Patterns of energy availability in free-living athletes and the physiological effects of controlled low energy availability in males

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A thesis submitted in partial fulfilment of the requirements of Liverpool John Moores University for the degree of *Doctor of Philosophy*.

April 2024.

COVID-19 preamble – Lead Supervisor Statement:

The COVID-19 pandemic has had a significant impact on many people's lives. Academia and research have not been exempted from this pandemic, and for many research students, this period of time has affected the progress and outputs of their research programme.

Harry's PhD studies were directly affected by national lockdowns given that the main aim of his PhD plans was to study the effects of low energy availability in males in controlled conditions. Harry's PhD started in October 2019 and data collection for his first laboratory-based study was scheduled to start in the first week of April 2020. Start of national lockdowns in March 2020 and closure of laboratories meant that we could not access the laboratories to work with human participants until August 2021 (1.5 years later than originally planned). This time was fraught with uncertainty not only because of the possibility of further lockdowns but also because of the added stress and strain by the layers of permissions and precautions necessary to conduct research in laboratory conditions with humans.

Fortunately, we were able to access a group of male elite road cyclists based in Norway, who continued to train normally during the lockdowns that were affecting a good part of the globe, allowing us to conduct research aligned with Harry's area of work. This opportunity allowed Harry to keep developing his thesis research topic, while being unable to work on his thesis original objectives due to reasons that were beyond our control. While unable to collect laboratory-based physiological data, as planned in his original thesis objectives, data collection for this first study happened in a nearly ideal conditions utilising remote data collection methodology and technology including the remote food photography method and estimates of energy expenditure from crank-based power meters, possibly the best methodology to assess energy availability in free-living conditions.

This is the reason why the first study of his thesis (Chapter 4) may appear slightly disjointed, in regard to methodology and objectives, from the other two experimental studies (Chapter 5 and 6). Nonetheless, data collected for chapter 4, were very useful to inform the experimental design and research questions of chapters 5 and 6, as the examiners and readers will appreciate.

Importantly, it also worth highlighting that despite the 1.5 years delay in data collection, Harry's PhD scholarship was extended only by 6 months, putting further pressure to complete complex and demanding experimental projects that were meant to be the core work of his thesis. Between August 2021 and September 2023, Harry has been relentless in his pursuit of excellence and high-quality data collection, achieving what I believe is the equivalent of nearly a full thesis worth of data, even while working part-time after the end of his scholarship.

This preamble is not to justify any sub-par work presented in this thesis, the work presented herein is of the highest quality, as the examiners and readers will appreciate, also evidenced by multiple conference presentations and important awards received at a leading conference during his thesis. However, it is worth a reminder of the historical context under which this work took place. A PhD is already a challenging endeavour, and I would like to express my appreciation and admiration, and invite the reader to do so as well, for Harry's capacity of doing this PhD work in the historical context that he did, in the face of added uncertainty and strain.

Dr. José L Areta
Director of Studies (lead supervisor) for Harry Taylor

Abstract:

The physiological effects of short-term low energy availability have been characterised in females; however, the responses of males are less well understood. The aim of this thesis was to examine the daily patterns of energy availability in free-living athletes and subsequently, to determine the endocrine, metabolic, and physiological effects of low energy and low carbohydrate availability under controlled experimental conditions in males. **Study 1** demonstrated that elite male road cyclists only partially compensate for greater daily exercise energy expenditure levels with increasing energy intake, leading to a fluctuating pattern of daily energy availability across a week of free-living training during pre-season. These findings provide novel insights into ecologically valid patterns of energy availability for experimental research. **Study 2** investigated the physiological effects of low energy availability under controlled experimental conditions using a heterogenous pattern of daily energy availability. Energy availability was reduced from 45 to 10 kcal•kg FFM^{-1}•day^{-1} for five days, with concomitant aerobic exercise. Low energy availability elicited changes in endocrine, metabolic, and physiological markers indicative of energy preserving mechanisms in healthy, active males. Circulating glycerol, NEFA, and D-3-hydroxybutyrate concentrations increased and triiodothyronine, leptin, and IGF-1 concentrations reduced in response to low energy availability. Concentrations of bone resorption marker ß-CTx increased and of bone formation marker P1NP reduced during low energy availability. Total testosterone concentrations and rates of muscle protein synthesis were unaffected by low energy availability. The findings of this study demonstrate that exercising males are sensitive to the effects of short-term low energy availability in a similar manner to that reported in females. **Study 3** investigated the endocrine, metabolic, and physiological effects of reduced carbohydrate availability under conditions of energy balance. Reducing carbohydrate intake from 7.7 to 1.5 g•kg^{-1}•day^{-1}, with an energy availability of 45 kcal•kg FFM^{-1}•day^{-1}, for four days led to increased circulating glycerol, non-esterified fatty acid, and D-3-hydroxybutyrate concentrations, in healthy active males. Concentrations of bone resorption marker ß-CTx increased with low carbohydrate availability, but bone formation marker P1NP concentrations were unchanged. Further markers of energy preservation typically associated with low energy availability, including triiodothyronine, leptin, insulin, and resting metabolic rate were unchanged by low carbohydrate availability. The findings of this study support the notion that bone metabolism may be affected by carbohydrate availability. Further endocrine, metabolic, and physiological responses typically associated with low energy availability appear sensitive to energy itself, rather than carbohydrate availability. **In summary**, free-living athletes experience heterogenous patterns of daily energy availability that have not been reflected in laboratory-based low energy availability research to-date. Low energy availability in males elicited endocrine, metabolic, and physiological changes indicative of energy preservation, similar to previously characterised in females. Some of these observed effects, such as changes in bone metabolism, may be mediated by carbohydrate availability. Whether the physiological effects of short-term low energy availability are predictive of long-term health and performance consequences requires further research.

Acknowledgements:

Firstly, I would like to thank my supervisors Dr Jose Areta, Dr Carl Langan-Evans, and Prof. James Morton for your support and guidance throughout my PhD. Your expertise, feedback, and encouragement have been invaluable over the past four years. Beyond your academic support, you have helped guide me and this PhD through a pandemic, university closures, and numerous delays outside of our control. Thank you. I would also like to thank my former supervisor Dr Tom Brownlee for encouraging me to pursue a PhD in the first place, and for your friendship and mentoring in the years since. Furthermore, a sincere thank you to Prof. Jatin Burniston for allowing me to attend your proteomics team meetings over the last year of my PhD. I have learned so much in this time and I will forever be thankful for the opportunity.

I am immensely grateful to LJMU School of Sport and Exercise Sciences (RISES) for supporting me with a PhD scholarship and for providing funding to attend conferences over my time at the university. Further, to the staff at LJMU who have helped me undertake this research (the following list is not exhaustive!), Dr Jules Strauss, Dr Sam Shepherd, Dr Matt Cocks, and Dr Julien Louis for your technical assistance with collecting muscle biopsies for the experimental studies. To the technicians who work tirelessly to ensure that research runs smoothly, including Dr Gemma Miller, Mr Dean Morrey, Mr George Savage, Dr Andy Moss, Dr Karl Gibbon, Mr Mark Doyle, and Dr Joe Maxwell amongst others over the years. I must also thank Dr Andy Moss, Prof. Neil Walsh, Dr Jason Edwards, Dr Dan Kashi, and Erick Mosquera for your time and technical assistance with sample analyses. Similarly, to Prof. Greg Atkinson for your help and guidance with statistics throughout my time at LJMU. Equally, to Dr Yusuke Nishimura for your collaboration on the proteomic analyses, Mr Wee Lun Foo for your tireless assistance with Study 2, Dr Jamie Pugh for being a constant source of advice, Dr Marcelle Ribeiro for collaborating on Study 3 and for your friendship, and to all the MSc students who assisted with data collection. My sincerest thanks to you all.

To my peers in the post-graduate office. Over the past four years you have never failed to help when I needed an extra pair of hands in the laboratory, you have infused me with a constant supply of coffee (FP!), and you have generally made living and working in Liverpool such an enjoyable experience. Special thanks to Dr Jen Barrett, Harvey, and Robyn for your assistance with data-collection and analyses, and to Connor and Dr Sam Bennett for your help with proteomics.

As my esteemed post-graduate colleagues like to remind me, my road to LJMU spanned many years. So, a further thank you to my friends, colleagues, and mentors who inspired me to pursue my passion for sports nutrition along the way before this PhD; in particular, my good friend Jack Wilson, Eliot Challifour and everyone at the Porsche Human Performance laboratory, as well as my MSci supervisors at the University of Bath, Professor James Betts, and Professor Javier Gonzalez.

Finally, this thesis would not have been possible without the limitless love and support of my parents. Thank you for being there for me at every turn and in every aspect of my life without fail. My loving partner Emma, who has celebrated the smallest of highs, been a soundboard for every low, and ensured that there was no chance of low energy availability in the writing of this thesis. My brother, Jack, for your love, and for teaching me that resilience is about much more than being brittle. And Emma's parents Tom and Helen, who have provided sage advice whenever I have needed it. Thank you, Tom, for reading this thesis countless times and for all your feedback.

A most heartfelt thank you to you all!

Dedications:

To my parents, to whom I owe everything.

To Emma, for always knowing when we should take a breath of fresh air.

And to the rest of my family, for your unwavering love and support.

Declaration:

I declare that the work in this thesis, which I now submit for assessment on the programme of study leading to the award of Doctor of Philosophy is entirely my own. All attempts have been made to ensure that the work is original and does not, to the best of my knowledge, breach any copyright laws and has not been taken from the work of others, apart from the works that have been fully acknowledged within the text. No portion of the work referred to in the thesis has been submitted in support of an application for another degree or qualification of this or any other university or other institute of learning. Elements of the work presented herein have been published in peer-reviewed journals. Where this is the case, acknowledgements have been provided.

Publications:

Publications of the work listed within this thesis are as follows:

1. Areta, J. L., Taylor, H. L., & Koehler, K. (2021). Low energy availability: history, definition, and evidence of its endocrine, metabolic, and physiological effects in prospective studies in females and males. *European Journal of Applied Physiology*, *121*(1), 1–21. https://doi.org/10.1007/s00421-020-04516-0.

2. Taylor, H. L., Garabello, G., Pugh, J., Morton, J., Langan-Evans, C., Louis, J., Borgersen, R., & Areta, J. L. (2022). Patterns of energy availability of free-living athletes display day-to-day variability that is not reflected in laboratory-based protocols: Insights from elite male road cyclists. *Journal of Sports Sciences,* *40*(16), 1849–1856. https://doi.org/10.1080/02640414.2022.2115676.

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List of Abbreviations:

ß-CTX	β-Carboxyl-Terminal Cross-Linked Telopeptide of Type I Collagen
ACSM	American College of Sports Medicine
AT	Adaptive Thermogenesis
AUC	Area Under the Curve
BIA	Bio-electrical Impedance Analysis
BM	Body Mass
BMD	Bone Mineral Density
BSI	Bone Stress Injury
CHO	Carbohydrate
CNS	Central Nervous System
DE	Disordered Eating
DW	Dry Weight
DLW	Doubly Labelled Water
DXA	Dual X-Ray Absorptiometry
EA	Energy Availability
ECLIA	Electrochemiluminescence Immunoassay
ED	Eating Disorder
EDTA	Ethylenediaminetetraacetic Acid
EEE	Exercise Energy Expenditure
EEE_{gross}	Gross Exercise Energy Expenditure
EEE_{net}	Net Exercise Energy Expenditure
EHMC	Exercise Hypogonadal Male Condition
EI	Energy Intake
ELISA	Enzyme-Linked Immunosorbent Assays
FDR	False Discovery Rate
FSH	Follicle Stimulating Hormone
FSR	Fractional Synthetic Rate
FFM	Fat Free Mass
GDF-15	Growth/Differentiation Factor-15
GH	Growth Hormone
GnRH	Gonadotrophin-Releasing Hormone
HPG axis	Hypothalamic-Pituitary-Gonadal axis
HPT axis	Hypothalamic-Pituitary-Thyroid axis
IGF-1	Insulin-like Growth Factor-1
IOC	International Olympic Committee

LBM	Lean Body Mass
LCHF	Low-Carbohydrate, High-Fat
LEA	Low Energy Availability
LH	Luteinising Hormone
LHep	Lithium Heparin
LT	Lactate Threshold
MEI	Metabolisable Energy Intake
MET	Metabolic Equivalent of Task
MPS	Myofibrillar Protein Synthesis
NEFA	Non-Esterified Fatty Acid
NTX	N-terminal telopeptide of type-1 collagen
OC	Osteocalcin
E_2	Oestradiol
P1CP	Procollagen Type 1 Carboxy-terminal Propeptide
P1NP	Procollagen type 1 N-terminal Propeptide
PPO	Peak Power Output
PRO	Protein
REDs	Relative Energy Deficiency in Sport
RER	Respiratory Exchange Ratio
RISES	Research Institute for Sport and Exercise Sciences
RMR	Resting Metabolic Rate
TBW	Total Body Water
TEE	Total Energy Expenditure
TSS	Training Stress Score
T_3	Triiodothyronine
T_4	Thyroxine
VL	Vastus Lateralis

List of Operational Definitions and Key Terms:

The following definitions refer to common key-terms used throughout this thesis and in the context of the studies being discussed:

Acute	≤ one-week intervention duration (in the context of controlled energy availability studies)
Adaptive Thermogenesis	The suppression in resting energy expenditure independent of alterations to body fat mass and fat free mas (Müller et al., 2015) OR: the regulatory heat-production in response to cold (i.e., cold-induced thermogenesis) or over-eating (to dissipate excess EI) (Müller & Bosy-Westphal, 2013) OR: the adjustment of all components of daily energy expenditure (i.e., both resting- and non-resting energy expenditure) to enhance metabolic efficiency (Hall, 2006; Müller & Bosy-Westphal, 2013)
Energy Availability	The amount of energy remaining for essential physiological processes once the energetic cost of exercise has been accounted for (Loucks, 2020). Typically expressed as the difference between energy intake and net exercise energy expenditure, relative to an individual's lean body mass
Energy Balance	The difference between energy intake and total energy expenditure (Burke, Lundy, et al., 2018; Siedler et al., 2023)
Female Athlete Triad	A condition typically afflicting active girls and women, incorporating any one of the three components of, 1) low energy availability, 2) menstrual dysfunction, and 3) low bone mineral density (De Souza et al., 2014; Nattiv et al., 2007)
Long-term	> one-week intervention duration (in the context of controlled energy availability studies)
Male Athlete Triad	*A syndrome of the three inter-related conditions of 1: LEA/energy deficiency (with or without an associated ED/DE), 2: impaired bone health, and 3: suppression of the HPG axis* (Nattiv et al., 2021)
Pattern	*A regular and intelligible form or sequence discernible in certain actions or situations; esp. one on which the prediction of successive or future events may be based (Oxford English Dictionary. Retrieved 27th March, 2024, from https://www.oed.com/dictionary/pattern_n?tab=meaning_and_use#31803324).* In this context, the pattern of energy availability derived from the relationship between daily exercise energy expenditure and energy intake (i.e. the pattern of increased exercise energy expenditure without compensatory increases in energy intake resulting in reduced energy availability)

Prolonged	> one-week intervention duration (in the context of controlled energy availability studies)
Relative Energy Deficiency in Sport (REDs)	*A syndrome of impaired physiological and/or psychological functioning experienced by female and male athletes that is caused by exposure to problematic (prolonged and/or severe) low energy availability. The detrimental outcomes include, but are not limited to, decreases in energy metabolism, reproductive function, musculoskeletal health, immunity, glycogen synthesis and cardiovascular and haematological health, which can all individually and synergistically lead to impaired well-being, increased injury risk and decreased sports performance* (Mountjoy et al., 2023)
Short-term	≤ one-week intervention duration (in the context of controlled energy availability studies)

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Chapter 1:

General Introduction

This chapter provides a broad overview of the concept of energy availability and the purported role of low energy availability in physiological sequelae associated with the Female Athlete Triad, Male Athlete Triad, and Relative Energy Deficit in Sport models.

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1.1. Background:

Insufficient dietary energy to maintain normal physiological functioning results in a state of low energy availability (*LEA*), which is believed to be the aetiological factor behind endocrine, metabolic, and physiological dysregulations observed in exercising individuals (Loucks, 2020). Low energy availability is therefore a core concept underpinning theoretical models that are contemporary topics in sports nutrition, such as the *Female Athlete Triad* (De Souza, et al., 2014), the *Male Athlete Triad* (Fredericson et al., 2021; Nattiv et al., 2021), and *Relative Energy Deficiency in Sport* (*REDs*; Mountjoy et al., 2023).

These models propose that chronic low energy availability affects a broad range of tissues and systems, including bone, muscle, the endocrine axis, and the immune system amongst others, ultimately affecting an athlete's health and performance (De Souza, Nattiv, et al., 2014; Mountjoy et al., 2023; Nattiv et al., 2021). Indeed, there is currently experimental evidence supporting the effects of low energy availability upon the disruption of menstrual function and markers of bone resorption and formation in females, as reviewed by Areta et al. (2021) and Loucks (2020). However, it is apparent that limited causal data linking low energy availability to many other physiological effects exists. Instead, much of the data underpinning these models is derived from cross-sectional or observational research (Areta et al., 2021). Within this research, energy availability (*EA*) is often measured in the field and this methodology has been shown to have significant limitations due to the error associated with assessing energy intake (*EI*) and expenditure, as reviewed by Burke, Lundy, et al. (2018). The validity of extending field-based measurements of energy availability to resulting physiological dysregulations is therefore questionable. Such observations are crucial in developing hypotheses relating to the presentation of athlete health and performance impairment. However, rigorously controlled laboratory-based experiments using carefully manipulated energy availability values are required to develop our understanding of the causal role of LEA upon physiological functioning (Areta et al., 2021).

Furthermore, the *Triad* and *REDs* models purport that low energy availability is pervasive in athletes due to under-fuelling for a given amount of exercise energy expenditure (*EEE*) (De Souza, Nattiv, et al., 2014; Mountjoy et al., 2023; Nattiv et al., 2021). However, the relationships between exercise energy expenditure, energy intake, and subsequent energy availability in free-living athletes are poorly characterised. Understanding the patterns of energy availability that athletes experience under free-living conditions might therefore inform future LEA-related research methodologies and enhance the ecological validity of this work.

The basis of our current understanding of the effects of low energy availability is grounded in laboratory-based research. Seminal work from Professor Anne Loucks sought to establish the cause of impaired function of the hypothalamic-pituitary-ovarian and hypothalamic-pituitary-adrenal axes

observed in amenorrhoeic female athletes. This research led to the discovery that low energy availability, and not the stress of exercise, was the main cause and is succinctly summarised in Loucks (2020). It is most likely due to the high incidence of stress fractures and menstrual dysfunction in females that research in low energy availability has predominantly been developed in females. Despite the *REDs* and *Male Athlete Triad* models asserting that exercising males may suffer similar afflictions (Mountjoy et al., 2023; Nattiv et al., 2021), it is apparent that equivalent high-quality research establishing the effect of low energy availability upon endocrine, metabolic, and physiological responses in males is very limited (Areta et al., 2021; De Souza, Williams, et al., 2014). Tightly controlled, laboratory-based studies are therefore required to establish further the physiological impact of low energy availability in males.

Finally, whilst energy itself appears to be a major disruptor of endocrine and physiological responses, most of the laboratory-based research in the area has used concomitant and equal reductions in carbohydrate, protein, and fat to reduce energy availability (Areta et al., 2021). It is therefore difficult to determine whether a reduction in one of these macronutrients specifically has a major role in influencing the physiological responses to low energy availability. Some research suggests that low carbohydrate availability may play a prominent role in the endocrine, metabolic, and physiological responses typically attributed to low energy availability. For example, recent studies have shown that markers of bone resorption and formation are affected by carbohydrate restriction independently from an energy deficit (Fensham et al., 2022; Hammond et al., 2019; Heikura et al., 2020). Further research investigating the isolated effects of reduced carbohydrate availability upon physiological markers typically associated with low energy availability is therefore warranted.

1.2. Aims and Objectives:

This thesis therefore has two primary aims; 1: to investigate the dietary and training practices of elite athletes in the field to characterise patterns of daily energy availability, and 2: to investigate the endocrine, metabolic, and physiological responses of healthy adult males to acute (< 1 week) periods of low energy availability under controlled laboratory conditions. These aims will be achieved by the completion of a series of inter-linked studies with the following objectives:

1. To examine the habitual exercise and dietary patterns of a cohort of elite (professional) male cyclists under free-living conditions during a 7-day pre-season training period. This will allow us to characterise the relationship between energy intake, exercise energy expenditure, and subsequent energy availability in a cohort of athletes that are commonly identified as being at particularly high-risk of LEA.

2. To investigate the effects of short-term low energy availability, elicited through caloric restriction with concomitant aerobic exercise, on markers of the hypothalamic-pituitary-gonadal and hypothalamic-pituitary-thyroid axes, markers of bone resorption and formation, and further key endocrine, metabolic, and physiological responses.

3. To investigate the physiological effects of short-term low carbohydrate availability, under conditions of energy balance, upon the same endocrinological, metabolic, and physiological parameters related to the regulation of bone health, the hypothalamic-pituitary-thyroid and hypothalamic-pituitary-gonadal axes. The objective of this study is to provide insight into the mediating role of carbohydrate availability in the physiological responses to low energy availability in males.

Chapter 2:

Literature review

This chapter provides a synthesis of research findings that have led to the formation of the concept of energy availability that underpins the LEA-related models of the Female Athlete Triad, Male Athlete Triad, and Relative Energy Deficiency in Sport. A review of the literature in relation to the physiological effects of low energy availability from research studies performed under laboratory-based controlled conditions then follows, with a key focus upon male responses to LEA.

