minute) (ANOVA: time, p≤0.001) (B) carbohydrate oxidation (g/kg/minute) (ANOVA: time, p≤0.001) (C) fat oxidation

AUC (D) and carbohydrate oxidation AUC. FOX, fat oxidation; COX, carbohydrate oxidation; AUC, area under the curve. *$p \leq 0.05$ compared to baseline (-30 minute) *for* high fitness status. † $p \leq 0.05$ compared to baseline (-30 minute) *for low fitness status.*

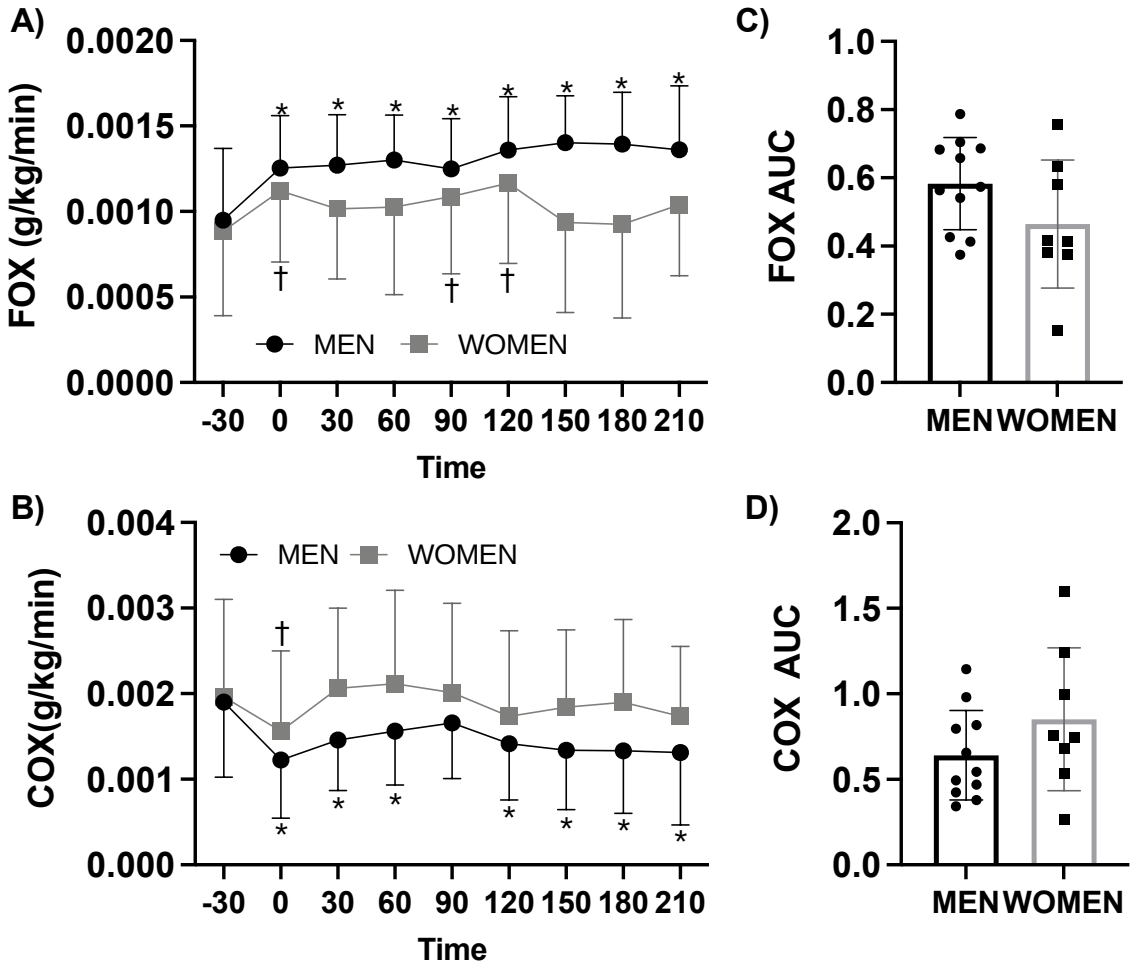


Figure 1.7. Baseline and postprandial response to high-fat meal challenge by Sex controlled for total kg body weight (Men n=11, Women n=8) (ANOVA; *time,* p≤0.001, n=19). (A) Fat oxidation (g/kg/minute) (ANOVA: *time*, p≤0.001; *group*, p=0.007) (B) carbohydrate oxidation (g/kg/minute) (ANOVA: *time*, *p*≤0.001) (C) Fat

oxidation AUC and (D) Carbohydrate oxidation AUC. HFM, high-fat meal challenge; FOX, fat oxidation; COX, carbohydrate oxidation; AUC, area under the curve. *p≤0.05 compared to baseline (-30 minute) *for Men.* † p≤0.05 compared to baseline (-30 minute) for *women*.

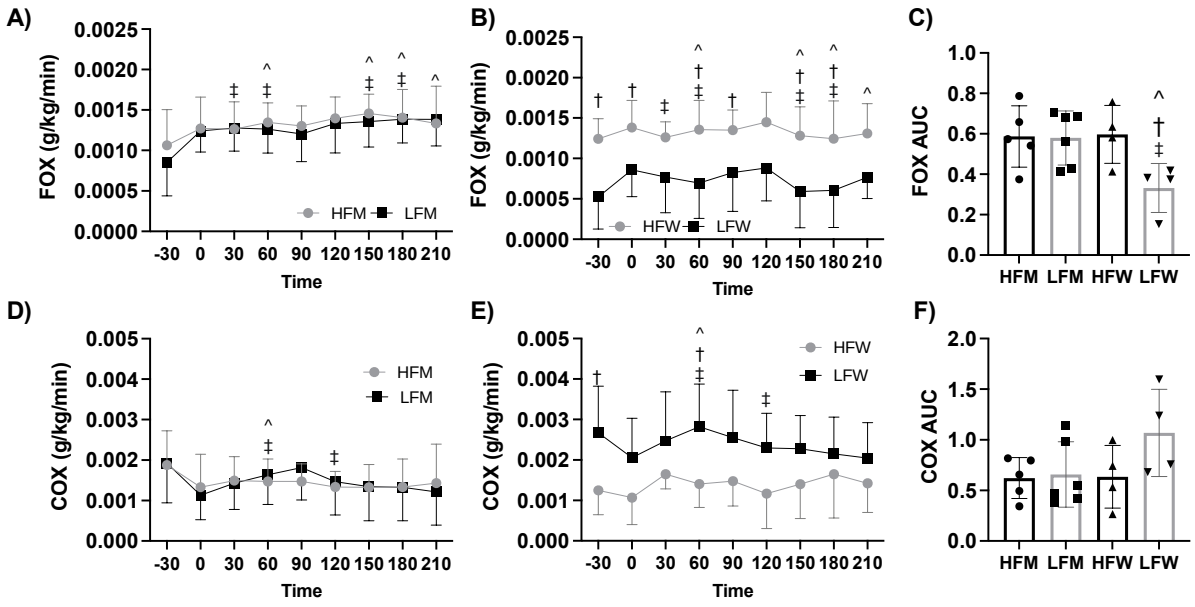


Figure 1.8. Baseline and postprandial response to high-fat meal challenge by fitness within sex controlled for total kg body weight (HFM n=5, LFM n=6, HFW n=4, LFW n=4) (ANOVA; *time* p≤0.001, n=19). (A) fat oxidation (g/kg/min) (ANOVA: *time*, p≤0.001, *group*, p=0.050) (B) carbohydrate oxidation (g/kg/min) (ANOVA: *time*, p≤0.001) (C) Fat oxidation AUC (Students paired *t* test: p≤0.05) and (D) Carbohydrate oxidation AUC (Students paired *t* test: p≤0.05). FOX, fat oxidation; COX, carbohydrate oxidation; AUC, area under the curve. **p≤ 0.05 HFM VS. LFM, # p≤0.05 HFM VS. HFW*, ^*p≤ 0.05 LFM VS. LFW*, †*p≤ 0.05 HFW VS. LFW*, ‡*p≤ 0.05 HFM VS. LFW*, ¥*p≤0.05 LFM VS. HFW*

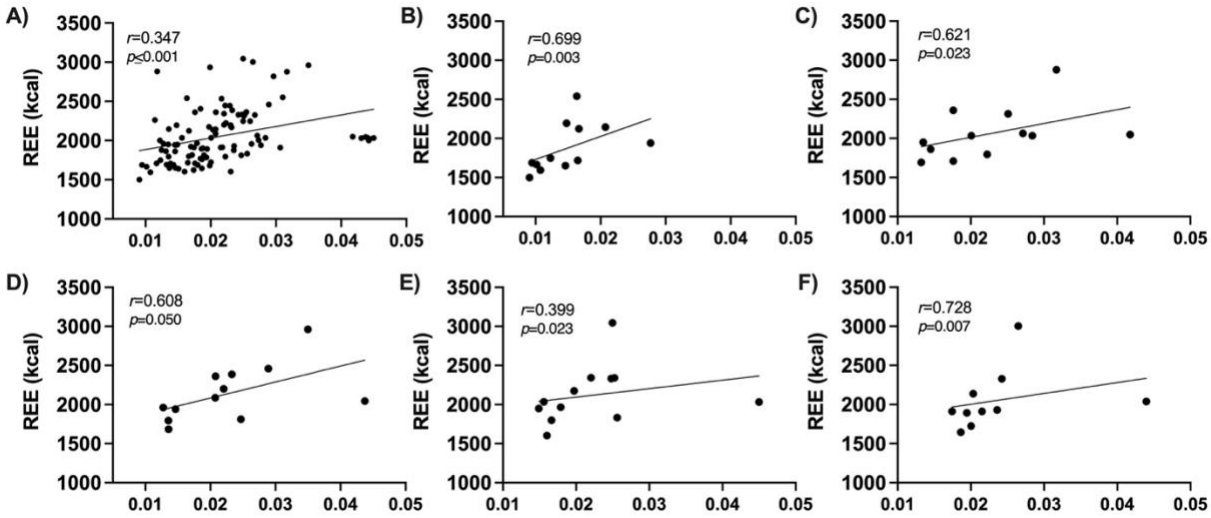
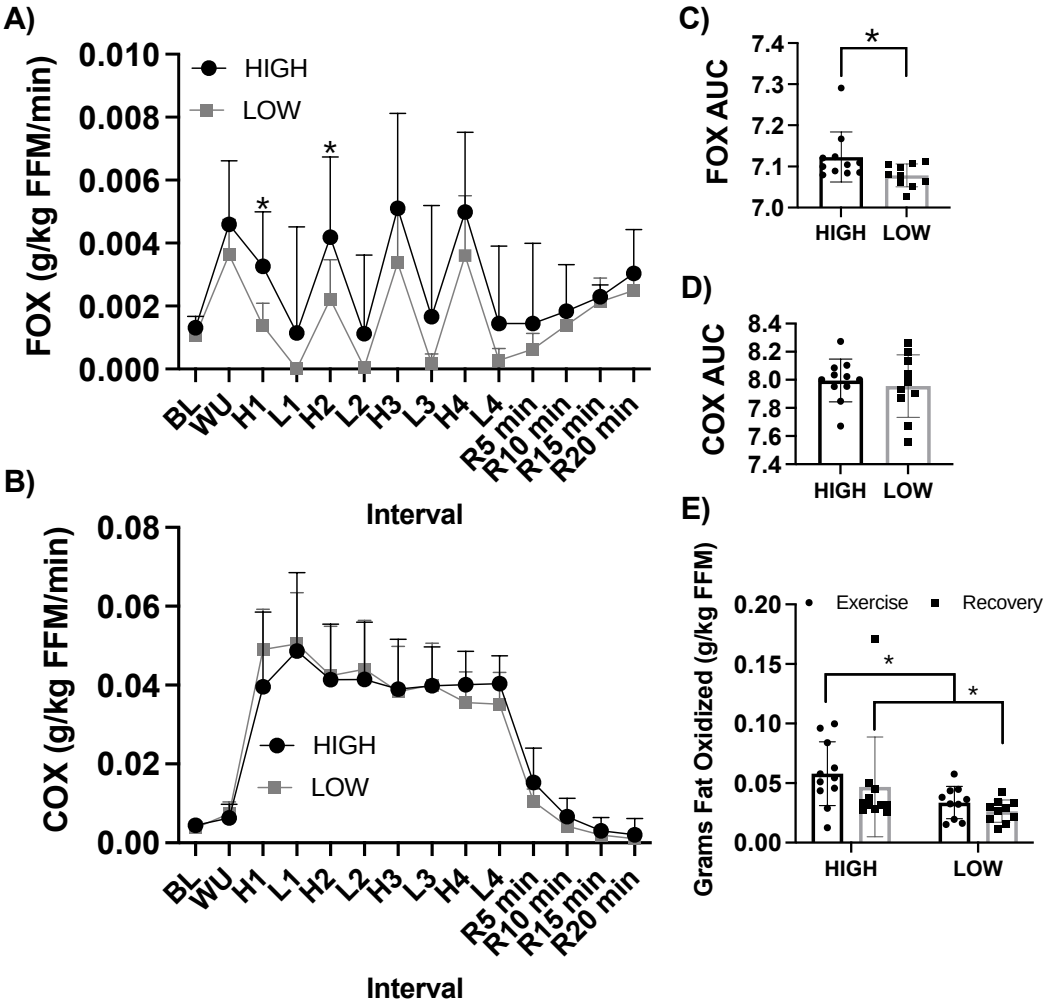
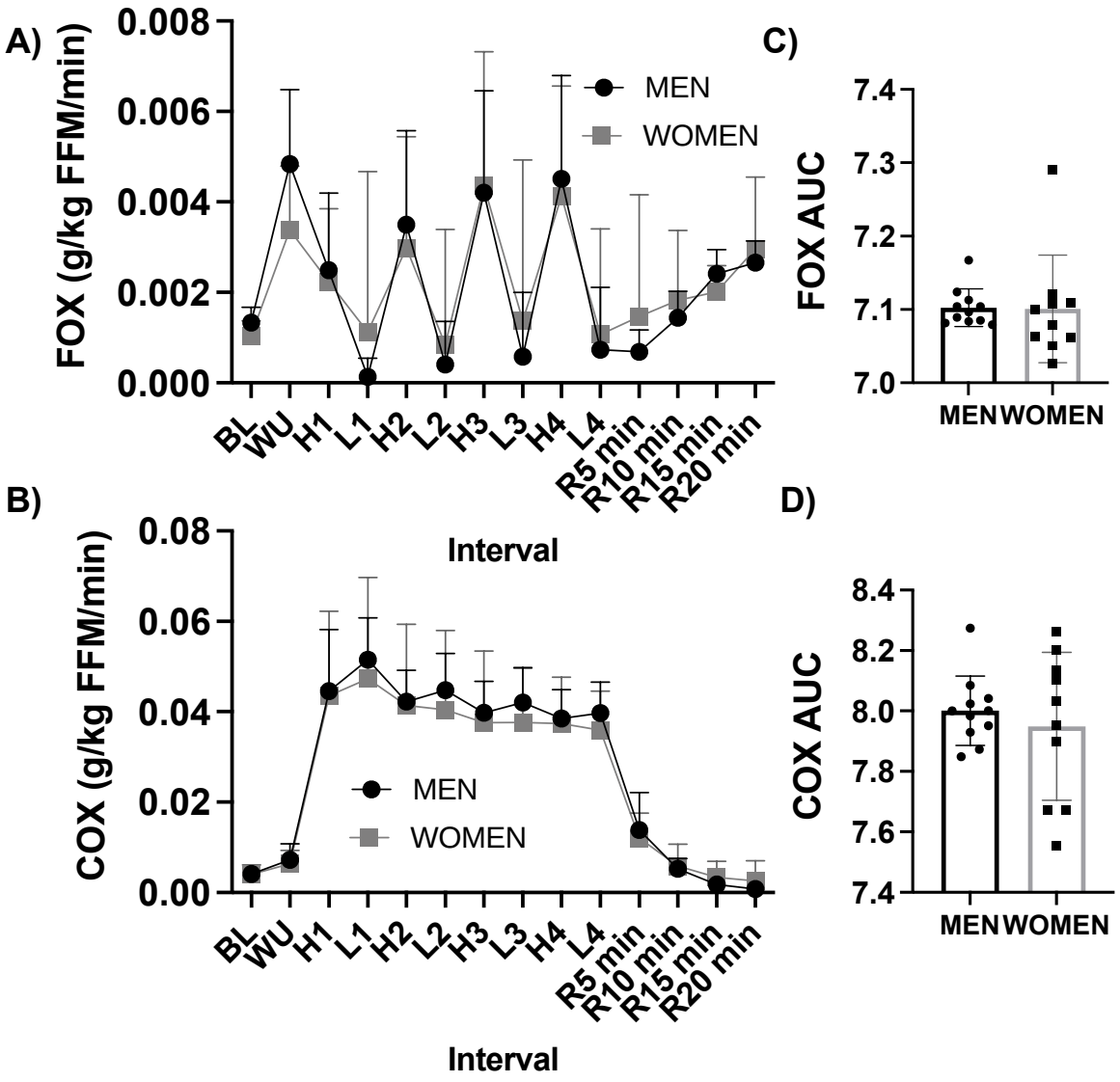


Figure 1.9. Correlations between resting energy expenditure and muscle metabolism (1/sec) across the (A) full high-fat meal challenge session (r=0.347, p≤0.001; n=12) (B) at baseline (-30 minute) (r=0.699, p=0.003; n=12) (C) at 30 min postprandial (r=0.621, p=0.023; n=12) (D) at 60 minute postprandial (r=0.608, p=0.050; n=12) (E) at 90 minute postprandial (r=0.399, p=0.023; n=12) (F) at 120 minute postprandial (r=0.728, p=0.007; n=11).



