HUMAN SUBSTRATE METABOLISM AT UPPER OXIDATIVE CAPACITIES: HOW INTENSITY AND PRE-EXERCISE NUTRITION AFFECT THE OXIDATIVE REGULATION OF CARBOHYDRATE AND FAT DURING METABOLIC TARGETED RUNNING

A DISSERTATION PRESENTED

BY

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TO

THE FACULTY OF HUMAN SCIENCES

PROFESSORSHIP FOR SPORTS MEDICINE AND SPORTS ORTHOPEDICS

FOR THE DEGREE OF DOCTOR OF PHILOSOPHY IN THE SUBJECT OF CLINICAL EXERCISE SCIENCE

UNIVERSITY OF POTSDAM

GERMANY, 2017

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Date of defense: 18.01.2019

Published online at the
Institutional Repository of the University of Potsdam:
https://nbn-resolving.org/urn:nbn:de:kobv:517-opus4-423338
https://doi.org/10.25932/publishup-42333

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Hereby, I declare that this thesis entitled “HUMAN SUBSTRATE METABOLISM AT UPPER OXIDATIVE CAPACITIES: HOW INTENSITY AND PRE-EXERCISE NUTRITION AFFECT THE OXIDATIVE REGULATION OF CARBOHYDRATE AND FAT DURING METABOLIC TARGETED RUNNING”, or parts of the thesis, have not yet been submitted for a doctoral degree to this or any other institution neither in identical nor in similar form. The work presented in this thesis is the original work of the author. I did not receive any help or support from commercial consultants. All parts or single sentences, which have been taken analogously or literally from other sources, are identified as citations.

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Abstract

Introduction: Carbohydrate (CHO) and fat are the main substrates to fuel prolonged endurance exercise, each having its oxidation patterns regulated by several factors such as intensity, duration and mode of the activity, dietary intake pattern, muscle glycogen concentrations, gender and training status. Exercising at intensities where fat oxidation rates are high has been shown to induce metabolic benefits in recreational and health-oriented sportsmen. The exercise intensity (Fat_{peak}) eliciting peak fat oxidation rates is therefore of particular interest when aiming to prescribe exercise for the purpose of fat oxidation and related metabolic effects. Although running and walking are feasible and popular among the target population, no reliable protocols are available to assess Fat_{peak} as well as its actual velocity (V_{PFO}) during treadmill ergometry. Moreover, to date, it remains unclear how pre-exercise CHO availability modulates the oxidative regulation of substrates when exercise is conducted at the intensity where the individual anaerobic threshold (IAT) is located (V_{IAT}). That is, a metabolic marker representing the upper border where constant load endurance exercise can be sustained, being commonly used to guide athletic training or in performance diagnostics. The research objectives of the current thesis were therefore, 1) to assess the reliability and day-to-day variability of V_{PFO} and Fat_{peak} during treadmill ergometry running; 2) to assess the impact of high CHO (HC) vs. low CHO (LC) diets (where on the LC day a combination of low CHO diet and a glycogen depleting exercise was implemented) on the oxidative regulation of CHOs and fat while exercise is conducted at V_{IAT}. **Methods:** Research objective 1: Sixteen recreational athletes (f=7, m=9; 25 ± 3 y; 1.76 ± 0.09 m; 68.3 ± 13.7 kg; 23.1 ± 2.9 kg/m²) performed 2 different running protocols on 3 different days with standardized nutrition the day before testing. At day 1, peak oxygen uptake (VO_{2peak})

and the velocities at the aerobic threshold (V_{LT}) and respiratory exchange ratio (RER) of 1.00 (V_{RER}) were assessed. At days 2 and 3, subjects ran an identical submaximal incremental test (Fat-peak test) composed of a 10 min warm-up (70% V_{LT}) followed by 5 stages of 6 min with equal increments (stage 1 = V_{LT}, stage 5 = V_{RER}). Breath-by-breath gas exchange data was measured continuously and used to determine fat oxidation rates. A third order polynomial function was used to identify V_{PFO} and subsequently Fat_{peak}. The reproducibility and variability of variables was verified with an intraclass correlation coefficient (ICC), Pearson's correlation coefficient, coefficient of variation (CV) and the mean differences (bias) ± 95% limits of agreement (LoA). Research objective 2: Sixteen recreational runners (m=8, f=8; 28 ± 3 y; 1.76 ± 0.09 m; 72 ± 13 kg; 23 ± 2 kg/m²) performed 3 different running protocols, each allocated on a different day. At day 1, a maximal stepwise incremental test was implemented to assess the IAT and V_{IAT}. During days 2 and 3, participants ran a constant-pace bout (30 min) at V_{IAT} that was combined with randomly assigned HC (7g/kg/d) or LC (3g/kg/d) diets for the 24 h before testing. Breath-by-breath gas exchange data was measured continuously and used to determine substrate oxidation. Dietary data and differences in substrate oxidation were analyzed with a paired *t*-test. A two-way ANOVA tested the diet X gender interaction ($\alpha = 0.05$). **Results:** Research objective 1: ICC, Pearson's correlation and CV for V_{PFO} and Fat_{peak} were 0.98, 0.97, 5.0%; and 0.90, 0.81, 7.0%, respectively. Bias ± 95% LoA was -0.3 ± 0.9 km/h for V_{PFO} and -2 ± 8% of VO_{2peak} for Fat_{peak}. Research objective 2: Overall, the IAT and V_{IAT} were 2.74 ± 0.39 mmol/l and 11.1 ± 1.4 km/h, respectively. CHO oxidation was 3.45 ± 0.08 and 2.90 ± 0.07 g/min during HC and LC bouts respectively ($P < 0.05$). Likewise, fat oxidation was 0.13 ± 0.03 and 0.36 ± 0.03 g/min ($P < 0.05$). Females had 14% ($P < 0.05$) and 12% ($P > 0.05$) greater fat

oxidation compared to males during HC and LC bouts, respectively. **Conclusions:** Research objective 1: In summary, relative and absolute reliability indicators for V_{PFO} and Fat_{peak} were found to be excellent. The observed LoA may now serve as a basis for future training prescriptions, although fat oxidation rates at prolonged exercise bouts at this intensity still need to be investigated. Research objective 2: Twenty-four hours of high CHO consumption results in concurrent higher CHO oxidation rates and overall utilization, whereas maintaining a low systemic CHO availability significantly increases the contribution of fat to the overall energy metabolism. The observed gender differences underline the necessity of individualized dietary planning before exerting at intensities associated with performance exercise. Ultimately, future research should establish how these findings can be extrapolated to training and competitive situations and with that provide trainers and nutritionists with improved data to derive training prescriptions.

Zusammenfassung

Einleitung: Kohlenhydrate (CHO) und Fett sind die bedeutendsten Energieträger bei anhaltender Ausdauerbelastung. Diese Substrate haben individuelle Oxidationsmuster, die von Intensität, Dauer und Art der Aktivität, sowie Nahrungszufuhr, Muskelglykogen-konzentration, Geschlecht und Trainingsstatus abhängen. Es ist bekannt, dass körperliche Aktivität unter hohen Fettverbrennungsraten, vorteilhafte metabolische Effekte bei freizeitaktiven und gesundheitsorientierten Sportlern hervorrufen. Die durch Belastungsintensität (Fat_{peak}) hervorgerufene höchste Fettoxidationsrate ist daher von besonderer Bedeutung für Empfehlungen von Fettverbrennungsaktivitäten und hierzu gehörigen metabolischen Effekten. Obgleich Joggen und Laufen als praktikabel und verbreitet in entsprechenden Zielgruppen angesehen wird, existieren keine reliablen Protokolle um Fat_{peak} und die hierzu spezifische Geschwindigkeit (V_{PFO}) bei einer Laufbandergometrie zu bestimmen. Darüberhinaus, ist bis heute ungeklärt, inwiefern die Verfügbarkeit von CHO vor körperlicher Belastung, die oxidative Regulation von Substraten beeinflusst, wenn die Belastungsintensität bei der individuelle anaeroben Schwelle (IAT) durchgeführt wird (V_{IAT}). Die IAT beschreibt hierbei einen metabolischen Schwellenwert, bis zu welchem eine konstante Ausdauerleistung aufrecht erhalten werden kann. Dieser Schwellenwert wird üblicherweise zur Trainingssteuerung oder Leistungsdiagnostik herangezogen. Die Forschungsziele der hier vorgelegten Thesis sind daher: 1) Die Überprüfung der Reliabilität und Variabilität von V_{PFO} und Fat_{peak} während einer Laufbandergometrie; 2) Die Überprüfung des Einflusses von kohlenhydratreicher (HC) im Vergleich zu kohlenhydratarmer (LC) Nahrungszufuhr auf die Regulierung der Oxidation von Kohlenhydraten und Fetten während körperlicher Aktivität bei V_{IAT}. Hierbei wurde am LC Tag eine Kombination von geringer CHO Zufuhr und einer Glykogen verarmenden

Belastung implementiert. **Methoden:** Forschungsziel 1: Sechszehn Freizeitsportler (f=7, m=9; 25 ± 3 y; 1.76 ± 0.09 m; 68.3 ± 13.7 kg; 23.1 ± 2.9 kg/m²) durchliefen 2 verschiedene Laufprotokolle an drei verschiedenen Tagen unter standardisierter Nahrungszufuhr vor den Untersuchungen. Am Tag 1 wurden die höchste Sauerstoffaufnahme (VO_{2peak}) und die Geschwindigkeiten bei der aeroben Schwelle (V_{LT}), sowie beim respiratorische Quotient (RER) von 1.00 (V_{RER}) erfasst. Am Tag 2 und 3 absolvierten die Probanden einen identischen submaximalen Stufentest (Fat-peak test), welcher aus einer 10 min Erwärmungsphase (70% V_{LT}) gefolgt von 5 gleichmäßig ansteigenden Stufen à 6 min (Stufe 1 = V_{LT}, Stufe 5 = V_{RER}) bestand. Atemgasdaten wurden durch „Breath-by-breath"-Analyse kontinuierlich gemessen und herangezogen um Fettoxidationsraten zu bestimmen. Aus diesen Daten wurde über eine Polynomfunktion dritter Ordnung V_{PFO} und folglich Fat_{peak} identifiziert. Die Reproduzierbarkeit und Variabilität dieser Parameter wurde mittels des Intraklassen-Korrelationskoeffizient (ICC), des Pearson's Korrelationskoeffizient, des Variabilitätskoeffizient (CV) und der mittleren Differenz (bias) ± 95% „limits of agreement" (LoA) überprüft. Forschungsziel 2: Sechzehn Freizeitläufer (m=8, f=8; 28 ± 3 y; 1.76 ± 0.09 m; 72 ± 13 kg; 23 ± 2 kg/m²) durchliefen 3 verschiedene Laufprotokolle an jeweils unterschiedlichen Tagen. Am Tag 1 wurde ein maximaler Stufentest durchgeführt, um die IAT und V_{IAT} zu erfassen. Am zweiten und dritten Tag absolvierten die Probanden eine Laufeinheit (30 min) in konstanter Geschwindigkeit bei V_{IAT}, unter Berücksichtigung einer 24 h zuvor durchgeführten und zufällig zugewiesenen Diät mit entweder HC (7g/kg/d) oder LC (3g/kg/d). „Breath-by-breath"-Atemgasdaten wurden kontinuierlich erfasst und für die Bestimmung der Substratoxidation herangezogen. Ernährungsdaten und Unterschiede in Substratoxidation wurden mittels *t*-test für abhängige Probanden analysiert. Interaktionseffekte zwischen Diät und Geschlecht wurden mittels einer Zwei-Wege

ANOVA getestet (α = 0.05). **Ergebnisse:** Forschungsziel 1: ICC, Pearson's Korrelationskoeffizient und CV von V_{PFO} und Fat_{peak} waren 0.98, 0.97, 5.0%; und respektive 0.90, 0.81, 7.0%. Bias ± 95% LoA betrug -0.3 ± 0.9 km/h für V_{PFO} und -2 ± 8% von VO_{2peak} für Fat_{peak}. Forschungsziel 2: Insgesamt waren IAT 2.74 ± 0.39 mmol/l und V_{IAT} 11.1 ± 1.4 km/h. CHO Oxidation war 3.45 ± 0.08 g/min während HC und respektive 2.90 ± 0.07 g/min während LC Laufeinheiten ($P < 0.05$). Gleichermaßen war Fettoxidation 0.13 ± 0.03 und 0.36 ± 0.03 g/min ($P < 0.05$). Frauen hatten im Vergleich zu Männern 14% ($P < 0.05$) und 12% ($P > 0.05$) größere Fettoxidation in HC und respektive LC Laufeinheiten. **Schlussfolgerungen:** Forschungsziel 1: Zusammenfassend wurde eine exzellente relative und absolute Reliabilität für V_{PFO} und Fat_{peak} gezeigt. Die gefundenen LoA könnten als Basis für zukünftige Trainingssteuerungsempfehlungen genutzt werden, obwohl Fettoxidationsraten bei anhaltenden Ausdauertrainingseinheiten bei dieser Intensität noch untersucht werden müssen. Forschungsziel 2: Hohe CHO-Zufuhr 24 h vor Belastung führt zu einhergehenden höheren CHO-Oxidationsraten und -verwertung, wobei eine niedrige systemische CHO-Verfügbarkeit den Anteil an Fettoxidation im gesamten Energiemetabolismus signifikant erhöht. Die beobachteten Geschlechtsunterschiede unterstreichen die Notwendigkeit einer individualisierten Ernährungsplanung im Vorfeld einer Belastung unter leistungsorientierten Intensitäten. Schlussendlich sollte zukünftige Forschung feststellen, wie hiesige Ergebnisse in Training und Wettkampfsituationen umgesetzt werden können, um somit Trainern und Ernährungswissenschaftlern verbesserte Daten für Trainingsempfehlungen bereitzustellen.

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List of Abbreviations

Acetyl-CoA: Acetyl-coenzyme A

ADP: Adenine diphosphate

ALB: Albumin

AMP: Adenine monophosphate

ATP: Adenosine triphosphate

Bias: Mean differences

BMI: Body mass index

CHO: Carbohydrate

CV: Coefficient of variation

FABP: Fatty acid binding protein

FFAs: Free fatty acids

Fat_{peak}: Intensity eliciting peak fat oxidation rates

GLUT4: Glucose transporter protein 4

Glyc depl: Glycogen depleting bout

G-1-P: Glucose-1-phosphate

G-6-P: Glucose-6-phosphate

HC: High carbohydrate

HIIT: High intensity interval training

HK: Hexokinase

HR: Heart rate

HR_{max}: Maximal heart rate

IAT: Individual anaerobic threshold

ICC: Intraclass correlation coefficient

IMTG: Intramuscular Triacylglycerol

LC: Low carbohydrate

LoA: Limits of agreement

MAP: Maximal aerobic power

Med: Medical check

MM: Mitochondrial membrane

NADH: Nicotinamide adenine dinucleotide

Pi: Inorganic phosphate

PDH: Pyruvate dehydrogenase

PFK: Phosphofructokinase

PFO: Peak fat oxidation

PHOS: Glycogen phosphorylase

PM: Plasma membrane

P_{max}: Maximal power

P3: Third polynomial

RER: Respiratory exchange ratio

SD: Standard deviation

VCO_2: Carbon dioxide output

V_E: Ventilation

V_{IAT}: Individual anaerobic threshold's intensity

V_{LT}: Velocity at aerobic threshold

VO_2: Oxygen uptake

VO_{2peak}: Peak oxygen uptake

V_{peak}: Peak running velocity

V_{PFO}: Velocity at which peak fat oxidation occurs

V_{RER}: Velocity at respiratory exchange ratio of 1.00

%BF: Percentage body fat

%TEM: Technical error of measurement

1. Introduction

Carbohydrate (CHO) and fat are the main substrates to fuel prolonged endurance exercise, each having its oxidation patterns regulated by several factors such as intensity, duration and mode of the activity, dietary intake pattern, muscle glycogen concentrations, gender and training status (Brooks & Mercier 1994, Weltan et al. 1998, Achten et al. 2003, Pendergast et al. 2011, Gonzales & Stevenson 2012, Gmada et al. 2012). When described as a sole function of exercise intensity, the oxidative metabolism of these two substrates has a clear pattern. Fat oxidation (intramyocellular lipids and plasma free fatty acids (FFAs)) will augment as intensity increases from low to moderate levels, achieving peak oxidation rates between 45 to 65% of peak oxygen uptake[1] (VO_{2peak}), then to become minimal at intensities above 85% of VO_{2peak} (Gonzales & Stevenson 2012, Achten & Jeukendrup 2003, Zehnder et al. 2005, Brun et al. 2007). CHO metabolism (blood glucose and stored muscle glycogen) increases parallel to exercise intensity and predominates at times of high physical exertion (Achten & Jeukendrup 2003, Zehnder et al. 2005, Brun et al. 2007). Steady-state exercise on the other hand (i.e. an exercise level that can be maintained for a prolonged period of time), normally favors fat oxidation (Brun et al. 2007). Based upon these regulatory mechanisms and depending on individual goals, athletes may be advised to vary their training regimen around different intensities (using and conditioning both aerobic and anaerobic energetic pathways), while aiming to expand endurance capacity, power and performance (Karakoç et al. 2012).