Elements of this chapter have resulted in the following publication:

Areta, J. L., Taylor, H. L., & Koehler, K. (2021). Low energy availability: history, definition, and evidence of its endocrine, metabolic, and physiological effects in prospective studies in females and males. *European Journal of Applied Physiology*, *121*(1), 1–21. https://doi.org/10.1007/s00421-020-04516-0

2.1. Low energy availability, a key topic in sports nutrition:

From a bioenergetic standpoint, once energy has been used for one process within the body, it cannot be used for another. As a finite resource, the body must therefore prioritise its use of energy for certain physiological processes above others (Bronson, 1985; Loucks, 2020). Homeostasis in humans can be disrupted by insufficient dietary intake, with this effect exacerbated by the energetic demands of undertaking physical activity. Considering the energetic cost of sport and exercise training, athletic individuals have therefore sometimes been observed to present with impairment of reproductive function and bone health. These impairments have been related to alterations of the endocrine milieu and metabolism triggered by insufficient energy availability, (Areta et al., 2021). Energy availability is defined as the amount of energy remaining for essential physiological processes once the energetic cost of exercise has been accounted for (Loucks, 2020). In exercising individuals, insufficient energy intake, a high exercise energy expenditure, or a combination of these factors can lead to a state of low energy availability. Under such conditions, it has been proposed that intra-organism competition for the limited energetic resources available lead to *trade-offs* in physiological function to maximise survival, and ultimately, reproductive success (Areta, 2023; Oliveira-Junior et al., 2022; Shirley et al., 2022). Through this *Life History Theory* perspective, phenotypic plasticity to down-regulate non-essential energy consuming processes would be considered a non-pathological essential survival trait. When sustained for extended periods, however, low energy availability may produce adverse effects upon health and performance (Shirley et al., 2022).

There is more research investigating the physiological effects of low energy availability in females than there is in males. As female participation in sport grew across the 1960's and '70's (Slater et al., 2017), so too did the observations of menstrual disturbances. This led to the emergence of a new research area that sought to investigate the prevalence of amenorrhea, its underlying mechanistic cause, and any further physiological dysregulations that may occur in athletic female populations (Loucks, 2020; Loucks & Horvath, 1985; Slater et al., 2017). Such research eventually led to the consensus that energy availability was the underlying cause of disruptions to reproductive function and bone health in exercising females, culminating in the synthesis of the *Female Athlete Triad* (De Souza, et al., 2014) model. The topic of low energy availability has continued to grow as a research area, with further models expanding the endocrine, metabolic, and physiological 'dysregulations' of LEA to exercising males in the *Male Athlete Triad* (Fredericson et al., 2021; Nattiv et al., 2021), and *REDs* models (Mountjoy et al., 2023). *REDs* in particular has become one of the key contemporary topics in sports nutrition research today. Since 2018, there has been more than 170 original research publications on *REDs* and/or LEA, yet nearly 75 % of these studies were cross-sectional or observational in design (Mountjoy et al., 2023). Despite the clear and growing interest in the perceived negative effects of low energy availability and its prevention, however, a recent narrative review has highlighted that there is a

lack of causal evidence underpinning its relationship with physiological dysregulations, particularly in males (Areta et al., 2021).

2.2. The origin of energy availability in animal models:

Prior to being applied in the sports science context, the concept of energy availability developed through observations of reproductive fitness in mammals in ecological settings and experimental models in rodents. From an ecological perspective, greater energy provision tends to lead to greater reproductive success, as summarised by Bronson (1985). As outlined previously, mammals must divide energy between numerous physiological functions and the expenditure of energy performing one function renders it unavailable for another (Loucks, 2020). Attaining energy for key metabolic processes that enhance survival chances has therefore been recognised to take precedence above growth and reproduction. More specifically, mammals must first satisfy the energetic demands of cellular maintenance, thermoregulation, and locomotion for food procurement from the energy and nutrients available to them. Once these energetic costs are satisfied, any remaining energy can be apportioned to growth, reproduction, and in conditions of energetic surfeit, stored as fat (Bronson, 1985).

In a landmark paper, Schneider & Wade (1989) conducted a series of experiments to investigate the influence of body mass and fuel availability on shifts in the reproductive status of Syrian hamsters. In these experiments, the pharmacological blockade of oxidisable fuels led to the impairment of reproductive function in 83% of the hamsters studied, despite no change in body mass. The authors subsequently concluded that the 'general availability of metabolic fuels', and not body mass, was the key factor determining reproductive status outcomes (Schneider & Wade, 1989). Two key follow-up papers strengthened this idea. Firstly, greater prior body mass and the provision of *ad libitum* glucose or fat solutions during food-deprivation were shown to preserve the reproductive status of golden hamsters, despite similar reductions in body mass in the latter trial, suggesting that the interplay of endogenous and exogenous metabolic fuel supply mediates reproductive function (Schneider & Wade, 1990a). Secondly, a reduction in metabolic fuel availability in Syrian hamsters via cold-induced thermogenesis and increased lab-simulated foraging requirements induced similar impairments in reproductive function (Schneider & Wade, 1990b). However, there was no quantification of the parameter of 'energy availability' as it is contemporarily viewed within these studies. Similarly, whist insightful, the study of small mammals is not directly transferrable to *Homo sapiens*. Larger mammals are typically more reproductively robust to transient fluxes in energy and nutrient availability, due to their lower metabolic rates and thermoregulatory cost, as well as greater fat stores (Bronson, 1985). Furthermore, considering the comparatively larger energy reserves, it remained unclear whether observations from small mammals and the role of 'metabolic fuel availability' could explain losses of ovulation in large mammals and humans into the 1990's (Bronson & Manning, 1991).

2.3. Establishing low energy availability as a concept affecting humans:

As participation rates in female sports increased, so too did research characterising the prevalence of amenorrhoea and further physiological dysregulations in athletic female populations. Such observations have been reviewed by Loucks & Horvath (1985) and Slater et al. (2017). Whilst the mechanistic cause of this phenomena was not well understood, body composition, training regimen, reproductive maturity, diet, sport specificity, psychological stress and 'multiple factors' had all been cited as possible causes of secondary amenorrhea observed in athletic female populations by the mid 1980's. One such commonly held belief that body composition is key in driving physiological function was dismissed on the grounds that some lean females were cyclic, other higher-fat athletes were not, and some athletes regained menstruation without discernible changes in body mass or body fat (Loucks & Horvath, 1985).

In contrast, the 'stress of exercise' and 'energy drain' became key aetiological suspects as two key papers emerged. Feicht et al. (1978) surveyed 128 female USA college track, field, and cross-country athletes, observing a positive correlation between weekly training mileage and secondary amenorrhea, up to a prevalence of 43% in athletes running 60 – 80 miles per week. In a sub-set of 56 middle-distance runners, amenorrhoeic athletes also reported a delayed menarche, more intense training months per year, and a quicker personal best 1500m time compared to eumenorrheic athletes. However, there was no difference in mean body mass or height between the amenorrhoeic and eumenorrheic runners, suggesting that body mass was not causative of the divergent menstrual status in these athletes (Feicht et al., 1978). Secondly, Warren (1980) observed adolescent ballet dancers over a period of four-years to monitor puberty progression and reproductive status. Compared to a control group, the ballet dancers had reduced body mass and estimated body fat at all ages, experienced a significant delay in menarche, and most dancers also subsequently developed secondary amenorrhoea. The key observation, however, was that amenorrhea occurrence increased with activity, whilst pubertal progression was apparent when activity levels decreased. As these changes occurred in the absence of changes in body mass, the authors therefore concluded that *energy drain* from exercise led to prolongation of the prepubertal state (Warren, 1980). Based in part upon the findings of Feicht et al. (1978) and Warren (1980), Winterer et al. (1984) proposed the 'caloric balance' theory, which appears to be the first testable energy availability hypothesis in humans. They suggested that the human brain, due to its high metabolic demand, monitors the balance of endogenous and exogenous caloric availability and depending on this flux, controls the release of gonadotrophin releasing hormone (*GnRH*). The subsequent inhibition or disinhibition of reproductive function then serves to preserve energy for the brain, much in line with the bioenergetic principles outlined by Bronson (1985), Schneider & Wade (1989) and others (Areta, 2023; Oliveira-Junior et al., 2022; Shirley et al., 2022).

It was nearly a decade until the first controlled energy availability study was conducted in humans, however. In their seminal study, Loucks & Callister (1993) used the first algebraic equation of energy availability (see Section 2.5) to investigate the impact of altering EA upon physiological responses in healthy, sedentary females. Participants completed two four-day periods of controlled dietary provision, with either 1: no exercise, 2: exercise to expend 1300 kcal at 40% maximal aerobic capacity, or 3: exercise to expend 1300 kcal at 70% maximal aerobic capacity. Dietary provision was manipulated in all groups to elicit energy availabilities of ~29 kcal•kg^{-1}•day^{-1} (*energy balance*) and ~8 kcal•kg^{-1}•day^{-1} (*LEA*) in the two trials. Serum triiodothyronine (T_3) concentrations were reduced after four days of LEA, but T_3 did not change following energy balanced trials irrespective of the exercise provision. Therefore, changes in energy availability, and not the stress of exercise, were attributed to the differences in circulating T_3 concentrations (Loucks & Callister, 1993).

To re-affirm that physiological perturbations are caused by low energy availability and not the stress of exercise, Loucks, Verdun, & Heath (1998) recruited nine healthy sedentary females to complete four days of EA at 45- (*EB*) and 10 kcal•kg lean body mass (*LBM*)$^{-1}$•day^{-1} (*LEA*). Target EA was achieved through daily exercise (running at 70% $\dot{V}O_{2max}$) to expend 30 kcal•kg LBM^{-1}•day^{-1}, equivalent to ~15 miles running per day, with total dietary provision. The data was then pooled with the findings of Loucks & Heath (1994a), which had investigated the effects of LEA on luteinising hormone (*LH*) pulsatility through dietary restriction alone, permitting the authors to isolate the effects of LEA from the stress of exercise. LEA achieved with the inclusion of daily exercise led to a significant reduction in 24-hr LH pulse frequency of 10%, but this effect was significantly smaller than when LEA was achieved by dietary restriction alone. LH pulse amplitude was significantly increased by LEA whether this was achieved with or without exercise. Similarly, when comparing the responses of the exercise and non-exercise cohorts, there were no differences between 24-hr LH pulse frequency, amplitude or transverse mean following either EB or LEA. Intense exercise, therefore, had no effect on LH pulsatility beyond its associated energetic cost and subsequent impact upon EA (Loucks et al., 1998).

Subsequent research has since established the role of energy availability in further physiological perturbations. This work led to the defining of low energy availability as the aetiological factor underpinning the *Female Athlete Triad* model (Nattiv et al., 2007). In the following years, the *Male Athlete Triad* (Fredericson et al., 2021; Nattiv et al., 2021) and *REDs* (Mountjoy et al., 2023) models have extended the proposed physiological effects of LEA to include males. Furthermore, the *REDs* model proposes that low energy availability is associated with a plethora of health and physical performance problems beyond its effects upon reproductive function and bone metabolism (Mountjoy et al., 2023). However, the lack of scientific support substantiating many of the further effects proposed in the *REDs* model has been met with scepticism by the scientific community (De Souza, Williams, et al., 2014).

2.4. The *Female Athlete Triad*, the *Male Athlete Triad*, and *REDs:*

The Female Athlete Triad:

In 1992, the American College of Sports Medicine (*ACSM*) convened a panel of experts to synthesise the first iteration of the *Female Athlete Triad*. The model identified the inter-related conditions of eating disorders (*ED*), amenorrhea, and osteoporosis affecting athletic female populations, but particularly in 'appearance' and 'endurance' athletes. Whilst the contributing role of 'inadequate nutrition' to the triad was raised at the ACSM conference, energy availability was not integrated as part of the model at this point (Yeager et al., 1993). Having subsequently been identified as a possible mechanistic driver of the *Female Athlete Triad* in the first official ACSM position stand (Otis et al., 1997), the seminal findings from Professor Loucks' research group provided fundamental evidence of a causal relationship between low energy availability with the impairment of reproductive function and bone turnover markers.

In 2007, a position stand from the American College of Sports Medicine (*ACSM*) was the first to officially state that LEA is the key driver of physiological dysregulations observed in female athletes (Nattiv et al., 2007). This was retained for a subsequent version in the mid-2010's (De Souza, Nattiv, et al., 2014). The *Female Athlete Triad* is defined as a condition typically afflicting active girls and women, incorporating any singular or combination of three factors of, 1) LEA, 2) menstrual dysfunction, and 3) low bone mineral density (*BMD*) (De Souza et al., 2014; Nattiv et al., 2007). The model stipulates that exercising females exist on a spectrum from optimal energy availability, through sub-optimal EA, down to low energy availability as a product of their dietary and training practices. Sub-optimal or low EA can arise with or without the presence of a clinically diagnosed *ED* or disordered eating (*DE*) (De Souza, Nattiv, et al., 2014; Nattiv et al., 2007). In a state of 'optimal' energy availability, the model (Figure 2.4.1) predicts normal menstrual function (eumenorrhea) and optimal bone health that female athletes should be advised to strive to attain where possible. As energy availability is reduced, sub-optimal EA may lead to endocrine and physiological responses that can be identified by changes in the hormonal milieu and ultimately result in sub-clinical menstrual disturbances (e.g., *Oligomenorrhoea:* menstrual cycles > 35 days, anovulation, and/or luteal phase deficiency), altered hormonal profiles (e.g., suppression of oestrogen), and uncoupling of bone turnover (formation and resorption). The latter of which, if sustained, is proposed to lead to impaired bone health and low BMD - defined as a BMD z-score between -1.0 to -2.0 in conjunction with a history of nutritional deficiencies, hypoestrogenism, stress fractures, and/or other secondary clinical fracture risk factors (Nattiv et al., 2007).

At the opposite end of the continuum from a healthy state, functional hypothalamic amenorrhoea and osteoporosis may be observed. Short-term LEA has been directly linked to impairment of reproductive function, as LEA affects the pulsatile release of GnRH from the hypothalamus and the subsequent pulsatility and amplitude of LH release (Loucks et al., 1998; Loucks & Heath, 1994a; Loucks & Thuma,

2003; Loucks & Verdun, 1998), with prolonged LEA proposed to manifest in functional hypothalamic amenorrhoea. Similarly, LEA may lead to changes of key bone-protective hormones in the endocrine milieu that ultimately contributes to the uncoupling of bone resorption and formation (Ihle & Loucks, 2004), which is thought to cause osteoporosis at the extreme end of the *Female Athlete Triad* continuum. The position stand defines osteoporosis as a BMD Z-score of ≤ -2.0, with corresponding secondary risk factors, such as chronic malnutrition, ED, hypogonadism, glucocorticoid exposure, and/or previous fractures. For female athletes in 'weight-bearing sports', a Z-score of < -1.0 is defined as a low BMD and this warrants further investigation. The factors identified within the *Female Athlete Triad* may manifest through repeated and/or chronic exposure to a state of low energy availability. Impairment of reproductive and bone health because of low energy availability can take months and years to manifest, respectively (Nattiv et al., 2007).

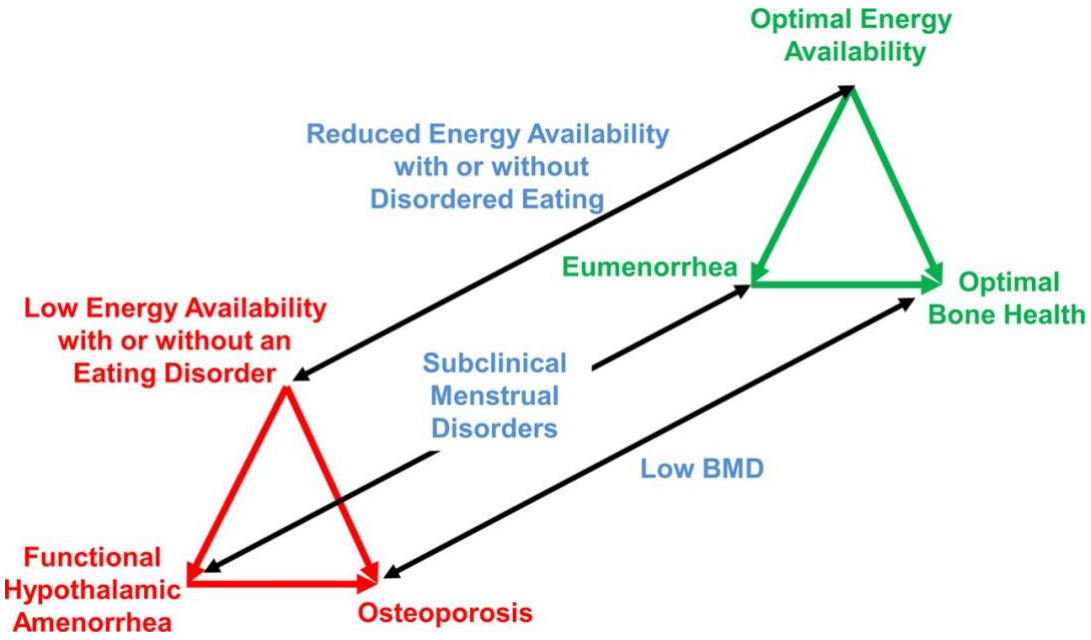


Figure 2.4.1. The *Female Athlete Triad* model: At the top-right corner of the spectrum, optimal EA corresponds to a normal menstrual status (eumenorrhea) and optimal bone health that female athletes should be advised to strive to attain where possible. As EA is reduced, sub-optimal EA may lead to sub-clinical menstrual disturbances (e.g., *Oligomenorrhoea:* menstrual cycles >35 days, anovulation, and/or luteal deficiency), impairment of hormonal profiles (e.g., suppression of oestrogen), and uncoupling of bone turnover (formation and resorption). The latter of which, if sustained can lead to impaired bone health and low BMD. The bottom-left corner of the figure reflects the extreme endpoint of negative consequences for female athletes from sustained LEA, culminating in functional hypothalamic amenorrhoea and osteoporosis. Prolonged LEA is purported to directly lead to impairment of reproductive health, as short-term LEA affects the pulsatile release of GnRH from the hypothalamus. Subsequently, the pulsatility and amplitude of luteinising hormone (*LH*) release also becomes impaired, manifesting in functional hypothalamic amenorrhoea. Amenorrhoea can be defined as *Primary* (a delay in the age of menarche >15 years of age) or *secondary* (the absence of menstrual cycle for ≥3 months). In respect to bone health, the resultant extreme of the female athlete triad is the diagnosis of osteoporosis. The ACSM defines osteoporosis as a BMD z-score of ≤ -2.0, with corresponding secondary risk factors, such as chronic malnutrition, ED, hypogonadism, glucocorticoid exposure, and/or previous fractures. Chronic LEA is proposed to influence bone health directly and indirectly. Directly, LEA appears to reduce bone formation, possibly via its effect on hormones that promote bone formation, including insulin, insulin-like growth factor-1 (*IGF-1*), T_3, and/or cortisol. Indirectly, the influence of LEA upon reproductive status also appears to correspond to decreases in hormones such as oestradiol (E_2), which inhibit bone resorption (De Souza, Nattiv, et al., 2014; Nattiv et al., 2007).

(Image source: De Souza et al., 2014)

Relative Energy Deficiency in Sport (REDs):

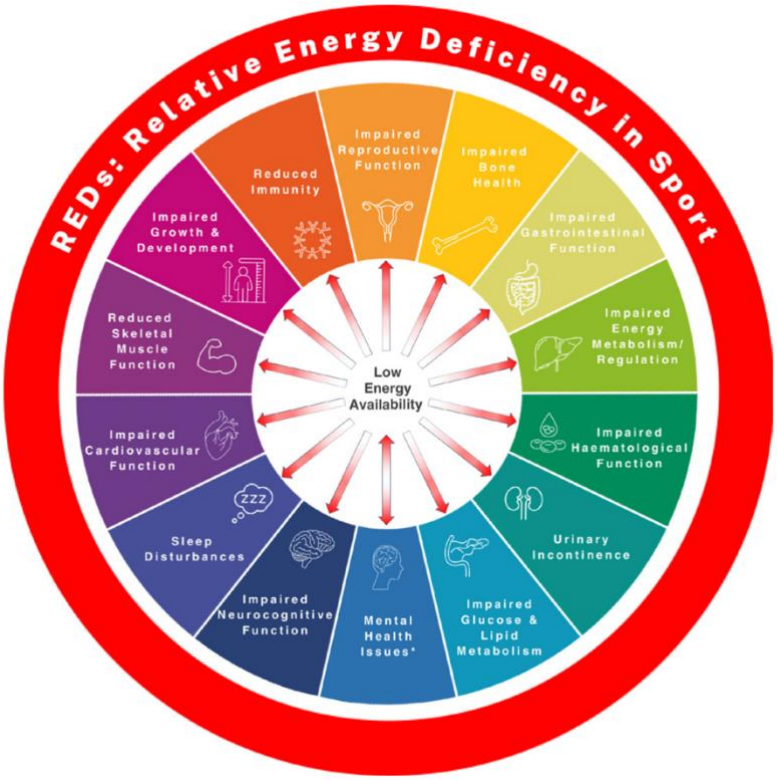
In 2014, the International Olympics Committee (*IOC*) released a consensus statement proposing a model aiming to progress the considerations of the *Female Athlete Triad*. The *REDs* model was proposed on the grounds that the consequences of LEA are not limited to the two factors detailed within the *Female Athlete Triad,* but rather that LEA results in a wider range of health and performance related consequences affecting both females and males. The original *REDs* model and a 2018 update therefore incorporated the dysregulations (and underpinning mechanisms) outlined within the *Female Athlete Triad,* but expanded the effects of LEA to a total of 10 health-related and 10 performance-related consequences (Mountjoy et al., 2014, 2018). In 2023, the IOC published a second update to the *REDs* model (Mountjoy et al., 2023). Amongst notable changes was the introduction of the concept of 'adaptable' vs 'problematic' low energy availability. *Adaptable* low energy availability refers to expected alterations in physiological functioning resulting from acute exposure to energetic trade-offs, in line with the concept of phenotypic plasticity or expected energetic trade-offs. In contrast, *problematic* low energy availability is concerned with negative health and/or performance outcomes associated with prolonged and/or severe energy deficits. As such, the 2023 definition of REDs has been updated to the following:

> *"A syndrome of impaired physiological and/or psychological functioning experienced by female and male athletes that is caused by exposure to problematic (prolonged and/or severe) low energy availability. The detrimental outcomes include, but are not limited to, decreases in energy metabolism, reproductive function, musculoskeletal health, immunity, glycogen synthesis and cardiovascular and haematological health, which can all individually and synergistically lead to impaired well-being, increased injury risk and decreased sports performance."* (Mountjoy et al., 2023, pp. 1,075).