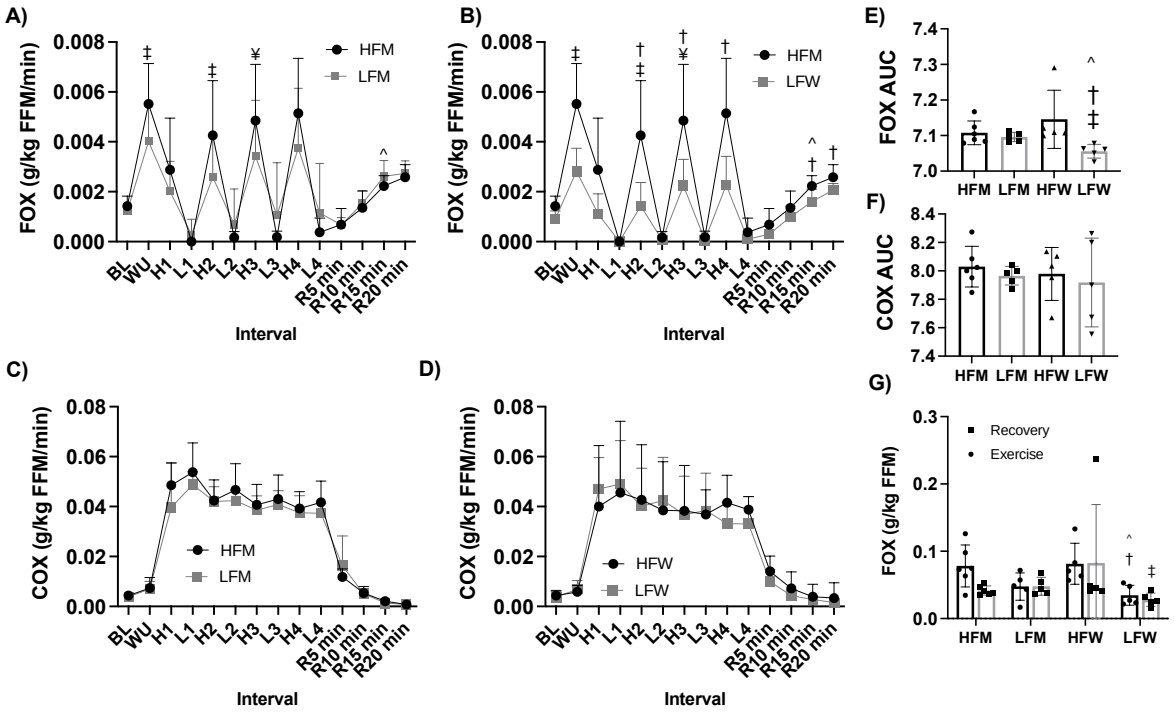
Supplemental Figure 1.1. Baseline, exercise and recovery fat and carbohydrate oxidation in response to HIIE by fitness status controlled by kg fat free mass (high n=11, low n=10) (A) Fat oxidation (g/kg FFM/min) (ANOVA: *time*, $p \leq 0.001$) (B) Carbohydrate oxidation (g/kg FFM/min) during HIIE (ANOVA: *time*, $p \leq 0.001$) (C) Fat oxidation AUC during HIIE (Students paired *t* test: p=0.047) (D) Carbohydrate oxidation AUC during HIIE (E) Total grams of fat oxidized (g/kg FFM) during HIIE stratified by during exercise alone (Students paired *t* test, p=0.025) and exercise and recovery (Students paired *t* test, $p=0.029$) HIIE, high intensity interval

exercise; FOX, fat oxidation; COX, carbohydrate oxidation; BL, baseline, WU, warmup; H1-4, HIIE 1-4; L1-4, low intensity intervals; R5-15, post exercise recovery minutes 5-15. *$p \leq 0.05$ high vs. low fitness status.



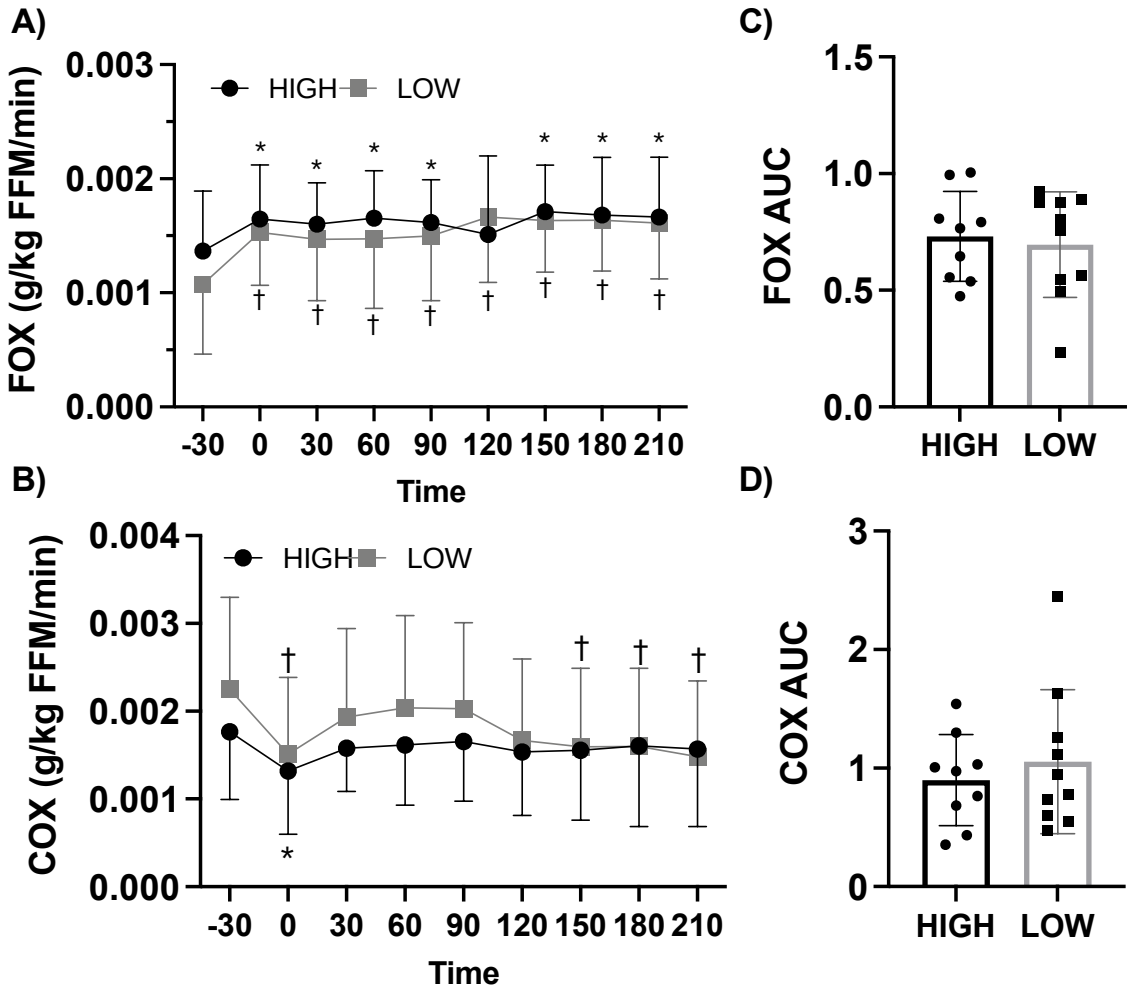
Supplemental Figure 1.2. Baseline, exercise and recovery fat and carbohydrate oxidation in response to HIIE by sex controlled for by kg FFM (men n=11, women n=10) (A) Fat oxidation (g/kg FFM/min) during HIIE (ANOVA: *time*, $p \leq 0.001$) (B)

Carbohydrate oxidation (g/kg/min) (ANOVA: *time*, $p \leq 0.001$) (C) Fat oxidation AUC during HIIE (D) Carbohydrate AUC during HIIE. HIIE, high intensity interval exercise; FOX, fat oxidation; COX, carbohydrate oxidation; BL, baseline, WU, warmup; H1-4, HIIE 1-4; L1-4, low intensity intervals; R5-15, post exercise recovery minutes 5-15. *$p \leq 0.05$ Men vs. Women.

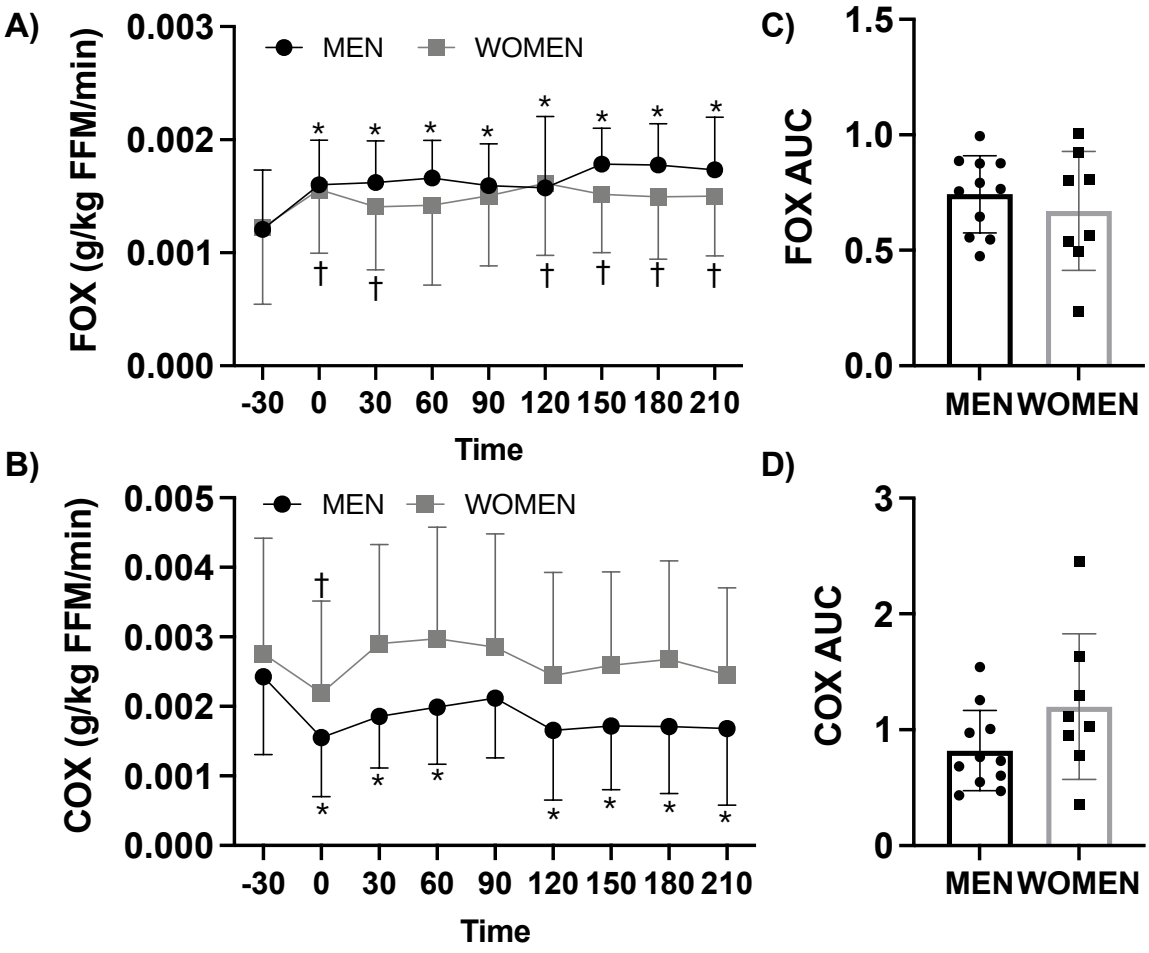


Supplemental Figure 1.3. Baseline, exercise and recovery fat and carbohydrate oxidation in response to HIIE by fitness status controlled for by kg FFM (HFM n=6, LFM n=5, HFW n=5, LFW n=5) (ANOVA; *time, p*≤0.001, n=21) (A) Fat oxidation (g/kg FFM/min) during HIIE for men (ANOVA: *time*, $p \leq 0.001$) and (B) Fat oxidation (g/kg FFM/min) during HIIE for women (ANOVA: *time*, $p \leq 0.001$) (C) Carbohydrate oxidation (g/kg FFM/min) during HIIE for men (ANOVA: *time*, $p \leq 0.001$) and (D) Carbohydrate oxidation (g/kg FFM/min) during HIIE for women (ANOVA: *time*,