In recent years there has been emerging interest in the improvement of training

[1] Throughout the text the terminology "peak oxygen uptake" will be solely and synonymously used instead of "maximal oxygen uptake" in order to preserve the reading flow.

prescriptions for metabolic-guided exercise bouts and nutritional practices (Brun et al. 2007, Mosler 2016, Thomas et al. 2016). For instance, exercising at intensities where fat oxidation rates are high has been advocated to induce metabolic changes that benefit both professional and recreational endurance athletes, as well as health-oriented exercisers (Gonzales & Stevenson 2012, Tolfrey et al. 2010, Romain et al. 2012). Subsequently, reliably identifying the intensity at which fat metabolism reaches peak oxidation levels is crucial when prescribing exercise for the purpose of fat oxidation and related metabolic effects (Chenevière et al. 2009). The reproducibility of the intensity eliciting peak fat oxidation (PFO) rates (i.e. Fat_{peak}, but also referred to as Fat_{max} or LIPOXmax) has been reported for a variety of submaximal incremental protocols (Gmada et al. 2012, Achten & Jeukendrup 2003, Pérez-Martin et al. 2001, Michallet et al. 2008, Meyer et al. 2009, Croci et al. 2014). However, all reliability studies to date have used cycle ergometry as the exercising method of choice, which in turn may limit a valid transferability from any of the previously tested protocols and their respective reproducibility indicators into other types of exercise. Yet, despite running and walking being feasible and popular modalities among different target populations (Mendelson et al. 2012), there are to date no reliability data on the estimations of Fat_{peak} during treadmill ergometry. Additionally, only a few studies have performed comprehensive statistical assessments as recommended by the guidelines for reliability assessment in sports medicine (Atkinson & Nevill 1998). These would include for instance, the establishment of both relative and absolute reliability indicators for key variables related to Fat_{peak} estimations, such as the actual velocity at which PFO rates occur (i.e. V_{PFO}), as well as the computation of its respective intrasubject (day-to-day) variability.

Nutrition as previously mentioned, also has the potential to alter the metabolic regulation of substrates with the intake of CHOs in particular, being not only crucial to fuel exercise at intensities above 65% of VO_{2peak}, but also directly assisting in the post-exercise recovery phase (Pendergast et al. 2011, Romijn et al. 1993). For instance, CHO-loading strategies (7-10 g/kg/d) may increase not only glycogen storage (up to 42% post-prandial) but also its overall usage, which in turn delays fatigue allowing exercise to be prolonged and endurance performance to be improved (Pendergast et al. 2011, Neufer et al. 1987, Chryssanthopoulos et al. 2004, Andrews et al. 2003, Wee et al. 2005). Still, these latter mechanisms are somewhat restricted to the male athletic population as females are well known for having a greater reliance on fat metabolism compared to males (Tarnopolsky 2008). In addition, female athletes have had mixed results when it comes to increasing muscle glycogen storage capacity and/or enhancing endurance exercise performance (i.e. despite CHO-loading equivalent to ~75% of the energy intake during 4-6 days) (Zehnder et al. 2005, Andrews et al. 2003, Roepstorff et al. 2002, Ruby et al. 2002). Currently under great publicity, are also strategies that aim to improve fat metabolism at higher exertion levels via metabolic adaptations induced by a systemic manipulation of CHO availability through diet and exercise intensity (Mosler 2016, Thomas et al. 2016). For example, when conducting key, high intensity training bouts on either fasted and/or low CHO dietary states, as well as under glycogen depletion (Mosler 2016). Yet, it remains unclear how pre-exercise CHO intake modulates the oxidative regulation of CHOs and fat, when exercise is conducted at the intensity/velocity where the individual anaerobic threshold (IAT) is located (V_{IAT}). Namely, a metabolic marker delineating the upper levels of endurance capacity in which a shift in the oxidative regulation of substrates is expected favoring a CHO driven

metabolism (Fröhlich 1989, Billat et al. 2003, Faude et al. 2009, Péronnet 2010). The IAT represents the upper border where constant load endurance exercise can be sustained, being commonly used to guide athletic training (e.g. when aiming to improve endurance capacity) or in performance diagnostics (Faude et al. 2009, Péronnet 2010, Kindermann 2004, Stegmann et al. 1981). Exertion at V_{IAT} can be generally sustained for up to 60 minutes, though the average speed of a marathon is reported to be only slightly under it (Billat et al. 2003, Faude et al. 2009). Consequently, in order to assist coaches, trainers and nutritionists in their planning of metabolic-guided training and/or pre-exercise nutritional strategies, it is necessary to first investigate how intensity, exercise mode (i.e. running) and pre-exercise nutrition (especially CHO intake) affect the metabolic regulation of substrate use at upper oxidative levels as individuals attempt to exercise in accordance to the above mentioned biomarkers of metabolic performance (i.e. V_{PFO} and Fat_{peak}) and exercise capacity (i.e. V_{IAT}). Therefore, in this current thesis the following research objectives were postulated:

Research objective 1

Establish the reliability and day-to-day variability of V_{PFO} and Fat_{peak}. Thus, contributing to the improvement of training prescriptions in running to enhance fat metabolism.

Research objective 2

Assess the impact of high CHO (HC) vs. low CHO (LC) diets (i.e. systemic CHO availability) on the oxidative regulation of CHOs and fat while moderately endurance-trained males and females run at V_{IAT}. Thus, providing a greater comprehension of the human substrate metabolism at a performance-related exertion level, and under two

different metabolic (nutritional) states often used when deriving training prescriptions for metabolic targeted exercise programs.

The outcomes of the presented research objectives have been independently published (peer-reviewed) at the Journal of the International Society of Sports Nutrition and Gavin Journal of Food and Nutritional Science. Original citations are as follow:

De Souza Silveira R, Carlsohn A, Langen G, Mayer F, Scharhag-Rosenberger F (2016) Reliability and day-to-day variability of peak fat oxidation during treadmill ergometry. *J Int Soc Sports Nutr* 13:1-7.

De Souza Silveira R, Kopinski S, Mayer F, Carlsohn A (2016) Influence of High vs. Low Carbohydrate Ingestion on Substrate Oxidation Patterns of Males and Females During Running Bouts at the Individual Anaerobic Threshold. *Gavin J Food Nutrit Sci* 1:1-8.

In order to further enlighten this thesis's scientific background and research problematic, a literature review has been elaborated and is presented in the upcoming section.

2. Literature Review

2.1. Mechanisms Comprising the Regulation of Energy Metabolism During Exercise

2.1.1. Physiological Overview

CHO and fat are unarguably the main fuel sources for aerobic adenosine triphosphate (ATP) production during exercise and the pathways that metabolize these fuels must be heavily up regulated to meet the increased demand for energy generation (Spriet & Watt 2003). The interaction between CHO and fat oxidation at a given exercise intensity is dependent on intracellular and extracellular metabolic environments (Spriet 2014). Moreover, the availability of substrate, both from inside and outside of the muscle, the intensity, duration and mode of exercise, as well as gender and physical conditioning (all of which are discussed separately throughout this review) will affect these environments (Gonzales & Stevenson 2012, Spriet 2014). As described in the pioneer works from Péronnet & Massicotte (1991), Romijn et al. (1993) and Brooks & Mercier (1994), during exercise urinary nitrogen excretion is negligible (indicative of nearly no protein oxidation) and endogenous energy sources originate from CHO (blood glucose, muscle and liver glycogen, as well as blood, muscle and liver lactate) and fat (adipose and intramuscular triglycerides as well as blood-borne FFAs and triglycerides) metabolism. Even in extreme conditions (e.g. prolonged exercise in fasting conditions), amino acid oxidation represents only a small fraction of total substrate utilization (<10%) (Jeukendrup & Aldred 2004).

When expanding into the physiological mechanisms comprising the oxidative regulation of substrate metabolism, Spriet & Watt (2003) infer, that any fat-induced shift in CHO metabolism is expected to target the enzymes that take key responsibilities in regulating

CHO metabolism and oxidation. E.g. inside the muscle these could include glucose uptake (glucose transporter protein 4 (GLUT4)) and phosphorylation (hexokinase), glycogenolysis (glycogen phosphorylase), glycolysis (phosphofructokinase) and the conversion to acetyl-coenzyme A (CoA; (pyruvate dehydrogenase)). Likewise, the same mechanisms would be expected for a CHO-induced down regulation of fat metabolism and oxidation. E.g. targeting the transport of long chain fatty acids into the cell (fatty acid translocase CD36) and the release of fatty acids from intramuscular triacylglycerol (hormone sensitive lipase), as well as the transport into the mitochondria (carnitine palmitoyl transferase complex) (Spriet & Watt 2003). In addition, endocrine mediation for instance, via the release of growth hormone, insulin-like growth factor, sex steroids and the catecholamines, are all hormonal activities that can influence energy metabolism during exercise (Brandou et al. 2006, Riddell 2008).

Ultimately, the discovery of proteins that assist in the transportation of fat across the plasma and mitochondrial membranes, the ability of these proteins to translocate to the membranes during exercise, as well as endocrine mediated responses, have enabled a more thorough understanding of the relationship between fat and CHO metabolism during exercise (Riddell 2008, Spriet 2014). Furthermore, these enable mechanistic proposals to explain the down regulation of fat metabolism when CHO availability is augmented and when moving from moderate to intense aerobic exercise modes (Spriet 2014).

2.1.2. Exercise Intensity and Energy Metabolism

The rate of energy turnover increases in parallel with increased exercise intensity and is during low-moderate intensity associated with an augmented utilization of both CHO

and fat (Sahlin et al. 2008). The oxidation of any one fuel at rest or during exercise does not occur in isolation, with many metabolic aspects being simultaneously active at given points in time (Spriet 2014). While fatty acids provide ~60% of the energy needs of skeletal muscle during rest in healthy adults (Cortright et al. 2006), the overall availability of substrate in the blood (i.e. glucose and fatty acid availability) is also a major aspect determining fuel utilization at both rest and during low-intensity exercise (Sahlin 2008). Moreover, fuel shifts occur at rest despite a generally unchanged metabolic demand (e.g. increasing the availability of blood glucose increases the uptake and oxidation of CHO in skeletal muscle, with a simultaneous decrease in the availability and oxidation of fat, and only minor changes in the metabolic rate) (Spriet 2014).

If portrayed as a sole function of exercise intensity, the oxidative metabolism of these two substrates has a clear pattern. Fat oxidation will augment as intensity increases from low to moderate levels, achieving peak oxidation rates between 45 to 65% of VO_{2peak}, then to become minimal at intensities above 85% of VO_{2peak} (Gonzales & Stevenson 2012, Achten & Jeukendrup 2003, Zehnder et al. 2005, Brun et al. 2007). CHO metabolism on the other hand, increases parallel to exercise intensity and will prevail at times of high physical exertion (Achten & Jeukendrup 2003, Zehnder et al. 2005, Brun et al. 2007). However, once a steady state at a given aerobic exercise intensity and metabolic demand has been established, there can be reciprocal shifts in the proportion of CHO and fat that are oxidized, which then normally favors a fat driven metabolism (Brun et al. 2007, Holloway & Spriet 2012, Spriet 2014) Figure 1 displays a contemporary view of the reciprocal relationship between CHO and fat oxidation during

exercise at power outputs of 40%, 65% and approximately 80% of VO_{2peak} (Spriet 2014).

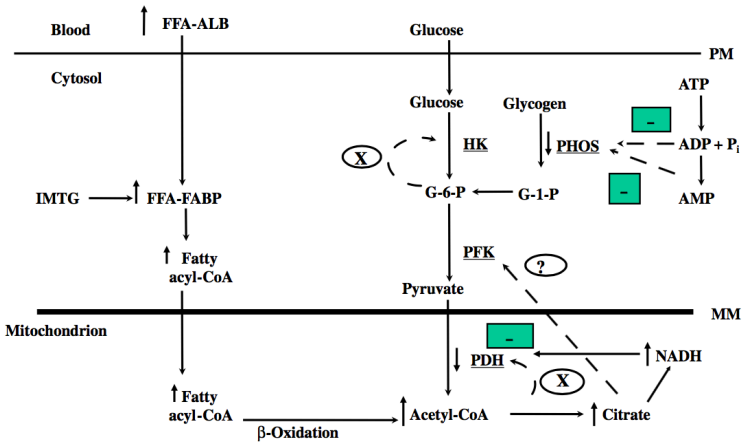

Figure 1: A contemporary view of the reciprocal relationship between carbohydrate and fat oxidation during exercise at power outputs of 40%, 65%, and approximately 80% of VO_{2peak}. Increasing the availability of plasma FFAs had no effect on acetyl-CoA and glucose-6-phosphate (G-6-P) contents (X = no effect) at any power output and increased citrate content only at 40% and 65% of VO_{2peak}. Reduced FFA availability did reduce pyruvate dehydrogenase (PDH) activity at 40% and 65% of VO_{2peak} and the flux through glycogen phosphorylase (PHOS) at all power outputs. The effect on phosphorylase flux was dominant at approximately 80% of VO_{2peak} and was less important at 40% and 65% of VO_{2peak}. The accumulation of free adenine diphosphate (ADP), adenine monophosphate (AMP) and inorganic phosphate (P_i) was reduced during exercise (as indicated by dashes) in the presence of increased FFA availability. Mitochondrial nicotinamide adenine dinucleotide (NADH) may be more abundant with high fat provision at the onset of exercise, increasing the aerobic production of ATP and reducing the mismatch between ATP demand and supply and accounting for the reduced accumulation of ADP, AMP, and Pi. (Albumin (ALB), fatty acid binding protein (FABP), glucose-1-phosphate (G-1-P), hexokinase (HK), intramuscular triacylglycerol (IMTG), mitochondrial membrane (MM), phosphofructokinase (PFK), plasma membrane (PM); Adapted from Spriet 2014)).