The revised *REDs* model suggests that responses to low energy availability exist on a continuum with each identified parameter potentially requiring varied exposure (i.e., severity/duration) to low energy availability to shift responses from 'adaptable' to 'problematic' LEA. Although there is no operational definition or criteria provided to differentiate when LEA is 'adaptable' compared to when it has become 'problematic' within the statement. The proposed consequences of 'problematic' low energy availability include health-related parameters, such as impairments to reproductive status, bone health, gastro-intestinal function, energy, glucose and lipid metabolism, growth and development, immune function, and sleep, amongst further factors (Figure 2.4.2.A). Proposed inter-related negative performance outcomes include time-loss to training (due to health problems and/or injury), diminished training response and muscle function, inadequate recovery, and reduced endurance/power performance capacity (Figure 2.4.2.B). However, the statement acknowledges the need for further long-term prospective studies and controlled intervention studies to develop our understanding of these

parameters, as much of the data underpinning the proposed decrements is of cross-sectional and/or observational nature, from clinical populations such as individuals with anorexia nervosa or other eating disorders, and/or from research using questionnaire-based assessment of symptoms proposed to relate to low energy availability (Mountjoy et al., 2023). This final methodology is flawed as such symptoms may have myriad contributing factors other than LEA. For example, the HPG and hypothalamic-pituitary-adrenal axes are tightly linked, and psychological stress has been shown to influence reproductive function in rodent models, as reviewed by (Acevedo-Rodriguez et al., 2018).

A.



B.

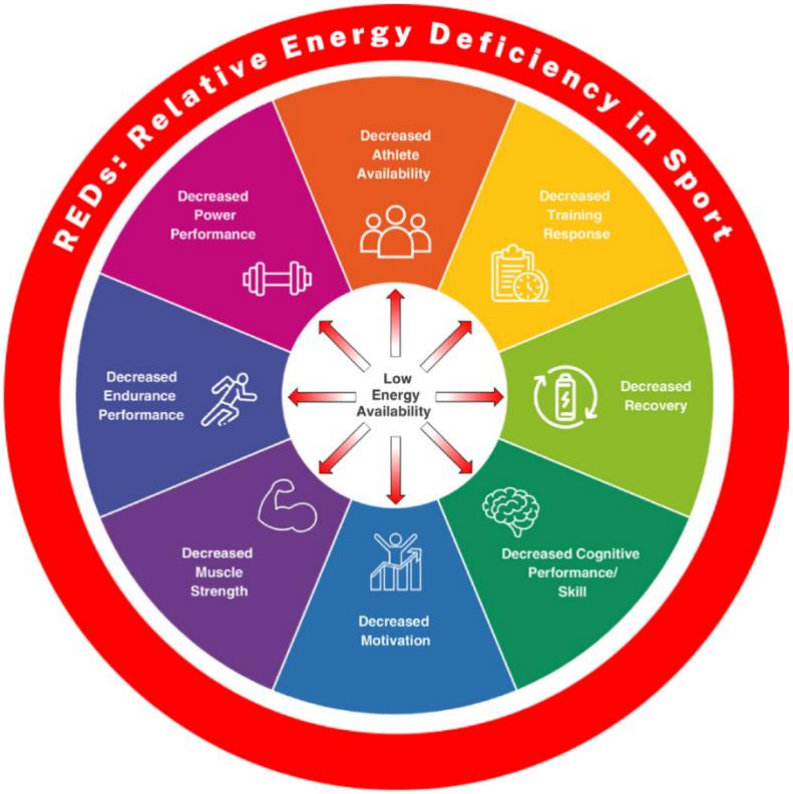


Figure 2.4.2. The (A) Health and (B) performance consequences associated with 'problematic' low energy availability proposed in the 2023 *REDs* model. Graded arrows denote that responses may begin as 'adaptable but progress to 'problematic' LEA with severe/prolonged exposure.

(Image source: Mountjoy et al., 2023)

The Male Athlete Triad:

Based upon more recent considerations and cross-sectional data suggesting that exercising males may also be susceptible to impairment of the hypothalamic-pituitary-gonadal (*HPG*) axis and low bone mineral density, as summarised by De Souza et al. (2014), the same proponents of the *Female Athlete Triad* have proposed an equivalent and standalone *Male Athlete Triad* model (Fredericson et al., 2021; Nattiv et al., 2021). The *Male Athlete Triad* model is defined as a syndrome of the three conditions of 1: LEA/energy deficiency (with or without an associated ED/DE), 2: impaired bone health, and 3: suppression of the HPG axis (Figure 2.4.3.). 'LEA/energy deficiency' refers to an energetic status where presentation of physiological signs of energy sparing are apparent, such as suppression of resting metabolic rate (*RMR*), low T_3, plateauing of reductions in body mass, low BMI, and/or reduced leptin concentrations (Nattiv et al., 2021).

Within the *Male Athlete Triad* model (Figure 2.4.3), a state of 'optimal' energy availability in males is associated with corresponding optimal bone health and HPG axis function that exercising males should aim to attain. Transient or prolonged sup-optimal LEA can lead to alterations in the endocrine milieu and physiological responses that ultimately perturb the HPG axis and uncouple bone turnover, increasing the risk of bone stress injury (*BSI*). At present, this 'middle-zone' of subclinical effects is poorly defined, however. Under conditions of prolonged or repeated exposure to low energy availability/energy deficiency, the extreme endpoint of the *Male Athlete Triad* spectrum may lead to the severe health and performance consequences. LEA/energy deficiency is proposed to directly impair functioning of the HPG axis, manifesting in hypogonadotropic hypogonadism characterised by 1: suppressed testosterone and LH concentrations/pulsatility, 2: decreased testosterone and decreased post-training gonadotropin responsiveness to GnRH stimulation, 3: impaired spermatogenesis, and 4: possibly decreased libido. Chronic LEA/energy deficiency may also lead to osteoporosis, with or without BSI, in male athletes. This may arise as a direct result of the influence of LEA/energy deficiency upon bone turnover, and indirectly, via the suppression of the HPG-axis with subsequent alterations in reproductive hormone concentration and responsiveness (Nattiv et al., 2021).

Given the lack of prospective data available from well-controlled studies in males, further research is required to develop the *Male Athlete Triad* towards a more definitive model of male physiological responses to low energy availability. A strength of the position stand is the clear classification of the quality of scientific data available for the proposed components, underpinning mechanisms, diagnosis, and treatment protocols for the syndrome. Similarly, key research topics that would facilitate progression towards a more definitive model are also outlined. To-date, only a handful have investigated the physiological effects of low energy availability in males under controlled experimental conditions. Fewer studies still have directly compared the physiological responses of males and females to controlled low energy availability interventions. Whilst limited, equivocal findings from controlled low

energy availability studies incorporating male participants suggest that males may be more robust to transient periods of low energy availability than females (Areta et al., 2014; Hammond et al., 2019; Ishibashi et al., 2020; Kojima et al., 2020; Murphy et al., 2021; Murphy & Koehler, 2020; Papageorgiou et al., 2017). The *Male Athlete Triad* therefore calls for more prospective research into the physiological effects of LEA upon reproductive function and bone health, the magnitude of LEA required to elicit perturbations and whether this occurs below a threshold EA, and for the development of screening tools and treatment protocols for at-risk male athletes (Fredericson et al., 2021; Nattiv et al., 2021).

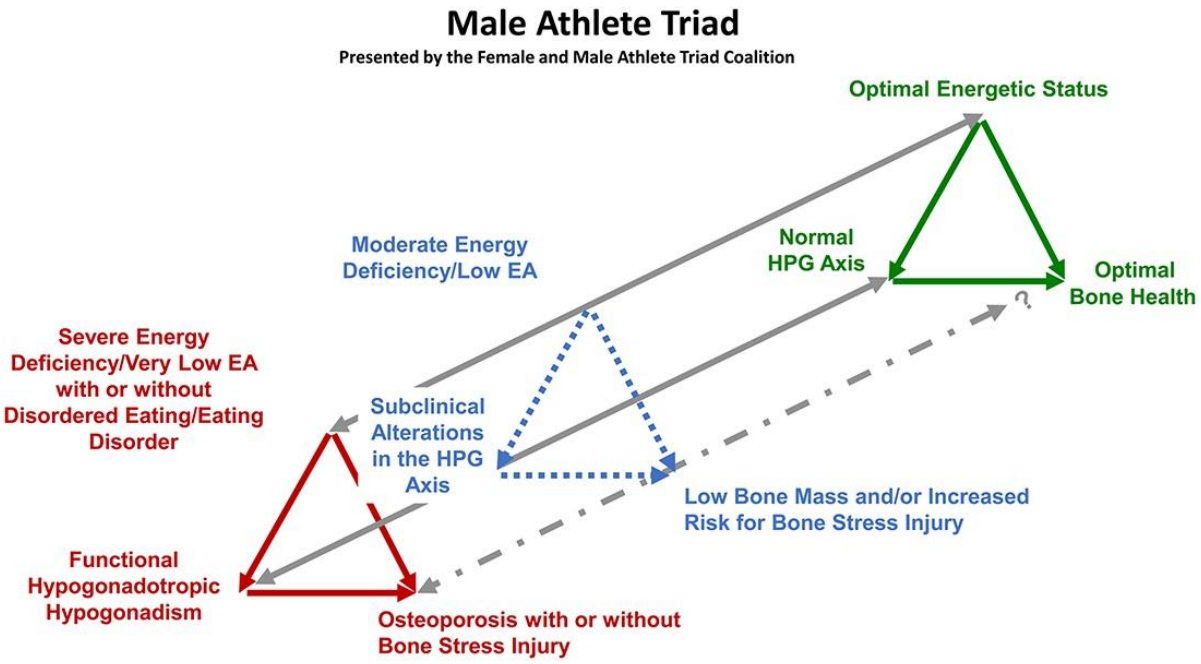


Figure 2.4.3. The Male Athlete Triad model: energetic status, hypogonadal status and bone health exist on a spectrum from optimal- to impaired state. Severe energy deficit/LEA influences the HPG axis directly and bone health directly and indirectly, via impaired HPG axis functioning.

(Image source: Nattiv et al., 2021)

Understanding LEA and relative energy deficiency further; a need for further research:

Despite intensive scientific debate and initial controversy regarding the *REDs* model, it is important to note that all these models share a central underpinning philosophy; attempting to optimise the health, well-being, and performance of athletes and exercising individuals. Further research is therefore required to establish whether a causal relationship between LEA and physiological dysregulations exists in males. In the case of *REDs*, the need for further research linking LEA to the broader physiological impairments proposed applies to both females and males. These issues are complex, and each model has its own strengths and limitations. All three should be investigated further and integration of the intellectual progress within each model can synergistically enhance our understanding of all three concepts (De Souza et al., 2022).

Causes of Low Energy Availability:

The *Female Athlete Triad*, *Male Athlete Triad*, and *REDs* models recognise that disordered eating/eating disorders or further mental health disorders (e.g. compulsive exercise/exercise addiction) can contribute to the generation of a state of low energy availability. However, they also recognise that further factors may contribute to exercising individuals experiencing LEA, either with or without concomitant DE/ED (De Souza, Nattiv, et al., 2014; Mountjoy et al., 2023; Nattiv et al., 2021). Regarding energy intake, Burke, Lundy, et al., (2018) and Nattiv et al. (2007) summarise that restrictive dietary habits beyond DE/ED, such as vegetarianism (or other restrictive diets), fasting, or the intentional and sometimes justified restriction of dietary intake to reduce body mass/fat, or limit weight gain during periods of injury can contribute to states of low energy availability. Similarly, a lack of education or knowledge of contemporary nutritional guidelines (Burke, Lundy, et al., 2018; Sampson et al., 2023), or even misguided nutrition beliefs and a fear of objective (e.g. team weigh-ins and skinfold assessments) or subjective (e.g. parental/coach comments on body) appraisals of body mass (McHaffie et al., 2022) may contribute to reduced dietary intake and subsequent low energy availability in exercising individuals.

Conversely, athletes with a high exercise energy expenditure due to their training and/or competition requirements may inadvertently experience LEA due to their extreme exercise commitments. This may be further compounded by an unawareness or inability to correspondingly increase energy intake to match this expenditure, as outlined by Burke, Lundy, et al., 2018 and Loucks et al. (2011). For example, both Heikura et al. (2019) and Moss et al. (2020) have observed that cohorts of elite cyclists and female professional footballers, respectively, have a reduced energy availability on competition (and training days for the footballers) days, compared to days of rest. Suggesting that the athletes either inadvertently or intentionally under-fuelled on days with greater exercise energy expenditure. For example, on days of high training volume (i.e. multiple hours performing exercise) athletes may encounter limited time and feeding opportunities outside of their exercise session on ‘long’ days (Westerterp & Saris, 1991), that compound the reduction in energy availability produced by their exercise load. However, there is limited data available regarding the impact that these factors collectively have upon the day-to-day variability in energy availability that athletes experience under free-living conditions. Further research may therefore seek to better understand the relationships between exercise energy expenditure, energy intake, and subsequent energy availability in free-living athletes and the factors that underpin these relationships. These data might then be used to inform interventions aimed at ‘optimising’ energy availability in exercising individuals and even inform future methodological considerations for laboratory-based controlled experiments examining the physiological effects of low energy availability.

2.5. Calculating energy availability:

In its most recent and current application to human research models, energy availability is typically defined as the difference between energy intake and net exercise energy expenditure (EEE_{net}), relative to an individual's lean body mass. The single numerical value attained represents the amount of energy remaining for all physiological processes once the energetic cost of EEE has been removed (Loucks, 2020). This definition has evolved over time, through three sequential algebraic formulas for calculating energy availability (Table 2.5.1) that have progressively represented the dietary energy available for the metabolically active tissues better (Loucks, 2020).

In the first instance, energy availability was defined and calculated as energy intake, minus gross exercise energy expenditure, relative to total body mass (Loucks & Callister, 1993). However, recognition that little energy expenditure occurs in body fat and that lean body mass was the relevant, active, tissue of interest led to the second definition equation of energy availability (Loucks, 2020). Energy availability was therefore calculated as energy intake, minus the total exercise energy expenditure, divided by 'lean body mass' in the second equation (Loucks & Heath, 1994b). Finally, the distinction that if a participant had not been performing an exercise session, they would still have had a degree of non-exercising energy expenditure in this time, led to a third equation for calculating energy availability. This was a critical insight, as the calculated exercise energy expenditure for training would otherwise be artificially increased, compared to the net energetic cost of training, by the contribution of basal metabolism and other routine activities. Accordingly, the third equation for energy availability is calculated as energy intake, minus net exercise energy expenditure (exercise energy expenditure, minus the equivalent basal cost of metabolism), divided by 'lean body mass' (Loucks et al., 1998). The differences in the variables of these equations lead to stepwise increases in calculated energy availability values across the first to third equations sequentially for a given combination of exercise energy expenditure, energy intake, and fat free mass (*FFM*), as outlined in Table 2.5.1. However, the existence of the different equations is rarely specified in the experimental or field-based research literature to-date, leading to potential under/over-estimation of energy availability and/or misinterpretation of data, as reviewed by Areta et al. (2021). Thus, some caution must be exercised when comparing energy availability values across different studies.

It should be highlighted that the equations detailed in Table 2.5.1. refer to 'Lean Body Mass' as the active portion of tissue measured. However, Loucks & Heath (1994b) and Loucks et al. (1998) used a hydrostatic weighing protocol which provides a two-compartmental assessment of body composition (Kasper et al., 2021). Thus, the equations refer to FFM, rather than LBM. The values of FFM and LBM differ slightly but have been used interchangeably in LEA literature (Areta et al., 2021). As such, studies are described verbatim to their original reporting of FFM or LBM within this thesis.

Table 2.5.1. The evolving definition and equations of energy availability (adapted from Areta et al., 2021)

	Original Concept	**1st Algebraic Definition**	**2nd Algebraic Definition**	**3rd Algebraic Definition**
Taxon of Focus:	Mammals (mainly rodents)	Humans	Humans	Humans
Novel characteristic in concept, definition, or formula:	Reproductive function and behaviour of mammals is influenced by the availability of 'metabolic fuels'. Exogenous and endogenous fuel availability influence rodent models. Blockade of endogenous fuel supply and food deprivation impairs reproductive function in rodents.	Equation provides first quantifiable and therefore measurable definition of EA in humans. EA refers to exogenous fuel provision. Exercise energy expenditure (EEE_{gross}) defined as gross amount of energy expended during exercise. EA expressed relative to total body mass.	EA now calculated relative to lean body mass. Exercise energy expenditure (EEE_{gross}) still defined as gross amount of energy expended during exercise.	To provide a value for the true energetic cost of an exercise session, net exercise energy expenditure calculated as: EEE_{net} = EEE_{gross} – non-exercise energy expenditure (RMR + NEAT).
Primary reference:	Bronson (1985); Schneider & Wade (1989).	Loucks & Callister (1993)	Loucks & Heath (1994b)	(Loucks et al., 1998)
Algebraic Formula:	None	$EA = \frac{(EI - EEEgross)}{BM}$	$EA = \frac{(EI - EEEgross)}{LBM}$	$EA = \frac{(EI - EEEnet)}{LBM}$
EI (kcal•day^{-1})	-	3700	3700	3700
EEE (kcal•day^{-1})	-	700	700	585
Body Mass/Lean Body Mass* (kg)	-	75 (BM)	60 (LBM)	60 (LBM)
Example EA†:	-	**<u>40</u>**	**<u>50</u>**	**<u>52</u>**

* Body Mass (*BM*). 'Lean body mass' (*LBM*) refers to the sum of active protoplasm within the body. Whilst acknowledging that a small difference may exist between this and 'fat free mass' (*FFM*), these terms may be used interchangeably, as it has been within the EA literature. †Example EA values are based upon an individual of 70kg body mass, with 20% body fat, with an example EI of 3700 kcal•day^{-1} and performing an hour of exercise with an EEE_{gross} of 700 kcal•hr^{-1}. EEE_{net} was calculated using a nominal resting metabolic rate value of 2000 kcal•day^{-1} and a physical activity level scaling of 1.4, eliciting ~115 kcal•hr^{-1} basal energy requirement.

2.6. Sources of error in the free-living quantification of energy availability:

By its very nature, estimating energy availability involves the quantification of its individual components; energy intake, exercise energy expenditure, and lean body mass/fat free mass. However, as reviewed by Burke, Lundy, et al. (2018), there are no established guidelines for quantifying these factors in free-living athletes and there is inherent error associated with the measurement of each of these variables

In sports nutrition, and nutrition more generally, assessing energy intake is a challenging and inaccurate process. Assessing energy intake is therefore recognised as a key limitation in the free-living assessment of energy availability (Tarnowski et al., 2023), with misreporting commonly introducing error into energy intake estimations (Poslusna et al., 2009). Inaccuracy in the field-based quantification of EI is therefore likely to lead to some of the largest discrepancies in calculated EA (Loucks, 2020) and there is currently no 'gold-standard' practice for measuring free-living energy intake (Capling et al., 2017). Assessment of dietary intake is typically conducted prospectively or retrospectively, using time and labour-intensive food-recording procedures. Such methods include written food-diaries, food frequency questionnaires, electronic tools (e.g., smartphone applications), and/or photographing meals pre- and post-consumption (Burke et al., 2018; Capling et al., 2017; De Souza et al., 2014; Tarnowski et al., 2023). However, these procedures are prone to under-reporting of portion sizes, total EI, and/or of foods deemed 'unhealthy', can lead to overall changes in food intake habits, and over-reporting of the intake of 'healthy' foods (Burke, Lundy, et al., 2018). Overall, when compared to doubly labelled water (*DLW*) measurements of total energy expenditure (*TEE*), a meta-analysis has recently shown that self-reported energy intake was typically under-reported by 19%, equating to approximately 600 kcal (Capling et al., 2017). Under-reporting of energy intake via prospective weighed food records specifically ranges from ~10 – 20% (Poslusna et al., 2009).

Error in the estimation of exercise energy expenditure may also lead to inaccuracies in the quantification of energy availability both in free-living and laboratory-based studies. The 'gold standard' for quantifying total energy expenditure in the field is the DLW method (Poslusna et al., 2009). However, this technique is both expensive and not specific to exercise energy expenditure, as stipulated in the energy availability algebraic equations (Burke, Lundy, et al., 2018; Poslusna et al., 2009). Research investigating energy availability in free-living athletes has therefore typically used alternative methods of assessing exercise energy expenditure, such as through the use of heart-rate monitors, accelerometers, GPS devices, and/or power meters (Burke et al., 2018; De Souza et al., 2014). However, as suggested by Burke, Lundy, et al., (2018), these devices may be better suited to simple exercise tasks, such as running or cycling, rather than more complex or field-based tasks, including team sports and resistance exercise. The direct measurement of mechanical work done using power-meters can provide an objective assessment of exercise energy expenditure However, the use of this technique is similarly

limited to certain exercise types, such as cycling. Furthermore, without the assessment of an individual's gross efficiency, the precision of the assessment may be compromised (Haakonssen et al., 2013). Accelerometers have been shown to underestimate free-living energy expenditure (Murakami et al., 2016) and exercise energy expenditure at higher exercise intensities (Abel et al., 2008), possibly limiting their application in athletic populations. Alternatively, exercise energy expenditure can also be estimated indirectly via the calculation of metabolic equivalent of task (*MET*) from the compendium of physical activities (Ainsworth et al., 2000, 2011) or similar, based upon training logs (Burke et al., 2018; De Souza et al., 2014). However, each of these methods is prone to measurement error and bias, can place further burden upon the participant and the researcher. Each would also have enhanced precision and accuracy when used in conjunction with indirect calorimetry assessments, which are not typically measured in the field (Burke, Lundy, et al., 2018; De Souza, Nattiv, et al., 2014). Furthermore, the use of predictive equations to calculate RMR and, subsequently, net exercise energy expenditure may lead to the underestimation of exercise energy expenditure in athletes with a suppressed metabolism. Corresponding energy availability calculations may then be overestimated, compared to the use of RMR measured via indirect calorimetry (Burke, Lundy, et al., 2018).