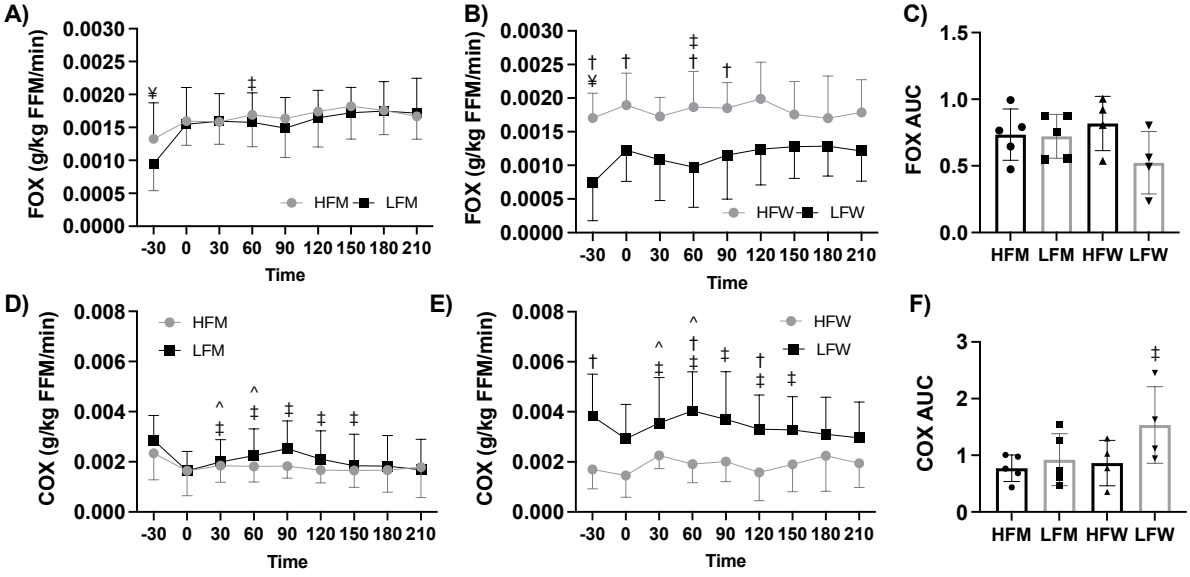
p≤0.001) (E) Fat oxidation AUC (Students paired *t* test: *p*≤0.05) (F) Carbohydrate oxidation AUC (G) Total grams of fat oxidized (g/kg FFM) during HIIE stratified by during exercise alone (Students paired *t* test: *p*≤0.05) and exercise and recovery (Students paired *t* test: p=0.050). HIIE, high intensity interval exercise; FOX, fat oxidation; COX, carbohydrate oxidation; HFM, high fitness men; LFM, low fitness men; HFW, high fitness women; LFW, low fitness women; BL, baseline, WU, warmup; H1-4, HIIE 1-4; L1-4, low intensity intervals; R5-15, post exercise recovery minutes 5-15. **p≤0.05 HFM VS. LFM*, #*p≤0.05 HFM VS. HFW*, ^*p≤0.05 LFM VS. LFW*, †*p≤0.05 HFW VS. LFW*, ‡*p≤0.05 HFM VS. LFW*, ¥*p≤0.05 LFM VS. HFW*



Supplemental Figure 1.4. Baseline and postprandial response to a high-fat meal challenge by Fitness Status controlled for kg fat free mass (high n=9, low n=10) (ANOVA; time p≤0.001, n=19) (A) Fat oxidation (g/kg FFM/minute) (ANOVA: time, p≤0.001) (B) carbohydrate oxidation (g/kg FFM/minute) (ANOVA: time, p≤0.001) (C) Fat oxidation AUC (D) and Carbohydrate oxidation AUC. FOX, fat oxidation; COX, carbohydrate oxidation; AUC, area under the curve. *p≤0.05 compared to baseline (-30 minute) *for* high fitness status. † p≤ 0.05 compared to baseline (-30 minute) *for low fitness status.*

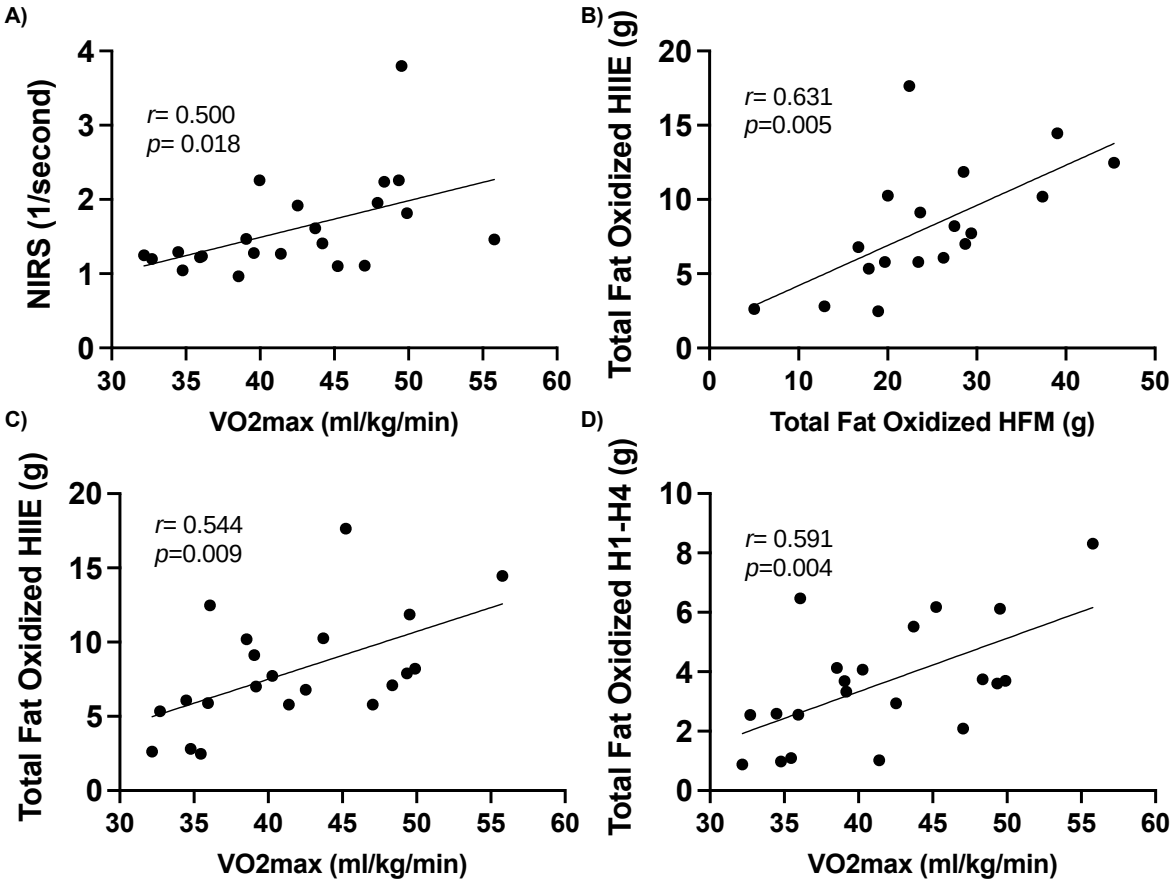


Supplemental Figure 1.5. Baseline and postprandial response to high-fat meal challenge by Sex controlled for fat free mass (Men n=11, Women n=8) (ANOVA; *time,* p≤0.001, n=19). (A) Fat oxidation (g/kg FFM/minute) (ANOVA: *time*, p≤0.001) (B) Carbohydrate oxidation (g/kg FFM/minute) (ANOVA: *time*, *p*≤0.001) (C) Fat oxidation AUC and (D) Carbohydrate oxidation AUC. HFM, high-fat meal challenge; FOX, fat oxidation; COX, carbohydrate oxidation; AUC, area under the curve. **p*≤0.05 compared to baseline (-30 minute) *for Men.* † *p*≤0.05 compared to baseline (-30 minute) for *women*.



Supplemental Figure 1.6. Baseline and postprandial response to high-fat meal challenge by fitness within sex controlled for kg fat free mass (HFM n=5, LFM n=6, HFW n=4, LFW n=4) (ANOVA; *time* p≤0.001, n=19). (A) fat oxidation (g/kg FFM/min) (ANOVA: *time*, *p*≤0.001, *group*, *p*=0.007) (B) carbohydrate oxidation (g/kg FFM/min) (ANOVA: *time*, *p*≤0.001) (C) Fat oxidation AUC (Students paired *t* test: *p*≤0.05) and (D) Carbohydrate oxidation AUC (Students paired *t* test: *p*≤0.05). FOX, fat oxidation; COX, carbohydrate oxidation; AUC, area under the curve.

* *p≤0.05 HFM VS. LFM*, #*p≤0.05 HFM VS. HFW*, ^*p≤0.05 LFM VS. LFW*, †*p≤0.05 HFW VS. LFW*, ‡*p≤0.05 HFM VS. LFW*, ¥*p≤0.05 LFM VS. HFW*



Supplemental Figure 1.7. Correlations between measures of metabolism, skeletal muscle mitochondrial oxidative capacity and fitness (A) correlation between skeletal muscle mitochondrial oxidative capacity (NIRS) (1/sec) and $\dot{V}O_{2peak}$ (mL/kg/min) (*r*=0.500; *p*=0.018; n=21) (B) correlation between total grams fat oxidized during HIIE and total grams fat oxidized during high-fat meal challenge (*r*=0.631; *p*=0.005; n=18). (C) correlation between total grams of fat oxidized during HIIE and $\dot{V}O_{2peak}$ (mL/kg/min) (*r*=0.544; *p*=0.009; n=21) (D) correlation between total grams of fat oxidized during the exercise portions of HIIE (H1-H4) and $\dot{V}O_{2peak}$ (mL/kg/min) (*r*=0.591; *p*=0.004; n=21).

CHAPTER 4

IMPACT OF AEROBIC FITNESS STATUS, MENSTRUAL CYCLE PHASE, AND ORAL CONTRACEPTIVE USE ON EXERCISE METABOLIC FLEXIBILITY.

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[1] Olenick et. al, 2022. To be submitted to Experimental Physiology.

Impact of aerobic fitness status, menstrual cycle phase, and oral contraceptive use on exercise metabolic flexibility.

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Abstract

We assessed the impact of menstrual cycle and fitness status on exercise metabolism, metabolic flexibility, blood lactate during high-intensity interval exercise (HIIE) in 25 women (24.4(3.6)yrs; 28.6(5.0) %BF, 38.8(6.6)mL/kg/min) experiencing a natural menstrual cycle (*n* = 14) or using oral contraceptives (*n* = 11). Participants were categorized by aerobic fitness status (High fit women (HFW): 43.7(4.7)mL/kg/min vs. Low fit women (LFW): 33.6(3.9)mL/kg/min; *P* = 0.001). Participants completed a single bout of HIIE during the follicular phase (FOL: day 2-7 or sugar pills) and luteal phase (LUT: day ~21 or 3rd week of active pills). HIIE was 4x4:3-min work:recovery (0W) intervals on a cycle ergometer at a power output of halfway between ventilatory threshold and $\dot{V}O_{2peak}$. Substrate oxidation was assessed via indirect calorimetry. Blood lactate was taken via finger stick. Recovery skeletal muscle oxidative metabolism ($m\dot{V}O_2$) was assessed using near-infrared spectroscopy. HFW oxidized more fat ($g \cdot kg^{-1}$) during the full session (FOL: *P* = 0.050, LUT: *P* = 0.001), high intervals (FOL: *P* = 0.048, LUT: *P* = 0.001), and low intervals (FOL: *P* = 0.032, LUT: *P* = 0.024) during FOL and LUT, and recovery during LUT (*P* = 0.033). Carbohydrate oxidation AUC ($g \cdot kg^{-1}$; *P* = 0.049, *P* = 0.024) was greater in HFW during FOL, but not LUT. Blood lactate was impacted by fitness in LFW during exercise during FOL (*P* ≤ 0.050), but not LUT. Metabolic flexibility quantified as change in rate of fat oxidation ($\Delta\ g \cdot kg^{-1} \cdot min^{-1}$) during HIIE intervals was greater in HFW than LFW

during intervals 2-3 in FOL and all intervals in LUT ($P \leq 0.050$). Recovery $m\dot{V}O_2$ was greater in HFW than LFW ($P = 0.036$). Overall, these data suggest fitness status more positively influences exercise metabolic flexibility and substrate oxidation at high exercise intensities than cycle phase or OC use alone.

Keywords

Metabolic Flexibility, Menstrual Cycle, Fat Oxidation, High Intensity Interval Training

Abbreviations

FOL	Follicular Phase
HIIE	High Intensity Interval Exercise
High	High Fit Women
Low	Low Fit Women
LUT	Luteal Phase
$m\dot{V}O_2$	Skeletal Muscle Oxidative Metabolism
NIRS	Near-infrared spectroscopy
NOC	Non-Oral Contraceptive User
OC	Oral Contraceptive User
RPE	Rating of Perceived Exertion

1. Introduction

Metabolic flexibility is the ability to efficiently adapt metabolism by substrate sensing, trafficking, storage, and utilization, dependent on availability and requirement [1]. Metabolic flexibility is essential for maintaining energy homeostasis in nutrient excess or energy demand and relies on a systemic control between systems such as endocrine, muscle and mitochondria [1, 2]. Communication between these systems is vital in maintaining energy balance. Disruptions in metabolic flexibility can have implications for exercise performance and development of metabolic disease [1-3]. Metabolic flexibility is traditionally examined during the transition between fasted and feeding, which requires a cellular cascade dependent on endocrine and mitochondrial shifts [1]. Another robust test of metabolic flexibility is the onset of exercise, as it requires appropriate shifting of skeletal muscle metabolic pathways to support activity demands [1, 2].Mechanisms that contribute to increased metabolic flexibility during exercise are involved in managing body mass, metabolic health, and exercise performance [4]. Aerobic fitness status also plays an important role in metabolic flexibility [5-7] during high-intensity interval exercise (HIIE) with higher fitness status correlating to increased fat oxidation [8, 9]. Furthermore, HIIE can be used as a test of an individual's exercise metabolic flexibility as shown by recent findings from our laboratory (Olenick et al. unpublished data).

Metabolism may also be impacted by the menstrual cycle or oral contraceptive use due to changes in estrogen and progesterone across the month [10]. Women have reported higher fat oxidation during exercise than men

[11], but the impacts of the menstrual cycle and hormonal contraceptive use on exercise substrate oxidation are less clear. Changes in hormonal concentrations may impact exercise performance and influence exercise substrate metabolism. Low hormones and elevated estrogen in the luteal phase may increase carbohydrate oxidation and reliance on glycogen stores [10, 12-16]. Furthermore, the high hormone luteal phase with both elevated progesterone and estrogen may increase reliance on fat oxidation and blunt carbohydrate metabolism. [10, 12-16].With whole body substrate differences in oral contraceptive users being less clear [17] Due to these previously observed changes in substrate metabolism, it seems plausible that exercise metabolic flexibility as quantified by total increase in fat oxidization during HIIE intervals may also be impacted by monthly hormonal changes.

Previous studies assessing metabolism during exercise across the menstrual cycle use continuous steady state, incremental or submaximal exercise protocols [18-20]. Recent work has shown differences in fat oxidation during HIIE according to fitness status in men [8, 9], and women during the follicular phase (Olenick et al., unpublished data). Lastly, no studies at this time have assessed the impact of the menstrual cycle or oral contraceptive use on substrate oxidation or metabolic flexibility during HIIE. Therefore, we aimed to assess substrate oxidation and metabolic flexibility during HIIE in the follicular and luteal phase of the menstrual cycle in naturally menstruating women and oral contraceptive users. We also explored blood lactate and muscle metabolism via near-infrared spectroscopy (CW-NIRS) in response to HIIE and their relationship

to substrate oxidation. We hypothesized that (i) hormonal changes across the menstrual cycle will impact substrate oxidation during HIIE, favoring carbohydrate in the follicular phase and fat oxidation in the luteal phase (ii) high fit women will have greater exercise metabolic flexibility than low fit women (iii) lower blood lactate levels will be associated with increased exercise metabolic flexibility.