2.1.3. Exercise Duration and Energy Metabolism

Whenever referring to the metabolic regulation of substrates during exercise, and as mentioned in the paragraphs above, the pattern of substrate oxidation is subject to exerting duration, with continuous aerobic exercise normally favoring oxidative pathways for fat metabolism. As precisely alluded in the latest joint position statement authored by the Academy of Nutrition and Dietetics, Dietitians of Canada, and the American College of Sports Medicine (Thomas et al. 2016): Exercise is fueled by an integrated series of energy systems which include anaerobic/non-oxidative (phosphagen and glycolytic) and aerobic/oxidative (CHO and fat oxidation) pathways, using substrates that are both endogenous and exogenous in origin. ATP and phosphocreatine provide a rapidly available energy source for muscular contraction, but not at sufficient levels to provide a continuous supply of energy for longer than ~10 sec. The anaerobic glycolytic pathway rapidly metabolizes glucose and muscle glycogen through the glycolytic cascade and is the primary pathway supporting high-intensity exercise lasting 10-180 sec. Eventually, since neither the phosphagen nor the glycolytic pathway can sustain energy demands to allow muscles to contract at a very high rate for longer lasting activities, oxidative pathways deliver the primary fuels for events lasting longer than ~2 min (Thomas et al. 2016).

As a practical example, Spriet (2007) alludes that during a marathon race, slower recreational runners perform at 60-65% of VO_{2peak} for ~3:45 h, while faster athletes will run at 70-75% of VO_{2peak} for ~2:45 h. The author goes on to emphasize, that all these runners rely on CHO and fat during the marathon, with slower runners having an average respiratory exchange ratio (RER) of ~0.90 in the last half of the marathon and the faster runners ~0.95-0.97. Hawley (2000) on the other hand, report that elite runners

run a marathon at intensities equivalent to 80-90% of VO_{2peak} within 2:05 h and 2:20 h. Although, no direct measurements of their RER values have been published, it seems possible that such high-level athletes could complete a marathon using nearly only CHO as a fuel source (i.e. in spite of an accentuated capacity for fat oxidation) (Spriet 2007). Still, exercise duration and the related regulatory (metabolic) mechanisms remain quite intricate as shown across the literature. Moreover, for health-oriented training practices, focus has often been placed on the actual construct of exercise bouts (i.e. multiple, relatively short exercise bouts, against single long enduring sessions) (Goto et al. 2011). For instance, Miyashita et al. (2006) have found similar reductions on postprandial lipemia in young healthy men following either a single 30 min bout of exercise, or ten 3 min bouts with rests in between at the same relative intensity (i.e. running at 70% of VO_{2peak}). These findings imply that comparable metabolic responses can be either way obtained as long as total exertion time and intensity remain the same (Goto et al. 2011). In contrast, a variety of investigations report on greater exercise-induced fat mobilization following several (shorter) bouts of exercise with breaks in between, compared with a single (longer) bout (Goto et al. 2011). More specifically, Goto et al. (2007) reported on greater increases in FFAs, glycerol and ketone body concentrations, but also in fat oxidation during the 60 min following two 30 min bouts of exercise with 20 min rests in between, compared with a single 60 min bout. All sessions were conducted on a cycle ergometer at 60% of VO_{2peak}. Using a fairly different approach, Talanian et al. (2007) showed that repeated bouts of high intensity interval training (HIIT) (ten 4 min bouts with 2 min rests in between bouts) evoked evident increases in whole-blood glycerol concentrations, as well as in fat oxidation by 36%. All sessions were conducted on a cycle ergometer at 90% of VO_{2peak}. In elaboration out of these

findings, Goto et al. (2011) found that three 10 min bouts of exercise with 10 min breaks in between produced a greater exercise-induced fat oxidation (~15% postprandial), compared to a single 30 min bout. All sessions were conducted on a cycle ergometer at 60% of VO_{2peak}. Altogether, these latter findings indicate that intermittent exercise sessions can be a good alternative when aiming to increase exercise-induced fat mobilization. This on the other hand, may impose convenient strategies for health-oriented sportspersons (e.g. sedentary, overweight and obese) looking for the benefits of an increased fat metabolism and greater exercise compliance, without having the pressure of performing long lasting endurance bouts. Still, the longer time spent exercising (especially at higher intensities and training levels), the greater is the contribution of substrates (CHO and fat) into the overall energy metabolism. In this sense, training strategies of optimal duration and intensity should be elaborated upon individual needs and goals, and ideally under the supervision of accredited physical-trainers and/or nutritionists (Thomas et al. 2016).

2.1.4. Exercise Mode and Energy Metabolism

Throughout the past years, several investigations have underlined that the type of exercise being performed also influences the pattern of substrate oxidation and the interconnected regulatory mechanisms. In studies assessing the physiological effects of endurance exercise, running and cycling have been the most commonly used exercise modes (Achten et al. 2003). Hereby, higher fat oxidation during treadmill compared with cycling exercise over a wide range of intensities has been consistently found in children, as well as in moderately- and well-trained adults (Glass et al. 1999, Achten et al. 2003, Capostagno & Bosch 2010, Lafortuna et al. 2010, Zakrzewski & Tolfrey

2012). Physiological explanations show that VO_{2peak} is typically 7-10% higher for treadmill compared with cycling exercise in untrained individuals (Mácek et al. 1976, Millet et al. 2009), thus the higher absolute VO_2 during treadmill exercise could in part explain the differences in substrate oxidation between exercise modes (Zakrzewski & Tolfrey 2012). Chenevière et al. (2010) also infer on additional explanations for such differences, including a smaller muscle mass involved in cycling, differences in muscle contraction regimens (i.e. concentric vs. eccentric), and a greater mechanical efficiency in running due to the stretch-shortening cycle.

Collectively, these findings indicate that running may have an edge over cycling in terms of a greater exercise-induced fat metabolism. Moreover, based on own findings Lafortuna et al. (2010) infer that, compared with cycling, exercising on a treadmill allows for the attainment of any given energy expenditure at a lower average heart rate (HR; (or in a shorter time)), with lower blood lactate concentrations and higher fat oxidation, and possibly also with a lower effort perception. Nevertheless, whether in health- or performance-oriented training practices (as the current literature does not offer a definite consensus), the most appropriate mode of activity needs to be conveyed individually and upon personal requirements and goals.

2.1.5. Endurance Training and Energy Metabolism

The health-related benefits from popular physical activity recommendations (20-60 min of continuous exercise of moderate-to-vigorous intensity, once a day and three times per week) and/or acute exercise training (high-intensity or with a prolonged duration ≥60 min) are undisputable. E.g. by means of improving cardiovascular fitness, preventing obesity and related diseases, including diabetes, hypertension and dyslipidemia, as well

as reducing body weight, body fat and systolic and diastolic blood pressures (Goto et al. 2011).

Now, when relating the effects of endurance training to the regulation of energy metabolism, a clear pattern can be observed. Brooks & Mercier (1994), have underlined in their renowned work to the cross-over concept (i.e. the power output at which energy from CHO-derived fuels predominates over energy from fat; Figure 2), that endurance training results in muscular biochemical adaptations that enhance fat oxidation as well as decrease the sympathetic nervous system responses to given submaximal exercise stresses (i.e. low- to moderate-intensity). In contrast, higher exercise intensities are conceived to increase contraction-induced muscle glycogenolysis, alter the pattern of fiber type recruitment, and increase sympathetic nervous system activity. Subsequently, the authors go on to conclude, that the pattern of substrate utilization in an individual at any point in time depends on the interaction between exercise intensity-induced responses (which increase CHO utilization) and endurance training-induced responses (which promote fat oxidation; Figure 2).

At a molecular level, an enhanced capacity for CHO oxidation following an exercise bout is associated with enhanced insulin receptor substrate-1 and phosphatidylinositol 3-kinase signaling, and increased GLUT4 (Civitarese et al. 2005). Likewise, improvements in fat metabolism after endurance training are facilitated by the up-regulation of the fatty acid transport protein CD36, the mitochondrial transporter carnitine palmitoyltransferase I, the uncoupling protein-3, and β-hydroxyacyl-CoA dehydrogenase (Civitarese et al. 2005).

Conclusively, regular endurance exercise training modulates numerous aspects of cellular biochemistry in multiple tissues, with rapid and marked increases in the

oxidative capacity of skeletal muscle (Camera et al. 2010). Ultimately, the shift in efficiency for metabolizing fat by skeletal muscle may occur as early as within 7-10 days of regular aerobic exercise training, since post-absorptive fat oxidation in this time-frame has been shown to increase independently of body composition changes (Cortright et al. 2006).

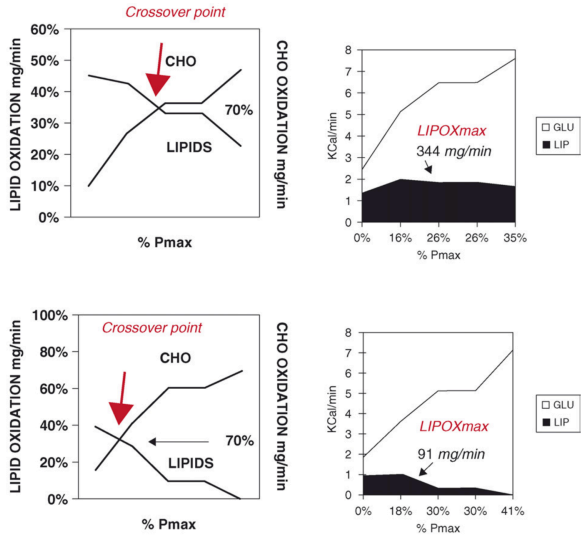

Figure 2: Example of the two opposite profiles of exercise calorimetry. Upper panel, metabolically enduring subjects, able to oxidize high quantities of lipids (i.e. fat) during exercise. Lower panel, glucodependent subjects, as are usually obese, diabetic, with hypothyroidism, but also athletes training at high-intensity or practicing intermittent exercise. LIPOXmax = Fat_{peak}; P_{max} = Maximal power. (Adapted from Brun et al. 2007).

2.1.6. Nutrition (CHO & Fat Manipulation) and Energy Metabolism

Nutrition has the potential to alter the metabolic regulation of substrates with an athlete's skeletal muscle having the remarkable plasticity to respond quickly to nutrient availability, which result in condition-specific metabolic and functional adaptations

(Hawley et al. 2011, Thomas et al. 2016). Subsequently, these adaptations may induce up- or down-regulation in the oxidative metabolism of both CHO and fat.

The intake of CHO in particular, is not only crucial to fuel exercise at intensities above 65% of VO_{2peak}, but also directly assisting in the post-exercise recovery phase (Pendergast et al. 2011, Romijn et al. 1993). For instance, CHO-loading strategies (7-10 g/kg/d) may increase not only glycogen storage (up to 42% post-prandial) but also its overall usage, which in turn delays fatigue allowing exercise to be prolonged and endurance performance to be improved (Pendergast et al. 2011, Neufer et al. 1987, Chryssanthopoulos et al. 2004, Andrews et al. 2003, Wee et al. 2005). However, these latter findings are somewhat restricted to the male athletic population as females are well known for having a greater reliance on fat metabolism compared to males (Tarnopolsky 2008). In addition, female athletes have had mixed results when it comes to increasing muscle glycogen storage capacity and/or enhancing endurance exercise performance (i.e. despite CHO-loading equivalent to ~75% of the energy intake during 4-6 days) (Zehnder et al. 2005, Andrews et al. 2003, Roepstorff et al. 2002, Ruby et al. 2002).

Eventually, the magnitude of the effect that CHO intake has depends on several factors including the type and amount of CHO, and very importantly, timing (i.e. whether CHO is ingested in the hours before exercise, from the start of exercise, or at any point during exercise) (Achten & Jeukendrup 2004). In accordance to the literature appraisal compiled by Achten & Jeukendrup (2004), the following patterns have been reported: When CHO is ingested before the start of exercise, RER is significantly higher than during fasted conditions in most investigations. This general suppression of fat oxidation (lasting at least 6 h) has also been observed over a wide range of intensities

(e.g. in moderately trained men, fat oxidation rates decreased by almost 30% from 50% until 70% of VO_{2peak}). After the start of exercise, the effect of CHO ingestion on fat oxidation depends on exercise intensity. During low- and moderate-intensity exercise, CHO ingestion has been reported to reduce fat oxidation compared with fasting conditions and almost to the same extent as when CHO is ingested before exercise. During high-intensity exercise however, the majority of studies reported no differences in fat oxidation between the fasted and fed states. Achten & Jeukendrup (2004) further underline, that the changes in substrate oxidation as a result of CHO intake are triggered by a number of mechanisms such as, adipose tissue lipolysis and fatty acid delivery to the muscle, hydrolysis of intramuscular triacylglycerols and fatty acid movement across the mitochondrial membranes. In summary, recommendations for CHO intake typically range from 3-10 g/kg/d (and up to 12 g/kg/d for extreme and prolonged activities), depending on the fuel demands of training or competition, the balance between performance and training adaptation goals, the athlete's total energy requirements and body composition goals (Thomas et al. 2016).

Pre-exercise fat ingestion (i.e. long-chain triacylglycerol ingestion 1 to 4 h before exercise), medium-chain triacylglycerols, fish oil, and conjugated linoleic acid have been suggested to alter metabolism to achieve weight loss, alter lipid profiles, or improve performance (Jeukendrup & Aldred 2004). However, as highlighted by Jeukendrup & Aldred (2004) in a comprehensive review, studies have demonstrated that ingestion of meals with long-chain triacylglycerols before exercise has little or no effect on metabolism and does not alter subsequent exercise performance. Additionally, medium-chain triacylglycerol supplementation before or during exercise has not been shown to be ergogenic and is often related to gastrointestinal discomfort (Jeukendrup &

Aldred 2004). Moreover, the consumption of high-fat diets in which more than 60% of the energy is derived from fat has been shown to decrease fat oxidation rates during exercise, even if consumed for only 2 to 3 days (Achten & Jeukendrup 2004).

Conclusively, for most athletes, fat intakes associated with eating styles that accommodate dietary goals typically range from 20%-35% of total energy intake (Thomas et al. 2016). Furthermore, while consuming ≤20% of energy intake from fat does not benefit performance and extreme restriction of fat intake may limit the food range needed to meet overall health and performance goals, the claims that extremely high-fat, carbohydrate-restricted diets provide a benefit to the performance of competitive athletes are not supported by current literature (Thomas et al. 2016).

Also, under great publicity are strategies that aim to improve fat metabolism at higher exertion levels via metabolic adaptations induced by a combined systemic manipulation of CHO availability through diet and exercise intensity (Mosler 2016, Thomas et al. 2016). For example, when conducting key, high intensity training bouts on either fasted and/or low CHO dietary states, as well as under glycogen depletion (Mosler 2016). Although the latter may successfully increase fat oxidation in the short term, prolonged effects on the energy metabolism as well as positive effects on exercise performance are still to be proven.

Finally, Thomas et al. (2016) reinforce that athletes need to consume energy that is adequate in amount and timing of intake during periods of high-intensity and/or long duration training to maintain health and maximize training outcomes. Low energy availability can result in unwanted loss of muscle mass; menstrual dysfunction and hormonal disturbances; sub-optimal bone density; an increased risk of fatigue, injury, and illness; impaired adaptation and a prolonged recovery process.

2.1.7. Gender and Energy Metabolism

Research in Western populations throughout the past decades has often demonstrated a gender effect on substrate metabolism during exercise (Tarnopolsky et al. 1995, Janyacharoen et al. 2009). As revised by Chenevière et al. (2011), while a few studies have not shown any gender differences in substrate utilization during submaximal exercise (Roepstorff et al. 2002, Ruby et al. 2002), the majority have reported a greater reliance on fat oxidation in women than in men for a given relative exercise intensity (Tarnopolsky et al. 1995, Friedlander et al. 1998, Carter et al. 2001, Devries et al. 2007).