Finally, a variety of techniques are available for the measurement of body composition and specifically, fat free mass. These include, dual X-ray absorptiometry (*DXA*), bio-electrical impedance analysis (*BIA*), air-displacement plethysmography, and surface anthropometrics/skin-fold assessments, although the latter of these is not recommended for estimating fat free mass (Burke et al., 2018; De Souza et al., 2014). Whilst care should be taken to adhere to best-practice procedures (Burke, Lundy, et al., 2018), a 2% error rate is not unexpected for body composition assessments, though Loucks (2020) argues that this would likely have a negligible effect on energy availability calculations.

It has therefore been questioned whether we are adequately able to quantify or detect meaningful differences in energy availability and, by extension, diagnose LEA and its physiological outcomes from data collected in the field (Burke, Lundy, et al., 2018). Considering these factors, inferences drawn regarding the physiological effects of LEA from datasets collected in free-living athletes should be correspondingly cautious. Conversely, tightly controlled laboratory-based studies can mitigate many of these limitations, particularly when all food is provided to participants, body composition is assessed using standardised protocols, and exercise energy expenditure and resting metabolic rates are measured rather than estimated.

2.7. Energy availability vs energy balance:

It is often necessary to stress that low energy availability is not synonymous to an energy deficit and does not always lead to a corresponding reduction in body mass (Burke, Lundy, et al., 2018). It is therefore critical to highlight some key distinctions between the concept of energy availability and the model of energy balance (*EB*) that has more commonly been used in the fields of dietetics and sports nutrition (Loucks et al., 2011).

Energy Balance and Energy Availability: Similar, But Not the Same Concept:

Whilst energy balance and energy availability appear to be relatively similar concepts, their focus is fundamentally different. Energy balance is defined as the difference between energy intake and total energy expenditure (Burke, Lundy, et al., 2018; Siedler et al., 2023) and is calculated using the equation:

$$EB = EI - TEE$$

Total energy expenditure can be further sub-divided into its main components, namely RMR, cold-induced thermogenesis (*CIT*), dietary induced thermogenesis (*DIT*), and activity energy expenditure, which is sub-categorised into exercise energy expenditure and non-exercise activity thermogenesis (*NEAT*; Müller & Bosy-Westphal, 2013). The energy balance equation therefore represents the net *output* of the physiological system once all daily functions have been performed, thus representing the loss or gain from the body's energy stores each day (Loucks et al., 2011). From a thermodynamic perspective, a negative energy balance value would therefore theoretically equate to a loss in body mass and, similarly, a positive value should lead to weight gain (Burke, Lundy, et al., 2018). In contrast, the energy availability equation only considers a single component of total energy expenditure; exercise energy expenditure (see Table 2.5.1.). As the expenditure of energy for one physiological process (e.g., exercise locomotion) cannot subsequently be used for another (Loucks, 2020), the single numerical value provided from this calculation represents the energy left over for the body to perform all other physiological processes aside from exercise. Energy availability therefore represents the *input* of energy into the physiological system (Loucks, 2007a, 2007b; Loucks et al., 2011). The distinction between energy balance and energy availability is outlined in Figure 2.7.1.

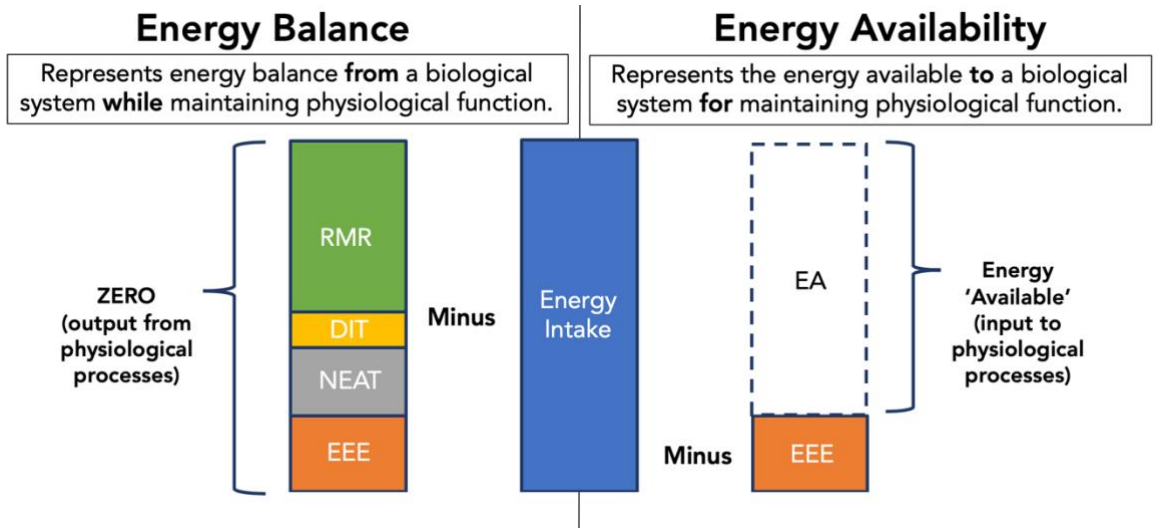


Figure 2.7.1. Unitless illustration of the conceptual difference underpinning the concept of energy balance (left side) compared to energy availability (*EA*; right side) for an individual in energetic equilibrium (EB = 0 kcal). Energy intake and exercise energy expenditure are equal for both concepts, but energy balance also accounts for the remaining components of total energy expenditure; non-exercise activity thermogenesis (*NEAT*), dietary induced thermogenesis (*DIT*), resting metabolic rate (*RMR*). Values within the illustration are proportional to a hypothetical day for an individual performing ~1 hour of moderate-vigorous exercise, with dietary induced thermogenesis and non-exercise activity thermogenesis accounting for 10% of energy intake and 40% of RMR, respectively. Cold-induced thermogenesis is assumed negligible and not included in this hypothetical scenario.

(Image and description source: Areta et al., 2021).

Adaptive Thermogenesis: The Conceptual Differentiator?

The phenomenon of metabolic suppression is often referred to as 'adaptive thermogenesis' (*AT*) or 'metabolic adaptation' (Müller & Bosy-Westphal, 2013). Adaptive thermogenesis has been equivocally defined as the adaptive suppression in resting energy expenditure independent of alterations to body fat mass and fat free mas (Müller et al., 2015), the regulatory heat-production in response to cold (i.e., cold-induced thermogenesis) or over-eating (to dissipate excess EI) (Müller & Bosy-Westphal, 2013), or most broadly, the adjustment of all components of daily energy expenditure (i.e., both resting- and non-resting energy expenditure) to enhance metabolic efficiency (Hall, 2006; Müller & Bosy-Westphal, 2013). The final definition of AT is suggestive that these mechanisms serve to reduce the rate of energy metabolism and thus preserve the body's lean and fat tissue (Müller & Bosy-Westphal, 2013)

The ability to store fat likely provided a key survival advantage throughout periods of sparse energy availability, however, these energy stores appear to be robustly defended against perturbation by mechanisms mediated via the central nervous system (*CNS*) in both lean and obese individuals (Rosenbaum & Leibel, 2010). These 'energy saving' mechanisms restore energetic equilibrium by

reducing the energy deficit from various energy expenditure outlets. Total energy expenditure is reduced via down-regulated allocation of energy to maintain the physiological functioning of organs and tissues, manifesting in a decline in resting metabolic rate. Reductions in RMR have been observed to range from ~8 - 10% in amenorrhoeic athletes (Koehler et al., 2016), lean males (Müller et al., 2015), and lean and obese males and females (Rosenbaum et al., 2008), or up to ~20% in individuals with anorexia nervosa (Kosmiski et al., 2014). Similarly, reductions in non-resting energy expenditure may occur via absolute reductions in physical activity levels/NEAT (Rosenbaum et al., 2008; Rosenbaum & Leibel, 2010), and/or via increased efficiency of movements during light activity (Hall, 2006; Müller et al., 2015; Müller & Bosy-Westphal, 2013; Rosenbaum et al., 2003; Rosenbaum & Leibel, 2010). These adaptations restore energy balance and consequently, weight stability is achieved at a lower set-point.

The importance of the conceptual difference between energy balance and energy availability is manifest during periods of energy deficiency and subsequent metabolic adaptation/AT, as an energy balance of zero does not necessarily equate to a healthy metabolic status. As reviewed by Aronne et al. (2021), and Müller & Bosy-Westphal (2013), when energy expenditure exceeds energy intake and an individual is in a state of negative energy balance, they may experience suppression of metabolic processes to increase energy balance back towards zero despite no changes to energy intake. When the negative energy balance is sustained for a prolonged period, these metabolic adaptations serve to reduce energy expenditure and maintain endogenous energy stores, likely as an evolutionary adaptation to increase survival chances (Shirley et al., 2022). Thus a new equilibrium between energy intake and expenditure restores energy balance to zero and it is therefore possible for an individual to be in a state of energy balance and low energy availability simultaneously (Burke, Lundy, et al., 2018; Loucks et al., 2011).

For example, in the seminal *Minnesota Semi-Starvation Experiment*, Keys et al. (1950) observed stabilisation of body mass and reduced RMR in the latter stages of a 24-week controlled semi-starvation period, where mean caloric intake was reduced from 3490 to 1570 kcal•day^{-1}. Subsequent computational modelling of this dataset demonstrated that total energy expenditure gradually declines over time to eventually match metabolisable energy intake (*MEI*; Figure 2.7.2.). In the model, during 'B' energy availability is 'adequate' and the resultant energy balance might be regarded as 'healthy'. However, at the end of 'SS', the apparent reductions in total energy expenditure ('metabolic adaptation') restore energy balance despite continued energy deficit/LEA, likely representing a state of 'non-healthy' energy balance. In this example, it is also evident that in a prolonged period of energy deficit, total energy expenditure progressively reduces to the point that body mass is stabilised despite continued low energy availability (Hall, 2006).

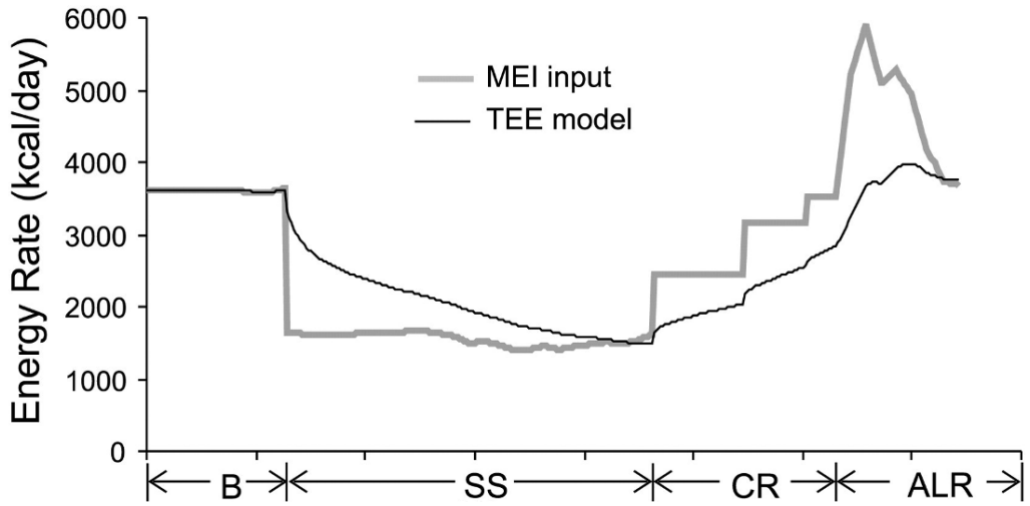


Figure 2.7.2. Computational modelling of total energy expenditure (*TEE*) relative to metabolisable energy intake (*MEI*) over the time-course of the *Minnesota Semi-Starvation Experiment*. *B* = Baseline, *SS* = semi-starvation, *CR* = controlled re-feeding, *ALR* = *Ad libitum* refeeding.

(Image source: Hall, 2006)

Forty-five is conceptually equal to zero:

As energy availability represents the *input* to the physiological system, its numerical value is inherently independent of all other factors of energy expenditure and therefore unaffected by AT/metabolic adaptation. Consequently, there is a large discrepancy between the value associated with a stable body mass between energy balance and energy availability calculations. An energy balance of 0 kcal•day^{-1} represents a thermodynamic equilibrium between the output of energy expended and consumed. Conversely, Loucks et al. (2011) outline that an equivalent energy availability associated with stable body mass and optimal physiological functioning is approximately 45 kcal•kg FFM^{-1}•day^{-1}, assuming no AT/metabolic adaptation has occurred.

This value appears to have been derived simply from early energy availability research (e.g., Hilton & Loucks, 2000; Ihle & Loucks, 2004; Loucks et al., 1998; Loucks & Heath, 1994b; Loucks & Thuma, 2003), which established that an EA of 45 kcal•kg FFM^{-1}•day^{-1} is associated with weight maintenance and optimal physiological function. However, little explanation of how this value was identified is provided. Sleeping metabolic rate has been shown to equal approximately 30 kcal•kg FFM^{-1}•day^{-1} (Loucks et al., 2011), and it is possible that the energetic cost of awakening, plus performing day-to-day tasks (e.g., NEAT) account for a further energy demand that means that ~45 kcal•kg FFM^{-1}•day^{-1} is required to ensure optimal physiological functioning and weight stability. In support of this, we have shown that an energy availability of 45 kcal•kg FFM^{-1}•day^{-1} is broadly associated with stable body mass (displayed as the reference point of '0' in Figure 2.7.3), whilst a strong linear relationship exists between body mass reductions with energy availability values below 45 kcal•kg FFM^{-1}•day^{-1} (Areta et al., 2021).

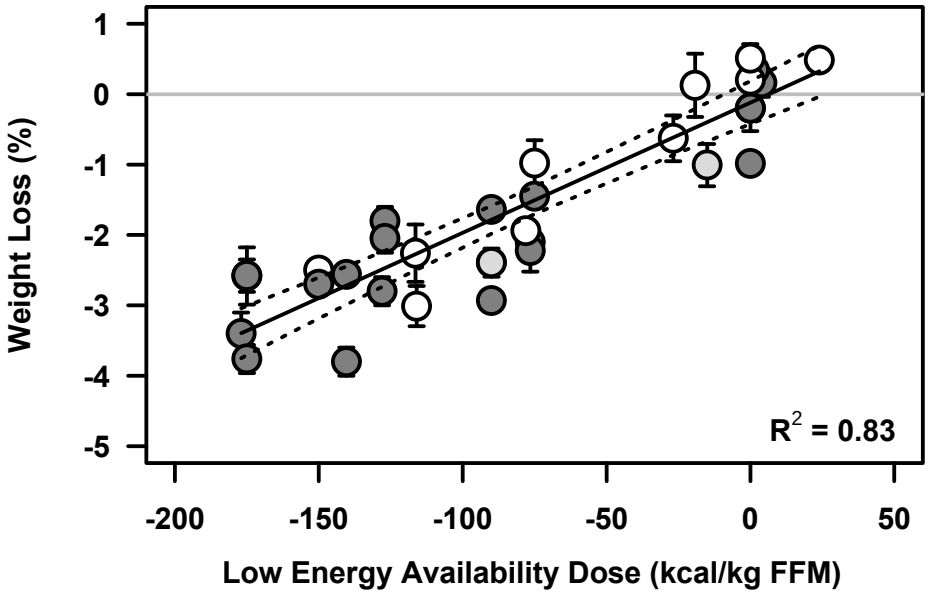


Figure 2.7.3. The relationship between energy availability and body mass changes after 3-5 days applying the concept of a low energy availability 'dose' to summarise the data from prospective studies manipulating energy availability and measuring body mass pre-post intervention. Low energy availability dose is defined as the total energy availability < 45 kcal•kg LBM^{-1}•Day^{-1}. Dark grey circles denote female-only studies, light grey circles a mixed-sex sample, and white circles a male-only study population. Error bars denote standard error of the mean weight loss where available.

(Image source: Areta et al., 2021).

2.8. The physiological effects of low energy availability:

Despite the purportedly broad physiological effects of low energy availability, much of the data underpinning the *Female Athlete Triad* (De Souza et al., 2014), *Male Athlete Triad* (Fredericson et al., 2021; Nattiv et al., 2021), and *REDs* (Mountjoy et al., 2023) models is derived from cross-sectional and field-based observational studies from athletes deemed to be 'at risk' of low energy availability. Such studies are useful for establishing a possible relationship between low energy availability and alterations to physiological function. However, they provide no evidence of the direct physiological effects of low energy availability or its associated mechanisms. Furthermore, in many of these studies energy availability values are estimated, which has associated methodological difficulties and a corresponding degree of error in the values attained (see section 2.6). On the other hand, the manipulation of energy availability under tightly controlled laboratory conditions represents a more appropriate experimental methodology to establish a causal role between LEA and its physiological responses. However, this approach can be costly and burdensome for both participants and researchers and is difficult to perform over longer durations. As such, there is correspondingly limited research available from well-controlled experiments that serve to establish causal links between low energy availability and its physiological effects. Furthermore, much of this research is limited to experimental periods of 3 – 5 days and has predominantly been conducted in female participants. This section will therefore summarise the experimental evidence currently available regarding the effects of low energy availability on the key endocrine, metabolic, and physiological parameters.

The hypothalamic-pituitary-gonadal axis:

Due to the high energetic cost of producing and rearing offspring, suppression of the reproductive system during periods of scarce energy provision represents a key energy saving mechanism in larger mammals to enhance reproductive fitness (Bronson, 1985; Shirley et al., 2022). The hypothalamic-pituitary-gonadal axis (Figure 2.8.1.) governs reproductive function through the coordinated, pulsatile, release of gonadotropin-releasing hormone (GnRH). GnRH stimulates secretion of the gonadotropins luteinising hormone and follicle stimulating hormone (*FSH*) from the anterior pituitary gland, hormones which act upon the gonads to stimulate gamete production and sex steroid hormones, such as testosterone, oestradiol (E_2), and progesterone. In turn, the steroid hormones also act as modulating factors, providing a negative feedback loop to upstream factors in the HPG axis (Acevedo-Rodriguez et al., 2018; Hackney, 2020).

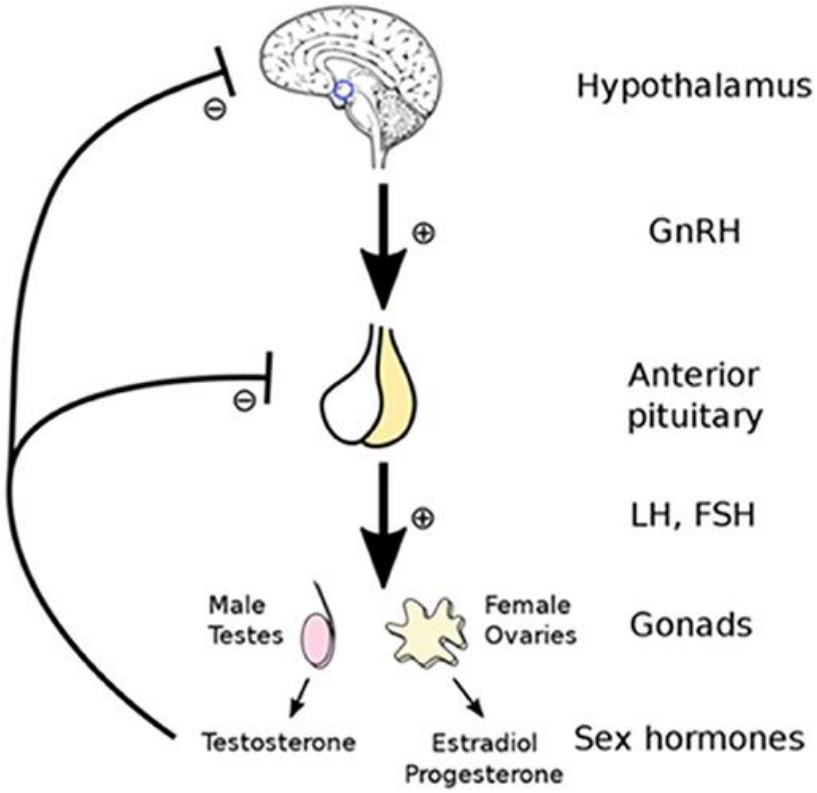


Figure 2.8.1. The hypothalamic-pituitary-gonadal axis: gonadotropin-releasing hormone (GnRH) is released from the hypothalamus in a pulsatile manner, stimulating the release of gonadotropins luteinising hormone (*LH*) and follicle stimulating hormone (*FSH*) from the anterior pituitary gland. The gonadotropins act upon the gonads to stimulate sex steroid hormone production, which contribute to HPG axis regulation through a negative feedback loop.

(Image source: Hackney, 2020)

Loucks & Heath (1994a) conducted the first prospective study to link LEA with dysregulation of LH pulsatility that reflected observations from females with hypothalamic amenorrhea. Compared to a normal energy availability trial (43 kcal•kg LBM^{-1}•day^{-1}), four days of controlled LEA (10 kcal•kg LBM^{-1}•day^{-1}) achieved through dietary restriction elicited a 23% reduction in LH pulse frequency and a 40% increase in LH pulse amplitude over the course of 24 hours in a cohort of sedentary females. These findings reflect the patterns of LH pulsatility observed in amenorrhoeic females (Khoury et al., 1987). In a follow-up study, Loucks, Verdun, & Heath (1998) used a similar four-day protocol, but elicited low energy availability through the incorporation of exercise to expend 30 kcal•kg LBM^{-1}•day^{-1}. The LEA trial elicited reduced LH pulse frequency, albeit to a smaller extent than through dietary restriction alone, and increased LH pulse amplitude. Taken together, these findings determined that low energy availability is the major factor underpinning the disruption of the HPG axis and not the stress of exercise. This conclusion would be stronger if the participants had completed all four trial arms, rather

than the authors combining the results of two separate controlled energy availability studies that elicited LEA with or without an exercise component. Loucks & Thuma (2003) subsequently compared the responses of sedentary females to five days of LEA set at 10, 20, or 30 kcal•kg LBM^{-1}•day^{-1}, achieved through dietary control and exercise, compared to a control trial of 45 kcal•kg LBM^{-1}•day^{-1}. LH pulse frequency was decreased, and pulse amplitude increased in the 10 and 20 kcal•kg LBM^{-1}•day^{-1} trials but remained unchanged in the 30 kcal•kg LBM^{-1}•day^{-1} intervention. These findings suggested that the disruption of the HPG axis occurs below a threshold of low energy availability of < 30 kcal•kg LBM^{-1}•day^{-1}. However, in these studies, other markers of the HPG axis such as FSH and E_2 were largely unaffected, or only reduced at the most extreme reductions of energy availability.