2. Methods

2.1. Ethical Approval. The study was approved by the University of Georgia Institutional Review Board (study no. 3287) with written informed consent obtained prior to any experimental procedures. The study conformed to the standards set by the *Declaration of Helsinki*, except for registration in a database.

2.2. Participants. Twenty-Five healthy, non-smoking, recreationally active (cardiovascular exercise ≥150 min/wk and resistance exercise ≥2 h/wk) women were recruited for three testing sessions. Participants either were experiencing a normal regular menstrual cycle for at least 12 mo with no history of birth control use in the 6 months prior (NOC; n = 14) or taking mono or triphasic based oral contractive pills (OC; n = 11). All participants were assigned female at birth and identify as female. Participants were free of any history of cardiovascular, metabolic, or musculoskeletal disease. Participants were not taking medications that affect metabolism, or any other supplements or vitamins during the study period. For purposes of this study, we operationally categorized fitness by

splitting our group in half when organized by $\dot{V}O_{2peak}$ and defined “high fitness women” (HFW) as having a $\dot{V}O_{2peak}$ of 43.7 (4.7) mL/kg/min, and “low fitness women” (LFW) as 33.6 (3.9) mL/kg/min.

2.3. Study design. Participants completed three trials consisting of a baseline testing followed by two separate HIIE session. Baseline testing consisted of anthropometrics, body composition, a skeletal muscle mitochondrial oxidative capacity test and $\dot{V}O_{2peak}$ testing. Baseline testing occurred at any point of the menstrual cycle or oral contraceptive cycle since differences in VO2max across the menstrual cycle appear to be minimal [21, 22]. HIIE sessions occurred during the early follicular (FOL) and mid luteal phases (LUT) of each cycle. FOL and LUT sessions were identical and included a single bout of HIIE. FOL and LUT sessions were randomized. All sessions were separated by >48h and completed within 2 mo. Participants refrained from exercise and alcohol ingestion for 24h before each trial and were fasted overnight for 10-12h prior to the HIIE sessions. Subjects were instructed to maintain current dietary habits. Dietary records were collected for 3d prior to each session and assessed using the United States Department of Agriculture National Nutrient Database for Standard Reference (**http://www.nal.usda.gov/fnic/foodcomp/search/**) to ensure that quantity of macronutrients and total energy consumed did not vary significantly between visits. Participants were instructed to consume a standard pre-fasting meal, consisting of 30% predicted resting energy expenditure (50% carbohydrate, 30% fat and 20% protein) [23]. One HIIE session was scheduled days 2-7 following the onset of the menstrual cycle or days 2-7 of one’s non-

hormonal (‘sugar pill’) pill week. The other HIIE session was scheduled ~6-8d following ovulation in NOC and during the third week of active pills in OC women (days 15-22) [24]. In the NOC group, ovulation was confirmed via urine analysis strips. Ovulation testing began on day 10 of the menstrual cycle through confirmed ovulation. Participants were emailed a daily reminder to test ovulation and photos confirming ovulation sticks were sent to study team members for confirmation.

2.4. Baseline testing session. Participants’ height, weight, body composition (via dual-energy X-ray absorptiometry, Horizon® DXA System, Hologic, Inc., Marlborough, MA, USA), blood pressure and resting heart were assessed. Skeletal muscle mitochondrial oxidative capacity test and $\dot{V}O_{2peak}$ were performed.

Mitochondrial oxidative capacity was determined by measuring changes in CW-NIRS (PortaMon, Artinis Medical Systems, Einsteinweg, The Netherlands) signals during periods of ischemia [21]. Each participant laid supine on a padded table with both legs fully extended (0° of flexion) with a CW-NIRS optode placed on the right Vastus Lateralis, approximately 2/3 of the way down from the greater trochanter to the patella and secured using elastic pre-wrap and an elastic bandage to reduce transient light. The knee extensors were stimulated percutaneously by two rectangular electrodes (2 x 4 in) placed over the belly of the Vastus Lateralis (Theratouch 4.7, Rich-mar, Inola, OK, USA) proximal and distal to the CW-NIRS probe. A rapid inflating pneumatic cuff (Delfi V34, Medical Innovations Inc., Vancouver, BC, CA and D.E. Hokanson Inc., Bellevue, WA, USA) was placed proximal to the CW-NIRS optode with enough separation to prevent

mechanical influence from inflation. CW-NIRS signals were sampled at 10 Hz and laser diodes at three wavelengths (905, 850, and 760 nm) corresponding to the absorption wavelengths of oxygenated hemoglobin. Resting measurements of mVO2 were assessed by inflation (250–300 mmHg) for 30 seconds. To assess exercise mVO2, 30 s of twitch neuromuscular electrical stimulation (NMES; biphasic pulse, duration/interval = 200/50 μs) was administered at 6.0 Hz. The intensity was adjusted for each subject to produce twitch contractions at the maximal tolerable level. To measure the rate of recovery of muscle oxygen uptake back to resting levels, four mitochondrial oxidative metabolism tests were performed consisting of a series of six brief occlusions (5 s on/ 5 s off of 250-300 mmHg) following 30 s of twitch NMES.

$\dot{V}O_{2peak}$ measured via a ramp protocol on an electromagnetically braked cycle ergometer (Lode Excalibur Sport, Groningen, Netherlands). Participants were fitted with a mask to collect respiratory gasses via indirect calorimetry (TrueOne 2400, Parvo Medics, Sandy, UT, USA). Participants completed a warmup by cycling for 3-minutes at 20 watts, after which work rate increased 20 W/min until volitional fatigue or participants could no longer sustain a cadence of ≥60 revolutions per min. $\dot{V}O_{2peak}$ was confirmed by a respiratory exchange ratio of ≥1.10, rating of perceived exertion (RPE) ≥18/20, and blood lactate ≥7 mmol assessed via finger stick (Lactate Plus, Nova Biomedical).

2.5. High-Intensity Interval Exercise Session. Participants arrived at the laboratory in the morning after an overnight fast (10-12h) to undergo a HIIE session. Participants were fitted with a mask to collect respiratory gasses for

analysis via indirect calorimetry (TrueOne 2400, Parvo Medics, Sandy, UT, USA). Exercise was performed on an electromagnetically braked cycle ergometer pre-programmed to the previously determined power output (Lode Excalibur Sport, Groningen, the Netherlands). After 3-min of seated rest, a 3-min warmup at 50W was performed. High-intensity intervals were four minutes long at an individualized intensity of the halfway point between gas exchange threshold and $\dot{V}O_{2peak}$. V-slope method was used to determine gas exchange threshold and independently verified by two study team members, as previously described [25]. Low intervals were three minutes of seated recovery (0W). Participants were asked to maintain a cadence of ≥60 RPM across all intervals. If a participant could not sustain ≥60 RPM power output, it was decreased in 5W increments till the participant could complete the work. If adjustments were made in the first session, they were matched in the second session (n = 5). Seated recovery was assessed for 20min post-exercise. Blood lactate via finger stick and rating of perceived exertion (RPE) were collected following each interval and lactate every five minutes during recovery.

2.6. Fat and carbohydrate oxidation assessment. Substrate oxidation analyses were analyzed with data expressed relative to total body weight ($g \cdot kg^{-1} \cdot min^{-1}$). Macronutrient oxidation rate was assessed for HIIE using equations developed by Jeukendrup [26] and Frayn [27].. For HIIE sessions, macronutrient oxidation rate was averaged for the duration of each interval. Total grams of substrate oxidized was calculated by multiplying the rate of substrate oxidation by time duration and summed for total exercise (high interval 1, 2, 3 and 4),

recovery (low 1, 2, 3, 4, and recover 5-20 min), and full session. Rate of substrate oxidation was multiplied by time during individual high intervals and recovery to estimate total amount of respective substrate oxidized. Changes in rate of fat oxidation ($g \cdot kg^{-1} \cdot min^{-1}$) during high intensity intervals (Δ) were calculating by subtracting the lowest fat oxidation value from the highest achieved from each participant during exercise intervals (high 1, 2, 3 and 4).

2.7 Post Exercise Resting Muscle Oxygen Uptake Assessments. Muscle oxygen uptake ($m\dot{V}O_2$) was assessed at 5, 10 and 15 minutes following the HIIE session. Muscle oxygen uptake of the vastus lateralis was determined by measuring changes in continuous-wave near-infrared spectroscopy (CW-NIRS) (CW-NIRS; PortaMon, Artinis Medical Systems, Einsteinweg, Netherlands) signals during 30 second periods of ischemia from a rapid inflating blood pressure cuff (Hokanson SC12; D.E. Hokanson Inc., Bellevue, WA). CW-NIRS device was placed on the Vastus Lateralis, approximately 2/3 of the way down from the greater trochanter to the patella and secured using elastic pre-wrap and an elastic bandage to reduce transient light. Three ischemic cuffs were completed (30 seconds on, 90 seconds off with an occlusion pressure of 250-300 mmHg) at 5, 10 and 15 minutes of recovery. The slope of CW-NIRS signals calculated resting metabolism following occlusion [28]. Data were analyzed using Matlab-based analysis software MATLAB® R2018b (MathWorks Inc., Natick, MA) as previously described [29]. The average slope (sec^{-1}) of the three cuffs was recorded.

2.8 Data analysis. Exercising muscle oxygen monitor signals provided included: muscle oxygen saturation percent (SmO_2%) and total hemoglobin (tHb).

Macronutrient oxidation rate was assessed for the entire HIIE trial. Frayn equations were used for all rest and recovery data, Jukendrup equations for 40-50% $\dot{V}O_{2peak}$ were used for low intensity intervals, and 50-75% $\dot{V}O_{2peak}$ for all high intensity intervals equations.

Frayn:

$$\text{Fat } (g \cdot min^{-1}) = (1.67 * VO_2\ (L \cdot min^{-1})) - (1.67 * VCO_2\ (L \cdot min^{-1}))$$

$$\text{Carbohydrate } (g \cdot min^{-1}) = (4.55 * VCO_2\ (L \cdot min^{-1})) - (3.21 * VO_2\ (L \cdot min^{-1}))$$

Jukendrup 40-50% $\dot{V}O_{2peak}$:

$$\text{Fat } (g \cdot min^{-1}) = (1.695 * VO_2\ (L \cdot min^{-1})) - (1.701 * VCO_2\ (L \cdot min^{-1}))$$

$$\text{Carbohydrate } (g \cdot min^{-1}) = (4.344 * VCO_2\ (L \cdot min^{-1})) - (3.061 * VO_2\ (L \cdot min^{-1}))$$

Jukendrup 50-75% $\dot{V}O_{2peak}$:

$$\text{Fat } (g \cdot min^{-1}) = (1.695 * VO_2\ (L \cdot min^{-1})) - (1.701 * VCO_2\ (L \cdot min^{-1}))$$

$$\text{Carbohydrate } (g \cdot min^{-1}) = (4.210 * VCO_2\ (L \cdot min^{-1})) - (2.962 * VO_2\ (L \cdot min^{-1}))$$

Oxidation values calculated as a negative value were replaced with a zero. Macronutrient oxidation rate was averaged for the duration of each interval. Total grams of substrate oxidized was calculated by multiplying the rate of substrate oxidation by time duration and summed for the full exercise bout, exercise (high-intensity intervals 1-4), and rest (low-intensity intervals 1-3, and recovery). Metabolic flexibility (MetFlex) was calculated as the difference in fat oxidation from lowest and maximal values (Δ) during high intensity intervals 1-4.

2.9 Statistical analysis. A two-way repeated-measures ANOVA was conducted to assess the statistical significance of time (*HIIE*), condition (*Cycle Phase*: FOL vs LUT) and group (*Fitness Status and OC Status*) (*HIIE* Cycle Phase*Fitness Status*OC Status*) on fat oxidation, carbohydrate oxidation, blood lactate, metabolic flexibility, and post exercise m$\dot{V}O_2$. Fat oxidation, carbohydrate oxidation and lactate area under the curves (AUC) were calculated using the trapezoid rule [30]. Student's paired t-test was used to compare total fat and carbohydrate oxidized calculations, total fat and carbohydrate oxidation AUC, lactate AUC, m$\dot{V}O_2$, and all group characteristics. Substrate oxidation analyses were analyzed with data expressed relative to total body weight ($g \cdot kg^{-1} \cdot min^{-1}$). Assumptions of normality were verified for all outcome measures. Statistical significance was accepted at $P \leq 0.05$. Data are presented as means (SD). All statistical analyses were performed with SPSS Statistics version 25.0 (IBM Corp., Armonk, NY, USA).

3. Results

3.1. Participant characteristics. Thirty participants were recruited. Twenty-five participants completed baseline and both HIIE sessions (24.4 (3.6) yrs; 28.6(5.0) %BF; 38.8 (6.6) mL/kg/min). Two participants were removed from the study, one for an abnormal cycle length, another due to not completing all sessions in 2mo. Three other participants were unable to complete the study for personal reasons. Only data from participants who completed both sessions was

included in final analysis. Participant characteristics are presented in Table 2.1. Participants all consumed a similar diet across all trials (Supplemental Table 2.1)

3.2. All Women. There was a significant effect of HIIE on fat oxidation ($g \cdot kg^{-1} \cdot min^{-1}$) (ANOVA: *Time*, $P \leq 0.001$, $\eta_p^2 = 0.993$, Figure 2.1), carbohydrate oxidation ($g \cdot kg^{-1} \cdot min^{-1}$) (ANOVA: *Time*, $P \leq 0.001$, $\eta_p^2 = 0.990$, Figure 2.2), blood lactate (mmol/L) (ANOVA: *Time*, $P = 0.036$, $\eta_p^2 = 0.978$, Figure 2.3), and $m\dot{V}O_2$ (sec^{-1}) (ANOVA: *Time*, $P \leq 0.001$, $\eta_p^2 = 0.773$, Supplemental Figure 2.5) for all participants, and conditions. There was no effect of menstrual cycle phase on fat oxidation ($g \cdot kg^{-1} \cdot min^{-1}$) (ANOVA: *Condition, P* = 0.933, $\eta_p^2 = 0.000$, Figure 2.1), carbohydrate oxidation ($g \cdot kg^{-1} \cdot min^{-1}$) (ANOVA: *Condition, P* = 0.661, $\eta_p^2 = 0.009$, Figure 2.2), or blood lactate (mmol/L) (ANOVA: *Condition, P* = 0.275, $\eta_p^2 = 0.084$, Figure 2.3).