Hormonal variability in women throughout the menstrual cycle has often been appointed in the literature as the main regulatory mechanism driving the observed differences. For instance, estrogen may promote endurance performance by altering CHO and fat metabolism with progesterone often appearing to act antagonistically (Oosthuyse & Bosch 2010, Vaiksaar et al. 2011). More specifically (and following the trend to contradictory findings), several studies did not find any (or only small) differences in substrate oxidation throughout the menstrual cycle, while others have suggested that during moderate-intensity exercise (<70% of VO_{2peak}), women have higher lipid utilization and a lesser reliance on CHO in the luteal compared to the follicular phase (Vaiksaar et al. 2011, Chenevière et al. 2011). Besides the possible influence of the phases of the menstrual cycle, additional factors could be involved in the fat metabolism gender dimorphism (Chenevière et al. 2011). These include a basal greater amount of body fat and intramuscular triacylglycerol stores and a larger percentage of type I fibers in women than in men, but also differences in circulating catecholamine concentrations and increases in transport or oxidation of available FFAs

(Chenevière et al. 2011). Furthermore, well-established factors like physical fitness, but also ethnicity and embedded nutritional habits my additionally contribute to substrate variability amongst men and women (Janyacharoen et al. 2009, Vaiksaar et al. 2011).

2.1.8. Contextual Synthesis: Metabolic Conditioning Through Exercise and Nutrition

Based upon the regulatory mechanisms presented in the above paragraphs, athletes and health-oriented exercisers may be advised to vary their training regimen around different intensities, durations, types of exercises and different nutritional strategies (using and conditioning both aerobic and anaerobic energetic pathways), while aiming to expand endurance capacity, power and performance, as well as improving on a variety of health-related characteristics (Karakoç et al. 2012, Romain et al. 2012).

One of the main outcomes of exercise training and regular physical activity has shown to be an improved ability to oxidize fat (Achten et al. 2004). Hence, in recent years there has been emerging interest in the improvement of training prescriptions for metabolic-guided exercise bouts (often combined with metabolic-guided nutritional practices) (Brun et al. 2007, Mosler 2016, Thomas et al. 2016). For instance, exercising at intensities where fat oxidation rates are high has been advocated to induce metabolic changes that benefit both professional and recreational endurance athletes, as well as health-oriented exercisers (Gonzales & Stevenson 2012, Tolfrey et al. 2010, Romain et al. 2012). In a meta-analysis from Romain et al. (2012), it was concluded that training targeted at the intensity eliciting PFO rates (i.e. Fat_{peak}) decreases fat mass and body weight and improves blood cholesterol. While these results are promising, most of all

for health-oriented exercisers, long-term effects of training at Fat_{peak}, especially in athletic populations, still need to be established.

Inevitably, a crucial aspect remains on how to reliably identify the intensity at which fat metabolism reaches peak oxidation levels when prescribing exercise for the purpose of fat oxidation and related metabolic effects (Chenevière et al. 2009). The reproducibility of Fat_{peak} has been reported for a variety of submaximal incremental protocols (Gmada et al. 2012, Achten & Jeukendrup 2003, Pérez-Martin et al. 2001, Michallet et al. 2008, Meyer et al. 2009, Croci et al. 2014). However, all reliability studies to date have used cycle ergometry as the exercising method of choice, which in turn may limit a valid transferability from any of the previously tested protocols and their respective reproducibility indicators into other types of exercise. Yet, despite running and walking being feasible and popular modalities among different target populations (Mendelson et al. 2012), there are to date no reliability data on the estimations of Fat_{peak} during treadmill ergometry. Additionally, only a few studies have performed comprehensive statistical assessments as recommended by the guidelines for reliability assessment in sports medicine (Atkinson & Nevill 1998). These would include for instance, the establishment of both relative and absolute reliability indicators for key variables related to Fat_{peak} estimations, such as the actual velocity at which PFO rates occur (i.e. V_{PFO}), as well as the computation of its respective intrasubject (day-to-day) variability.

Now, when addressing the upper levels of endurance exercise capacity (where energy metabolism is expected to be driven primarily by CHO oxidation), it remains unclear how the systemic availability of CHO modulates substrate oxidation while exercising at the IAT (i.e. namely at V_{IAT}). As previously stated, the IAT represents the upper border where constant load endurance exercise can be sustained, being commonly used to

guide athletic training (e.g. when aiming to improve endurance capacity) or in performance diagnostics (Faude et al. 2009, Péronnet 2010, Kindermann 2004, Stegmann et al. 1981). Exertion at V_{IAT} can be generally sustained for up to 60 minutes, though again, the average speed of a marathon is reported to be only slightly under it (Billat et al. 2003, Faude et al. 2009). Moreover, at V_{IAT}, CHO-derived energy sources (especially glycogen availability) will undertake the main role in the sustainment of exercise rate, and subsequently determine exercise performance (Billat et al. 2003).

Therefore (and in synthesis to the previously drawn research objectives), to better assist coaches, trainers and nutritionists in their planning of metabolic-guided training and/or pre-exercise nutritional strategies, it is necessary to first investigate how intensity, exercise mode (i.e. running) and pre-exercise nutrition (especially CHO intake) affect the metabolic regulation of substrate use at upper oxidative levels as individuals attempt to exercise in accordance to the above mentioned biomarkers of metabolic performance (i.e. V_{PFO} and Fat_{peak}) and exercise capacity (i.e. V_{IAT}). Subsequently, in order to precisely answer these questions the following research objectives were postulated: 1) Establish the reliability and day-to-day variability of V_{PFO} and Fat_{peak}. Thus, contributing to the improvement of training prescriptions in running to enhance fat metabolism; 2) Assess the impact of HC vs. LC diets (i.e. systemic CHO availability) on the oxidative regulation of CHOs and fat while moderately endurance-trained males and females run at V_{IAT}. Thus, providing a greater comprehension of the human substrate metabolism at a performance-related exertion level, and under two different metabolic (nutritional) states often used when deriving training prescriptions for metabolic targeted exercise programs.

3. Methods

3.1. Investigational Approach: Research Objective 1

3.1.1. Subjects

Sixteen healthy and active adults involved in the regular practice of different sports disciplines (i.e. running, cycling, rugby and weight-lifting) voluntarily took part in this investigation. The study was conducted in accordance with the declaration of Helsinki. The ethics committee from Potsdam University approved the study and participants gave their written informed consent after receiving detailed information about the investigational protocol and aims. Inclusion criterion was ≥3 h of training per week. The participants' anthropometric and training data are given in table 1.

Table 1: Anthropometric and training data of subjects.

	Overall (n=16)	Males (n=9)	Females (n=7)
Age (yrs.)	25 ± 3	26 ± 3	23 ± 2*
Height (m)	1.76 ± 0.09	1.81 ± 0.07	1.69 ± 0.06*
Weight (kg)	68.3 ± 13.7	81.9 ± 6.5	59.8 ± 7.1*
BMI (kg/m^2)	23.1 ± 2.9	24.8 ± 1.9	21.0 ± 2.0*
%BF	14.2 ± 3.7	12.3 ± 2.3	16.7 ± 2.8*
Training (h/week)	7 ± 2	7 ± 3	6 ± 2

All values are mean ± SD; BMI, Body mass index; %BF, Percentage body fat; * = $P < 0.05$ (gender comparisons only).

3.1.2. General Design

All examinations were conducted at Potsdam University's Outpatient Clinic. At day 1, a full medical check (anamnesis, anthropometrical assessment, physical examination, resting ECG) was carried out preceding the first exercise appointment as recommended

by the German Federation for Cardiovascular Prevention and Rehabilitation (Bjarnason-Wehrens et al. 2004). Subsequently, participants performed a maximal baseline running test to determine the exercise stages for the Fat-peak tests. On days 2 and 3, an identical submaximal incremental running test (Fat-peak test 1 and 2) was carried out on the same treadmill ergometer (0.4% inclination) (H/P/ Cosmos Pulsar Graphics. 2005 ®, Germany). A breath-by-breath Metamax 3B system (Cortex Biophysik GmbH. Leipzig, Germany) was used to monitor respiratory data and to determine fat oxidation rates via indirect calorimetry. Diet was controlled on the day prior to each of the submaximal tests. Participants performed all tests in a fasted state and were additionally advised to refrain from training during the 24h before each bout. Female's menstrual cycle was uncontrolled.

3.1.3. Baseline Test

The baseline test consisted of a stepwise incremental running bout until volitional exhaustion. The initial stage of 6 km/h, stage increments of 2 km/h and stage duration of 3 min were defined to exhaust subjects in not less than 4 stages (Meyer et al. 2009). Lactate concentrations were measured in between stages from capillary blood samples taken from the hyperemized earlobe (Biosen S line, EKF diagnostic GmbH. Magdeburg, Germany). Subsequently, the following parameters were determined: The velocities at the aerobic threshold (V_{LT}) (Dickhuth et al. 1999) and RER of 1.00 (V_{RER}), as well as VO_{2peak} and peak running velocity (V_{peak}).

3.1.4. Fat-peak Tests

Forty-eight hours after baseline, subjects performed the first submaximal incremental run. The bout lasted 30 min, i.e. 5 stages of 6 min, and was designed on an individualized basis, based on the recorded gas exchange and blood lactate variables from each participant (Meyer et al. 2009). The starting velocity was set at V_{LT} while the end velocity was V_{RER}. Hence, to obtain five stages of equal increment, the difference between end- and start-velocity needs to be divided by four (i.e. [(V_{RER} - V_{LT}) ÷ 4 = increment]). Before officially commencing the test, a 10 min warm up phase at 70% V_{LT} was implemented to stabilize cardiopulmonary parameters and reduce possible breathing artifacts that may arise at the beginning of exercise calorimetry (Xu & Rhodes 1999). The second (identical) submaximal bout was then carried out 48 to 72 h later at the same time for each participant (07:00, 8:00 or 9:00 am). Subsequently, the following parameters were determined: fat oxidation rates, PFO, V_{PFO}, oxygen uptake (VO_2) at V_{PFO} and HR at V_{PFO}.

It must be noted that the current protocol has been previously tested in a pilot investigation and eventually adapted into the current format. The results of this pilot investigation have been recently published elsewhere (De Souza Silveira 2017).

3.1.5. Dietary Control

For compliance control, food intake was documented in a standardized diet record form (Carlsohn et al. 2012) during the day before each submaximal run and analyzed later on. Participants were not given any specific dietary recommendations, but simply told to identically repeat their conventional nutritional plan at both days. A 12-hour overnight fast was also enforced before every running bout. Nutrient and energetic values,

including possible deviations within diet record forms were computed based on the German Nutrition database (PRODI 5.7, Nutri-Science GmbH. Hausach, Germany).

3.1.6. Gas Exchange Data Analysis

Gas exchange data were checked for plausibility and analyzed using the software Metasoft 3, version 3.9. VO_{2peak} was defined as the highest 30 sec average value during the baseline test. For the Fat-peak tests, fat oxidation rates were calculated from VO_2 and the non-protein RER (i.e. the ratio between VO_2 and carbon dioxide output (VCO_2)) according to Péronnet (Péronnet & Massicotte 1991).

Fat oxidation rate (mg/min $^{-1}$) = $-1.7012\ VCO_2 + 1.6946\ VO_2$

CHO oxidation rate (mg/min $^{-1}$) = $4.585\ VCO_2 - 3.2255\ VO_2$

This technique provides calculations for substrate oxidation under the assumption that urinary nitrogen excretion is negligible. Gas exchange data (viewed with time interval of 10 sec) were averaged over the last 30 sec of each stage. By applying a third polynomial (P3) function (Prism 6, GraphPad Software Inc.), a graphic depiction of fat oxidation rates as a function of exercise intensity was created for each individual and used to determine PFO, V_{PFO}, Fat_{peak} (Croci et al. 2014, Stisen 2006) and subsequently VO_2 and HR at V_{PFO}.

3.1.7. Statistics

All of the analyzed parameters are descriptively reported as mean ± standard deviation (SD). Statistical analysis was performed using SPSS, version 20, IBM, USA & Microsoft Excel 2011. Samples were checked for normality using the Shapiro-Wilk test. Gender differences in anthropometry, training and baseline performance data were

tested with an un-paired t-test. During the Fat-peak tests, differences in VO_2, RER, fat oxidation rates and HR were assessed with a two-way ANOVA for repeated measures (test X stage). A paired t-test assessed the in between test differences for V_{PFO}, PFO, $Fat_{peak,}$ VO_2 at V_{PFO}, HR at V_{PFO}, as well as the differences in the dietary data. Relative and absolute reliability of V_{PFO} and Fat_{peak} were verified with an intraclass correlation coefficient (ICC), the coefficient of variation (CV) and the Pearson's correlation coefficient. The day-to-day variability of V_{PFO} and Fat_{peak} was assessed with a Bland-Altman analysis by establishing the mean differences (bias) ± 95% limits of agreement (LoA). Significance was set at a α-level of 0.05 (reported to the second decimal case).

3.2. Investigational Approach: Research Objective 2

3.2.1. Subjects

Sixteen healthy recreational runners (8 males/8 females) voluntarily took part in this investigational study. The ethics committee of the University of Potsdam approved the study and participants gave their written informed consent after receiving detailed information on the investigational protocol and study aims. The study was conducted in accordance with the declaration of Helsinki. To increase the cohort's homogeneity in regards to physical conditioning, subjects, were only included if weekly training was ≥3 hours. Anthropometric characteristics are provided in table 2.

Table 2: Anthropometric data of subjects.

	Overall (n=16)	Males (n=8)	Females (n=8)
Age (yrs.)	28 ± 3	30 ± 3	26 ± 2*
Height (m)	1.76 ± 0.09	1.83 ± 0.08	1.70 ± 0.03*
Weight (kg)	72 ± 13	83 ± 8	61 ± 5*
BMI (kg/m^2)	23 ± 2	24.9 ± 1.1	21.2 ± 1.3*
%BF	14.7 ± 3.3	14.1 ± 3.5	15.3 ± 3.0

All values are mean ± SD; BMI, Body mass index; %BF, Percentage body fat; * = $P < 0.05$ (gender comparisons only).

3.2.2. General Design

All examinations were conducted at Potsdam University's Outpatient Clinic. A full medical check (anamnesis, anthropometrics, physical examination, resting ECG) was carried out preceding the first exercise appointment as recommended by the German Federation for Cardiovascular Prevention and Rehabilitation (Bjarnason-Wehrens et al. 2004). At day 1, participants performed a baseline running test in which the IAT

(Dickhuth et al. 1999), V_{IAT}, VO_{2peak} and maximal heart rate (HR_{max}) (RS 400, © Polar Electro, Finland) were determined. On days 2 and 3, a submaximal running test at V_{IAT} was carried out on the same treadmill ergometer (H/P/ Cosmos Pulsar Graphics 2005 ®, Germany). A breath-by-breath Metamax 3B system (Cortex Biophysik GmbH. Leipzig, Germany) was used to monitor respiratory data and to determine CHO and fat oxidation rates via indirect calorimetry (detailed below). For the 2 submaximal runs, HC (7g/kg/d) and LC (3g/kg/d) dietary protocols were prescribed for the 24 h preceding each test (detailed below). As part of the LC protocol, a glycogen-depleting running bout was additionally performed (60 min at 75% HR_{max} in the evening, 12 h prior to the actual submaximal running bout; Figure 3 depicts a flowchart of the investigational design). Participants were additionally advised to refrain from any other exercise practices during the 48 h preceding each submaximal bout.