Experimental research investigating the influence of low energy availability upon HPG axis function is much more limited in males than females. Despite this, exercising males deemed 'at risk' of experiencing low energy availability - typically endurance and weight-limited athletes - have been observed to display acute hypogonadism and/or the *Exercise Hypogonadal Male Condition* (*EHMC*; as reviewed by Hackney, 2020) as well as impaired reproductive status characterised by a reduced sperm count and reduced sperm motility (De Souza et al., 1994). However, this deductive link between low energy availability and alterations of the HPG axis in males is derived from cross-sectional and observational studies. For example, as a cross-sectional study, the small sample size of 9 – 11 participants per observation group in De Souza et al. (1994) represents a major limitation of a key study often cited to support the notion that LEA impairs HPG axis functioning in exercising males.

In contrast, data from limited short-term male-specific studies investigating the physiological effects of acute (≤ 7 days) low energy availability under controlled experimental conditions are somewhat more equivocal. Koehler et al. (2016) assessed the physiological effect of four-days of LEA at ~16 kcal•kg FFM^{-1}•day^{-1}, with and without an exercise (cycling) component, compared to four days of controlled normal EA (~38 - 40 kcal•kg FFM^{-1}•day^{-1}) in six healthy males. However, the LEA intervention did not produce changes in concentrations of total testosterone. Similarly, Kojima et al. (2020) investigated the effect of three days of LEA (~19 kcal•kg FFM^{-1}•day^{-1}) compared to normal EA (~53 kcal•kg FFM^{-1}•day^{-1}) in a cohort of seven healthy well-trained male runners. LEA reduced body mass, fat free mass, and resting respiratory exchange ratio. However, there were no significant differences in serum free testosterone, creatine kinase, or plasma IGF-1 concentrations between the LEA and normal EA interventions (Kojima et al., 2020). Taken together, the findings of these two studies suggest that the HPG axis may not be sensitive to low energy availability. Alternatively, other factors may contribute to alterations in the HPG axis observed in exercising males. For example, in a cohort of elite male race walkers, six days of training (performing ~18 – 19 km•day^{-1} of training) reduced testosterone concentrations in an 'adequate' energy availability high carbohydrate control (40 kcal•kg FFM^{-1}•day^{-1}) group, an isocaloric low-carbohydrate high-fat (*LCHF*) group (< 50 g•day^{-1}), and an LEA intervention

group (15 kcal•kg FFM^{-1}•day^{-1}) alike. Factors outside of the dietary manipulation, such as the training intervention therefore likely contributed to this disruption of the HPG axis (Mckay et al., 2022a). Taken together, the degree and duration of LEA required to elicit perturbation of the HPG axis in males remains unclear at present (Nattiv et al., 2021), if such a relationship exists. Further research is therefore required to understand the relationship between these factors further.

Hypothalamic-pituitary-thyroid axis:

The hypothalamic-pituitary-thyroid (*HPT*) axis is central in the regulation of energy expenditure and adaptive thermogenesis (Kim, 2008; McAninch & Bianco, 2014). Thyroid hormones are secreted from the thyroid gland under control of the hypothalamic-pituitary axis and influence key metabolic pathways that control energy storage, expenditure and therefore energy balance. The thyroid hormones exert a regulatory effect upon most tissues, but primarily impact the brain, adipose tissue, skeletal muscle, liver, and pancreas. Under hypothyroid conditions, for example, a review by Mullur et al. (2014) outlines that resting energy expenditure, lipolysis and gluconeogenesis are all reduced and body mass increases. Amenorrhoeic female athletes present with significantly reduced T_3 concentrations compared to cyclic female athletes and sedentary controls (Loucks et al., 1992).

In the seminal energy availability study conducted in humans, Loucks & Callister (1993) investigated the role of four-days of low energy availability (~8 kcal•kg^{-1}•day^{-1}) induced through dietary restriction alone, or with exercise at 40% or 70% of maximal aerobic capacity, upon thyroid hormone concentrations. The LEA interventions increased total thyroxine (T_4) and reverse triiodothyronine (rT_3), but reduced serum total and free T_3 concentrations regardless of exercise provision. The same parameters were unchanged in the energy balanced intervention (~29 kcal•kg^{-1}•day^{-1}). Collectively, these findings provided early evidence that the HPT axis was sensitive to low energy availability and that the stress of exercise did not influence this beyond its effect upon energetic status (Loucks & Callister, 1993). A follow-up study then determined that the HPT axis was disrupted below a threshold of low energy availability following four days of LEA in a similar cohort of sedentary females. Total and free T_3 concentrations were reduced below a threshold of ~25 kcal•kg LBM^{-1}•day^{-1}, whilst free T_4 and rT_3 concentrations were increased at a lower threshold of LEA below ~19 kcal•kg LBM^{-1}•day^{-1} (Loucks & Heath, 1994b). Most controlled studies in females thereafter have demonstrated that low energy availability induces reductions in T_3 (Loucks, 2006; Loucks et al., 1998; Loucks & Heath, 1994a; Loucks & Thuma, 2003; Loucks & Verdun, 1998; Oxfeldt et al., 2023; Papageorgiou et al., 2018), suggesting that this is a reliable marker of LEA in females.

However, there is significantly less data available from controlled studies investigating the effects of low energy availability upon the HPT axis in males. Papageorgiou et al. (2017) investigated the responses of 11 trained males and females (N = 22) to five days of low energy availability (15 kcal•kg

FFM^{-1}•day^{-1}) compared to normal (45 kcal•kg FFM^{-1}•day^{-1}), achieved through daily running exercise with dietary manipulation. However, T_3 area under the curve was unchanged in both the male and female cohorts between the LEA and normal EA trials, and there was no difference in responses between sexes. It is noteworthy that participants were only provided with diet plans adapted from their habitual dietary intakes for the intervention phases, rather than receiving a standardised study diet. There may therefore have been differences in nutritional content between participant diets that introduced artefact to the data from this study. Similarly, four days of low energy availability providing ~16 kcal•kg FFM^{-1}•day^{-1}, achieved through reduced energy intake or the addition of cycling exercise, did not elicit any changes in serum T_3 concentrations compared to normal energy availability trials providing ~40 kcal•kg FFM^{-1}•day^{-1} in a cohort of six exercising males (Koehler et al., 2016). Whilst limited, present data therefore suggest that the HPT axis of males is more robust to acute periods of low energy availability than females.

Bone metabolism:

The cycle of bone remodelling is a slow process that occurs continually through the coupled resorption and formation of its matrix (Figure 2.8.2.). Exercise is generally thought to be beneficial to bone health. However, its effects are complex and influenced by myriad factors including age, exercise type, and nutrition status (Dolan et al., 2020). Considering the observed association between reduced bone mineral density, osteoporosis, and stress fracture prevalence in both female and male populations 'at risk' of chronic LEA (De Souza, Nattiv, et al., 2014; Nattiv et al., 2021), bone metabolism has been a topic of focus in low energy availability research. However, detecting changes in bone mineral density using imaging techniques may take months or years. Experimental studies investigating effects of LEA over acute periods (typically 3 – 5 days) under controlled conditions have therefore focused on evaluating changes in blood-borne markers of bone/collagen formation and resorption, which can be predictors of long-term changes in bone mineral density (Dolan et al., 2020; Villareal et al., 2016). A major caveat to this methodological approach is that whilst bone responses to mechanical loading appear to be site-specific (Kannus et al., 1994), analysing changes in concentrations of markers of bone/collagen formation and resorption relies on the assessment of total systemic concentrations. This approach therefore lacks specificity and cannot determine changes in bone remodelling at individual sites of interest, such as those loaded by any given exercise modality (Dolan et al., 2020).

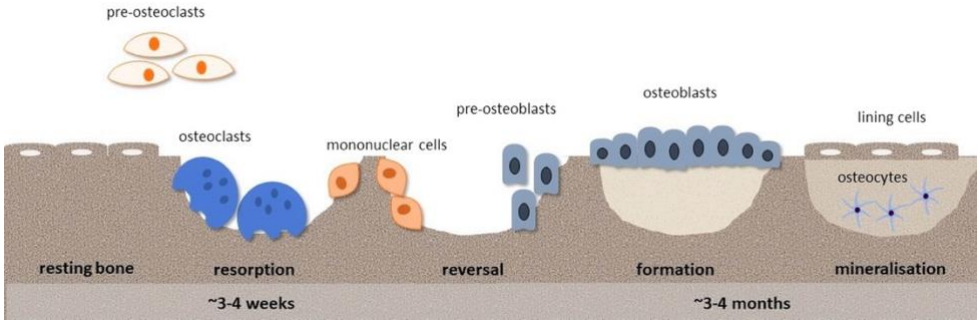


Figure 2.8.2. The bone remodelling cycle. Bone remodelling is a coupled, synchronised process in which bone is activated. Resorption, reversal and then formation of bone then follows. The basic multicellular unit, consisting of osteoblasts, osteoclasts, and osteocytes coordinates this process.

(Image source: Dolan et al., 2020)

A handful of studies have investigated the causal influence of low energy availability on markers of bone 'turnover' under experimental conditions. Ihle & Loucks (2004) reduced energy availability to 10, 20, or 30 kcal•kg LBM^{-1}•day^{-1} for five days and compared the responses of markers of bone resorption and formation to a control condition providing an energy availability of 45 kcal•kg LBM^{-1}•day^{-1}. Concentrations of the bone resorption marker N-terminal telopeptide of type 1 collagen (*NTx*) only increased under the lowest energy availability condition of 10 kcal•kg LBM^{-1}•day^{-1}, however, markers of bone formation type I procollagen carboxy-terminal propeptide (*P1CP*) and osteocalcin (*OC*) were reduced in each of the low energy availability conditions. Whilst total OC is no longer strictly viewed as a marker of bone formation (Dolan et al., 2020), the progressive decline in P1CP as energy availability reduced was indicative of a causal role of LEA in the 'uncoupling' of bone resorption and formation. Furthermore, these findings suggest that bone formation is more sensitive to LEA than bone resorption (Ihle & Loucks, 2004). However, exercise was incorporated into each experimental condition, rendering it impossible to isolate the effects of exercise from energy availability upon bone turnover from this study.

To investigate the effect of low energy availability induced through dietary restriction alone or through exercise, Papageorgiou et al. (2018) recruited ten active females to complete three days of low energy availability (15 kcal•kg LBM^{-1}•day^{-1}) induced with and without running exercise and compared responses to a control trial (45 kcal•kg LBM^{-1}•day^{-1}**).** Low energy availability induced through diet alone reduced concentrations of the bone formation marker procollagen type 1 N-terminal propeptide (*P1NP*), compared to the control condition, but P1NP was unaffected during the LEA with exercise trial. However, there were no significant differences in bone turnover marker responses between the LEA conditions and it was suggested that the exercise bout provided some osteoprotective effects during LEA. β-Carboxyl-Terminal Cross-Linked Telopeptide of Type I Collagen (*ß-CTx*)

concentrations were unchanged across all conditions, providing further evidence that bone formation is more sensitive to LEA than bone resorption.

These findings may only be applicable to females, however, as current evidence suggests that males and females may exhibit divergent bone responses to acute periods of controlled low energy availability. Papageorgiou et al. (2017) compared the responses of 11 male and 11 female healthy habitual runners to five days of low energy availability (15 kcal•kg FFM^{-1}•day^{-1}) and five days of control EA (45 kcal•kg FFM^{-1}•day^{-1}), achieved through dietary manipulation and controlled running exercise. Whilst females displayed increases in ß-CTx concentrations and reduced P1NP concentrations, leading to a corresponding overall reduction in bone metabolism (bone turnover marker ratio) following the LEA intervention, no changes were observed in the male cohort for the same parameters. A strength of this study outcome was the provision of a multivitamin, reducing potential for confounding between the effects of low energy availability and micronutrient deficits, such as calcium or vitamin D. Although this multivitamin only provided 25% of the calcium nutrient reference value (Papageorgiou et al., 2017). In a mixed cohort of 5 males and 2 females, no overall changes in P1NP concentrations were observed following three days of LEA (15 kcal•kg FFM^{-1}•day^{-1}) induced via dietary restriction and following a single bout of resistance exercise on day 3 of the intervention (Murphy & Koehler, 2020). It had therefore been suggested that males may be more robust to the physiological impact of acute periods of low energy availability upon bone responses (Areta et al., 2021). However, a more recent study from Fensham et al. (2022) has shown that six days of low energy availability (15 kcal•kg FFM^{-1}•day^{-1}) reduced fasting P1NP concentrations in a cohort of elite male race-walkers, compared to a control trial. However, it is noteworthy that a concurrent LCHF intervention group displayed similar reductions in fasting P1NP concentrations to the LEA cohort and in fact were the only group to display reduced P1NP area under the curve (*AUC*) across a 25-km race-walking test. Both LEA and LCHF groups did display increased ß-CTx AUC in the exercise test, whilst the control group was unchanged. Similarly, whilst not strictly an energy availability study, the reduction of energy intake to 50% of energy requirements for three days, alongside 60-mins of daily running, has been shown to reduce P1NP concentrations compared to an energy balanced control condition, in a cohort of 8 trained male runners. However, no changes in bone resorption markers NTx or deoxypyridinoline were apparent (Zanker & Swaine, 2000).

These findings show that bone resorption and formation in males may indeed be affected by low energy availability, but that this process may also be carbohydrate-mediated. Both factors highlight the need for further research in exercising male populations to establish the magnitude and duration of low energy availability required to perturb bone metabolism, as well as the potential mediating role of carbohydrate availability. Similarly, as the *REDs* and the *Male Athlete Triad* models are applied to athletic individuals, there is a need for further research in trained and male athletes. Although the feasibility of conducting long-term, controlled, studies in such populations is questionable.

Leptin:

Leptin is an anorexigenic adipokine primarily expressed from the adipose tissue (Blüher & Mantzoros, 2009; Elliott-Sale et al., 2018). As a key energy sensing hormone, its main function is to communicate to multiple organs whether there is adequate energy to sustain life, or more specifically, when energy storage is inadequate (Blüher & Mantzoros, 2009). Under conditions of energy deficit, reductions in concentrations of leptin is the key endocrine signal that triggers increases in energy intake and enhances the efficiency of energy utilisation (Ravussin et al., 2014). Considering the influence of leptin upon metabolic processes and energy expenditure, as well as the presence of leptin receptors in the hypothalamus and multiple other organs, leptin has been shown to play a key mediating role in central pathways that have been shown to be disrupted by low energy availability (Figure 2.8.3.). These include the hypothalamic-pituitary-gonadal, hypothalamic-pituitary-thyroid, and growth hormone-insulin-like growth factor-1 axes. For example, leptin stimulates secretion of GnRH from the hypothalamus (Blüher & Mantzoros, 2009). As such, the response of leptin to low energy availability has been of interest within the research area.

Where assessed, leptin has appeared to consistently be reduced in female participants in response to controlled low energy availability (Hilton & Loucks, 2000; Loucks, 2006; Loucks & Thuma, 2003; Papageorgiou et al., 2017, 2018). Comparatively, there is a lack of data available from controlled studies in males. Moreover, sexual dimorphism may exist regarding leptin responses to low energy availability. For example, whilst leptin concentrations were reduced following five-days of LEA in trained females, they were unaffected in males and a significant main effect of sex suggested differing responses between males and females (Papageorgiou et al., 2017). Conversely, in a cohort of seven active males, five days of LEA (15 kcal•kg FFM^{-1}•day^{-1}) with both low and high protein intake reduced leptin concentrations by 55% and 65%, respectively, compared to the normal EA (40 kcal•kg FFM^{-1}•day^{-1}) trial (Murphy et al., 2021). Similarly, when comparing low energy availability (four days of ~16 kcal•kg FFM^{-1}•day^{-1}) induced through exercise or dietary restriction in males, both interventions led to a reduction in leptin concentration compared to the control EA condition (Koehler et al., 2016). These data extend the findings of Hilton & Loucks, (2000) and Papageorgiou et al. (2018), who demonstrated that reductions in leptin are elicited by low energy availability and not the stress of exercise in females, demonstrating that the same is true for males. However, whilst the leptin response to low energy availability in females appears to be well established, equivalent data from male studies is much more limited and conflicting. Considering the key energy-sensing role of leptin and its influence upon myriad physiological systems affected by low energy availability, further controlled research in males is necessary to better determine its relationship to low energy availability.

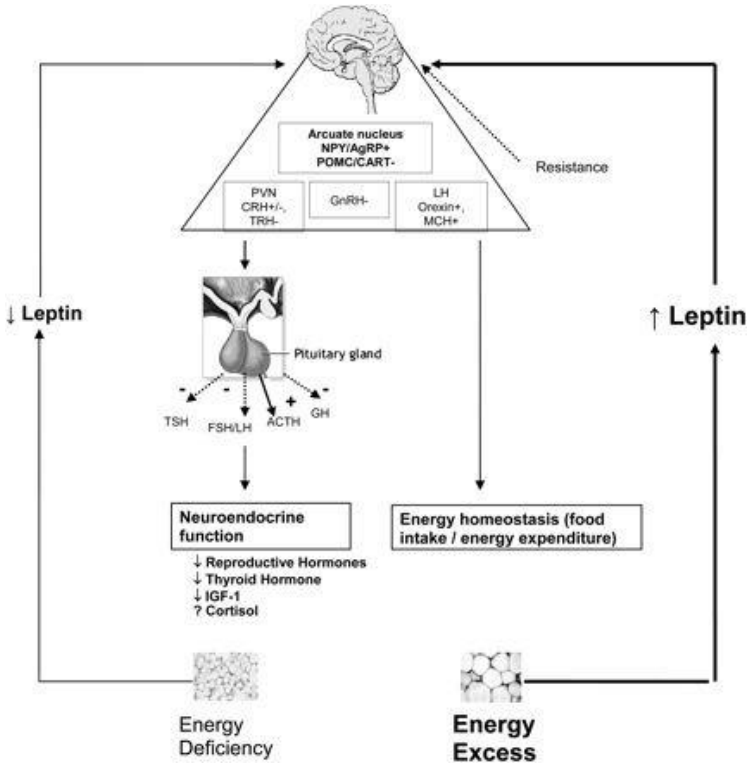


Figure 2.8.3. Under conditions of energy deficit, circulating leptin concentrations are reduced. This reduction acts centrally upon neuroendocrine axes. Pituitary secretion of thyroid stimulating hormone (*TSH*), follicle-stimulating hormone (*FSH*), luteinising hormone (*LH*), and growth hormone (*GH*) are reduced, and adrenocorticotropic hormone (*ACTH*) increased. Subsequent concentrations of reproductive and thyroid hormones, as well as the anabolic hormone IGF-1 are reduced.

(Image source: Blüher & Mantzoros, 2009)

The growth hormone-insulin-like growth factor-1 axis:

Growth hormone (*GH*) is a peptide secreted from somatotroph cells in the anterior pituitary gland and is essential for muscle and bone anabolism and the metabolism of carbohydrates, lipids, and proteins. Most of the effects of GH are mediated by insulin-like growth factor 1 (*IGF-1*), a peptide produced in the liver, although GH may have IGF-1 independent effects upon carbohydrate and lipid metabolism (Elliott-Sale et al., 2018; Fazeli & Klibanski, 2014). Under conditions of energy balance, *GH* is released from the from the pituitary gland and stimulates the release of *IGF-1* from the liver. IGF-1 exerts its anabolic effects in most tissues and inhibits pituitary GH release through a negative feedback mechanism. However, IGF-1 is sensitive to nutritional cues and in states of undernutrition GH resistance manifests, whereby GH concentrations will either be normal or elevated with concomitant reductions in IGF-1 concentrations. In energy deficit, STAT-5 phosphorylation (the key mediator of IGF-1 production within the liver) is thought to be reduced largely through the release of two key inhibitors; fibroblast growth factor 21 (*FGF21*) and sirtuin 1 (*SIRT1*), reducing IGF-1 production and subsequent inhibition of GH release from the pituitary (Figure 2.8.4.). As reviewed by Fazeli & Klibanski (2014). this likely conferred a survival benefit under conditions of calorie deficit, given that

GH appears to play a key role in mobilising energy stores, whilst the action of IGF-1 increases energy expenditure**.**

Considering the evident link between the GH-IGF-1 axis and 'undernutrition', the responses of GH and IGF-1 has been of interest within the experimental low energy availability research to-date. In female studies, IGF-1 has consistently been shown to be reduced in response to low energy availability (Loucks, 2006; Loucks et al., 1998; Loucks & Heath, 1994a; Loucks & Thuma, 2003), and this response appears to occur whether LEA is induced with an exercise component or with dietary restriction alone (Papageorgiou et al. 2018). Of these studies, those that also investigated GH responses observed that concentrations were consistently increased by low energy availability in females (Loucks, 2006; Loucks et al., 1998), although in some cases this only occurred at an energy availability ≤ 20 kcal•kg LBM^{-1}•day^{-1} (Loucks & Thuma, 2003). Papageorgiou et al. (2017) did not find a significant reduction in IGF-1 AUC in their female cohort following LEA, compared to the control trial. Although they did find a significant main effect of sex, with females displaying lower IGF responses than males, for whom IGF-1 concentrations were also unchanged. Although whether the use of AUC over a timeframe of multiple days is the most appropriate statistical methodology might be questioned, particularly considering that diet was not standardised between participants.