3.3. *Fitness Status*

3.3.1 *Fat Oxidation*. With participants assessed based on fitness status (Fitness Status: HFW, *n* = 13 vs. LFW, *n* = 12) there was an effect of fitness status on fat oxidation ($g \cdot kg^{-1} \cdot min^{-1}$) (ANOVA: *Group, P* = 0.002, $\eta_p^2 = 0.386$, Figure 2.1) and cycle phase and fitness status in FOL (ANOVA: *Group*Condition, P* = 0.027, $\eta_p^2 = 0.231$, Figure 2.1) and LUT (ANOVA: *Group*Condition, P* = 0.005, $\eta_p^2 = 0.314$, Figure 2.1). Fat oxidation ($g \cdot kg^{-1} \cdot min^{-1}$) was significantly greater in HFW than LFW during FOL at high 2 (*P* = 0.040), low 2 (*P* = 0.032), high 3 (*P* = 0.023), low 3 (*P* = 0.023), high 4 (*P* = 0.005) and during LUT phase at baseline (P=0.030), high 2 (*P* = 0.009), high 3 (*P* = 0.005), low 3 (*P* = 0.033) and high 4 (*P* = 0.015), Figure 2.1a-b. Fat oxidation ($g \cdot kg^{-1} \cdot min^{-1}$) AUC was significant higher in HFW

than LFW during both FOL (AUC $P = 0.050$) and LUT (AUC $P = 0.001$), Figure 2.1a-b. Fat oxidized ($g \cdot kg^{-1}$) during the full session was significantly greater in HFW than LFW during the full session (FOL: $P = 0.050$, LUT: $P = 0.001$), high intervals (FOL: $P = 0.048$, LUT: $P = 0.001$), and low intervals (FOL: $P = 0.032$, LUT: $P = 0.024$) during both FOL and LUT, and recovery during LUT ($P = 0.033$), Figure 2.1c. Furthermore, difference in the total change in rate of fat oxidation ($g \cdot kg^{-1} \cdot min^{-1}$) ($\Delta$) during high intensity exercise intervals was significantly greater in HFW than LFW in the FOL at high 2 and 3 (H1: $P = 0.248$, H2: $P = 0.018$, H3: $P = 0.004$, H4: $P = 0.090$, Figure 2.4), and LUT phase across all intervals (H1: $P = 0.041$, H2: $P = 0.001$, H3: $P = 0.001$, H4: $P = 0.006$, Figure 2.4).

3.3.2. *Carbohydrate Oxidation.* There was no effect of fitness status on carbohydrate oxidation ($g \cdot kg^{-1} \cdot min^{-1}$) (ANOVA: *Group,* $P = 0.157$, $\eta_p^2 = 0.093$, Figure 2.1c-d). Carbohydrate oxidation ($g \cdot kg^{-1} \cdot min^{-1}$) AUC was significantly greater in HFW than LFW during FOL (AUC $P = 0.024$) but not LUT (AUC $P = 0.124$), Figure 2.2d-e. Carbohydrate oxidized ($g \cdot kg^{-1}$) during the full session was significantly greater in HFW than LFW women during the full session ($P = 0.049$) and high intervals ($P = 0.044$) during FOL, Figure 2.1f.

3.3.3. *Lactate Response.* There was a significant effect of fitness status (ANOVA: *Group* $P = 0.045$, $\eta_p^2 = 0.256$, Figure 2.3d-e) and cycle phase on blood lactate (mmol/L) in FOL (ANOVA: *Group*Condition,* $P = 0.048$, $\eta_p^2 = 0.252$, Figure 2.3d) but not LUT (ANOVA: *Group*Condition,* $P = 0.072$, $\eta_p^2 = 0.212$, Figure 2.3e). Blood lactate (mmol/L) was significantly higher in LFW than HFW

at low 2 (*P* = 0.036), high 3 (P= 0.038), high 4 (P =0. 044), low 4 (*P* = 0.041), and recovery 10 minutes (*P* = 0.016) in FOL, and at high 4 (*P* = 0.027) in LUT, Figure 2.3d. There were no differences in lactate AUC between groups or across cycle phases (*P* > 0.050, Figure 2.3f).

3.3.4. *$m\dot{V}O_2$.* There was a significant effect of time and fitness status (ANOVA: *Time*Group*, *P* = 0.043, η_p^2 = 0.325, Figure 2.5), fitness status overall (ANOVA: *Group*, *P* = 0.013, η_p^2 = 0.311, Figure 2.5) and fitness during LUT (ANOVA: *Group*, *P* = 0.007, η_p^2 = 0.357, Figure 2.5), but not FOL (ANOVA: *Group*, *P* = 0.053, η_p^2 = 0.203, Figure 2.5) on $m\dot{V}O_2$. Furthermore, HFW had significantly greater $m\dot{V}O_2$ than LFW during post HIIE recovery at 5 minutes (*P* = 0.007), 10 minutes (*P* = 0.007) and 15 minutes (*P* = 0.007) in LUT but only 10 minutes in FOL (*P* = 0.040) with 5 (*P* = 0.054) and 15 minutes (*P* = 0.076) trending greater.

3.4. *Oral contraceptive status.*

3.4.1 *Fat Oxidation.* With participants assessed based on oral contraceptive status (OC Status: NOC=14, OC=11) and assessed by menstrual cycle phase (Cycle Phase: FOL vs LUT), there was no effect of OC status on fat oxidation (ANOVA: *Group*, *P* = 0.141, η_p^2 = 0.100, Figure 2.2a-b) overall, in FOL (ANOVA: *Group*, *P* = 0.064, η_p^2 = 0.154, Figure 2.2a-b) or LUT (ANOVA: *Group*, *P* = 0.714, η_p^2 = 0.007, Figure 2.2a-b). NOC had a significantly higher fat oxidation ($g \cdot kg^{-1} \cdot min^{-1}$) than OC during high 4 (*P* = 0.038), recovery 5 (*P* = 0.013), recovery 10 (*P* = 0.012), and recovery 15 (*P* = 0.029) during FOL, but at no time point during LUT. Fat oxidized ($g \cdot kg^{-1}$) during recovery was significantly greater in NOC than

OC during FOL (P = 0.028, Figure 2.2c) and NOC during FOL than LUT (P = 0.048 Figure 2.2c).

3.4.2 *Carbohydrate Oxidation.* There was no effect of OC status on carbohydrate oxidation ($g \cdot kg^{-1} \cdot min^{-1}$) (ANOVA: *Group, P* = 0.900, η_p^2 = 0.001, Figure 2.2c-d). Carbohydrate oxidation ($g \cdot kg^{-1} \cdot min^{-1}$) was significantly greater in FOL in OC than NOC at Recovery 5 min (P = 0.038), Recovery 10 (P = 0.026) and Recovery 15 (P = 0.004), with no differences in the LUT, Figure 2.2d-e. NOC had a greater carbohydrate oxidation ($g \cdot kg^{-1} \cdot min^{-1}$) at recovery 15 ($P$ = 0.033) and lower carbohydrate oxidation ($g \cdot kg^{-1} \cdot min^{-1}$) during warmup ($P$ = 0.031) and at recovery 10 minutes (P = 0.030) during FOL than LUT. When total grams of carbohydrate oxidized ($g \cdot kg^{-1}$) during recovery was significantly greater in NOC than OC during FOL (P = 0.007, Figure 2.2f).

3.4.3 *Blood Lactate.* When There was no effect of OC status on blood lactate (mmol/L) (ANOVA: *Group, P* = 0.618, η_p^2 = 0.012, Figure 2.3). NOC had greater blood lactate than OC during warmup in the FOL (P = 0.025), and OC had greater blood lactate at recovery 15 (P = 0.018) during FOL than LUT, with no other differences. There were no differences in blood lactate AUC between groups or phases (P > 0.050, Figure 2.3c).

4. Discussion

In the current study we assessed the impacts of menstrual cycle phase, oral contraceptive use and fitness status on metabolic responses to HIIE in young healthy women. Additionally, we assessed measures of muscle

mitochondrial capacity. The major findings include: 1) Fitness status plays an important role in exercise fat oxidation, carbohydrate oxidation, blood lactate and skeletal muscle metabolism during HIIE in young women; 2) High fitness women exhibit greater exercise metabolic flexibility than their low fit counterparts; 3) Differences in exercise metabolism may be unique to the menstrual cycle phase, with low fitness women having poorer exercise metabolic flexibility in the follicular phase; and 4) Exercise metabolism during HIIE is not significantly impacted by menstrual cycle phase or oral contraceptive use independent of fitness status.

A primary outcome of this study was to see the role fitness status played on exercise metabolic flexibility. We find that fitness status plays a significant role in women's exercise metabolism. High fitness women utilized more fat during HIIE in both the follicular and luteal phases and tend to increase fat oxidation during high intensity intervals to a great extent (Figures 2.1 & 2.4). While the ability to oxidize fat is thought to be decreased above 65-70% $\dot{V}O_{2peak}$ [32], current data challenges this by displaying an increase capacity to oxidize fat across the entire exercise period in high fitness individuals. A high level of aerobic fitness is associated with a significantly higher utilization of fat oxidation, but similar carbohydrate oxidation rates (g/min) during HIIE [8, 9] (Olenick et al., unpublished data). Here we find greater carbohydrate oxidation AUC and grams oxidized during exercise and the full HIIE session in high fitness women during the follicular phase, but not luteal phase (Figure 2.1). Elevated carbohydrate oxidation here may be due to the increased fitness status and calorie expenditure accompanying that but support the proposed increased carbohydrate oxidation in

the of the follicular phase [14, 16, 33, 34]. Interestingly, we see greater blood lactate in low fitness women during the follicular phase, despite lower carbohydrate reliance [14, 16, 33, 34], which appears to be negatively related to fat oxidation (Supplemental Figure 2.1). Low fitness in women appears to blunt the metabolic response to HIIE in the follicular phase, with high fitness women having a greater capacity to switch fat oxidation across both phases, but especially in the luteal phase (Figure 2.1 & 2.4). Higher circulating estrogens of the luteal phase are related to elevated fat oxidation and lower carbohydrate oxidation, with the opposite being seen in the follicular phase where hormone concentrations are lower [14, 16, 33, 34]. Fat oxidation differences between fitness status noted here may be magnified then, by these underlying hormonal fluctuations between groups.

Exercise presents a unique stress to the body and muscle tissue [1, 35]. Previous work from our lab found that HIIE can be used as a measure to assess metabolic flexibility and that this was more drastically reduced in untrained women (Olenick et al., unpublished data). Our present data show an increased capacity to upregulate fat metabolism during HIIE in high fitness women, which is enhanced by the luteal phase (Figure 2.4) and blunted in the follicular phase in low fitness women. Metabolic flexibility is strongly tied to fitness status and overall metabolic health and can predict future weight gain and metabolic dysregulation [1, 2, 5, 36]. Here, our low fitness women had greater fat mass, body weight, body composition, and visceral adipose tissue, less muscle tissue and a lower VO2max (Table 2.1). Aerobic training improves mitochondrial

content, oxidative capacity and fatty acid delivery, although we did not see significantly greater mitochondrial capacity between our groups [37] (Table 2.1). High fitness women appear to benefit in the face of hormone fluctuations, adjusting their substrate oxidation more favorably compared to low fit women in each phase (Figure 2.1 & 2.4).

Estrogen receptors located within type I muscle fibers play a role in the fatty acid oxidation pathways [4] that are also impacted by aerobic training [38]. Some evidence indicates that compared to men, women have more type 1 muscle fibers, exhibit a greater capacity to store and breakdown intramuscular triglycerides as well as greater whole body fat oxidation, and have enhanced mitochondrial sensitivity to lipid oxidation [39-42]. Our data suggest that these benefits may compound, or be more apparent, following aerobic training. Women undergoing endurance training increases whole body and skeletal muscle fatty acid oxidation during exercise [43] and significantly increase intramuscular lipid density following training and to men [44]. Here we see that high fit women also increase whole body fat oxidation during HIIE than low fit women, while adjusting their substrate utilization in favor of the underlying hormone changes through increase carbohydrate oxidation without subsequent lactate rises in the follicular phase, and greater fat oxidation capacity in the luteal phase. Low fit women, however, are negatively impacted by the carbohydrate reliance often see with the lower hormone follicular phase by producing more lactate and oxidizing less fat. However, it appears that regardless of training status, the high hormone luteal phase is associated with greater exercise metabolic flexibility during HIIE in

young healthy women. Unfortunately, there are few published data on differences in effects of exercise training between men and women [45]. While future research is warranted on this topic, the protective effects of estrogen and their interaction with lipid oxidation pathways may play an important role in female health, performance, and exercise substrate oxidation. Our present data, taken with our previous work, suggest the importance of fitness status in even young, healthy, women. Impairments in exercise metabolic flexibility appear during exercise in young healthy women during exercise, which may differ across the menstrual cycle phase. Future work should expand on the interaction fitness and hormonal status plays on women's metabolic health and exercise fat oxidation.

We found no within group differences in fat oxidation, carbohydrate oxidation or blood lactate during the follicular or luteal phase of the menstrual cycle in all women (NOC, OC, HFW or LWF) (Figures 2.1 & 2.3). These data indicate that menstrual cycle phase impacts on exercise metabolism may not be apparent during high intensity interval exercise, beyond the fitness status differences noted. Our findings are supported by other data showing no differences in exercise fat or carbohydrate metabolism in women between menstrual cycle phases [46-48]. Conversely, previous data has also indicated that it that the higher circulating estrogens of the luteal phase are related to elevated fat oxidation and lower carbohydrate oxidation, with the opposite being seen in the follicular phase where hormone concentrations are lower [14, 16, 33, 34]. However, many of these studies looking at menstrual cycle effects on substrate oxidation were performed at low to moderate exercise intensities of 30-

60% VO_{2max} [14, 20, 33, 49]. Interestingly, we found significantly greater fat oxidation in NOC women during the recovery period, which contrasts previous data indicating greater fat oxidation in the luteal phase [14, 16, 33, 34]. While estrogens affect lipid oxidation, sparing carbohydrate, in the luteal phase we see the opposite [4]. However, these differences in post exercise fat oxidation responses and are potentially of interest from a recovery, health, and body composition management standpoint.

While our study assessed the impact of interval exercise, a few studies have assessed this at higher intensities, similar to our participants whose average high intensity interval %VO_{2max} ranged from 65-75% during each session (Supplemental Table 2.1). During exercise, fat oxidation peaks (FatMax) then drop off during exercise above 65-70% VO_{2max} [50-54], with no difference FatMax in women across cycle phases [55]. Greater fat and lower carbohydrate oxidation during the mid-luteal phase than mid follicular phase have been observed in women cycling at 35% 60% [34] and 70% VO_{2max} [33, 34], but this difference diminishes at 75% VO_{2max} [34]. Furthermore, intensity increases fat and carbohydrate oxidation more than cycle phase in women transitioning from 45 to 65% VO_{2max} [20]. Overall, the prevailing data suggest that the metabolic demands of exercise intensity above a certain point (~75% VO2max) may override menstrual cycle impacts on substrate utilization. This threshold phenomenon may explain the lack of differences between cycle phases observed herein (Figure 2.1-2) [13, 16, 34].

We found no differences in fat oxidation, carbohydrate oxidation or blood lactate in all women in women taking oral contraceptives in either cycle phase (LUT: active vs FOL: inactive pills) (Figure 2.2) or compared to non-users (NOC) (Figure 2.2). Indicating that during HIIE pill phase or oral contraceptive use may have no meaningful impact on exercise metabolism. Supporting our current findings, women cycling at 45% and 85% VO2max for 30 minutes have greater carbohydrate oxidation with increased intensity, but no differences in active vs. inactive pill phases on RER or compared to their NOC counterparts [56], with additional studies showing no differences in fat oxidation or RER in women between active or inactive pill phases or following the use of oral contraceptives at 45 or 65% VO2max [17, 57-60] . Interestingly, we found significantly greater fat oxidation and lower carbohydrate oxidation in NOC women than OC women during the recovery period of the Luteal phase (Figure 2.2). Women using oral contraceptives may have a higher lipid oxidation rate [61], elevated free fatty acid kinetics [17, 56, 59], and decreased glucose flux [56, 59] during exercise, despite no differences in whole body oxidation compared to eumenorrheic women. Therefore, while our study shows no differences at the whole-body level, glucose and lipid kinetics may differ during HIIE in OC users and has yet to be assessed.