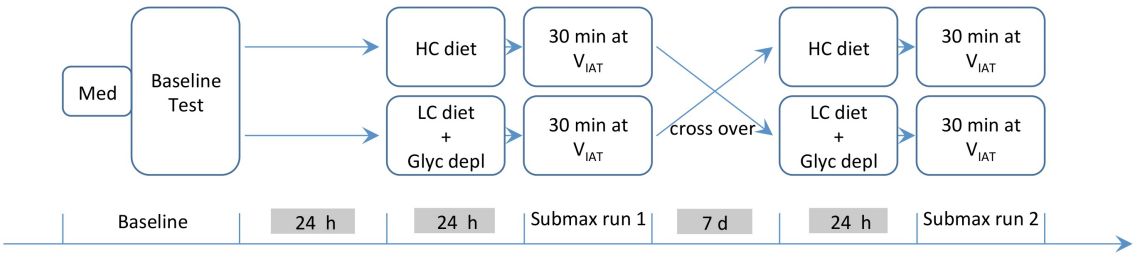

Figure 3: Flowchart depicting investigational design of the randomly assigned protocols (Med, Medical check; Glyc depl, Glycogen depleting bout).

3.2.3. Baseline Test

Subjects performed a stepwise incremental test until volitional exhaustion. The initial stage (6 km/h), stage increment (2 km/h) and stage duration (3 min) were defined to exhaust subjects in not less than 4 stages (Meyer et al. 2009). Lactate concentrations

were measured in between stages from capillary blood samples taken from the hyperemized earlobe (Biosen S line, EKF diagnostic GmbH. Magdeburg, Germany).

3.2.4. Submaximal Runs

Forty-eight hours after the baseline test, subjects performed the first submaximal run. This bout was composed of a 30 min, constant-pace endurance run at V_{IAT}. The second submaximal bout was then carried out 7 days later at the same time for each participant (07:15, 8:00 or 8:45 am). Before commencing the tests, a 3 min run at 80% V_{IAT} served as a warm up not only so subjects could adapt to the forthcoming brisk exercise pace, but also to stabilize cardiopulmonary parameters and reduce possible breathing artifacts that may arise at the beginning of exercise testing (Xu & Rhodes 1999).

3.2.5. Nutritional Intervention & Managing CHO Availability

The HC and LC dietary protocols were randomly assigned for the 24 h preceding each submaximal run. This one day nutritional intervention has its caloric content calculated for each individual based on the basal metabolic rate and the World Health Organization's PAL-Score (Harris & Benedict 1918, Human energy requirements. Scientific background papers from the Joint FAO/WHO/UNU Expert Consultation 2005). Dietary protocols were only prescribed with no food being supplied throughout the investigation. Therefore for compliance control, food intake was documented in a standardized diet record form (Carlsohn et al. 2012) and analyzed later on. Nutrient and energetic values, including possible deviations from the prescribed protocols were computed based on the German Nutrition database (PRODI 5.7, Nutri-Science GmbH. Hausach, Germany). The dietary plan was designed for breakfast, lunch and dinner

(plus in between snacks), and consisted of foods typically eaten in Germany. The plan was standardized with no caffeine (with the exception of a standardized morning coffee) alcohol or supplements included, and individually adapted to body mass to achieve CHO aims. As part of the LC protocol, an exercise bout with duration and intensity proven to deplete glycogen stores was implemented (Costill et al. 1971, Krssak et al. 2000). This bout combined to the LC diet (which subsequently avoids glycogen recovery or super compensation) (Philp et al. 2012, Jensen & Richter 2012), would then create a metabolic state where low CHO availability can be assumed. The amounts of CHO intake (i.e. 7 vs. 3 g/kg/d) were chosen, as these are common thresholds used in both clinical and scientific settings.

3.2.6. Gas Exchange Data Analysis & Calculations

Values from respiratory volume and gas concentrations were transmitted directly to the analysis software (Metasoft 3, version 3.9). All tests had the investigated gas exchange parameters viewed with an average time interval of 10 sec. VO_{2peak} was defined as the highest VO_2 recorded during the baseline test within a period of 30 sec. For the two submaximal runs, calculations of CHO and fat oxidation rates were performed using stoichiometric equations in accordance to the non-protein RER technique (Péronnet & Massicotte 1991). Markers were set every 5 min during the possible 30 min of each submaximal exercise bout. Respiratory data as well as CHO and fat oxidation values were averaged from the last 30 sec preceding every marker.

3.2.7. Statistics

All of the analyzed parameters are descriptively reported as mean ± SD. Statistical analysis was performed using a commercial software package SPSS, version 20, IBM, USA and Microsoft Excel 2011. Samples were checked for normality using the Shapiro-Wilk test. Gender differences in anthropometry, baseline parameters and within nutritional protocols were tested with an unpaired *t*-test. Differences in dietary data, cardiopulmonary parameters as well as differences in substrate oxidation between the trials with different nutritional protocols (including gender comparisons) were computed with a paired *t*-test. The interaction of the gas exchange variables between diet and gender was analyzed with a two-way ANOVA for repeated measures (diet x gender). Significance was set at an alpha level of 0.05 (reported to the third decimal case).

4. Results

4.1. Results: Research Objective 1

4.1.1. Baseline Characteristics

As presented in table 3, the overall values for VO_{2peak}, V_{peak}, V_{LT} and V_{RER} were 47 ± 6 ml/min/kg, 15.8 ± 1.6 km/h, 8.2 ± 0.9 km/h and 12.8 ± 1.6 km/h respectively. Significant gender differences were observed for all variables, except V_{LT}.

Table 3: Baseline performance data.

	Overall	Males	Females
VO_{2peak} (ml/min/kg)	47 ± 6	51 ± 3	42 ± 2*
V_{peak} (km/h)	15.8 ± 1.6	16.7 ± 1.0	14.6 ± 0.9*
V_{LT} (km/h)	8.2 ± 0.9	8.5 ± 0.5	8.0 ± 0.7
V_{RER} (km/h)	12.8 ± 1.6	13.8 ± 1.1	11.4 ± 0.4*

All values are mean ± SD; * = P < 0.05 (gender comparisons only).

4.1.2. Dietary Intake

There were no significant differences (overall and individually) for any of the calculated variables in the reported dietary intake during the 24 h preceding the Fat-peak tests (*P > 0.05*). Mean values for energy, carbohydrate, fat and protein intake were 2507 ± 561 kcal, 345 ± 118 g, 73 ± 34 g and 106 ± 28 g, respectively.

4.1.3. Fat-peak Tests

Individual values for start and end velocities ranged from 6.5 to 10.4 km/h and from 10.9 to 15.6 km/h, respectively. Likewise, stage increments ranged between 0.7 and 1.7 km/h. As shown in figures 4-7, there were no significant differences recorded for VO_2

(*P = 0.20*), RER (*P = 0.58*), fat oxidation rates (*P = 0.79*) and HR (*P = 0.13*) during the two Fat-peak tests. Also with no significant systematic differences between bouts, mean V_{PFO} was 8.2 ± 1.9 and 7.9 ± 1.8 km/h (*P = 0.69*). The range in which individual means of V_{PFO} were detected varied from 5.7 ± 0.2 to 12.5 ± 0.3 km/h, with 11 subjects achieving V_{PFO} (in both tests) during the warm up phase (i.e. below V_{LT}). Accordingly, mean PFO was 0.58 ± 0.22 and 0.60 ± 0.22 g/min (*P = 0.85*). The respective range of individual means for PFO went from 0.30 ± 0.08 to 1.03 ± 0.08 g/min. Fat_{peak} averaged at 64 ± 7 and 62 ± 6 % of VO_{2peak} (*P = 0.35*), with individual means ranging from 50 ± 3 to 74 ± 2 % of VO_{2peak}. Mean VO_2 at V_{PFO} was 30 ± 6 and 29 ± 6 ml/min/kg during each of the Fat-peak tests respectively (*P = 0.61*). The corresponding individual means for VO_2 at V_{PFO} ranged between 21 ± 2 and 40 ± 2 ml/min/kg. Likewise, mean HR at V_{PFO} was 143 ± 11 and 140 ± 13 beats/min (*P = 0.46*), with range of individual means varying between 116 ± 1 and 162 ± 6 beats/min.

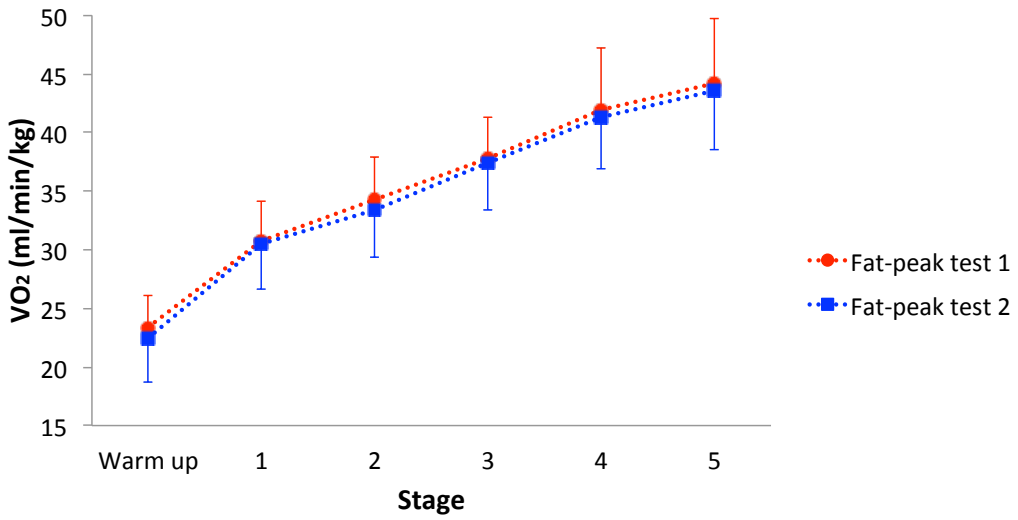

Figure 4: Average VO_2 during Fat-peak tests. All values are mean ± SD.

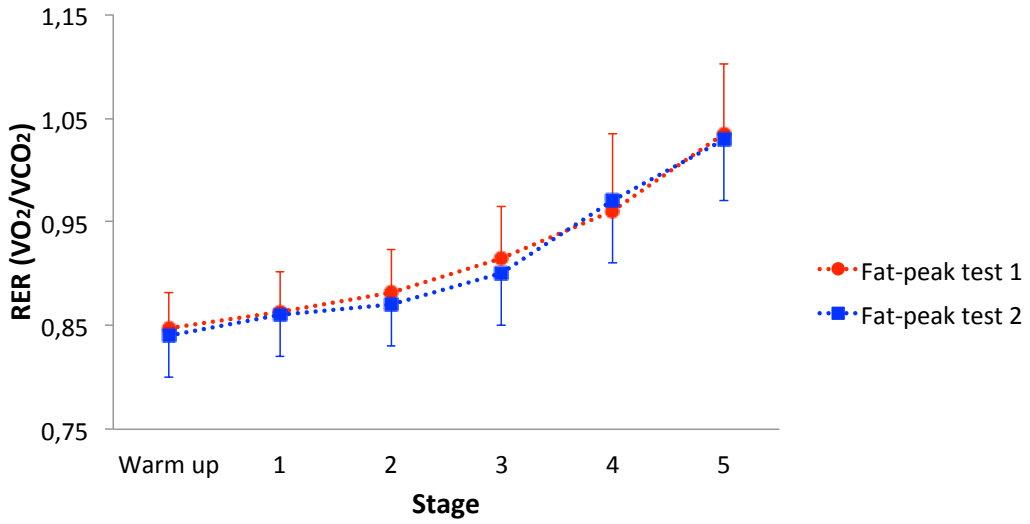

Figure 5: Average RER during Fat-peak tests. All values are mean ± SD.

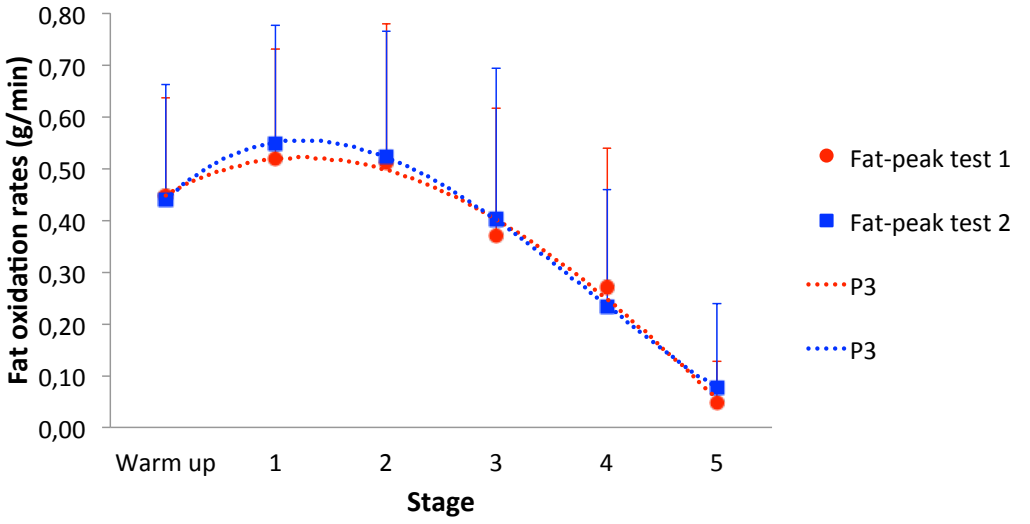

Figure 6: Average fat oxidation rates during Fat-peak tests (P3 interpolated). All values are mean ± SD.

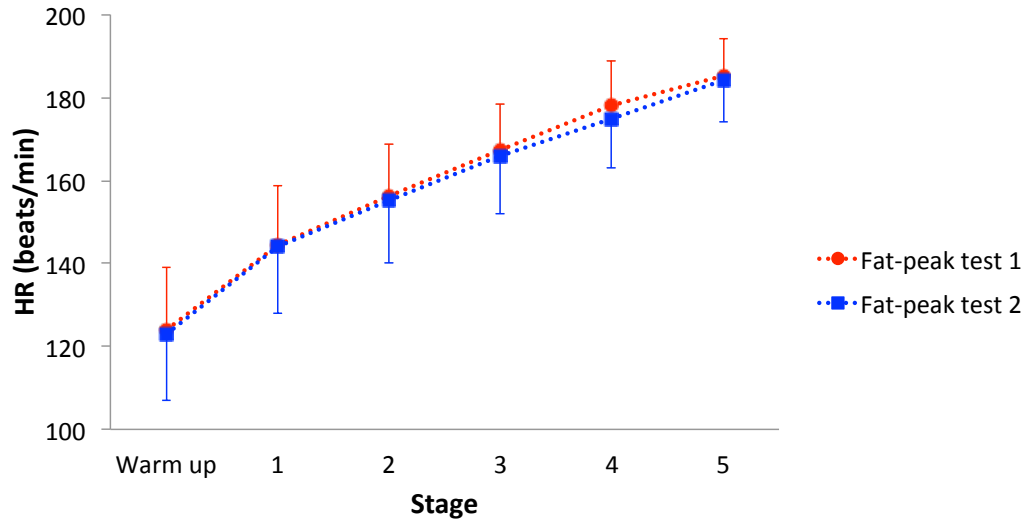

Figure 7: Average HR during Fat-peak tests. All values are mean ± SD.

4.1.4. Reliability & Day-to-day Variability Assessment of V_{PFO} & Fat_{peak}

ICC, Pearson's coefficient and the CV scored 0.98, 0.97 and 5.0% for $V_{PFO,}$ and 0.90, 0.81 and 7.0% for Fat_{peak} respectively. As shown in figure 8, the bias ± 95% limits of agreement for V_{PFO} were -0.3 ± 0.9 km/h (-2 ± 8% of VO_{2peak}). Thus, indicating that 95% of the intra-individual differences should be expected between -1.2 and +0.6 km/h (-10 and +6% of VO_{2peak}).