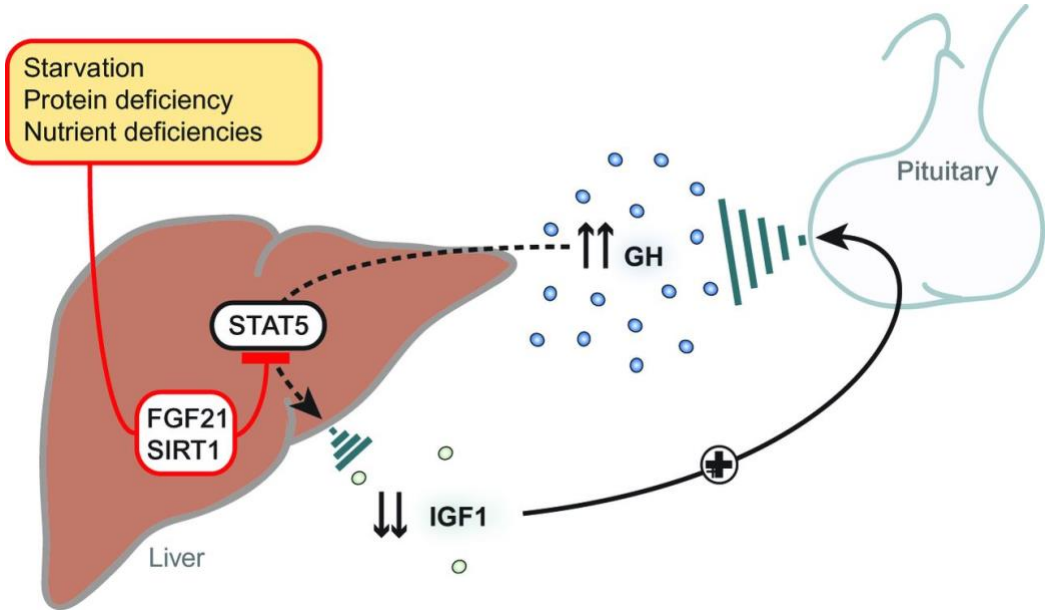


Figure 2.8.4. In energy balance, *GH* is released from the from the pituitary gland and, upon binding to its hepatocyte receptor, promotes the release of *IGF-1* from the liver by stimulating STAT5 phosphorylation (through the activation of JAK2). IGF-1 exerts anabolic effects in most tissues and inhibits pituitary GH release through a negative feedback loop. In energy deficit, FGF21 and Sirt1 concentrations increase, inhibiting STAT5 phosphorylation and subsequent IGF-1 production. The subsequent reduction in negative feedback to the pituitary then stimulates further GH release.

(Image source: Fazeli & Klibanski, 2014)

As suggested by the findings of Papageorgiou et al. (2017), the response of the GH-IGF-1 axis to low energy availability in males is less clear. Both Koehler et al. (2016) and Murphy et al. (2021) have also shown that four and five days of low energy availability (15 kcal•kg FFM^{-1}•day^{-1}), respectively, did not reduce fasting IGF-1 concentrations. However, these studies were conducted in small cohorts of six and seven males. In contrast, a similarly small cohort of seven trained male runners subjected to a shorter three-day intervention have shown reduced IGF-1 concentration in response to a low energy availability intervention providing < 20 kcal•kg FFM^{-1}•day^{-1} (Kojima et al., 2020). To-date, only one study appears to have assessed the concomitant responses of GH and IGF-1 to low energy availability in males, albeit in a mixed cohort of five males and two females. Three days of dietary restriction to produce an energy availability of 15 kcal•kg FFM^{-1}•day^{-1} produced significant reduction in GH concentrations and increased IGF-1 concentrations, indicating that the LEA intervention had elicited GH resistance (Murphy & Koehler, 2020). Given the equivocal nature of these findings, further research should seek to establish if more severe reductions in energy availability or interventions of longer duration produce more consistent effects in disruption of the male GH-IGF-1 axis. Considering the small populations studied to-date, future research should ideally also be conducted in larger cohorts of athletic males.

Metabolic substrates (glucose, non-esterified fatty acids, glycerol, ketones) and insulin:

Metabolic substrates and insulin are not strictly mediators of the physiological effects of low energy availability, rather their concentrations are altered as a product of substrate deprivation. However, these parameters serve a central role in metabolic regulation, and it is therefore noteworthy to catalogue the current observed responses of these factors in response to experimentally induced LEA. In both males and females, blood glucose and insulin concentrations have typically been reduced following LEA, whether assessed in the morning fasted state (Koehler et al., 2016; Loucks & Verdun, 1998; Oxfeldt et al., 2023), via 24-hr transverse mean values (Loucks, 2006; Loucks et al., 1998; Loucks & Thuma, 2003), or around repeated exercise sessions (Hammond et al., 2019). Although two studies have observed no change in fasting glucose concentrations (Kojima et al., 2020; Mckay et al., 2022a), whilst Papageorgiou et al. (2018) observed no change in insulin concentrations. Correspondingly, blood concentrations of ketone body ß-Hydroxybutyrate have consistently been shown to increase following LEA (Hammond et al., 2019; Loucks, 2006; Loucks & Thuma, 2003; Loucks & Verdun, 1998). Most studies have not examined the responses of free fatty acids and glycerol to assess for increased lipolytic activity, although (Koehler et al., 2016) observed that fasting glycerol concentrations increased in their cohort of six males following the LEA intervention. Furthermore, increased fat oxidation rates have also been observed during sub-maximal exercise following acute) exposure to low energy availability (< 20 kcal•kg LBM^{-1}•day^{-1}) for < 24 hours (Areta et al., 2020), as well as following nine days of a low (15 kcal•kg FFM^{-1}•day^{-1}) energy availability intervention (Burke et al., 2023). Taken together, these data support the notion that low energy availability also induces concomitant low-carbohydrate

availability. At present, the isolated effects of low energy compared to low carbohydrate availability upon the physiological parameters studied under experimental conditions remains a confounding factor.

Skeletal muscle responses:

Skeletal muscle is the essential tissue facilitating human movement and has a key influence upon health and performance. Its mass is ultimately determined by the net dynamic balance of protein synthesis and breakdown. Skeletal muscle serves essential primary functions under the three broad categories of mechanical force generation (for movement), metabolic regulation (through the release of myokines), and storage (of amino acids and energy as intramuscular triglycerides and skeletal muscle glycogen) (Shirley et al., 2022; Wolfe, 2006). The final of these functions is of profound importance during energy deficits, as the amino acids stored within the muscle are released as essential gluconeogenic precursors. During starvation, the diminishment of skeletal muscle reserves has been identified as the key determinant of death in extreme starvation and illness (Wolfe, 2006). Furthermore, considering the essential role of skeletal muscle in producing movement, this tissue has by extension been fundamental to the procurement of food throughout the evolutionary history of *Homo sapiens*. It has therefore been argued that under conditions of caloric deficit, energetic supply to the skeletal muscle is prioritised above less immediately essential processes, such as growth and reproduction, to sustain locomotion and ultimately food supply (Areta, 2023). Conversely, with a tissue-specific RMR of ~13 kcal•kg^{-1}•day^{-1}, the resting energetic cost of skeletal muscle is relatively low compared to internal organs. However, its relatively greater mass makes it an energetically costly tissue, accounting for approximately 16% and 24% of resting energy expenditure in females and males, respectively. The energetic cost of skeletal muscle is further augmented by physical activity and specific exercise. It may therefore be expected that reductions in skeletal muscle mass could save considerable energy during periods of energetic stress (Oliveira-Junior et al., 2022; Shirley et al., 2022). As reviewed by Shirley et al. (2022), the response of skeletal muscle to energy deficit may therefore be influenced by a variety of situational factors, including age, sex, training status, health, body composition, and nutritional status, encompassing the low energy availability ‘dose’ (the product of LEA severity and duration) and dietary macronutrient composition.

Perhaps due to the apparently conflicting influence of skeletal muscle in essential physiological functioning, compared to its high energetic demand, its responses to low energy availability are not well understood. Whilst there is a lack of experimental data on the topic, perhaps the most researched aspect of muscular responses to-date is the influence of LEA upon anabolic resistance. At the endocrine level, three days of LEA at 15 kcal•kg FFM^{-1}•day^{-1} has been shown to impair the negative feedback loop of the GH-IGF-1 axis following a single bout of resistance exercise, indicating impaired sensitivity to anabolic stimuli resulting from low energy availability. Although skeletal muscle responses were not examined to establish the effect of this hormonal disruption at the muscle tissue level (Murphy &

Koehler, 2020). In trained females, ten days of low energy availability has been shown to reduce bulk synthesis of myofibrillar and sarcoplasmic proteins with a corresponding reduction in nitrogen balance, compared to a parallel 'optimal' energy availability control trial-arm (Oxfeldt et al., 2023). Similarly, five days of LEA (30 kcal•kg FFM^{-1}•day^{-1}) was sufficient to reduce resting myofibrillar protein synthesis (*MPS*) rate in a mixed cohort of eight males and seven females (Areta et al., 2014). However, a single bout of resistance exercise restored basal MPS rates observed during energy balance, whilst progressive increases in protein consumption elevated MPS further in a step-wise fashion (Areta et al., 2014), supporting the notion that muscle responses to low energy availability vary depending on the stimuli of specific macronutrients and contractile activity. Interestingly, this intervention had no effect on the mTOR or AMPK pathways, mRNA expression of PGC-1α, or total p53 content/p53 phosphorylation. Similarly, autophagy signalling was also broadly unaffected, suggesting a limited role of autophagy in early LEA-induced proteolysis (Smiles et al., 2015). Taken together, acute LEA may therefore disrupt the anabolic response of skeletal muscle, but further research is required on this topic.

Conventional thinking has also stipulated that LEA is often associated with reductions in both endogenous (i.e., skeletal muscle and liver glycogen) and exogenous (i.e., blood glucose) fuel availability, with subsequent performance decrements occurring, as reviewed by Melin et al. (2023). Indeed, skeletal muscle glycogen concentrations have been shown to be reduced following repeated bouts of high-intensity exercise in a low energy availability trial, compared to a high carbohydrate control (Hammond et al., 2019). However, whilst both Kojima et al. (2020) and Ishibashi et al. (2020) also observed significant reductions in skeletal muscle glycogen content following three days of LEA < 20 kcal•kg FFM^{-1}•day^{-1}, this had no effect upon time to exhaustion in an exercise capacity test at 90% $\dot{V}O_{2max}$ compared to normal energy availability (Kojima et al., 2020). Similarly, 9 days of LEA (15 kcal•kg FFM^{-1}•day^{-1}) across an intense training phase led to similar improvements in performance compared to a normal EA control group in a cohort (19 M, 3 F) of highly-trained race-walkers (Burke et al., 2023), suggesting that acute LEA may not be detrimental to muscle function and/or performance. In support of this, Areta et al. (2020) observed that an overnight exposure to LEA (19 kcal•kg FFM^{-1}•day^{-1}) led to elevated p53 mRNA expression 3.5 hrs post-exercise the following day, compared to an energy balanced group achieved through high fat dietary intake, despite no differences in muscle glycogen content, AMPK or p38. In contrast, Hammond et al. (2019) did not observe significant changes in the mRNA expression of p38MAPK, PGC-1α or p53, key markers of mitochondrial biogenesis between their LEA, LCHF and control trials. Contemporary sports nutrition paradigms have suggested that 'train low' manipulations of glycogen availability may enhance adaptive responses to exercise (Hawley & Morton, 2014). By extension, acute LEA may therefore also enhance muscle oxidative capacity. However, continuous low energy availability over a sustained period may affect endurance performance or training adaptations (VanHeest et al., 2014). Longer-term controlled studies

are therefore still required to further understand the role of concomitant LEA and exercise upon muscle responses in healthy, athletic individuals.

Summary of the physiological effects of low energy availability:

Since the seminal work of Professor Loucks, interest in the effects of low energy availability has grown significantly, with many physiological effects now attributed to LEA. However, observational research has increased disproportionately to laboratory-based research. Therefore, there is still little experimental data demonstrating a causal influence of low energy availability upon alterations in physiological function, particularly in males. As such, further research is clearly required to understand this topic better and substantiate the assertions of the *Male Athlete Triad* and *REDs* models that exercising males are indeed susceptible to LEA-related health and performance decrements.

2.9. Necessary research directions:

Based upon the experimental data presented in section 2.8, low energy availability appears to be a potent stressor capable of disrupting the endocrine milieu, key neuroendocrine axes, and metabolic regulation within a matter of days. Yet there are numerous topics within the field of low energy availability research that must be addressed by future research. Some of these topics will briefly be discussed in the following section:

There is limited knowledge of male responses to low energy availability:

Due to the observations of a high prevalence of menstrual dysfunction and impaired bone health in exercising females in the 1970's and 1980's, most low energy availability research has been conducted in females. As such this is likely one of the few fields of exercise nutrition research where female responses have been investigated more than those of males (Cowley et al., 2021). Despite the synthesis of the *Male Athlete Triad* (Nattiv et al., 2021) and *REDs* (Mountjoy et al., 2023) models, which expand the physiological effects of low energy availability to encompass exercising males, we therefore do not have sufficient evidence of a causal relationship between LEA and the physiological alterations proposed to support this assertion at present.

Based upon the limited data from male participants on markers of bone metabolism (Murphy & Koehler, 2020; Papageorgiou et al., 2017) and endocrine responses (Koehler et al., 2016; Kojima et al., 2020), it would appear that male physiology is more resilient to LEA. Although limited data have shown bone resorption and formation markers and leptin to be altered following low energy availability in males responses (Murphy et al., 2021). The apparent sexual dimorphism in endocrine and physiological responses may be associated with the energetic demands of maintaining the reproductive system and gestation, which are significantly higher for females than males (Bronson, 1985). Female physiology may therefore be more sensitive to reductions in EA to ensure successful gestation in periods of reduced or low EA. Future research should first seek to establish if acute bouts of low energy availability can disrupt male endocrine systems in a similar manner to in females and replicate the seminal studies of Prof. Loucks in determining what levels of energy availability are likely to affect male physiology.

Patterns of energy availability in laboratory-based studies may be artificially homogenous:

Most controlled, laboratory-based, experiments investigating the physiological effects of low energy availability have clamped energy availability at a consistent level, with no day-to-day variability in energy intake or exercise energy expenditure. The relationship between energy intake, exercise energy expenditure, and resultant energy availability in free-living athlete populations is not well characterised. However, based upon limited data available, free-living athletes appear to experience considerable variability in daily energy availability levels (Heikura et al., 2019; Moss et al., 2020). The ecological validity of laboratory based prospective research investigating low energy availability is therefore

questionable and further research is necessary to determine the energy availability patterns of free-living individuals.

Low carbohydrate availability may be a confounding variable in existing research:

Most controlled energy availability studies have manipulated caloric intake whilst keeping a consistent relative contribution of each macronutrient. It is inherently impossible to create a caloric deficit without concomitantly creating a deficit in at least one macronutrient. Considering the putative role of carbohydrate availability in myriad physiological processes, as reviewed by Gonzalez et al. (2016) and Hearris et al. (2018), it is possible that a reduction in carbohydrate availability represents a significant confounding factor in energy availability research. For example, recent research investigating the effects of LCHF diets suggest that carbohydrate availability, not energy availability, may be the mediating factor of the responses of markers of bone resorption and formation (Fensham et al., 2022; Hammond et al., 2019; Heikura et al., 2020). This parallels the theory that the glucose requirements of the brain dictate a central neuroendocrine response, whereby glucose availability of less than approximately 130 $g \bullet day^{-1}$ elicits perturbation of the endocrine milieu (Loucks & Thuma, 2003). Future research should seek to understand the influence of varying macronutrient availabilities under conditions of low energy availability upon physiological responses.

Chapter 3:

General methods

This chapters describes the general methodologies and underpinning methodological theories used within this thesis.

3. General methodology

The *General methods* chapter outlines the common methodological approaches undertaken across the experimental chapters. For brevity, methods used universally across experimental chapters are detailed within this chapter, with reference to this section made as appropriate in each of the experimental chapters. As data was collected remotely for Study 1, most of the methods for doing so are outlined within this specific chapter (Chapter 4). Study 2 and Study 3 (Chapters 5 & 6) used similar experimental protocols; the methods outlined in this section predominantly relate to these chapters.

3.1. Location of testing:

Study 1 was an observational study conducted via remote data-collection, with free-living elite male road cyclists participating from their home environments within Europe. Studies 2 & 3 were laboratory-based experimental studies conducted in the exercise physiology and biochemistry laboratories of the Research Institute for Sport and Exercises Sciences (*RISES*), Liverpool John Moores University.

3.2. Participant characteristics:

All participants who volunteered to take part in the studies were either trained, elite-level cyclists (Chapter 4) or regularly exercising males aged 18 – 40 years old (Chapters 5 and 6). All participants provided written informed consent to participate in each study. Volunteers were generally healthy – i.e. free from any skeletal muscle abnormality, neurological condition or under pharmacological intervention - during any study. Participants were asked to avoid heavy exercise and alcohol consumption in the 24 hours preceding laboratory visits. Participation was voluntary and individuals were free to withdraw from the studies at any time. For Study 2 and Study 3, participants were recruited using circular recruitment emails, recruitment posters placed around campus and via word-of-mouth. Participants were eligible to take part in Study 2 and Study 3 if they met the following criteria: Healthy (not taking prescription medication), male, aged 18-40 years, weight-stable (± 2 kg) for previous 6 months and between 18 – 26% body fat (Study 2 only), completing 3+ sessions/hours of aerobic exercise per week, returned an eating attitude test (*EAT-26*) score < 20 (Garner et al., 1982), were a non-smoker, and had access to a smartphone. Individuals were excluded if they fell outside of these criteria, displayed any contraindication on the readiness to exercise questionnaire (PAR-Q), were following a restrictive diet (e.g. vegan, vegetarian, ketogenic/low carbohydrate), had a food allergy/intolerance, had an underlying medical condition, or were unwilling to adhere to the study protocol outlined in the participant information sheet.

3.3. Body mass and body composition:

Anthropometric assessments were conducted in the fasted state in the morning. Prior to assessments, participants voided their bladder and removed all jewellery and clothing, down to their undergarments.

In Study 1, participants were asked to measure their body mass at home (various weighing-scale brands, undisclosed) on Day 1 and immediately after the assessment period. The use of home-scales has been shown to be sufficiently accurate and consistent for research purposes (Yorkin et al., 2013). In Study 2 and Study 3, participant's height was measured to the nearest 0.1 cm with a stadiometer (SECA 213: SECA GMBH, Hamburg, Germany), in the anatomical position, with the posterior aspect (heels, gluteals and upper back) in contact with the vertical measuring rule. Participants performed an inhalation and exhalation prior to the researcher moving the rule sliding arm onto the crown of the head. Waist circumference was recorded to the nearest 0.1 cm using a tape measure (SECA 201: SECA GMBH, Hamburg, Germany).

Bio-electrical impedance analysis:

Participants completed a body composition assessment via bio-electrical impedance analysis using an 8-electrode BIA machine (SECA mBCA 515; SECA GMBH, Hamburg, Germany), following manufacturer guidelines and the computer-linked analysis software (SECA Analytics 115: SECA GMBH, Hamburg, Germany). Body mass was measured to the nearest 0.05 kg. The machine was then set to run a full body composition analysis and results saved to the computer software. Body composition was assessed via BIA in all laboratory visits during the intervention periods for Study 2 and 3 (Chapters 5 and 6).

Dual-energy X-ray absorptiometry (DXA) scans:

Body composition was measured via whole-body DXA scan (QDR Series Discovery A; Hologic Inc., Marlborough Massachusetts, USA, software version 12:4:3) using a fan beam. DXA scanning best-practice protocols were followed for all scans (Nana et al., 2015, 2016). Bone density Z-scores were classified relative to age, sex, and ethnicity. DXA system calibration was completed using an anthropometric spine and step phantom, with a subsequent radiographic uniformity test undertaken. Body mass, as measured to the nearest 0.05 kg via the BIA machine, was inputted into the DXA software and the participant was then positioned on to the centre of the DXA bed. Traction of the neck and legs was performed to ensure spinal alignment. Hands were positioned to the side of the participant's hips, with EVA foam 'spacers', with feet turned inwards to the centre line. Once in position, participants were instructed to remain still for the duration of the scan. Regional DXA data were segmented into trunk, left-, and right-side of the upper and lower limbs. All DXA scan positioning and subsequent scan analyses were completed by the same trained and experienced individual. Laboratory technical error of measurement (TEM) and coefficient of variation (CV) for DXA derived measures of FFM, FM and BF% has 0.44 kg:1.0%, 0.37 kg:1.9%, 0.41:1.9%, respectively and for BMD measures CV is <1.5% (Langan-Evans et al., 2021).

3.4. Controlled exercise protocols:

The laboratory-based studies reported in chapters 5 and 6 (Studies 2 & 3) involved supervised exercise sessions performing cycling ergometry (Lode Corival CPET, Lode, Groningen, Netherlands) as the exercise modality. Participant saddle height was recorded following their first laboratory visit and replicated for all future trials, but participants were freely able to adjust the handlebars to their comfort.

Exercise sessions were initiated with four three-minute stages of progressive workload at 50 W, 75 W, 100 W and 125 W to provide a standardised warm-up and to facilitate the assessment of sub-maximal cycling efficiency and substrate utilisation. After the standardised 12-minute warm-up, workload was set to the power output corresponding to 60% $\dot{V}O_{2max}$ (Study 2) or 95% of lactate turn-point 1 (Study 3). Participants kept the mouthpiece and nose clip on for a further 3-minutes, up to 15-minutes of exercise. HR and RPE were recorded in the final minute of each stage and substrate oxidation and net exercise energy expenditure was calculated from the mean values of expired O_2 and CO_2 from the final 60 seconds of each stage (See section 3.5).

Thereafter, participants were asked to insert the mouthpiece and fit the nose clip for the final 2.5 minutes of every 15 minutes of exercise, to allow the collection of two minutes of expired gas samples for every 15 mins of exercise (i.e., from 27.5 – 30 mins). Heart rate (*HR*) and rating of perceived exertion (*RPE*: Borg, 1970) were recorded in the final minute of each 15-minute exercise 'block', with substrate oxidation and net exercise energy expenditure calculated from the mean values of expired O_2 and CO_2 from the final 60s of each stage and extrapolated across the preceding 15 minutes of steady state exercise. Participants cycled until a net exercise energy expenditure equating to 15 kcal•kg FFM^{-1}, calculated from indirect calorimetry values, had been attained. Following 30- and 60-minutes of exercise, participants were asked to stop exercising and provided with a 5-minute rest period. Participants exercised continuously from 60-minutes onwards (Study 2, Chapter 5) or took a further 5-mins rest (Study 3, Chapter 6) at 90-minutes of exercise. Total exercise time and a final HR and RPE were recorded at the cessation of exercise. Water was permitted *ad libitum* and participants self-selected audio playback during exercise sessions.