We found no differences in blood lactate across high intensity exercise across the menstrual cycle in either group, or in oral contraceptive users; other than during the first high intensity interval in the follicular phase of NOC women. The lactate threshold may be slightly earlier in the follicular phase, which would

cause a more rapid rate of lactate production [22], potentially explaining why our only difference was elevated blood lactate in the first high intensity interval in the follicular phase compared to the luteal phase (Figure 2.3). Lactate may have no difference across the menstrual cycle during light exercise, but does at heavy and exhaustive exercise with lower luteal phase lactate [10, 22, 62-66] or no difference at all [31, 62, 67, 68]. Oral contraceptives use also appears to not impact exercise lactate levels [56, 59, 66]. Previous work that shows differences in lactate across the cycle are often done as incremental steady state or continuous time to exhaustion [10, 22, 62-66], whereas our study is the first to assess lactate responses during high HIIE. Our data suggest that HIIE responses may not be differently impacted by menstrual cycle phase or oral contraceptive use, potentially due to its intensity being well above the lactate threshold.

Low fitness women had significantly greater blood lactate across the exercise session in the follicular phase, and elevated but no significant greater higher lactate in the luteal phase (Figure 2.3), matching previous findings from our lab (Olenick et al., unpublished data). Untrained males also have elevated blood lactate during HIIE [8]. Additionally, blood lactate had a negative relationship with fat oxidation in women in the present study, but only in the follicular phase (Supplemental Figure 2.1). These data are consistent with a recent report suggesting that blood lactate levels can be used as an indirect measure of metabolic flexibility during cycling exercise [69]. Lactate appears to have an inverse relationship with fat oxidation during exercise [69], such as the relationship observed in the present study's low fit women and in the follicular

phase (Figure 2.3 & Supplemental Figure 2.1). These data suggest that fitness status plays a greater role in lactate metabolism during HIIE in women, with possible greater differences in the follicular phase, reflecting poorer metabolic flexibility in our untrained women.

An important point is that while there were no overall differences in exercise substrate oxidation across the menstrual cycle, there has been a bigger push in menstrual cycle research to examine individual cases rather than or in addition to group comparisons (Supplemental Figure 2.2), as menstruating individuals may experience cycle differences not only between participants, but month to month in their own cycles [10, 70]. As our data show, there is individual variation in response to the HIIE protocol between women, despite no overall differences between phases noted at the group level (Supplemental Figure 2.2). Although our study sample is somewhat limited, it appears that some individuals were ‘positive responders’ to menstrual cycle hormonal changes, while others were not. This may come down to individual hormone concentrations across the month. While data at the group level may mask individual differences, future work should assess women by positive vs non responders to highlight differences that may impact substrate oxidation, metabolic flexibility, health, or performance.

Our study is the first to our knowledge to use NIRS to assess exercise and post exercise muscle oxygen kinetics in women across the menstrual cycle. We found that post exercise $m\dot{V}O_2$ is significantly elevated in high fit women compared low fit women, indicating a significantly greater muscle oxygen uptake during recovery (Figure 2.5). Increased skeletal muscle oxidative capacity is a

favorable byproduct of aerobic training, supporting our findings here in high fit women [40]. These findings also parallel our metabolism findings, suggesting both at the whole-body level as well as muscular level, impairments in skeletal muscle oxidative capacity may play a role in poorer exercise metabolic flexibility in young healthy women.

Limitations

One limitation of our current study is that we had no biomarker measures of circulating progesterone in our female participants. Knowledge of both estrogen and progesterone in our participant could have addressed individual differences more clearly. Furthermore, another limitation is the lack of an ability to analyze the impact of fitness status within NOC and OC groups, since the majority of our high fitness participants were also on oral contraceptives. While this was not intentional, upward of 68% of sports women report preventative birth control use. OC use within our study sample reflected this population level data.[71]. Future work should further examine the impacts of fitness status in NOC and OC status alone. Further work is needed to determine the role fitness status plays in women's exercise substrate oxidation, specifically their ability to oxidize fat. The role that aerobic fitness training plays within substrate oxidation has impacts on both performance across the menstrual cycle and overall metabolic health in women. Future research is necessary to determine the impacts of an impaired capacity for fat oxidation during exercise on long-term

adiposity and metabolic health and its interaction with hormonal changes across the cycle [72, 73].

5. Conclusion

In summary, we found that fitness status plays an important role in exercise fat oxidation, carbohydrate oxidation and blood lactate during HIIE in young women. High fitness women exhibit greater exercise metabolic flexibility than their low fit counterparts. These differences may be unique to the menstrual cycle phase, with low fitness women having poorer exercise metabolic flexibility in the follicular phase. Furthermore, our findings suggest that a driving factor in fitness status differences may be in part explained by underlying skeletal muscle oxidative capacity. The authors suggest that aerobic training may enhance the effects of estrogen on whole body and muscle metabolism in women, and future research should look at the unique role fitness status plays on these adaptations in women and how these may differ across the cycle. Lastly, future research should explore the link between fitness status and women's metabolic health, in the context of female sex hormones across a variety of populations and throughout the lifespan.

COMPETING INTERESTS

N.T.J. reports a consulting agreement with Renaissance Periodization, LLC separate from the submitted work.

AUTHOR CONTRIBUTIONS

Experiments were conducted in the Integrative Cardiovascular Physiology Laboratory located in the Department of Kinesiology at the University of Georgia. A.A.O. and N.T.J. conceived and designed the research. A.A.O. collected data. A.A.O. and N.T.J. analyzed the data. All authors contributed to interpretation of the results. A.A.O. and N.T.J. drafted the manuscript. All authors edited and revised manuscript. All authors have read and approved the final version of the manuscript and agree to be accountable for all aspects of the work in ensuring the questions related to the accuracy or integrity of any part of the work are appropriately investigated and resolved. All persons designated as authors qualify for authorship, and all those who qualify for authorship are listed.

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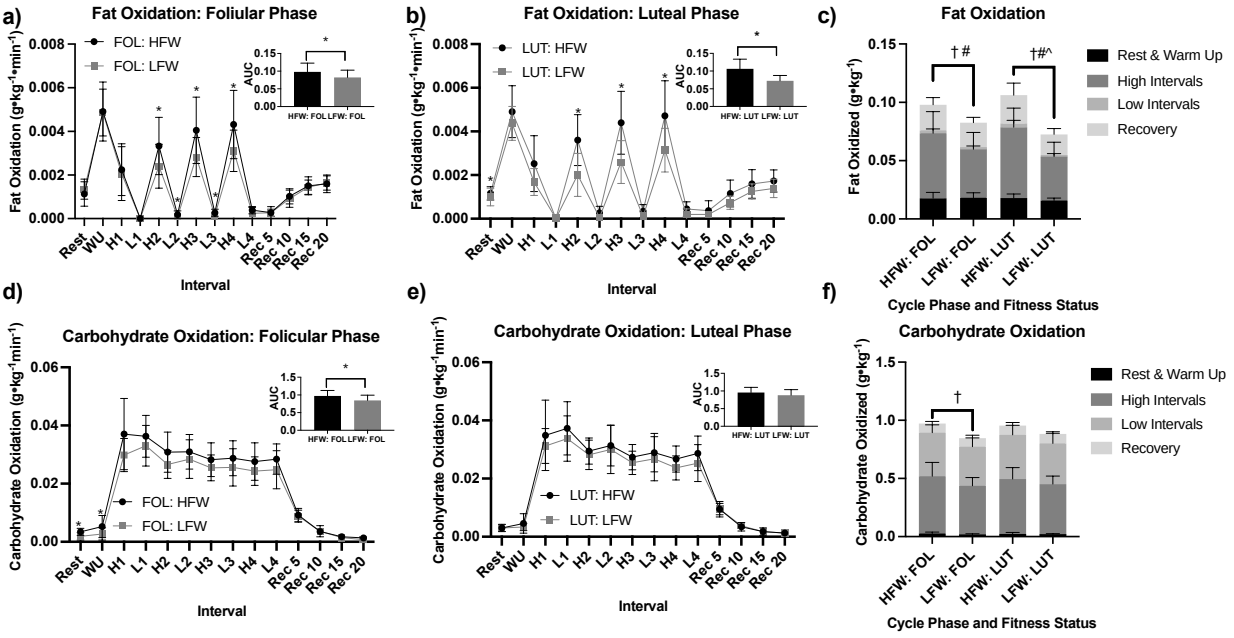
Table 2.1 Participant characteristics.

Measure	Overall (n = 25)	OC Status			Aerobic Fitness Status		
		NOC (n = 14)	OC (n = 11)	P-Value	HFW (n = 13)	LFW (n = 12)	P-value
Age (years)	24.4 (3.6)	24.7 (3.9)	24.2 (3.4)	0.727	24.0 (3.6)	25.0 (3.7)	0.507
Height (cm)	165.5 (6.9)	167.6 (6.0)	162.8 (7.4)	0.090	163.9 (7.9)	167.3 (5.6)	0.248
Baseline Weight (kg)	65.1 (10.5)	67.8 (12.1)	61.8 (7.3)	0.101	59.5 (9.3)	71.3 (8.3)	0.003*
Follicular Session (kg)	65.0 (10.1)	66.8 (12.1)	62.8 (6.41)	0.359	60.1 (9.1)	70.5 (8.3)	0.010*
Luteal Session (kg)	64.9 (10.4)	66.7 (12.2)	62.4 (7.3)	0.324	59.7 (9.5)	70.6 (8.4)	0.009*
Fat Free Mass (kg)	47.1 (5.9)	47.9 (6.4)	46.1 (5.5)	0.445	45.1 (6.7)	49.3 (4.4)	0.082
Fat Mass (kg)	19.4 (5.9)	21.3 (6.7)	17.0 (3.8)	0.073	15.7 (3.8)	23.5 (5.2)	0.001*
Body Fat (%)	28.6 (5.0)	30.1 (5 .2)	26.9 (4.3)	0.116	25.6 (3.6)	32.0 (4.2)	0.001*
Visceral Adipose Tissue (g)	168.9 (55.9)	180.1 (52.5)	154.6 (59.4)	0.268	147.1 (49.0)	192.5 (55.1)	0.040*
Waist-to-Hip Ratio	0.73 (0.04)	0.73 (0.04)	0.73 (0.03)	0.689	0.73 (0.04)	0.73 (0.04)	0.694
$\dot{V}O_{2peak}$ (mL/kg/min)	38.8 (6.6)	37.1 (7.1)	41.0 (5.5)	0.142	43.7 (4.7)	33.6 (3.9)	0.001*
Mitochondria oxidative capacity (1/sec)	1.39 (0.36)	1.36 (0.39)	1.43 (0.34)	0.632	1.43 (0.33)	1.35 (0.41)	0.655
Follicular Phase Session (Day)	4.5 (1.9)	5.4 (1.5)	3.4 (1.9)	N/A	4.1 (1.9)	5.0 (2.0)	0.253
Luteal Phase Session (Day)	20.0 (2.4)	21.2 (2.2)	18.5 (1.8)	N/A	19.4 (2.7)	20.6 (1.9)	0.247
Luteal Phase Session (Day)	6.5 (1.2)	6.7 (0.92)	N/A	N/A	6.3 (1.6)	6.6 (0.9)	0.737
Power Output High Intervals (W)	158.7 (20.8)	154.5 (23.3)	164.16 (16.8)	0.266	162.1 (21.6)	155.1 (20.3)	0.419
Max Power Output During $\dot{V}O_{2peak}$ test (W)	219.8 (28.7)	214.2 (31.2)	227.0 (24.6)	0.278	223.3 (30.1)	216.1 (27.8)	0.541

Note: OC, oral contraceptive users ; NOC, non-oral contraceptive users; HFW, high fitness women; LFW, low fitness women; cm, centimeters; g, grams; kg, kilograms; %, percent; mL, milliliters; min, minute; sec, second; W, watts; N/A, not available; *significant differences between groups ($P < 0.05$).

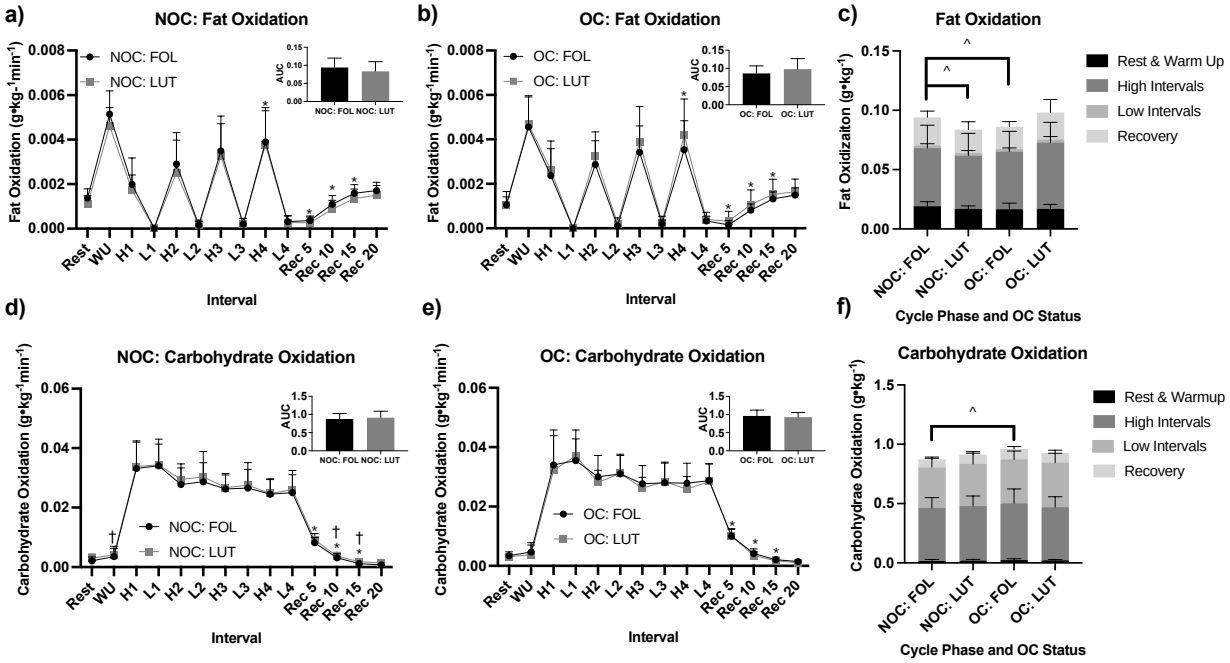
Figure 2.1. Fat and carbohydrate oxidation response during HIIE across the menstrual cycle by fitness status.