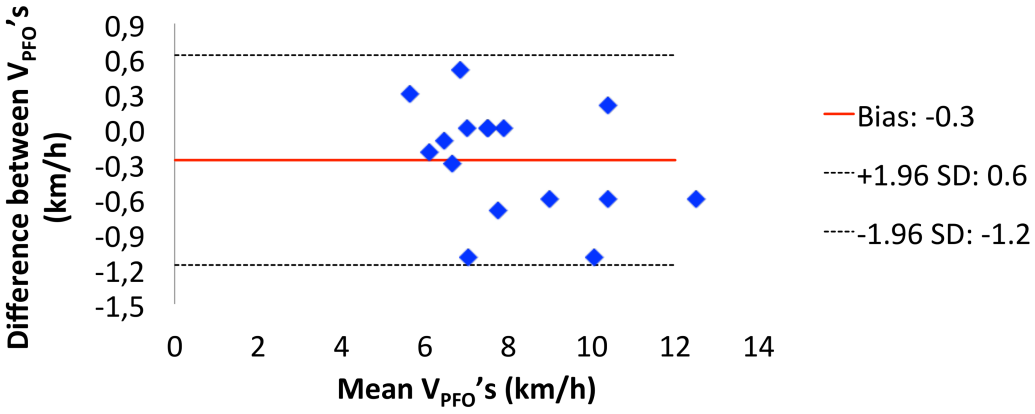

Figure 8: Bland-Altman plot for V_{PFO} during Fat-peak tests.

4.2. Results: Research Objective 2

4.2.1. Baseline Characteristics

As presented in table 4, the overall values for IAT, V_{IAT} and HR_{max} were 2.74 ± 0.39 mmol/l, 11.1 ± 1.4 km/h and 194 ± 10 beats/min respectively, with no significant gender differences. VO_{2peak} differed significantly between genders with males achieving 50 ± 0 ml/min/kg and females 44 ± 5 ml/min/kg.

Table 4: Baseline performance data.

	Overall	Males	Females
VO_{2peak} (ml/min/kg)	47 ± 5	50 ± 0	44 ± 5*
HR_{max} (beats/min)	194 ± 10	193 ± 12	195 ± 5
V_{IAT} (km/h)	11.1 ± 1.4	11.4 ± 0.8	10.7 ± 1.8
IAT (mmol/l)	2.74 ± 0.39	2.71 ± 0.43	2.77 ± 0.40

All values are mean ± SD; * = P < 0.05 (gender comparisons only).

4.2.2. Dietary Intake

Overall CHO intake differed significantly (*P = 0.005*) between HC (7.1 ± 1.5 g/kg/d) and LC (3.4 ± 0.8 g/kg/d) protocols (Figure 9). Accordingly, the mean deviations from the targeted CHO intake were 2 ± 21% (*P = 0.566*) and 15 ± 28% (*P = 0.001*) for HC and LC protocols respectively. As shown in figure 10, during the HC days, intake was 7.0 ± 1.4 g/kg/d for males and 7.2 ± 1.7 g/kg/d for females (*P = 0.661*), resulting in a deviation of 0.3 ± 19.5% (*P = 0.902*) and 3.3 ± 24.2% (*P = 0.871*) respectively. Accordingly, the intake during the LC days amounted to 3.1 ± 0.7 and 3.8 ± 0.9 g/kg/d (*P = 0.078*), which reflects a target deviation of 3 ± 24% (*P = 0.520*) and 26 ± 29% (*P = 0.003*) respectively.

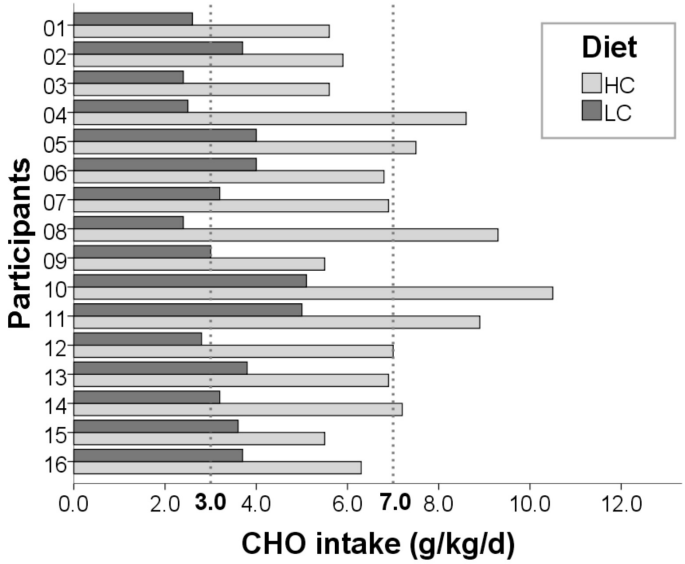

Figure 9: Reported CHO intake during HC and LC days. Dotted lines mark the targeted amounts for CHO intake.

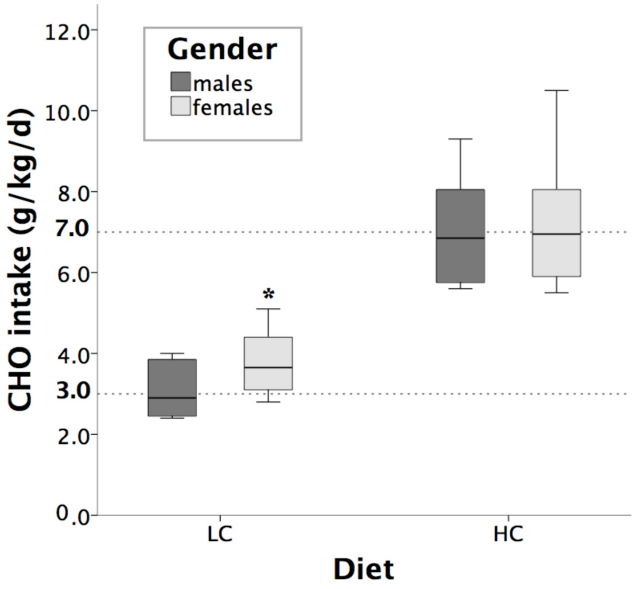

Figure 10: Gender comparisons of CHO intake within dietary protocols. Values are expressed as median, quartiles and extremes; * = $P < 0.05$.

4.2.3. Cardiopulmonary Parameters During HC & LC Bouts

As shown in figure 11, with no significant differences between bouts at any of the measured points (*P = 0.756* at rest; *P = 0.768* at 5 min; *P = 0.145* at 10 min; *P = 0.067* at 15 min; *P = 0.069* at 20 min; *P = 0.089* at 25 min; *P = 0.079* at 30 min), the overall HR ranged from 161 ± 11 (at 5 min) to 176 ± 13 beats/min (at 30 min; *P = 0.001*) during the HC bout, and from 165 ± 12 to 178 ± 11 beats/min (*P = 0.007*) during the LC bout respectively. Mean VO_2 was 38 ± 5 and 39 ± 5 ml/min/kg during HC and LC bouts respectively (*P = 0.086* at 5 min; *P = 0.060* at 10 min; *P = 0.189* at 15 min; *P = 0.518* at 20 min; *P = 0.059* at 25 min; *P = 0.132* at 30 min; figure 12). The RER was significantly higher during the HC bout at all measure points (*P = 0.006* at 5 min; *P = 0.000* at 10 min; *P = 0.003* at 15 min; *P = 0.001* at 20 min; *P = 0.007* at 25 min) but the last (*P = 0.059* at 30 min; figure 13).

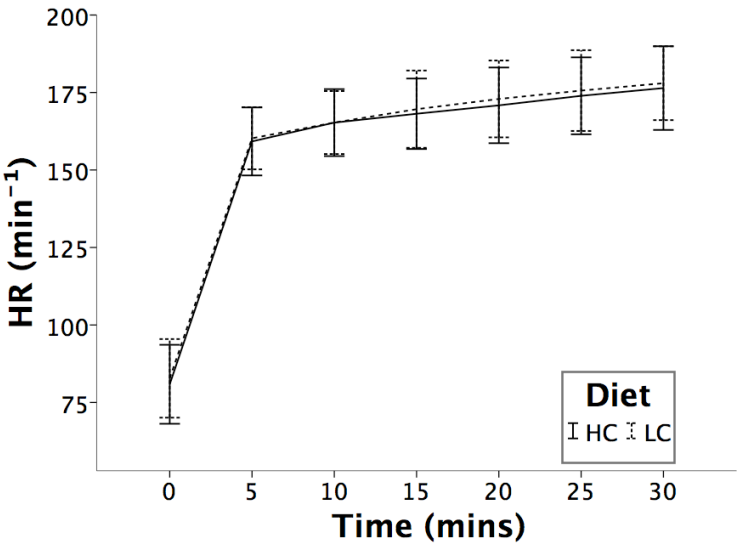

Figure 11: Average HR measured during submaximal runs at rest and each of the 5 min marker points. All values are mean ± SD.

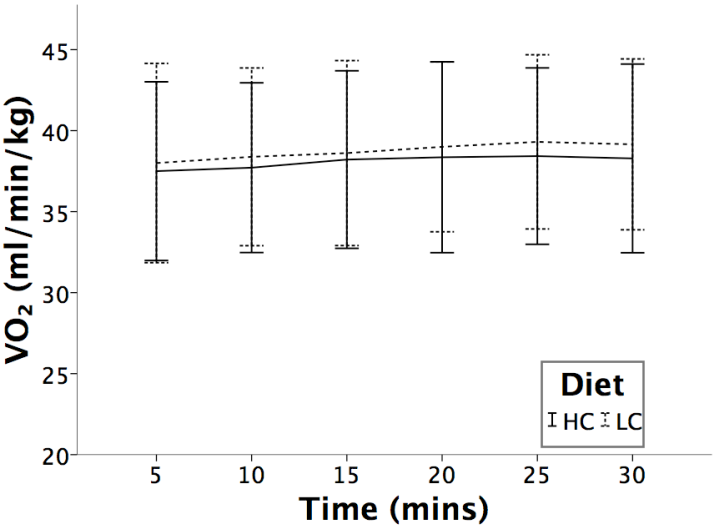

Figure 12: Average VO_2 measured during submaximal runs at each of the 5 min maker points. All values are mean ± SD.

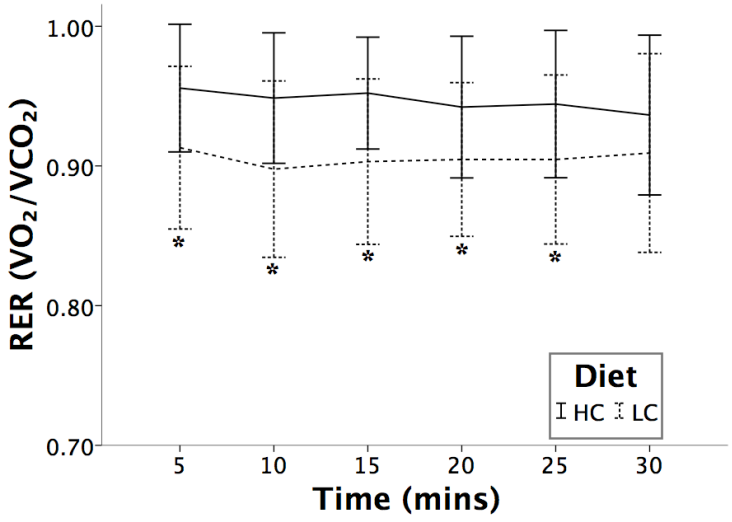

Figure 13: Average RER measured during the submaximal runs at each of the 5 min marker points; All values are mean ± SD; * = $P < 0.05$.

4.2.4. CHO & Fat Oxidation

Relative (%) and absolute (g/min) values for overall CHO and fat oxidation recorded during the two submaximal runs are presented in figures 14-17. Substrate oxidation differed significantly between HC and LC bouts at minutes 5 to 25 but not at minute 30 (*P = 0.000; P = 0.001; P = 0.001; P = 0.010; P = 0.003; P = 0.059*). CHO oxidation was on average 3.45 ± 0.08 and 2.90 ± 0.07 g/min (*P = 0.000*) during HC and LC bouts respectively. Likewise, fat oxidation rates were 0.13 ± 0.03 and 0.36 ± 0.03 g/min (*P = 0.000*). CHO metabolism accounted for 84 ± 15 and 72 ± 20% (*P = 0.000*) of the overall oxidized substrates during both HC and LC bouts respectively. Figures 18-21 display gender differences in the amount of oxidized substrates during the runs and relative to overall substrate use. When comparing CHO and fat oxidation within each of the two nutritional states, significant gender differences could only be shown during the HC run, and at the measurement times of 5, 10, 25 and 30 minutes (*P = 0.002; P = 0.004; P = 0.017; P = 0.006;*), but inconsistently at minutes 15 and 20 (*P = 0.066; P = 0.059*). The relative contribution of CHOs to the overall oxidative metabolism was greater in males compared to females (i.e., 90 ± 11% vs.76 ± 16% (*P = 0.033*) in the HC run and 77 ± 13% vs. 65 ± 24% (*P = 0.059*) in the LC run respectively). Consistently, the relative contribution of fat was higher in females compared to males (i.e., 24 ± 16% vs. 10 ± 11% (*P = 0.033*) in the HC run and 35 ± 24% vs. 23 ± 13% (*P = 0.059*) in the LC run respectively). The analysis of interaction effects between nutrition and gender resulted in non-significant findings (*P = 0.766*).

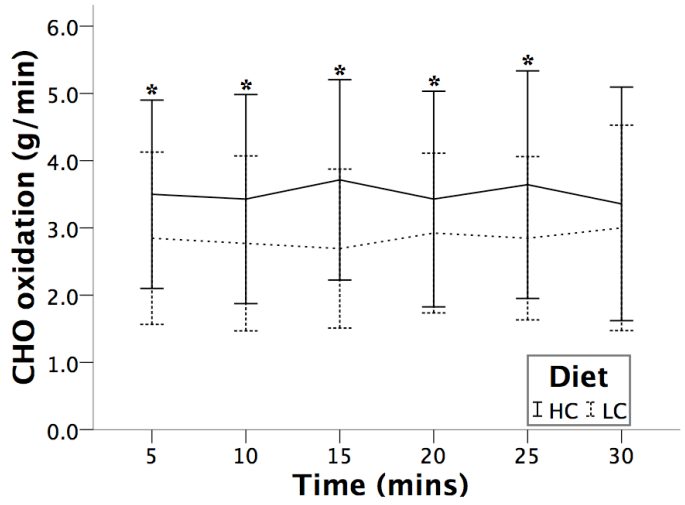

Figure 14: Absolute (g/min) CHO oxidation during HC and LC runs at each of the 5 min measurement points. All values are mean ± SD; * = P < 0.05.

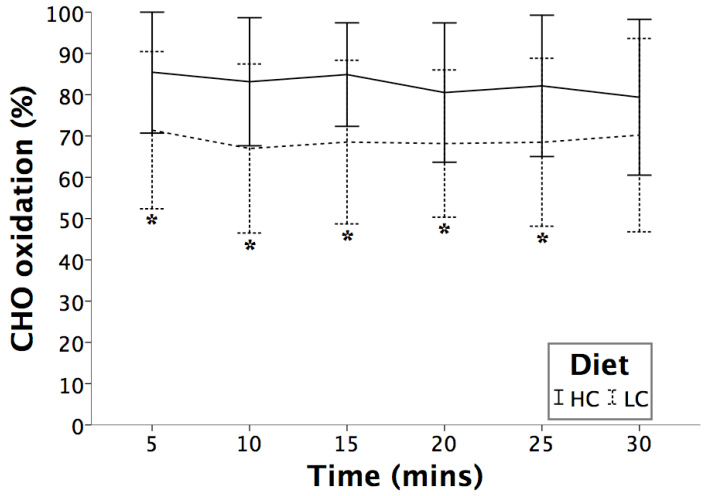

Figure 15: Relative (%) CHO oxidation during HC and LC runs at each of the 5 min measurement points (Raw data corrected: values > 100% [cutoff]). All values are mean ± SD; * = P < 0.05.