3.5. Physiological Measures:

Heart rate:

For all laboratory-based exercise sessions, participants were fitted with a short-range radio telemetry device (Polar H7, Polar, Kempele, Finland) for the measurement of heart rate. Data was recorded using the linked *Polar Beat* (Polar, Kempele, Finland) smartphone/tablet application, on individualised Polar accounts.

Indirect calorimetry - general principle:

The method of indirect calorimetry uses measurements of gas exchange at the lungs (whole body O_2 consumption and CO_2 production), from which estimates of substrate oxidation during rest and exercise can be derived. In this method, given that carbohydrates, fats, and proteins differ in their chemical composition and, therefore, the amount of O_2 required and CO_2 produced when oxidised, rates of substrate utilisation for energy production can therefore be estimated through the measurement of $\dot{V}O_2$ consumed and $\dot{V}CO_2$ produced at rest and during exercise.

Assessment of respiratory gases at rest:

In studies 2 and 3, RMR was assessed using indirect calorimetry open hood systems. Study 2 (Chapter 5) used a Gaseous Exchange Measurement (*GEM*) Open Circuit Indirect Calorimeter (GEMNutrition Ltd., Warrington, UK) to assess RMR. The GEM machine was warmed-up for ≥ 20 minutes prior to use and then calibrated (twice) using known concentrations of 20 % O_2 and 1 % nitrogen, and a zero (100% nitrogen) span gas (BOC Ltd., Woking, UK) in line with the manufacturer's recommendations. At the beginning of the study, the calibration was validated by conducting an ethanol burn to confirm an established RER value of 0.67. Study 3 (Chapter 6) used a Cosmed Q-NRG Metabolic Monitor (Cosmed Srl, Rome, Italy) to assess RMR. The Q-NRG was warmed-up for 20-minutes prior to use and calibrated monthly using known concentrations of 16 % O_2 and 5 % CO_2 (Cosmed Srl, Rome, Italy), in line with manufacturer guidelines.

To assess RMR, participants lay supine and quietly on a medical bed in a dimly lit room for five minutes. A transparent ventilated hood was then placed over the participant's head and shoulders and wrapped with a plastic sheet to minimise external atmospheric exchange. The participant was instructed to relax, lay as still as possible, and to breathe normally over the duration of the assessment. The RMR assessment was conducted over a further 25 minutes, with only the final 20 minutes of data used for data analysis (Iraki et al., 2023). $\dot{V}O_2$, $\dot{V}CO_2$, and subsequent RMR and respiratory exchange ratio (*RER*) data was averaged every 30 seconds by the respective GEM and Cosmed Q-NRG software, with the 20-minute average used to calculate the average resting energy expenditure ($kcal \bullet min^{-1}$) over the assessment period and extrapolated to reflect a 24 hour (daily) resting metabolic rate ($kcal \bullet day^{-1}$).

Assessment of respiratory gases during exercise:

Analyses of respiratory gases during laboratory exercise sessions were conducted on a Moxus modular metabolic system (AEI technologies, Pittsburgh, PA, USA). To ensure all inspired and expired gases were recorded, participants were fitted with a nose clip and a mouthpiece mounted upon a two-way T-shaped breathing valve (Hans Rudolph Inc. Shawnee, KS, USA). To determine ventilatory volume, the mouthpiece was connected via a 6ft tube to a turbine on the inspiratory side of the valve. Expired gases

pass from the other side of the valve to a 4.2 litre mixing chamber, connected via a separate 6ft tube. Samples were drawn from the mixing chamber continuously at a flow of 200 ml•min^{-1}, through a dual-stage Nafion dryer, into to the O_2 (S-3A/I with an N-22M O_2 sensor) and CO_2 (CD-3A with a P-61B sensor) analysers. The analysers use the zirconia and non-dispersive infrared methods for O_2 and CO_2 analysis, respectively. The Moxus instrument was calibrated in line with manufacturer guidelines prior to each day's exercise sessions; following a 16-hour warm-up, gas volume was calibrated using a high-precision 3 L calibration syringe (Hans-Rudolph Inc. Shawnee, KS, USA). Gas calibration was performed using a two-point calibration curve with gases of known-concentrations of 15.99% O_2 and 3.98% CO_2 and 21.00 % O_2 and 0.03 % CO_2 (AEI technologies, Pittsburgh, PA, USA). Expired gases were averaged over a 30-second period for $\dot{V}O_{2max}$ assessments, and over a 60-second period during sub-maximal and steady state exercise protocols.

Estimation of substrate utilisation and energy expenditure during exercise:

Rates of whole-body carbohydrate and fat oxidation during exercise (g•min^{-1}) were estimated from indirect calorimetry assessments and calculated using the equations of Jeukendrup & Wallis (2005) for exercise intensities of 50 – 75% $\dot{V}O_{2max}$. Carbohydrate (equation 1) and fat oxidation (equation 2) were therefore calculated using the following equations:

$$\mathbf{1}.\ \boldsymbol{Carbohydrate\ Oxidation\ Rate}\ (\boldsymbol{g \bullet min^{-1}}) = (4.210 \times \dot{V}CO_2) - (2.962 \times \dot{V}O_2)$$

$$\mathbf{2}.\ \boldsymbol{Fat\ Oxidation\ Rate}\ (\boldsymbol{g \bullet min^{-1}}) = (1.695 \times \dot{V}O_2) - (1.701 \times \dot{V}CO_2)$$

These equations estimate exercise substrate utilisation from $\dot{V}O_2$ and $\dot{V}CO_2$ measurements, both in L•min^{-1}, assuming that these values accurately reflect tissue O_2 consumption and CO_2 production and a negligible contribution of protein oxidation. In line with the recommendations of Jeukendrup & Wallis (2005), the oxidation of 1g of carbohydrate was assumed to be energetically equivalent to 4.07 kcal, using a 20% glucose:80% glycogen contribution ratio. Similarly, 1g of fat oxidation was assumed as equivalent to 9.75 kcal, with gross exercise energy expenditure (EEE_{Gross}) calculated as outlined in equation 3 (Jeukendrup & Wallis, 2005):

$$\mathbf{3}.\ \boldsymbol{EEE_{Gross}}\ (\boldsymbol{kcal \bullet min^{-1}}) = (0.550 \times \dot{V}CO_2) + (4.471 \times \dot{V}O_2)$$

Table 3.5.1. outlines the energy content and O_2 and CO_2 requirement and production for the oxidation of varying carbohydrate sources, fats, and amino acids. To calculate net exercise energy expenditure, the most recently measured RMR (kcal•min^{-1}) value was multiplied by the preceding exercise sampling period's duration (in mins) during steady state exercise (see Chapter 2.5 and Table 2.5.1).

Table 3.5.1. Energy and volumes of O_2 required and CO_2 produced in the oxidation of carbohydrate, fat, and amino acids. Adapted from Jeukendrup & Wallis (2005)

	Energy (kcal•g^{-1})	**O_2 Required (L•g^{-1})**	**CO_2 Produced (kcal•g^{-1})**	**RQ**	**Energy Equivalent of O_2 (kcal•L^{-1})**
Glucose	3.74	0.7455	0.7426	0.996	5.02
Glycogen	4.15	0.8283	0.8251	0.996	5.02
Fatty Acid	9.75	2.0092	1.4136	0.704	4.85
Amino Acid	4.09	0.9842	0.7931	0.807	4.16

Assessment of cycling gross efficiency:

Exercise Gross Efficiency (*GE*) was calculated as the ratio between the mechanical work (ergometer power) and the metabolic work (from indirect calorimetry EEE_{gross}) completed per unit of time (equation 4), as outlined by (Coyle et al., 1992).

$$\textbf{4.}\ \boldsymbol{Gross\ Efficiency}\ (\%) = \frac{\boldsymbol{Mechanical\ Work\ (kcal{\bullet}min^{-1})}}{\boldsymbol{Metabolic\ Work\ (kcal{\bullet}min^{-1})}}\ \boldsymbol{x\ 100}$$

Ergometer power (W) was converted to kcal•min^{-1}:

1: *1 Watt = 1 J•s^{-1}*

2: $\frac{J{\bullet}s^{-1}}{4.18} = cal{\bullet}s^{-1}$

3: $\frac{cal{\bullet}s^{-1}}{1000} = kcal{\bullet}s^{-1}$

4: *kcal•s^{-1} x 60 = kcal•min^{-1}*

Assessment of Lactate Thresholds, $\dot{V}O_{2max}$ and peak power output:

Participants attended the laboratory for a baseline test at least two days, and no more than seven days prior to their proposed start-date for the study intervention phase for studies 2 (Chapter 5) and 3 (Chapter 6). Participants refrained from eating for two hours prior to testing (Study 2) or were tested in in an overnight fasted state (Study 3).

Participants performed a two-part incremental cycle test to determine sub-maximal exercise $\dot{V}O_2$, lactate threshold (*LT*), maximal oxygen consumption ($\dot{V}O_{2max}$), and peak power output (*PPO*). For part one, in Study 2, participants commenced cycling at 75 W for three minutes. Power output was then increased by an increment of 25 W every 3-minutes until a target HR of 80% ($HR_{80\%}$) of age-predicted maximum (HR_{max} = 220 – participant age in years; Fox & Naughton, 1972) had been reached. Alternatively, in Study 3 participants commenced cycling at 90 W for 3-minutes. Power output was then increased by 30 W every 3-minutes until a 2 mmol•L^{-1} increase in blood lactate concentration from the previous stage had been attained. Lactate turn-point 1 was defined as the exercise intensity where blood lactate

concentrations increased by ≥ 1.0 mmol•L^{-1} across the 3-minute stage (Newell et al., 2015). Expired gases were collected continuously throughout exercise. HR, rating of perceived exertion (*RPE*), and a capillary blood sample (fingertip) were collected during the final 30 seconds of each stage. Blood lactate concentrations were assessed using a Biosen analyser (Biosen C-Line, EKF Diagnostic, Cardiff, UK).

Participants were then given 5 – 10 mins of self-selected recovery time prior to performing part two of the exercise test, in which time they were allowed to dismount the bike or cycle at 75 W. Water was provided *ad libitum* during this period.

For part two of the exercise test, the initial power output was set to the penultimate stage participants completed in part one. Participants completed one-minute exercise stages, with power increased by 25 W (Study 2) or 30 W (Study 3) every minute until volitional exhaustion was achieved. Expired gases were collected continuously, with HR and RPE collected in the final 15 seconds of each stage. A test was considered maximal if at least two of the following criteria were attained, 1: HR was within 10 beats•min^{-1} of age predicted maximum, 2: RER > 1.1, and 3: a plateau in $\dot{V}O_2$ despite increased workload. $\dot{V}O_{2max}$ was determined as the highest 30s average value attained during the exercise test. Test PPO was calculated using the following equation (Kuipers, Verstappen, Keizer, Geurten & Van Kranenburg, 1985):

$$PPO = W_{final} + ([t/60] \bullet PI)$$

Where W_{final} = power output from the final whole stage completed, t = time spent in the final incomplete stage (s), 60 = the stage duration (s) and, PI = the power increase between stages (W).

Finally, a simple linear regression equation using $\dot{V}O_2$ data from the sub-maximal exercise test (part one) was used to estimate cycling power outputs (W) that would correspond to 55, 60 and 65 % of $\dot{V}O_{2max}$ for each participant. Following a rest period of ≥ 20 mins, participants completed 20 minutes of cycling to serve as a brief familiarisation and to check that the estimated power outputs broadly corresponded to the measured $\dot{V}O_2$. Expired gases were collected continuously for 15 minutes and then from min 18 – 20. HR and RPE were also recorded at 3-minute intervals from 0 – 15 mins and for minute 20.

3.6. Psycho-physiological assessments:

Ratings of Perceived Exertion (RPE):

Participant RPE during exercise was reported using a 15-point Likert scale (Figure 3.6.1. Borg, 1970). Participants were familiarised to the scale during preliminary laboratory visits (i.e. baseline testing and familiarisation).

Rating	Description
6	No Exertion at All
7	Extremely Light
8	
9	Very Light
10	
11	
12	
13	Somewhat Hard
14	
15	Hard
16	
17	Very Hard
18	
19	Extremely Hard
20	Maximal

Figure 3.6.1. Borg Rating of Perceived Exertion (RPE) scale.

3.7. Dietary analysis and controls:

Dietary intake provision:

In Study 2 (Chapter 5) and Study 3 (Chapter 6), all dietary intake was provided for the experimental phases of the intervention periods. Briefly, dietary provision was planned and calculated using Nutritics™ (Nutritics Ltd, Dublin, Ireland) dietary analysis software, which calculated energy and macronutrient intake for each participant. The target caloric intake to produce the requisite energy availability (accounting for planned exercise energy expenditure) was determined using the fat free mass assessed in the pre-intervention laboratory visit (i.e. Day -5 in Study 2 and Day -4 in Study 3) of the first intervention period. All food was prepared by experienced members of the research team and weighed to the nearest 1 g using home kitchen scales.

Upon departure from the laboratory on testing mornings, participants were provided with all dietary intake for the period intervening lab-visits. Participants were instructed to refrain from consuming any further calorie-containing food or drinks, except for a single, unsweetened, black coffee or tea daily for habitual caffeine consumers. Meals were provided pre-packaged and where possible, pre-cooked. Any food that required heating was provided with full written cooking instructions. A meal-plan was provided with an overview of the dietary provision and participants were instructed to tick off or record the time of each meal consumption. Participants were also instructed to record fluid intake, any deviations from their dietary provision, and any leftover foods on their recording sheet. Members of the research team were available remotely (via smartphone messaging app. *WhatsApp*, Dublin, Ireland) to

answer any participant queries relating to the meal plan. Details of the individual study diets are outlined within their respective experimental chapters.

Energy availability calculations:

All calculations of energy availability within this thesis are calculated using the most recent definition of EA (see Chapter 2.5, and Table 2.5.1. '3rd algebraic definition'). Further details on study-specific energy availability calculations are outlined within their respective experimental chapters.

3.8. Collection, storage, and analysis of muscle and blood:

Collection of fasted venous blood samples:

In Study 2 and Study 3, participants had a fasting venous blood sample drawn from the ante-cubital vein by a trained phlebotomist using a 21G x ¾" x 12" safety-lok butterfly needle (Becton, Dickinson and Company, Franklin Lakes, NJ, USA). Approximately 18 – 26 ml of blood was drawn from each arm in alternate manner at each of the laboratory visits in the intervention phases of study 2 and 3. Samples were drawn into singular 6 ml whole blood (serum), 8 ml serum separator tube (*SST*. Study 2 only), 6 ml ethylenediaminetetraacetic acid (*EDTA*), and 6 ml lithium heparin (*LHep*) vacutainers (BD Vacutainer, Becton, Dickinson and Company, Franklin Lakes, NJ, USA). All vacutainers were inverted eight-times following collection, in line with our laboratory guidelines. Plasma samples (EDTA and LHep) were placed on ice immediately, whilst serum and SST vacutainers were allowed to clot at room temperature for 30 minutes, before being placed on ice until centrifugation. Blood samples were centrifuged for 10-minutes at 4˚C and a relative centrifugal force of 1200*g*, in line with laboratory standard operating procedures. Samples were then individually aliquoted into triplicate 1.5 ml Eppendorf tubes (Eppendorf UK Limited, Stevenage, Great Britain) per time-point and sample type and stored at -80°C for subsequent analysis.

Collection of skeletal muscle samples:

Skeletal muscle biopsies (100-150 mg) were obtained from the lateral portion of the vastus lateralis (*VL*) muscle in an overnight fasted state in Study 2 and Study 3. A single muscle biopsy was collected at each time-point from a separate incision site (2 – 3 cm from any previous biopsy sites) using the Weil-Blakesley conchotome technique. The Weil-Blakesley conchotome needle has a sharp biting tip with a 4-6 mm hollow tip. The needle can be finely manoeuvred for controlled tissue penetration and sample collection. The tip is opened and closed to 'grip' the muscle tissue and then rotated through 360˚ to cut the tissue sample. The use of this technique typically provides samples between 20 – 290 mg (Baczynska et al., 2016).

All skeletal muscle biopsies were performed by a trained member of the *RISES* research staff. Participants were asked to lay in a semi-recumbent position on a medical bed and the biopsy site of the

VL was prepared. Avoiding previous biopsy sites, the area for incision was shaved, washed with an alcohol swab, and then washed again with Hydrex surgical scrub (ECOLAB Ltd., Leeds, UK), before a sterile sheet was placed around the site. 1.5 – 2 ml of 5 mg•ml^{-1} local anaesthetic (0.5% Marcaine; Bupivacaine hydrochloride, Kays Medical, Liverpool, UK) was administered to the incision site to anaesthetise the area. A 5 – 10 mm incision through the skin and muscle fascia was then made using a sterile, single-use, disposable scalpel (needle size 10: Swann-Morton, Nu-Care Products Ltd., Bedfordshire, UK). A sterilised conchotome biopsy needle was then used to retrieve a muscle sample from the VL muscle of the participant. Post-biopsy treatment and follow-up aftercare was provided to all participants in line with *RISES* muscle biopsy standard operating procedures.

Muscle samples were blotted to remove excess blood and then dissected to remove any visible fat or connective tissue. Three ~30 – 40 mg sections of muscle sample were sliced using a sterile, single-use, scalpel (needle size 11: Swann-Morton, Nu-Care Products Ltd., Bedfordshire, UK) and placed into individual sterile screw-top 2 ml sterile freezing vials (Sigma-Aldrich, Dorset, UK). These samples were then immediately snap-frozen in liquid nitrogen to minimise tissue degradation and stored at -80˚C for future analysis.

3.9. Analytical techniques:

Spectrophotometry principle:

The spectrophotometry method works by measuring the amount of light that each sample absorbs, which is then used for the determination of plasma metabolite concentrations by plotting the absorbance value against the absorbance of standards of known concentration. Within the spectrometer, the lens transmits a straight beam of light (photons) that passes through the prism to be split into several component wavelengths. The desired wavelength is then selected by the silt and is transmitted through the sample in cuvette. The number of photons that are absorbed by the sample is then detected by the photometer (Figure 3.9.1).

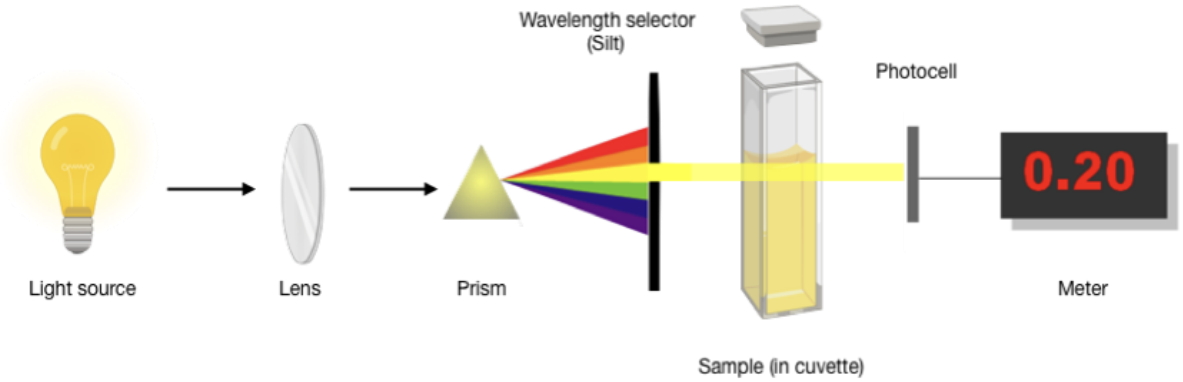


Figure 3.9.1. An overview of the general principle of spectrophotometry.

Analysis of serum and plasma metabolites and hormones:

Samples were analysed for plasma glucose, lactate, non-esterified fatty acids (*NEFA*) and glycerol concentrations using commercially available kits and a Randox Daytona spectrophotometer (Randox, Crumlin, Northern Ireland. UK). Mean coefficient of variation (*CV*) for duplicate measurements was < 5% for plasma glucose, lactate, NEFA and glycerol in both Study 2 and Study 3.

Commercially available enzyme-linked immunosorbent assays (*ELISA*) were used to measure serum (Study 2) and plasma (Study 3) triiodothyronine (DiaMetra S.r.l, Perugia, Italy. Mean intra-plate CV; Study 2: 4.4 %, Study 3: 6.1 %), serum insulin-like growth factor 1 (mean intra-plate CV; Study 2: 5.4 %, Study 3: 4.4 %), plasma insulin (Study 2 mean intra-plate CV: 6.7 %), and plasma leptin (mean intra-plate CV; Study 2: 5.4 %, Study 3: 7.4 %) (all DRG International Inc., Springfield, NJ, USA), erythropoietin (*EPO*; Abcam, Cambridge, UK. Mean intra-plate CV; Study 2 [plasma]: 4.8 %, Study 3 [serum]: 4.1 %) and serum growth/differentiation factor-15 (*GDF-15*; R&D Systems, Minneapolis, MN, USA. Mean intra-plate CV; Study 2: 3.1 %, Study 3: 3.8 %). Samples were run in duplicate except for EPO, which was run in triplicate. Absorption was determined using a microplate reader (Clariostar, BMG Labtech, Ortenberg, Germany) at the wavelengths specified by the kit manufacturer. Manufacturer instructions and quality control checks were followed and the coefficient of variation of standards and samples were < 20 % (Ouellet-Morin et al., 2011). Plasma insulin duplicate CV values exceeded 20% for four samples within one participant in Study 2. Due to budget and sample constraints, it was not possible to re-run these samples. These data points were retained within sample analyses. Where provided, control high and control low samples were run in duplicate, values attained were required to fall within the specified range on individual kit inserts for an assay to be considered valid. Manufacturer provided kit sensitivity and precision are summarised in Table 3.9.1. Due to time and budget constraints and the low throughput of each assay kit, it was not possible to establish 'in house' reliability and quality control statistics for the ELISAs conducted.