Note: (a) Fat oxidation (g•kg^{-1}•min^{-1}) response during HIIE (ANOVA: *Time*, $P \leq 0.001$, $\eta_p^2 = 0.993$; *Group* $P = 0.002$, $\eta_p^2 = 0.386$, n = 25) in FOL (ANOVA: *Group*Condition,* $P = 0.027$, $\eta_p^2 = 0.231$, n = 25). Fat oxidation AUC during HIIE (Student *t*-test: $P = 0.050$, HFW: $n = 13$; LFW: $n = 12$) (b) Fat oxidation (g•kg^{-1}•min^{-1}) (ANOVA: *Time*, $P \leq 0.001$, $\eta_p^2 = 0.993$; *Group* $P = 0.002$, $\eta_p^2 = 0.386$, n = 25) response during HIIE in LUT (ANOVA: *Group*Condition,* $P = 0.005$, $\eta_p^2 = 0.314$). Fat oxidation AUC during HIIE (Student *t*-test: $P = 0.001$, HFW: $n = 13$; LFW: $n = 12$) (c) Total grams of fat oxidized (g•kg^{-1}) during HIIE stratified by during the full session, seated rest and warm up, high intervals, low intervals, and recovery (Student *t*-test: $P < 0.050$, HFW: $n = 13$; LFW: $n = 12$) (d) Carbohydrate oxidation (g•kg^{-1}•min^{-1}) response during HIIE (ANOVA: *Time*, $P \leq 0.001$, $\eta_p^2 = 0.990$; *Group,*

P = 0.157, η_p^2 = 0.093, n = 25) in FOL. Carbohydrate oxidation AUC during HIIE (Student *t*-test: *P* = 0.024, HFW: *n* = 13; LFW: *n* = 12) (e) Carbohydrate oxidation (g•kg^{-1}•min^{-1}) response during HIIE (ANOVA: *Time*, *P* ≤ 0.001, η_p^2 = 0.990; *Group,* *P* = 0.157, η_p^2 = 0.093, n = 25) in LUT during HIIE. Carbohydrate oxidation AUC during HIIE (Student *t*-test: *P* = 0.124, HFW: *n* = 13; LFW: *n* = 12) (f) Total grams of carbohydrate oxidized (g•kg^{-1}) during HIIE stratified by during the full session, seated rest and warm up, high intervals, low intervals, and recovery (Student *t*-test: *P* < 0.050, HFW: *n* = 13; LFW: *n* = 12). HIIE, high intensity interval exercise; FOL, follicular phase; LUT, luteal phase; HFW, high fitness women; LFW, low fitness women; BL, baseline; WU, warmup; H1-4, HIIE 1-4; L1-4, low intensity intervals; R5-15, post exercise recovery minutes 5-15. * P ≤ 0.05 HFW vs LFW, * P ≤ 0.050 Full Session, † P ≤ 0.050 High Intensity Intervals, # P ≤ 0.05 Low Intensity Intervals, ^ P ≤ 0.05 Recovery

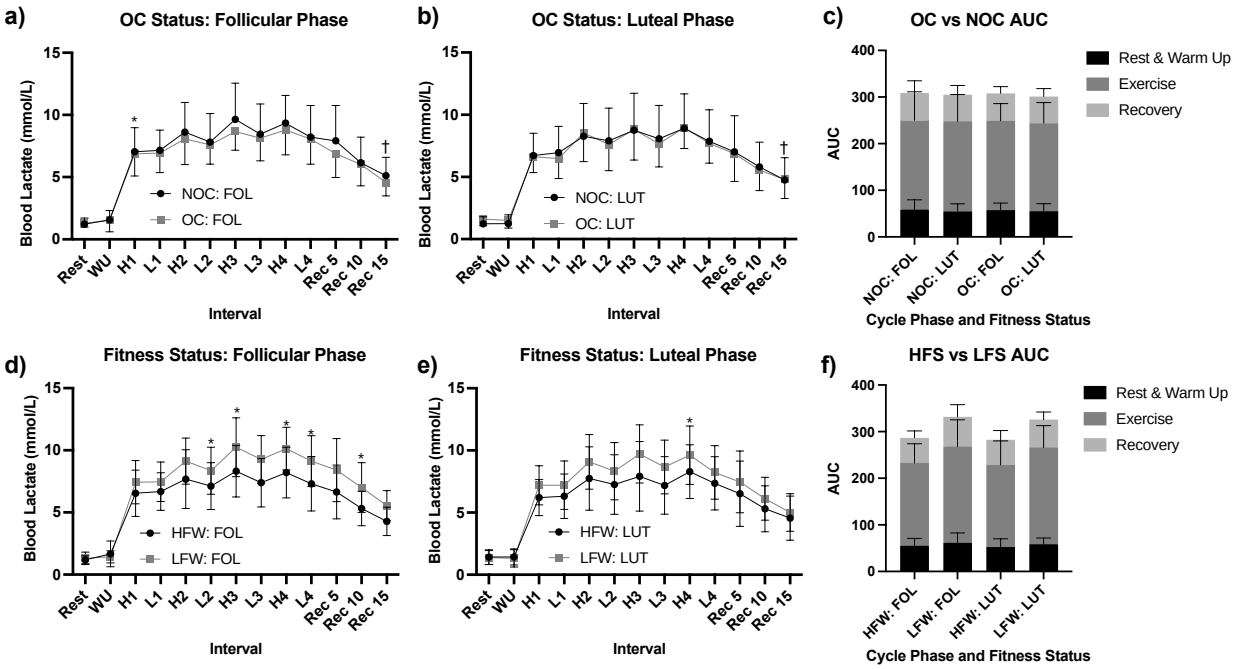
Figure 2.2. Fat and carbohydrate oxidation response during HIIE across the menstrual cycle by oral contraceptive status.



Note: (a) Fat oxidation (g•kg^{-1}•min^{-1}) response during HIIE (ANOVA: *Time*, $P \leq 0.001$, $\eta_p^2 = 0.993$; *Group FOL*, $P = 0.064$, $\eta_p^2 = 0.154$; *Group LUT*, $P = 0.714$, $\eta_p^2 = 0.007$, $n = 25$) in NOC. Fat oxidation AUC during HIIE (Student *t*-test: $P = 0.125$, NOC: $n = 14$; OC $n = 11$) (b) Fat oxidation (g•kg^{-1}•min^{-1}) response during HIIE in OC Fat oxidation AUC during HIIE (Student *t*-test: $P = 0.275$, NOC: $n = 14$; OC $n = 11$) (c) Total grams of fat oxidized (g•kg^{-1}) during HIIE stratified by during the full session, seated rest and warm up, high intervals, low intervals, and recovery (Student *t*-test, $P \leq 0.050$, NOC: $n = 14$; OC $n = 11$). (d) Carbohydrate oxidation (g•kg^{-1}•min^{-1}) (ANOVA: *Time*, $P \leq 0.001$, $\eta_p^2 = 0.990$, $n = 25$) response during HIIE in NOC. Carbohydrate oxidation AUC during HIIE (Student *t*-test: $P = 0.154$, NOC: $n = 14$; OC $n = 11$) (e) Carbohydrate oxidation (g•kg^{-1}•min^{-1})

response during HIIE in OC. Carbohydrate oxidation AUC during HIIE (Student *t*-test: $P = 0.341$, NOC: $n = 14$; OC $n = 11$) (f) Total grams of carbohydrate oxidized ($g \cdot kg^{-1}$) during HIIE stratified by during the full session, seated rest and warm up, high intervals, low intervals, and recovery (Student *t*-test, $P < 0.050$, NOC: $n = 14$; OC $n = 11$). HIIE, high intensity interval exercise; FOL, follicular phase; LUT, luteal phase; NOC, non-oral contraceptive user; OC, oral contraceptive user; BL, baseline; WU, warmup; H1-4, HIIE 1-4; L1-4, low intensity intervals; R5-15, post exercise recovery minutes 5-15. * P ≤ 0.050 NOC vs OC in FOL, † P ≤ 0.050 FOL vs LUT in NOC, ^ P ≤ 0.050 Recovery

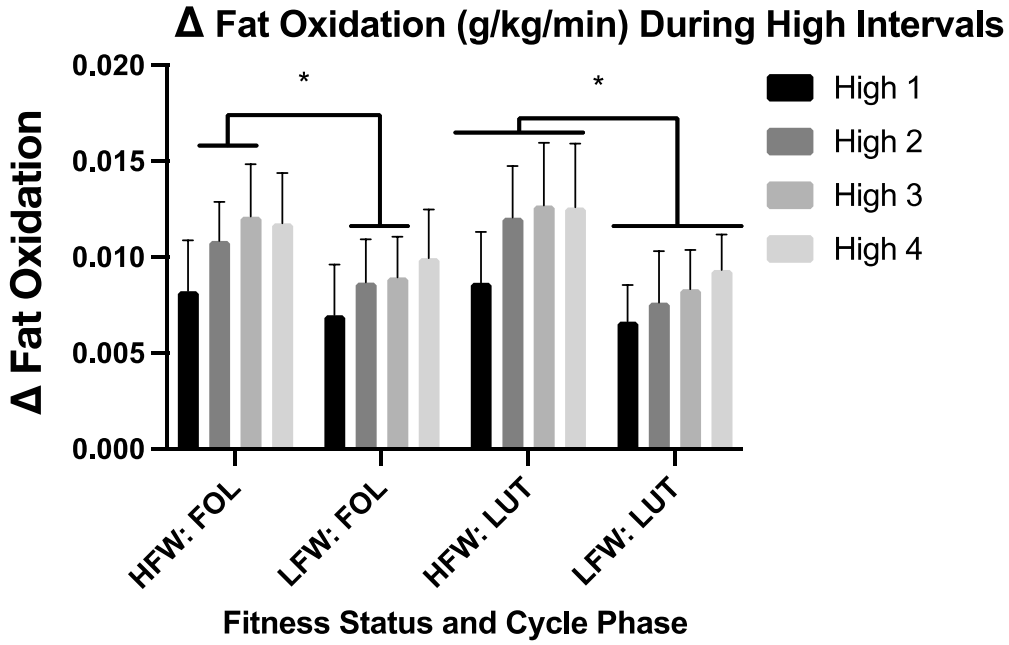
Figure 2.3. Blood Lactate response during HIIE across the menstrual cycle.



Note: (a) Blood Lactate (mmol/L) repones to HIIE (ANOVA: *Time*, $P = 0.036$, $\eta_p^2 = 0.978$, $n = 25$) by OC status during FOL (b) Blood Lactate (mmol/L) repones to

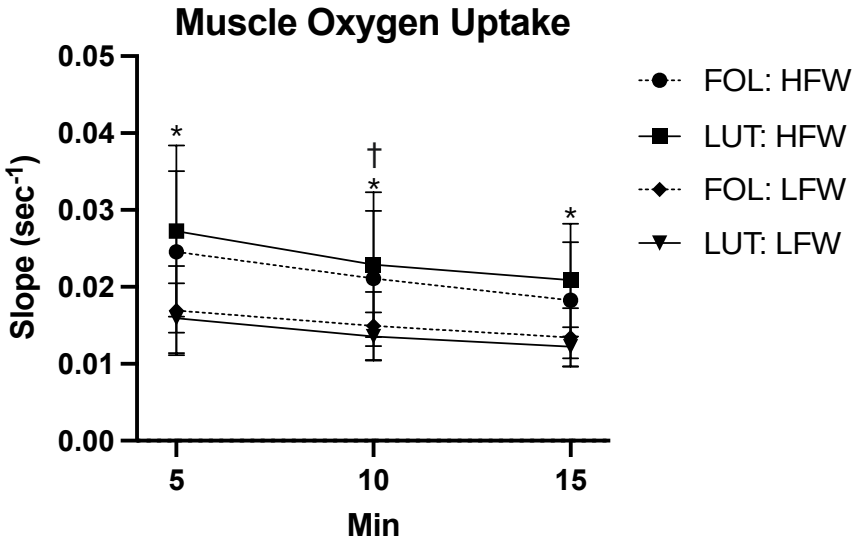
HIIE (ANOVA: *Time*, $P = 0.036$, $\eta_p^2 = 0.978$, $n = 25$) by OC status during LUT (c) Blood Lactate AUC by OC status stratified by seated rest and warm up, exercise, and recovery (Student *t*-test, $P > 0.050$, NOC, $n = 14$; OC $n = 11$) (d) Blood lactate (mmol/L) repones to HIIE (ANOVA: *Time*, $P = 0.036$, $\eta_p^2 = 0.978$, $n = 25$) by Fitness Status during FOL (ANOVA: *Time*, $P = 0.036$, $\eta_p^2 = 0.978$; *Group*Condition,* $P = 0.048$, $\eta_p^2 = 0.252$, $n = 25$) (e) Blood lactate (mmol/L) repones to HIIE by Fitness Status during LUT (ANOVA: *Time*, $P = 0.036$, $\eta_p^2 = 0.978$; *Group*Condition,* $P = 0.072$, $\eta_p^2 = 0.212$, $n = 25$) (f) Blood Lactate AUC by Fitness Status stratified by seated rest and warm up, exercise, and recovery (Student *t*-test, $P > 0.050$, HFW, $n = 13$ LFW $n = 12$) HIIE, high intensity interval exercise; FOL, follicular phase; LUT, luteal phase; NOC, non oral contraceptive user; OC oral contraceptive user; HFW, high fitness women; LFW, low fitness women; BL, baseline; WU, warmup; H1-4, HIIE 1-4; L1-4, low intensity intervals; R5-15, post exercise recovery minutes 5-15. * $P \leq 0.050$ NOC vs OC *or* HFW vs LFW, † $P \leq 0.050$ OC FOL vs OC LUT

Figure 2.4. Metabolic Flexibility during HIIE by fitness status.



Note: Metabolic flexibility assessed as total difference in fat oxidation from lowest and maximal values (Δ) during high intensity intervals in FOL and LUT between HFW and LFW. FOL, follicular phase; LUT, luteal phase; HFW, high fitness women; LFW, low fitness women. $P \leq 0.05$ *between groups*

Figure 2.5. Post Exercise Muscle Oxygen Uptake ($m\dot{V}O_2$)



Note: Post Exercise Muscle Oxygen Uptake ($m\dot{V}O$; sec^{-1}) in participants based on fitness status across the menstrual cycle (ANOVA: *Time*, $P \leq 0.001$, $\eta_p^2 = 0.773$; *Time*Group*, $P = 0.043$, $\eta_p^2 = 0.325$; *Group LUT*, $P = 0.007$, $\eta_p^2 = 0.357$; *Group FOL*, $P = 0.053$, $\eta_p^2 = 0.203$, $n = 25$). FOL, follicular phase; LUT, luteal phase; HFW, high fitness women; LFW, low fitness women. * $P \leq 0.050$ HFW vs LFW LUT, † $P \leq 0.050$ HFW vs LFW FOL

Supplemental Table 2.1. Participant % of VO2max, PRE and Heart Rate across exercise intervals.