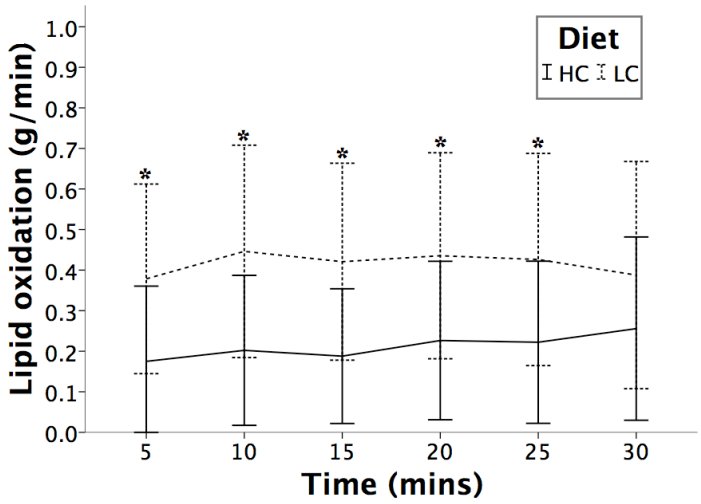

Figure 16: Absolute (g/min) fat oxidation during HC and LC runs at each of the 5 min measurement points (Raw data corrected: values < 0 g/min [cutoff]). All values are mean ± SD; Lipid = fat; * = P < 0.05.

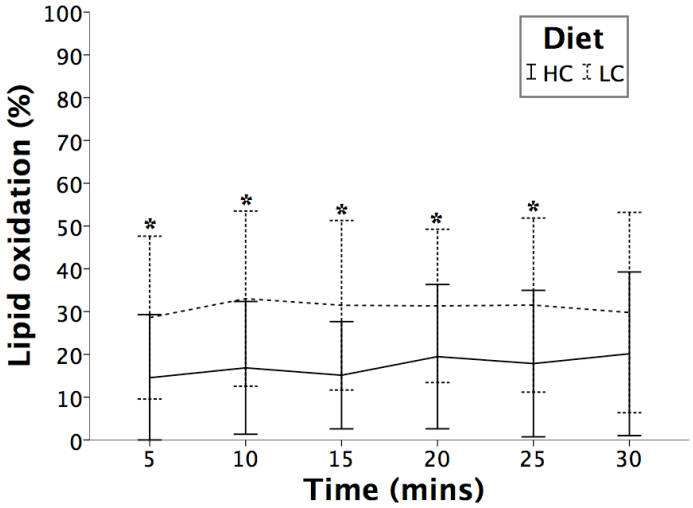

Figure 17: Relative (%) fat oxidation during HC and LC runs at each of the 5 min measurement points (Raw data corrected: values < 0% [cutoff]). All values are mean ± SD; Lipid = fat; * = P < 0.05.

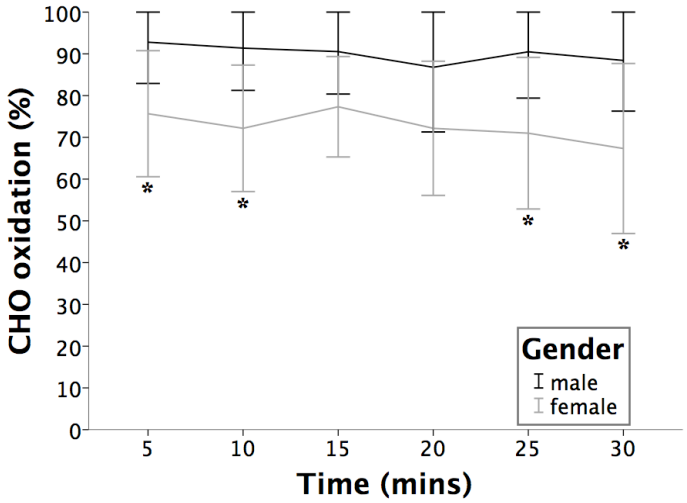

Figure 18: Gender comparison for CHO oxidation during HC run (Raw data corrected: values > 100% [cutoff]) and in relation to the relative overall amount of oxidized substrates. All values are mean ± SD; * = P < 0.05.

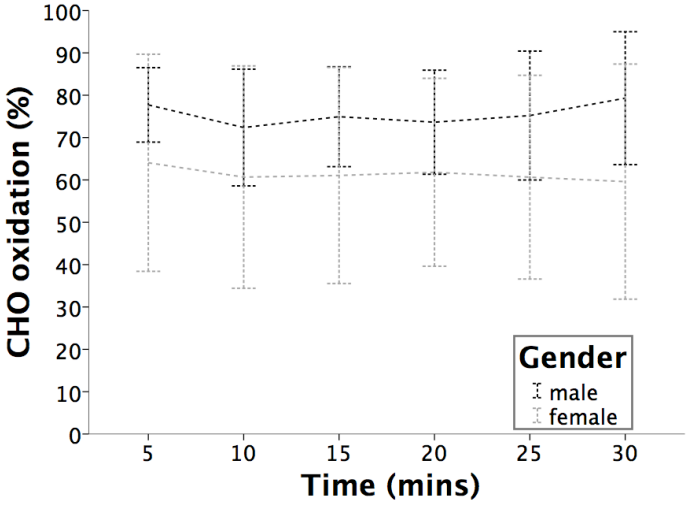

Figure 19: Gender comparison for CHO oxidation during LC run (Raw data corrected: values > 100% [cutoff]) and in relation to the relative overall amount of oxidized substrates. All values are mean ± SD.

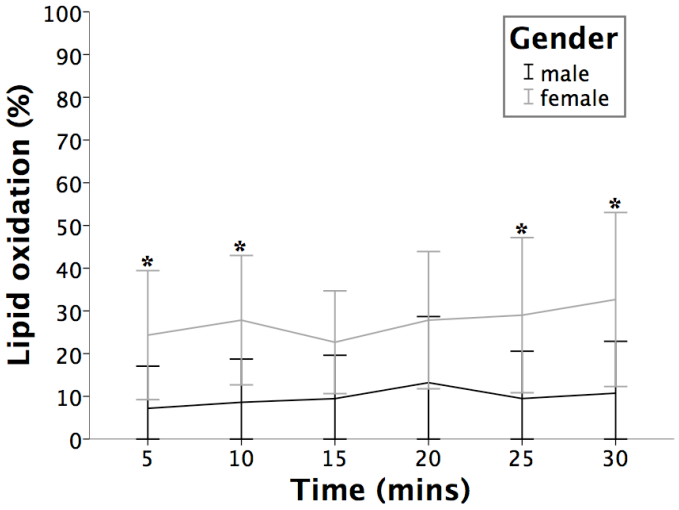

Figure 20: Gender comparison for fat oxidation during HC run (Raw data corrected: values < 0% [cutoff]) and in relation to the relative overall amount of oxidized substrates. All values are mean ± SD; Lipid = fat; * = P < 0.05.

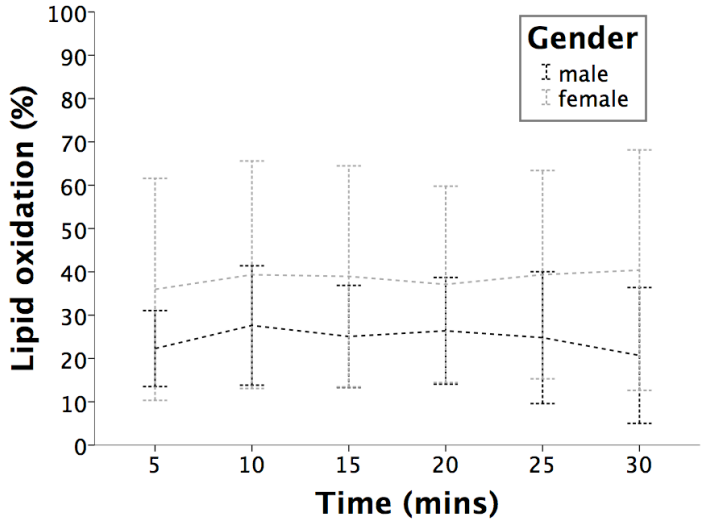

Figure 21: Gender comparison for fat oxidation during LC run (Raw data corrected: values < 0% [cutoff]) and in relation to the relative overall amount of oxidized substrates. All values are mean ± SD; Lipid = fat.

5. Discussion

5.1. Discussion: Research Objective 1

The aim of the current study was to establish the reproducibility of key parameters that correspond to PFO rates (i.e. V_{PFO} and Fat_{peak}) during treadmill ergometry. The main results of this investigation were the high ICC and Pearson's correlation coefficient computed for V_{PFO} and Fat_{peak}, in addition to the correspondingly low CV (i.e. 0.98, 0.97, 5.0%; and 0.90, 0.81, 7.0% respectively). Moreover, the performed Bland-Altman analysis has revealed a small bias of -0.3 km/h between Fat-peak tests, with 95% LoA for the reproducibility of V_{PFO} of 0.9 km/h (i.e. -2 ± 8% of VO_{2peak}).

To our knowledge, the present investigation is the first to report on the reproducibility and day-to-day variability of both V_{PFO} and Fat_{peak} during treadmill ergometry running. Hence, the current results reveal excellent values for the particular relative and absolute reliability indicators. The study group of Gmada et al. (2012) seems to be the first to have taken a more comprehensive statistical approach to assess the repeatability of Fat_{peak}. In their study, 12 sedentary, but otherwise healthy males performed a graded exercise test (5 stages of 6 min at 20, 30, 40, 50 and 60% of the maximal aerobic power (MAP)) after a 12-hour overnight fast. ICC and CV values for Fat_{peak} across test re-test trials separated by a time interval of 4 days were 0.97 and 5.0%, respectively. The mean differences ± 95% LoA for Fat_{peak} was 0.6 ± 7.2 W, indicating that 95% of the intra-individual differences should be contained between -6.6 and +7.7 W. Based on these values, relative and absolute reliability of Fat_{peak} were deemed as highly reliable by the authors. Unfortunately, no further appraisal has been made to address the physiological plausibility or applicability of the given LoA. Three other investigations have employed similar submaximal graded protocols (i.e. similar stage increment and duration, plus the

12-hour overnight food restriction prior to each bout), which were based either on the measured or on the theoretical MAP to establish the reproducibility of Fat_{peak}. Yet, conflicting findings have been reported. Pérez-Martin et al. (2001) report a CV of 11.4% for Fat_{peak}, and considered it satisfactory after assessing 10 overweight, but otherwise healthy male participants (no LoA analysis carried out). Similarly, Michallet et al. (2008) report on CV values between 7 to 12%. Here, the reproducibility of Fat_{peak} was assessed via two different gas exchange techniques in a group of 14 healthy and moderately trained participants (9 males, 5 females). More recently, Croci et al. (2014) assessed 15 healthy and moderately trained males, and computed CV values between 16 and 20% for Fat_{peak} while implementing three different data analysis procedures. The authors additionally report a high intra-individual variability with mean differences ± 95% LoA for Fat_{peak} (calculated with a P3 function) of -4 ± 32% of VO_{2peak}, indicating that 95% of the intra-individual differences should be expected between -37 and +28% of VO_{2peak}. Two other investigations using different methodological approaches have addressed the reliability and/or variability of Fat_{peak} estimations. Achten & Jeukendrup (2003) have advocated good reliability after assessing 10 healthy and moderately trained males as they performed an incremental test to exhaustion (test start: 95 W; stage increment and duration: 35 W every 3 min) on three different occasions and after a 12-hour overnight fast. The CV for Fat_{peak} (% of VO_{2peak}) was 9.6%. The authors additionally report a root mean square error (typical error) and 95% confidence interval for Fat_{peak} of 0.23 l/min (0.17 -0.34 l/min). Meyer et al. (2009) on the other hand, show a large intra-individual variability for Fat_{peak} after assessing 21 healthy participants (10 males, 11 females) of varying endurance capacities. Nutrition was moderately controlled, but with no fasting required prior to the exercise bouts. The implemented

incremental exercise protocol was nearly identical to the one currently used in our study (further appraisal on the protocol is given below). The mean differences ± 95% LoA for Fat_{peak} was -13 ± 0.91 l/min (-3.9 ± 28% of VO_{2peak}). Hence, 95% of intra-individual differences were to be expected between -1.04 and +0.78 l/min (-32 and +23% of VO_{2peak}). In this case, the large variability can be mostly attributed to the fact that only the end of each exercise stage was evaluated and not a continuous curve (i.e. whenever PFO switches from stage 2 to 3, for instance due to a small difference in the recorded rates, it will then result a large difference in the equivalent % of VO_{2peak}).

In the current study, the computed scores agree closely with those reported by Gmada et al. (2012), especially the CV, which has come noticeably lower then all of the other values reported in preceding analyses. As to the intra-individual (day-to-day) variability of Fat_{peak}, when expressed as % of VO_{2peak}, our LoA values have been distinctly lower then those observed by Meyer et al. (2009) and Croci et al. (2014). However, whilst these results enable closer comparisons to some of those from other investigations, making reasonable inferences as to the physiological plausibility and practical applicability of these LoA has shown to be a challenging task. As implied by Croci et al. (2014), previous studies have deemed an intra-individual variability of ± 10 beats/min for HR at V_{PFO} as acceptable, since this reflects a realistic margin in individuals who use HR for the monitoring of training intensity (Achten & Jeukendrup 2003, Meyer et al. 2009). Accordingly, in the present investigation this threshold has been sustained in most participants, with only three of them eventually exceeding the given cutoff (though by no more than 3 beats/min). Therefore, based on the strong aggregate of reliability indices and the generally lower intra-individual variability observed for the aforementioned physiological aspects (i.e. Fat_{peak} as % of VO_{2peak} and

HR at V_{PFO}), we consider the present Fat_{peak} estimations as the most reliable and coherent to date. Furthermore, the employed treadmill running protocol may be used as a reliable tool to identify Fat_{peak} in moderately trained individuals, and according to the reported intra-individual variability values, serve as the basis for future investigational research.

In spite of that, its applicability for athletic training is still questionable. For instance, the high day-to-day variability for PFO (g/min) remains largely unexplained. In the current study, PFO recordings between Fat-peak tests differed by a minimum of 0.01 g/min (1%) and a maximum of 0.28 g/min (45%) among the participants, which is consistent with inter- and intra-individual patterns described in previous investigations (Gonzales & Stevenson 2012, Meyer et al. 2009, Croci et al. 2014). On the grounds of this known variability for PFO, recent studies (Schwindling et al. 2014, Takagi et al. 2014) have questioned the practical applicability of prescribing exercise training based on Fat_{peak}, since it remains debatable whether prolonged exercise at Fat_{peak} can indeed be maintained with PFO rates. Therefore, it may be ultimately necessary for prospective studies (e.g. those looking at the sustainability of PFO during prolonged exercise bouts at Fat_{peak}) to consider the LoA (or simply the individual test re-test difference) for Fat_{peak}, V_{PFO} and PFO. Then, based on that, delineate the ± intensities in which exercise bouts should be performed and eventually evaluate how this impacts the sustainability of PFO (i.e. also in accordance to identified intra-individual variability of each person). Other questions in need of further research include: 1) What are the physiological determinants and additional intrinsic/extrinsic factors influencing the variability of fat oxidation rates during running, as well as in other types exercise? 2) How applicable,

versatile and reliable is the current protocol across different cohorts of people (e.g. patients, untrained persons or professional athletes)?