Phase			Follicular Phase								Luteal Phase							
Measure	Group	n	High 1	Low 1	High 2	Low 2	High 3	Low 3	High 4	Low 4	High 1	Low 1	High 2	Low 2	High 3	Low 3	High 4	Low 4
%VO2max	NOC	14	71.7 (5.2)	41.3 (7.7)	72.7 (5.0)	42.1 (7.7)	73.9 (5.2)	42.0 (7.1)	73.1 (5.7)	43.1 (5.9)	71.5 (4.4)	40.1 (6.7)	72.8 (3.9)	41.9 (7.3)	73.4 (4.3)*	41.9 (7.3)	72.9 (4.9)	42.5 (5.3)
	OC	11	67.1 (8.9)	39.6 (10.8)	67.3 (11.7)	41.4 (11.6)	67.5 (11.1)	41.4 (11.9)	69.1 (11.3)	44.1 (11.7)	66.9 (8.6)	41.5 (13.4)	66.7 (10.1)	42.8 (13.6)	67.4 (10.1)*	43.1 (13.9)	69.4 (10.5)	46.0 (13.2)
	HFW	13	68.0 (9.2)	38.2 (10.9)	67.5 (11.0)	39.4 (11.6)	67.3 (9.7)*	39.8 (11.6)	68.1 (10.1)*	41.5 (11.3)	66.6 (8.1)*	39.1 (13.0)	67.1 (9.6)*	40.4 (13.3)	67.9 (9.6)	41.0 (13.4)	69.1 (9.8)	43.3 (13.0)
	LFW	12	71.5 (4.0)	43.1 (5.9)	73.4 (4.2)	44.3 (5.8)	75.2 (5.2)*	43.8 (5.9)	74.9 (5.2)*	45.7 (4.0)	72.6 (3.4)*	42.5 (5.1)	73.4 (3.2)*	44.4 (5.5)	73.8 (3.8)	44.1 (6.2)	73.9 (4.2)	44.8 (3.5)
RPE	NOC	14	14.2 (1.4)	8.7 (3.3)	15.3 (1.5)	9.5 (2.9)	16.2 (1.8)	9.6 (3.0)	16.2 (1.8)	9.3 (2.8)	13.6 (1.3)	9.3 (2.8)*	14.8 (1.8)	9.6 (3.3)	15.8 (1.7)	9.4 (2.9)*	16.2 (1.7)	8.9 (2.9)*
	OC	11	13.8 (1.3)	10.5 (2.3)	15.2 (1.5)	11.6 (2.9)	15.5 (1.7)	11.7 (2.6)	15.9 (1.6)	11.5 (3.4)	13.6 (1.2)	11.5 (1.8)*	14.6 (1.3)	11.5 (2.6)	15.7 (1.5)	11.6 (2.2)*	16.1 (1.2)	12.0 (2.7)*
	HFW	13	14.2 (1.2)	9.4 (2.6)	15.4 (1.3)	10.6 (3.2)	15.5 (1.2)	10.7 (2.8)	15.8 (1.3)	10.0 (3.2)	13.9 (1.3)	10.5 (2.5)	14.9 (1.4)	10.5 (3.1)	16.0 (1.5)	10.7 (2.7)	16.2 (1.4)	10.4 (3.3)
	LFW	12	13.9 (1.5)	9.6 (3.4)	15.2 (1.8)	10.3 (3.0)	16.4 (2.2)	10.4 (3.2)	16.4 (2.1)	10.5 (3.3)	13.3 (1.2)	9.9 (2.7)	14.5 (1.8)	10.3 (3.2)	15.5 (1.6)	10.1 (3.0)	16.1 (1.7)	9.8 (3.1)
Heart Rate	NOC	14	154.2 (10.5)	145.4 (17.2)	163 (10.6)	149.1 (16.7)	165.7 (10.4)	152.1 (16.9)	165.6 (12.4)	156.4 (10.7)	145.5 (31.0)	134.2 (27.0)	153.4 (32.2)	142.9 (19.1)	156.2 (31.1)	145.2 (22.1)	157.9 (31.6)	154.8 (16.5)
	OC	11	142.4 (31.0)	132.2 (19.1)	157.4 (17.9)	141.6 (18.0)	161.4 (16.3)	145.6 (17.1)	165.1 (14.6)	143 (27.9)	150.7 (16.3)	139.8 (20.1)	156.2 (15.9)	146.8 (20.8)	160.5 (14.3)	150.7 (18.5)	164.0 (14.3)	148.2 (31.2)
	HFW	13	150.3 (16.7)	133.3 (16.4)	157.7 (16.4)	140.6 (16.1)	160.9 (14.8)	142.2 (15.5)*	162.8 (14.6)	144.7 (20.4)	141.7 (33.1)	128.7 (30.3)	145.9 (33.1)	138.7 (24.3)	149.1 (31.3)	140.2 (25.2)	152.7 (32.8)	142.6 (29.2)*
	LFW	12	147.5 (27.8)	146.5 (19.7)	163.6 (11.3)	151.5 (17.4)	167.1 (11.0)	156.8 (15.6)*	168.1 (11.3)	156.9 (20.1)	154.5 (10.2)	145.3 (9.5)	164.0 (8.9)	151.1 (10.2)	167.9 (8.3)	155.6 (8.7)	169.1 (7.7)	162.0 (9.2)*

Note: OC, oral contraceptive users ; NOC, non-oral contraceptive users; HFW, high fitness women; LFW, low fitness women; RPE, rating of perceived exertion. High, high intensity interval; Low, low intensity interval.

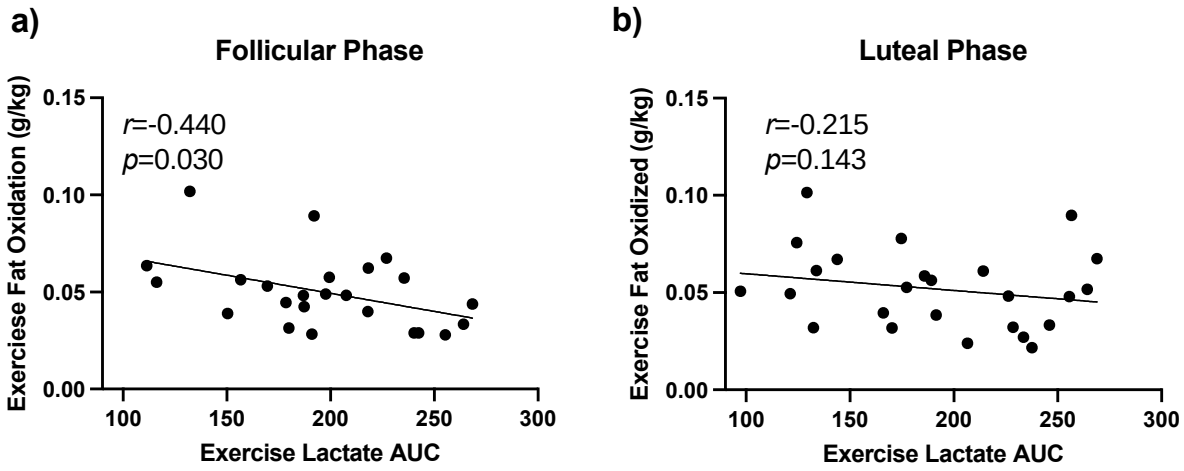
*significant differences between groups ($P < 0.05$).

Supplemental Table 2.2. Participants self-reported reported 3-day dietary intake prior to each testing session.

	Carbs (g)	Protein (g)	Fat (g)	Calories (kcal)
Follicular Phase	170.7 (48.0)	76.5 (48.7)	57.5 (37.0)	1883.5 (504.1)
Luteal Phase	159.1 (28.1)	68.0 (53.7)	Fat: 53.6 (41.1)	1947.9 (689.4)

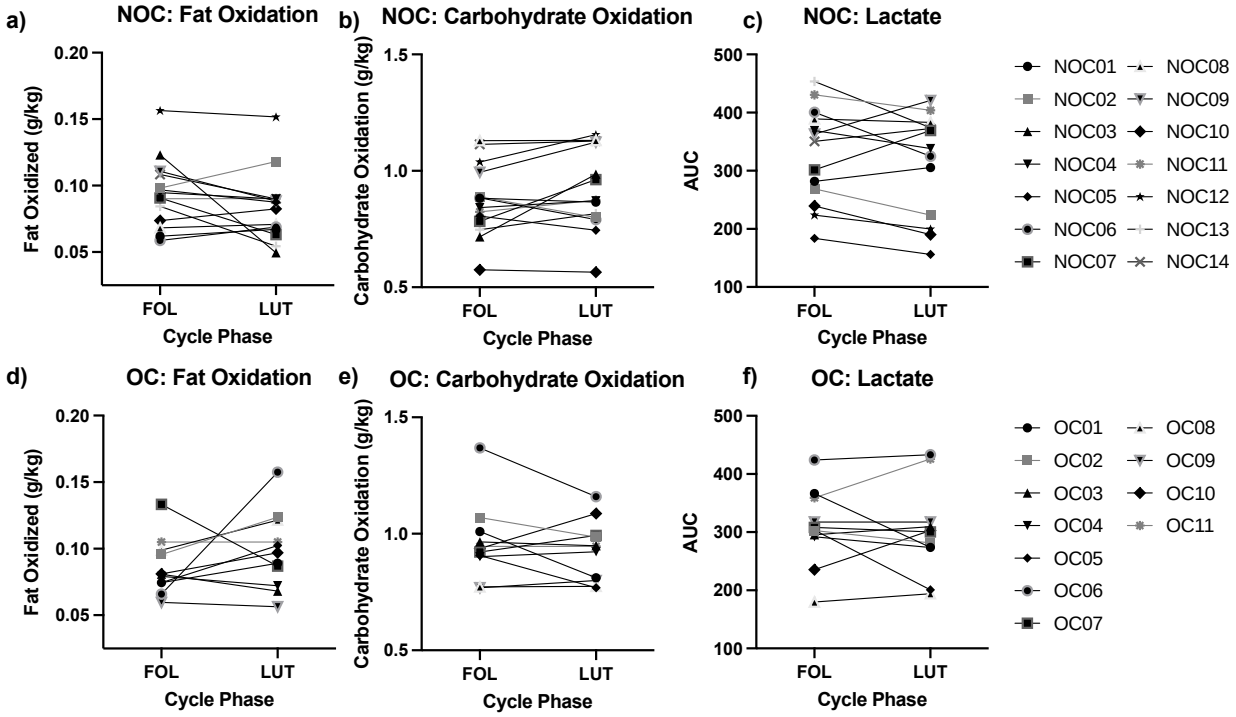
Note: (P > 0.05)

Supplemental Figure 2.1. Correlations between exercise fat oxidation and exercise blood lactate



Supplemental Figure 2.1. Correlations between exercise fat oxidation (g/kg) and exercise lactate AUC (H1-L4). During the (A) follicular phase (*r*=-0.440, *p*=0.030; n=25) and (B) luteal phase (-30 minute) (*r*=0.215, p=0.143; n=25)

Supplemental Figure 2.2. Individual differences



Note: Individual differences in exercise fat oxidation, carbohydrate oxidation and blood lactate in NOC (a-c) and OC (d-f) groups. FOL, follicular phase; LUT, luteal phase; NOC, non oral contraceptive user; OC oral contraceptive use.

CHAPTER 5

CONCLUSION

The results of this collective body of work highlight the influence that fitness status, sex, fitness, menstrual cycle and muscle play in whole body metabolism. Specifically, these studies enhance our understanding of metabolic flexibility beyond the post prandial state, classifying and demonstrating the efficacy of assessing it during high intensity interval exercise. Furthermore, this body of work adds to our current understandings of sex and menstrual cycle differences, or lack thereof, during high intensity exercise. Together our data suggesting that fitness status plays a more important role in women's metabolic response that menstrual cycle phase or oral contraceptive use, and may be more important to metabolic flexibility in women than men.

In our first study we demonstrate for the first time that the capacity to oxidize fat during HIIE and in response to a high-fat meal challenge are related. High intensity interval exercise may be useful as an additional assessment to assess metabolic flexibility. Our test used here was more sensitive in detecting underlying metabolic differences in young healthy individuals, otherwise not noted during a traditional post prandial response meal. Specifically, the individual variability within our data is wider in response to the high fat meal challenge, potentially masking differences the HIIE session was able to capture. Furthermore, while we found no

sex differences in our study, we noted the important role that fitness status plays in metabolism, specifically in women, where low fit women showed impaired metabolism compared to both high fit women and men. This data highlights the importance of one's response to exercise as being an often-overlooked aspect of our metabolic health. The unique stress high intensity exercise presents to the muscle metabolic system is an effective way of capturing underlying metabolic flexibility. Overall health is more than just how we respond to what we eat, but also how we move. The ability to capitalize on the fat oxidation pathways both following meals but also during exercise can lead to greater whole body metabolic health in the post prandial state, improved body composition, and long-term metabolic health. Future research should consider our HIIE test as an alternate or more sensitive way to assess metabolic flexibility in young healthy individuals, who's meal response may be more similar and variable.

In our second study, we followed up our first further looking at the discrepancies noted in lower fit women. However, this time we expanded our timeline to also account for the menstrual cycle and oral contraceptive use in women, all variables that may impact exercise, whole-body metabolism, and metabolic health. In this work, we used high intensity interval exercise for the first time to assess the impacts of the menstrual cycle, oral contraceptive status and fitness status in women. We found that high fitness women benefit from greater exercise metabolic flexibility than low fit women. Furthermore, that a driving factor in these differences may be in part explained by underlying skeletal muscle oxidative capacity. However, we found no differences within our groups across the

menstrual cycle phase or with oral contraceptive use, suggesting these do not negatively impact exercise metabolic response during high intensity interval exercise. Our findings, together with our first study, are important in the assessment of women's metabolic health and the impact that a higher fitness status may have on this. Furthermore, we show muscle metabolism may play a role in this, potentially driving these adaptations. We hypothesis that the benefits of estrogen on metabolic health may be enhanced by aerobic training in young healthy pre-menopause women. Together, our studies showing the importance of fitness status, which may contribute to exercise metabolic flexibility and long-term metabolic health.

Lastly, our studies together show the efficacy of using non-invasive muscle and mitochondrial tools to further assess muscle and metabolism during the post prandial period, post exercise, during exercise and independently. Here were demonstrated for the first time the assessment of muscle metabolism in the post prandial and post exercise states. Additionally, we demonstrated these measures are sensitive enough to detect differences within fitness status, and the menstrual cycle during exercise. The use of non-invasive tools to assess muscle oxygen uptake, metabolism, and mitochondrial capacity can transform both research and practice. These tools may be commercially available for consumers and researchers to assess underlying muscle physiology otherwise not able to be detected outside of invasive measures such as biopsy. Our collective data here demonstrate both the efficacy of these measures, as well as the relationship that muscle plays with whole body metabolism during feeding and exercise. Since

skeletal muscles is a major organ, housing large sums of our mitochondria driving oxidative metabolism, storage uptake and breakdown, being able to quantify their contribution may help shed light on underlying metabolic inefficiencies for sport and metabolic health.

Our work here is important to a growing body of literate focused on women's health and performance. Recent reports indicate that only 6% of current publications in our field focus on only on women, with only 20% of those looking at women specific topics such as menstrual cycle implications to training and health [159]. Historically, there has been inconsistencies in the impacts of the menstrual cycle on exercise metabolism, with no work looking at this during high intensity exercise or exercise metabolic flexibility, or regarding fitness status. Here we show the importance of aerobic fitness status in women, which influence exercise metabolism more than menstrual cycle phase or oral contraceptive use. In both our studies, women with a lower fitness status had both lower post prandial and exercise fat oxidation, during both the luteal and the follicular phase. Our results indicate there is no negative impact of menstrual cycle phase or oral contraceptive use on exercise metabolism, specifically during higher intensity interval exercise, in the early follicular and mid luteal phases.

Furthermore, our studies are the first to use high intensity interval exercise as a novel means of assessing exercise metabolism. The ability to switch between carb and fat oxidation, along with minimal lactate accumulation may be an important measure of metabolic flexibility and long-term metabolic health. The relationship between post prandial metabolic flexibility and exercise has not till

now, been established. Together our studies support the argument that exercise should not be left out of the equation when assessing metabolic health. Furthermore, that muscle metabolism and mitochondrial capacity may play important contributing roles to whole body metabolism. Since aerobic exercise stimulate the fat oxidation pathways and improve mitochondrial capacity and biogenesis, the role of exercise is vital in the maintenance of metabolic flexibility and health. The later here being especially important in the context of young healthy women. With so much emphasis on cycle or oral contraceptive effects, we suggest that the emphasis for general health and physical performance when it comes to exercise substrate utilization and metabolic flexibility should be placed on improving overall aerobic fitness status.

In conclusion, our studies show for the first time that one's metabolic flexibility following high fat feeding and exercise are related. Exercise plays a vital role in maintaining metabolic health and capacity to oxidize fat during both the post prandial state and during high intensity interval exercise. Furthermore, low fitness status in women impairs their metabolic flexibility, but exercise metabolism is not negatively impacted by menstrual cycle phase or oral contraceptive use in women. The ability to utilize fats is favorable for long term weight management, metabolic health and preventing metabolic disease. Our work emphases the importance of increased aerobic fitness in improving metabolism in women.

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