To date, there have been a few investigations assessing the reproducibility of Fat_{peak} (Gmada et al. 2012, Achten & Jeukendrup 2003, Pérez-Martin at al. 2001, Michallet et al. 2008, Meyer et al. 2009, Croci et al. 2014). Though the majority of those have failed to make thorough statistical analyses by not providing indicators of both relative and absolute reliability for Fat_{peak} estimations (i.e. the degree to which individuals/variables maintain their position in a sample with repeated measurements; or the degree to which repeated measurements vary for individuals/variables), in addition to practical information on the respective intra-individual (day-to-day) variability by establishing the LoA (i.e. the individual subject differences in a test re-test plotted against the respective individual means) (Atkinson & Nevill 1998, Altman & Bland 1983, Bland & Altman 1986, Baumgarter 1989). Hereto, previous studies suggest that an ICC greater than 0.90 is reflective of high relative reliability, while values between 0.80 and 0.90 should be rated as moderate, with figures under 0.80 being graded as not sufficient for physiological testing (Gmada et al. 2012, Vincent 1995). Additionally, a Pearson's coefficient greater than 0.80 is advocated as high (Atkinson & Nevill 1998), whereas a CV under 10% can be considered as an indicator for a reliable test, being a commonly used and accepted threshold for biological variables (Gmada et al. 2012, Vassault et al. 1986, Atkinson et al. 1999).

In the current study we have implemented rigid pre-testing conditions with standardized nutrition and exercise restraint for the 24 h prior to each submaximal bout. Yet, other methodological factors such as the elected exercise protocol, data analysis approach as well as the embedded equipment error may affect the determination of fat oxidation

rates and subsequently V_{PFO} (Croci et al. 2014). The currently employed exercise protocol intends to cover the realistic range for V_{PFO} determination and takes into account important physiological aspects in its design to ensure gas exchange maintains steady state for as long as possible (Meyer et al. 2009). The start velocity (V_{LT}) corresponds to the first increase in blood lactate and can be considered as the upper border for the conduction of regenerative training. The end velocity (V_{RER}) represents a metabolic state where energy supply is expected to yield solely from carbohydrate metabolism. Ultimately, three stages in between these metabolic markers should account for an accurate determination of V_{PFO} (Meyer et al. 2009, Xu & Rhodes 1999, Meyer et al. 2005, Jeukendrup & Wallis 2005). Additionally, we have chosen to create P3 curves, as it is a valid and widely used method that models the overall kinetics of fat oxidation for a more coherent representation of V_{PFO} and PFO (Chenevière et al. 2009).

Here we would like to comment on the 11 participants that had their V_{PFO} and Fat_{peak} computed during the warm up phase. One reason for this could of course be the rather moderate aerobic endurance capacity of participants, since in less trained individuals Fat_{peak} occurs at lower exercise intensities than in trained individuals (Jeukendrup & Wallis 2005). However, when looking at the individual raw fat oxidation rates, only 5 subjects have had indeed higher fat oxidation values during the warm up phase. The remaining 6, had their highest raw values recorded at the end of the first stage and were somewhat "drifted backwards" due to the applied P3 interpolation and how the curve-fit reacted upon the variables. Such a drift can also occur in the opposite way as depicted in figure 4, which in this case, was caused when curve-fitting the overall means for fat oxidation rates instead of individual values. This prompted the curve into a small elongation (likely driven by the subjects that had PFO rates at the latter stages of the

tests). Hence, the depiction of PFO rates that are slightly lower than the mean of individually interpolated values, and which also occur during the test phase and not the warm up. Still, the use of a mathematical model such as the P3, is a more consistent approach than just accounting for the raw measured values when analyzing data that does not align in a perfect curve (Chenevière et al. 2009). However, alternative ways of curve-fitting might be evaluated in the future.

At last, it must be noted that the total variation observed in our test re-test is a sum of both biological and equipment variation (error) (Meyer et al. 2009, Croci et al. 2014). Though analyzing the relative contribution of each of these parameters was beyond the scope of this study, the used gas exchange analyzer has been considered reliable (Macfarlane & Wong 2012). Ideal ICC values (1.00) were computed for ventilation (V_E) VO_2 and VCO_2. Respectively, the average intra-device technical error of measurement (%TEM) was 0.2, 1.4 and 1.1%.

5.2. Discussion: Research Objective 2

The present study analyzed the oxidative regulation of CHO and fat in a group of recreational runners as they performed 2 running bouts with standardized intensity at V_{IAT}. Participants were well fed with CHOs (HC protocol) or presumably, in a metabolic state of reduced CHO availability (LC protocol) before completing each bout. Baseline results indicate a fairly homogeneous physical conditioning among subjects as no significant gender differences were observed for IAT (mmol/l) or V_{IAT} (km/h). Throughout the 30 min submaximal runs, overall HR increased constantly and equally (~80 to 90% HR_{max}), with high but steady-state VO_2 recordings (~80% VO_{2peak}). Yet, as clearly depicted in the overall ventilatory response to exercise (see RER diagram on figure 13), the applied dietary scheme has influenced the oxidative regulation of substrates, with CHO metabolism prevailing throughout runs. Overall, CHO oxidation was 0.55 g/min (~16%; *P = 0.000*) greater during the HC compared to the LC run. Conversely, fat oxidation was 0.23 g/min (~64%; *P = 0.000*) greater during the LC compared to the HC bout. In relation to the overall energy metabolism, these differences reflect a significant increase of 12% in the oxidative activity of each substrate depending on which dietary protocol had been implemented.

At a gender level, CHO oxidation was also predominant, though females were able to consistently oxidize more fat than males under both conditions and throughout the entire duration of bouts (i.e. 14 and 12% greater fat oxidation during HC and LC bouts, respectively). Males on their side had higher CHO oxidation rates computed at both conditions, with highest and significant differences being recorded during the HC bout. It should be noted nonetheless, that during the LC bout fat oxidation might have even been suppressed in females, as CHO intake was exceeded in 26% (*P = 0.003*) compared

to only a 3% (*P = 0.520*) extrapolation by males. However, as glycogen itself was not measured, it cannot be completely assured whether the performed pre-exercise protocols had any effect on glycogen concentrations or its subsequent utilization during exercise. Nevertheless, as pointed out by Andrews et al. (2003), the significantly higher RER recorded in the HC bout are reflective of a higher rate of CHO metabolism, indicating that when CHO is made available through pre-exercise loading, one will also preferentially utilize CHO. In addition, a significantly higher VO_2 capacity from males at both baseline (12%) and during the submaximal bouts (~15%) could partly explain why males consistently oxidized more CHO (Billat et al. 2003), even though relative to VO_{2peak}, exercise was performed at the same intensity by both genders (i.e. 41 ± 2 vs. 35 ± 6 ml/min/kg and 42 ± 2 vs. 36 ± 5 ml/min/kg for males and females during HC and LC bouts respectively).

Physiological explanations to the observed findings suggest that increasing endogenous CHO availability will result in a greater muscle glycogenolysis and/or muscle glucose uptake, thus preventing a decline in blood glucose concentration during subsequent exercise, ultimately favoring a CHO driven metabolism while fat oxidation is partially inhibited (Andrews et al. 2003, Wee et al. 2005, Cermak & van Loon 2013). Moreover, during constant exercise at V_{IAT}, anaerobic glycolysis is enhanced (fuelled almost exclusively from plasma glucose entering the muscle fiber via facilitated diffusion and the glucose transporter type 4, or from glucose-phosphate provided through glycogenolysis from muscle glycogen), and provides a constantly increasing portion of the energy yield (Faude et al. 2009, Péronnet 2010). Still, why females burn more fat than males even at high exertion levels remains debatable. Plausible explanations in literature imply that a variety of factors such as the distribution and activation of α- and

β-adrenergic receptors, aerobic capacity but mostly endocrine mediated responses, predispose females to have a greater reliance on fat oxidation compared to males (Andrews et al. 2003, Braun & Horton 2001, Friedlander et al. 1998). Additionally, glycogen supercompensation occurs to a smaller extent in females compared to males (Walker et al. 2000), thereby directly affecting its subsequent availability for oxidation.

To our knowledge, the current investigation is the first to analyze how a simple, 24-hour manipulation of CHO intake may affect substrate oxidation during a constant, high-intensity running bout at V_{IAT}. In this sense, we would like to point out some plausible practical implications to our findings before making appraisals to previous investigations as well as raising a few prospective questions. Coaches, trainers and nutritionists should be aware of the reported oxidative patterns and how those ultimately influence the empting rates of glycogen (or glycogen sparing for that matter, as well as how those may influence high-intensity training and competition performance, which still remain to be established), and therefore, reinforce an individual and gender-based approach to pre-exercise nutrition. For example, as females show a greater reliance on fat metabolism compared to males, in spite of similar (or greater) systemic CHO-loading. In addition, they should be attentive when planning training strategies to the fact that, an identical bout of exercise may result in different metabolic reactions and may thus, cause different metabolic adaptations to training. Unfortunately, as shown by Wissman & Willoughby (2006), only a limited amount of studies (Walker et al. 2000, Wismann & Willoughby 2006, Tarnopolsky et al. 1995) have reported on substrate oxidation while combining CHO-loading strategies and exercising conditions that are similar to the ones applied in the present investigation. Tarnopolsky et al. (1995) reported on gender differences in substrate oxidation when CHO intake was increased

from 55 to 75% of the total energy intake during 4 days prior to exercise. They showed that when cycling at 75% VO_{2peak} for 60 min, females oxidized significantly more fat and less CHO compared to males. However, these findings should be critically interpreted as CHO intake was not prescribed relative to body weight, and consequently males ended up having a higher intake than females (i.e. 8.2 and 6.4 g/kg/d respectively). In one other study, Walker et al. (2000) used a CHO-loading strategy consisting of moderate (4.7 g/kg/d) and high (8.2 g/kg/d) intakes of CHO for 7 days before participants (females only) cycled at ~80% VO_{2peak} until volitional exhaustion. Results, which account for the first 75 min of exercise, reveal a significant increase of 0.44 g/min (~16%) in CHO oxidation during the high compared to the moderate intake bout. Conversely, fat oxidation was 0.17 g/min (~40%) greater during the moderate intake bout ($P < 0.05$). In this particular study, muscle glycogen increased 13% after the high compared to the moderate CHO protocol. Though significant, the magnitude of this supercompensation was still smaller than those previously observed in male athletes (Walker et al. 2000). Other investigations, for instance, the so-called "Train Low" studies, have reported on the effects of training in a glycogen-depleted state and found it to be an effective strategy to increase fat metabolism in athletes (Hawley & Burke 2010, Burke 2010). However, the benefits of such a protocol remains debatable, as no gains in endurance performance have been consistently observed (Scharhag-Rosenberger 2012, Hawley & Burke 2010, Burke 2010). Moreover, as highlighted by Scharhag-Rosenberger (2012), such a training strategy may induce a down-regulation in CHO metabolism, which consequently hinders the body's ability to make use of the potentially spared glycogen stores. Therefore, it would be of interest for prospective studies to investigate the effects of systematic training at V_{IAT} (e.g. on substrate

oxidation activity and adaptability over time, as well as against performance time-trials or bouts until volitional exhaustion) whilst subjects are well fed with CHOs or in a metabolic state of reduced CHO (glycogen) availability.

Lastly, we would like to acknowledge a few limitations of the current study. Female's menstrual cycle was not controlled. Therefore we cannot account on the eumenorrhoeic or amenorrhoeic status of the assessed female participants, as well as whether and how the follicular or luteal phases of their menstrual cycle could have influenced substrate oxidation. Dietary intake was only controlled for the 24 h prior to each submaximal exercise bout. In addition, due to methodological limitations, glycogen levels were not objectively assessed. Hence, we cannot assure if previous nutrient intake (i.e. outside of the controlled 24 h) would have resulted in significant additional accumulation of CHOs, or whether that could have influenced glycogen concentrations and subsequently substrate utilization. Future studies should in this case control for baseline glycogen levels before nutritional interventions are began and introduce longer dietary control periods. Still, our combined protocols of controlled dietary and exercise regimes have certainly brought subjects into the intended acute metabolic states of high and low systemic CHO availability.

6. Overall Conclusions

The current thesis aimed in assessing how intensity, exercise mode and pre-exercise nutrition affect the metabolic regulation of substrates at upper oxidative levels and in accordance to clear biomarkers of metabolic performance and exercise capacity. The first research objective, for the first time aimed at investigating the reliability and day-to-day variability of peak fat oxidation in treadmill running in moderately trained male and female recreational athletes, using appropriate statistical methods. In summary, the reproducibility of V_{PFO} and Fat_{peak} during treadmill ergometry was found to be excellent with ICC, Pearson's correlation coefficient and CV scoring at 0.98, 0.97, 5.0%; and 0.90, 0.81, 7.0% respectively. Fat_{peak} determined in a treadmill test might therefore serve as training prescription, although fat oxidation rates at prolonged exercise bouts at this intensity still need to be investigated.

The second research objective aimed in providing more evidence and a better understanding on the metabolic regulation of substrates when pre-exercise CHO intake is manipulated and exercise is performed at a level of upper endurance capacity. Our findings suggest that 24 h of high CHO consumption results in concurrent higher CHO oxidation rates and overall utilization, where as maintaining a low systemic CHO availability significantly increases the contribution of fat to the overall energy metabolism. The observed gender differences clearly underline the necessity of individualized dietary planning before exerting at intensities associated with performance exercise (e.g. prolonged exercise at V_{IAT}). Ultimately, future research should establish how these findings can be extrapolated to training and competitive situations and with that provide trainers and nutritionists with improved data to derive training prescriptions.

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Acknowledgements

First and foremost, I would like to express my appreciation and give special thanks to my advisor Prof. Dr. med. Frank Mayer for giving me the opportunity to develop and conclude my doctoral studies at the Professorship for Sports Medicine and Sports Orthopedics of the University of Potsdam. From day one he has been on my side, giving me all the necessary support and encouraging me to pursue my objectives, both in and out of the academic setting. His immense expertise and the given guidance throughout the vast fields of sports medicine, allowed me to develop into the clinical exercise scientist of today.

Further, I would like to thank Prof. Dr. Wolfgang Kemmler, who has been supporting my academic aspirations from the very first week I was in Germany (almost a decade ago). He is a true inspiration to me at the academic level, but most importantly, at a personal level, with his great charisma, politeness and humble character.

Special thanks go to Prof. Dr. Friederike Scharhag-Rosenberger, Junior-Prof. Dr. Anja Carlsohn and Dr. med. Michael Cassel for all the mentoring and support over the years. Many thanks also, to all my fellow colleagues from the Clinical Exercise Program as well as to the Outpatient Clinic's staff.

I also would like to acknowledge the support of the Potsdam Graduate School and thank them for all the funding and academic assistance.

Finally, I would like to give thanks and praise to my family members and friends around the world (especially to my loving wife Charlotte, my mother and father as well as my best friend Gerrit "G") for all their support throughout this journey.

Publication List

Conference Abstracts

Raul De Souza Silveira, Michael Cassel, Frank Mayer (2012) Does a spindle shape or enlarged Achilles tendon represent a problem in young athletes? *Muscles, ligament and Tendons Journal.* Poster presentation at the annual EFOST meeting.

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