For Study 2 and Study 3, analysis of serum total testosterone, plasma *ß-CTx*, and plasma P1NP concentrations was outsourced to a commercial clinical laboratory (Liverpool Clinical Laboratories, Liverpool, UK). For Study 3, serum insulin concentrations were also assessed by Liverpool Clinical Laboratories. All samples were assessed using electrochemiluminescence immunoassay (*ECLIA*) on a Roche Cobas e801 analyser with Roche assay kits and calibrators (Roche Diagnostics Ltd., West Sussex, UK), following completion of the laboratory's daily quality control protocols. Each morning, prior to running analyses, three levels (*low*, *medium*, and *high*) of quality control (Technopath IA plus, Technopath Distribution Limited, Limerick, Ireland) were run, with a single-level quality control protocol then performed once every six hours. Quality control values were required to fall within ± 2 SD of the specified target mean for data to be accepted. Roche diagnostic kit data informations sheets can be found in Appendix B.

Table 3.9.1. Reported sensitivity, intra-assay, and inter-assay for all ELISA and Randox kits used

Parameter	Kit Manufacturer	Sensitivity	Intra-assay precision (CV)	Inter-assay precision (CV)
T3	DiaMetra	5 ng•dl^{-1}	≤ 10.7 %	≤ 9.1 %
IGF-1	DRG	4.08 ng•ml^{-1}	< 7.4 %	< 14.8
Insulin	DRG	10.6 pmol•l^{-1}	< 2.6 %	< 6.0 %
Leptin	DRG	0.7 ng•ml^{-1}	< 9.6 %	< 9.1 %
EPO	Abcam	1 mIU•ml^{-1}	6.1 %	10 %
GDF-15	R&D Systems	4.4 pg•ml^{-1}	< 2.8 %	< 6.0 %
Glucose	Randox	0.35 mmol•l^{-1}	< 2.5 %	< 4.3 %
Glycerol	Randox	14.5 µmol•$^{-1}$	< 1.4 %	< 6.4 %
NEFA	Randox	0.072 mmol•l^{-1}	< 4.9 %	< 4.6 %
D-3-Hydroxybutyrate	Randox	0.05 mmol•l^{-1}	< 1.7 %	Not reported

Skeletal muscle glycogen concentrations:

Muscle glycogen concentration was determined using the acid hydrolysis method described by van Loon et al (2000). The acid hydrolysis method principle allows for the cleavage of glyosidic bonds which are necessary for the attachment of glucose molecules to one another, allowing for the breakdown of di- and polysaccharide chains into single sugar monosaccharides. Once boiled, an acid-base neutralisation reaction is performed with the addition of KOH, saturated with KCl to neutralise the pH of the sample.

Approximately 1 - 5 mg of freeze-dried tissue was powdered and dissected free of all visible non-muscle tissue and subsequently hydrolysed by incubation in 500 µl of 1 M HCl for 3 hours in a water bath set to 100°C. Samples were removed from the water bath every 45 minutes and briefly vortexed before being returned to boil. After cooling for 20 minutes at room temperature, samples were neutralised by the addition of 250 µl of 0.12 mol•l^{-1}/2.1 mol•l^{-1} KOH, saturated with KCl and centrifuged at 5000 *g* for 10 minutes at 4°C. Following centrifugation, 200 µl of supernatant was frozen at -80°C and subsequently later analysed in duplicate for glucose concentration according to the hexokinase method using a commercially available kit (GLUC-HK, Randox Laboratories, Crumlin, UK). Muscle glycogen concentration is expressed as mmol•kg^{-1} DW and the mean coefficient of variation between duplicates was 10 %. Due to a technical issue in the laboratory, skeletal muscle glycogen concentrations for Study 3 (Chapter 6) await analysis upon resolution of this problem.

Skeletal muscle protein synthetic rates:

The following protocol was conducted in collaboration with Professor Jatin Burniston and Dr Yusuke Nishimura, from LJMU *RISES* proteomics department. All sample and data processing was completed by the proteomics team at LJMU. This thesis has undertaken the biological interpretation of the skeletal muscle protein synthesis data, following the completion of these steps by the proteomics team.

Individual protein fractional synthetic rates were calculated based upon the precursor: product ratio of deuterium incorporation from the body water (precursor) into muscle proteins (product). In line with practices routinely used within our laboratory, protein extraction and quantification, were conducted as previously described. Precursor enrichment of D_2O was measured in blood plasma samples by gas chromatography-mass spectrometry conducted in line with previous research methodologies from our laboratory (Camera et al., 2017; Hesketh et al., 2020; Srisawat et al., 2023):

Calculation of D_2O Enrichment

Body water enrichment of D_2O was measured in plasma samples against external standards that were constructed by adding D_2O to 18 MΩ water over the range from 0.0 to 5.0 % in 0.5 % increments. D_2O enrichment of aqueous solutions was determined by gas chromatography-mass spectrometry after exchange with acetone (McCabe et al., 2006). Samples were centrifuged at 12,000 g, 4°C for 5 min, and 20 µl of plasma supernatant or standard was reacted overnight at room temperature with 2 µl of 10 M NaOH and 4 µl of 5% (v/v) acetone in acetonitrile. Acetone was then extracted in to 500 µl chloroform and water was captured in 0.5 g Na_2SO_4 before transferring a 200 µl aliquot of chloroform to an auto-sampler vial. Samples and standards were analysed in triplicate by using an Agilent 5973 N mass selective detector coupled to an Agilent 6890 gas chromatography system (Agilent Technologies, Santa Clara, CA, USA). A CD624-GC column (30 m 30.25 mm 31.40 mm) was used in all analyses. Samples (1 ul) were injected by using an Agilent 7683 auto sampler. The temperature program began at 50°C and increased by 30°C/min to 150°C and was held for 1min. The split ratio was 50:1 with a helium flow of 1.5 ml/min. Acetone eluted at 3 min. The mass spectrometer was operated in the electron impact mode (70 eV) and selective ion monitoring of m/z 58 and 59 was performed by using a dwell time of 10 ms/ ion.

Protein Extraction and Quantification

Proteins were extracted from muscle samples as previously described (Camera et al., 2017; Hesketh et al., 2020). Muscle samples were ground in liquid nitrogen, then homogenized on ice in 10 volumes of 1 % Triton X-100, 50 mM Tris, pH 7.4 (including complete protease inhibitor; Roche Diagnostics, Lewes, United Kingdom) using a PolyTron homogenizer (KINEMATICA, PT 1200 E) followed by sonication (Fisherbrand™). Homogenates were incubated on ice for 15 minutes, then centrifuged at 1000 x g, 4 °C, for 5 minutes to fractionate myofibrillar (pellet) from soluble (supernatant) proteins. Myofibrillar proteins were resuspended in a half-volume of homogenization buffer followed by centrifuged at 1000 x g, 4 °C, for 5 minutes. The washed myofibrillar pellet was then solubilized in lysis buffer (7 M urea, 2 M thiourea, 4% CHAPS, 30 mM Tris, pH 8.5). Aliquots of protein were precipitated in 5 volumes of ice-cold acetone and incubated for 1 h at -20 °C, and resuspended in 200 uL of lysis buffer. Proteins were cleared by centrifugation at 12,000 x g, 4 °C, for 45 minutes. Total

protein concentration (µg/µl) was quantified against bovine serum albumin (BSA) standards using the Bradford assay (Thermo Scientific, #23236), according to the manufacturer's instructions.

Protein Digestion

Tryptic digestion was performed using the filter-aided sample preparation (FASP) method (Wiśniewski et al., 2009). Aliquots containing 100 µg protein were washed with 200 µl of UA buffer (8 M urea, 100 mM Tris, pH 8.5). Proteins were incubated at 37 °C for 15 minutes in UA buffer containing 100 mM dithiothreitol followed by incubation (20 min at 4 °C) protected from light in UA buffer containing 50 mM iodoacetamide. UA buffer was exchanged for 50 mM ammonium bicarbonate and sequencing-grade trypsin (Promega, Madison, WI, USA) was added at an enzyme to protein ratio of 1:50. Digestion was allowed to proceed at 37 °C overnight then peptides were collected in 100 µl of 50 mM ammonium bicarbonate containing 0.2 % (v/v) trifluoroacetic acid. Samples containing 4 µg of peptides were de-salted using C18 Zip-tips (Millipore Billercia, MA, USA) and eluted in 40% (v/v) acetonitrile and 0.1 % (v/v) trifluoroacetic acid. Peptides were dried by vacuum centrifugation and resuspended in 20 µl of 2.5 % (v/v) acetonitrile, 0.1 % (v/v) formic acid containing 10 fmol/ µl yeast alcohol dehydrogenase (MassPrep standard; Waters Corp., Milford, MA).

Liquid Chromatography-Mass Spectrometry Analysis

Peptide mixtures were analysed using an Ultimate 3000 RSLC nano liquid chromatography system (Thermo Scientific) coupled to Q-Exactive orbitrap mass spectrometer (Thermo Scientific). Samples were loaded on to the trapping column (Thermo Scientific, PepMap^{TM} 100, 5 µm C18, 300 µm X 5 mm), using ulPickUp injection, for 1 minute at a flow rate of 25 µl/min with 0.1 % (v/v) TFA and 2% (v/v) ACN. Samples were resolved on a 500 mm analytical column (Easy-Spray C18 75 µm, 2 µm column) using a gradient of 97.5 % A (0.1 % formic acid) 2.5 % B (79.9 % ACN, 20 % water, 0.1 % formic acid) to 50 % A 50 % B over 150 min at a flow rate of 300 nl/min. The data-dependent selection of the top-10 precursors selected from a mass range of m/z 300-1600 was used for data acquisition consisted of a 70,000-resolution full-scan MS scan at m/z 200 (AGC set to 3^{e6} ions with a maximum fill time of 240 ms). MS/MS data were acquired using quadrupole ion selection with a 3.0 m/z window, HCD fragmentation with a normalized collision energy of 30 and in the orbitrap analyzer at 17,500-resolution at m/z 200 (AGC target 5^{e4} ion with a maximum fill time of 80 ms). To avoid repeated selection of peptides for MS/MS, the program used a 30 s dynamic exclusion window.

Measurement of protein turnover rates

Protein fractional turnover rates (FSR) were calculated consistent with our previous work (Hesketh et al., 2020). Mass isotopomer abundance data were extracted from MS spectra using Progenesis QI (Nonlinear Dynamics, Waters Corp., Newcastle, UK). The abundance of m_0–m_3 mass isotopomers was collected over the entire chromatographic peak for nonconflicting peptides that were used for label-free

quantitation. Mass isotopomer information was processed in R version 4.0.3 (R core team., 2016). Incorporation of deuterium into newly synthesized protein causes a decrease in the molar fraction of the monoisotopic (m_0) peak.

$$fm_0 = \frac{m_0}{m_0 + m_1 + m_2 + m_3}$$

Equation 1: fm_0 = molar fraction, m_0 = monoisotopic peak, m_1, m_2, m_3 = mass isotopomers 1 - 3.

Over the duration of the experiment, changes in mass isotopomer distribution follow a nonlinear exponential pattern as a result of the rise-to-plateau kinetics of D_2O-labelled amino acids into newly synthesized protein. The rate constant (k) for the decay of fm_0 was calculated as a first-order exponential spanning from the beginning (t) to end (t′) of the 24 h labeling period, using Equation (2).

$$k = \frac{1}{(t' - t)} \cdot -\ln\left(\frac{fm_{0t}}{fm_{0t'}}\right)$$

Equation 2: k = rate constant, t = first timepoint, t' = end timepoint, fm_{0t} = molar fraction at first timepoint, $fm_{0t'}$ = molar fraction at last timepoint.

The rate of change in mass isotopomer distribution is also a function of the number of exchangeable H sites, and this was accounted for by referencing each peptide sequence against standard tables (Price et al., 2012) that report the relative enrichment of amino acids by deuterium in humans. Peptide FSR was derived by dividing k by the molar percentage enrichment of ^2H in plasma (p) and the total number (n) of ^2H exchangeable H-C bonds in each peptide.

$$FTR = \frac{k}{(n \cdot p)}$$

Equation 3: k = rate constant, n = number of H-D exchange sites, p = precursor enrichment

The median FSR of peptides assigned to each protein was used to calculate the FSR for each protein in each individual sample. Decimal values were multiplied by 100 to give FSR in %/h.

Chapter 4:

Patterns of energy availability of free-living athletes display day-to-day variability that is not reflected in laboratory-based protocols: Insights from elite male road cyclists

This work was presented at the Future Physiology 2021 conference in poster format and is published in The Journal of Sports Sciences (2022):

Taylor, H. L., Garabello, G., Pugh, J., Morton, J., Langan-Evans, C., Louis, J., Borgersen, R., & Areta, J. L. (2022). Patterns of energy availability of free-living athletes display day-to-day variability that is not reflected in laboratory-based protocols: Insights from elite male road cyclists. *Journal of Sports Sciences, 40*(16), 1849–1856. https://doi.org/10.1080/02640414.2022.2115676.

4.1. Abstract:

The physiological effects of low energy availability (EA) have been studied using a homogenous daily EA pattern in laboratory settings. However, whether this daily EA pattern represents those of free-living athletes and is therefore ecologically valid is unknown. To investigate this, we assessed daily exercise energy expenditure, energy intake and EA in ten free-living elite male road cyclists (20 min Mean Maximal Power: 5.27 ± 0.25 $W \cdot kg^{-1}$) during seven consecutive days of late pre-season training. Energy intake was measured using the remote-food photography method and exercise energy expenditure estimated from cycling crank-based power-meters. Seven-day mean ± SD energy intake and exercise energy expenditure was 57.9 ± 10.4 and 38.4 ± 8.6 $kcal \cdot kg\ FFM^{-1} \cdot day^{-1}$, respectively. EA was 19.5 ± 9.1 $kcal \cdot kg\ FFM^{-1} \cdot day^{-1}$. Within-subjects correlation between daily energy intake and exercise energy expenditure was 0.62 (95% CI: 0.43 - 0.75; $P < .001$), and 0.60 (95% CI: 0.41 - 0.74; $P < .001$) between carbohydrate intake and exercise energy expenditure. However, energy intake only partially compensated for exercise energy expenditure, increasing 210 $kcal \cdot day^{-1}$ per 1000 $kcal \cdot day^{-1}$ increase in expenditure. EA patterns displayed marked day-to-day fluctuation (range: -22 - 76 $kcal \cdot kg\ FFM^{-1} \cdot day^{-1}$). The validity of research using homogenous low EA patterns therefore requires further investigation.

4.2. Introduction:

Laboratory-based research investigating the causal effects of low energy availability on endocrine, and physiological dysregulations has typically reduced energy availability to between 10 and 30 kcal•kg FFM^{-1}•day^{-1} for periods of three to six days, incorporating a steady pattern of LEA with no variation in EA values between days (Areta et al., 2021; Loucks, 2020). However, to date, there is limited evidence available systematically assessing the daily pattern of the two key parameters determining energy availability; namely EEE and dietary EI in athletes training under free living conditions. Thus, the patterns of EA that athletes experience during consecutive days of regular training remain poorly characterised, and there is little direct scientific evidence to support the idea that athletes with increased EEE experience reduced EA.

LEA, or insufficient dietary energy to maintain normal physiological function, is currently considered the key aetiological factor underpinning the *female-* and *male athlete triads*, and the *REDs* models (Areta et al., 2021). However, as reviewed by Areta et al. (2021) and Loucks (2020), most of the research describing the causal effects between LEA, endocrine and physiological dysregulations is based on well controlled laboratory-based studies in sedentary females, which induce a homogenous and constant state of LEA. Furthermore, limited field-based research available in athletic populations suggests that this pattern of energy availability does not reflect what athletes may be experiencing in the field (Heikura et al., 2019; Langan-Evans et al., 2021; Louis et al., 2020; Moss et al., 2020). For example, we have shown a high variability of EA with fluctuating daily training load in recent case-studies of a male combat sport athlete and a master's triathlete (Langan-Evans et al., 2021; Louis et al., 2020). Similar observations have also been reported in female football players (Moss et al., 2020), and in professional cyclists during competition (Heikura et al., 2019). However, the typical daily EA patterns of athletes under free-living conditions have not been addressed directly.

Thus, the potentially variable nature of daily EA in free-living athletes warrants further investigation. At present, the ecological validity of laboratory-based evidence establishing a causal link between EA and endocrine, metabolic, and physiological responses using constant exposure to LEA is unclear. Direct extrapolation of laboratory-based findings and EA threshold values to the field could therefore be troublesome (Heikura et al., 2021), given that the physiological responses to sustained LEA may not be the same as to when LEA is induced intermittently. Therefore, characterising the patterns of EA athletes typically experience in the field is pertinent to inform future laboratory-based LEA research. This will ensure that future experimental protocols more closely resemble field conditions, enhancing ecological validity.

With this in mind, we sought to characterise the relationship between EEE and EI, and the resultant variability in patterns of daily EA in athletes following their regular training and nutrition practices

during seven days of late pre-season training under free living conditions. We performed the current assessment on elite road-cyclists due to: 1) their typically high daily EEE (Jeukendrup et al., 2000), 2) the typically heterogenous nature of their daily EEE (Saris et al., 1989), and 3) the capacity to accurately estimate EEE in real-world conditions using crank-based power meters (Haakonssen et al., 2013). We hypothesised that athletes would fail to sufficiently match EI with EEE, with resultant EA patterns showing high day-to-day variability.

4.3. Methods:

Participants:

Ten highly trained elite road-cyclists of international standard, affiliated with a professional cycling team (continental level), took part in the assessment. Participant characteristics were as follows (mean ± SD): Age 22 ± 8 Years, Body Mass 75.1 ± 8.5 kg, Height 1.84 ± .05 m, absolute 5 min mean maximal power (*MMP*) 490 ± 46 W, absolute 20 min MMP 395 ± 37 W, relative 5 min MMP 6.54 ± .25 W/kg, relative 20 min MMP 5.27 ± .25 W/kg. Season's mean maximal power (MMP) values were determined from the athletes' online training logs (*TrainingPeaks*, Boulder, CO), based upon individual participants' power meter data (7 athletes = *Shimano*, Shimano Inc., Osaka, Japan; 2 athletes = *Quarq*, Quarq/SRAM LLC, Chicago, Ilinois, USA; 1 athlete = *4iiii*, 4iiii Innovations Inc. Cochrane, Alberta, Canada). Based upon the criteria presented by McKay et al. (2022b), all participants were classified as Tier 4 athletes.

Study Design:

Using an observational study-design, 10 male road cyclists completed a seven consecutive day period of assessments during a late pre-season training block. Participants' dietary and training habits were monitored remotely as part of athlete support undertaken by a professional cycling team over this period. Participants were following their coach's individualised training programme, and all participants were living and training separately from each other. For this specific cycling team, the 'late pre-season' training period is a phase of heavy training load (~20 hrs·week^{-1}, ~900 - 950 Training Stress Score [*TSS*]·week^{-1}) typically undertaken between January to March. This precedes a 'specific preparation' phase (~17 hrs·week^{-1}, ~850 - 900 TSS·week^{-1}) undertaken in March/April, prior to the start of the racing in the following weeks (data provided by team coach). Comparatively, the 'early pre-season' and annual (summary) training loads for the team were ~16 hrs·week^{-1}, ~750 - 800 TSS·week^{-1} and ~17 hrs·week^{-1}, ~700 - 750 TSS·week^{-1}, respectively. The current study is a retrospective analysis of data systematically collected in observation of the nutritional practices of our cohort of athletes. Individuals were encouraged to continue their normal dietary practices to provide a baseline assessment of their typical dietary intake. No experimental interventions were applied during the observation period. Ethical approval was granted by Liverpool John Moores University's Research Ethics Committee (Ref: 20/SPS/052).

Training:

All participants were undertaking late pre-season training and following individualised training plans (for training duration and intensity) designated by their team coach and there was no purposeful change of the training schedule during data collection. Weight-management was not an aim of the training period, as detailed by the head coach, and there was no direction to restrain dietary intake in combination with the training plans provided. The prescribed training plans varied between individuals but typically included six days of training, including interval sessions, low intensity short cycling sessions (< 3 hrs) and long cycling sessions (> 3 hrs), with one day of recovery. Most training sessions were allocated to road cycling. All training details were recorded online (*TrainingPeaks*, Boulder, CO, USA).

Quantification of Dietary Intake:

Self-reported energy and macronutrient intakes were assessed across the seven days using a modified version of the remote food photography method (RFPM), which has been shown to accurately measure the EI of free-living individuals (Martin et al., 2009). In short, athletes provided a photograph of their food and drink before and after consumption. Photographs were timestamped alongside a description of the food/drink (including information on quantities, brands, preparation, and cooking methods) and a known-size visual reference (e.g., credit card or tennis ball) and then sent to a smartphone app-based group chat (*WhatsApp*, Dublin, Ireland) with two trained researchers (athlete and two researchers per group).

Prior to data collection, all participants attended an online video meeting, during which the RFPM was explained in detail and all athletes were provided with the opportunity to ask questions. To ensure athletes did not omit any foods/drinks and increase the accuracy of the food records, researchers prompted the athlete for further information on any items that were difficult to identify, but no feedback was provided regarding type and/or quantity of foods selected during recording. Dietary intake was analysed individually by the two members of the research team (< 2 years' experience as a registered nutritionist) assigned to that participant, using dietary analysis software (*Nutritics*^{TM}, Nutritics Ltd. Dublin, Ireland), which calculated energy and macronutrient intake for each athlete. A third practitioner checked all inputs independently. Output values from the two practitioners were averaged to provide estimates of EI in kilocalories per day ($kcal \cdot day^{-1}$) and macronutrient intakes, reported in grams (g) and grams per kg body mass ($g \cdot kg^{-1}$).

To further assess the athlete nutrition behaviour, dietary intake data was also stratified into four categories dependent on daily training volume, based upon the current consensus of carbohydrate requirements for training load (Thomas et al., 2016). Based upon these guidelines, data were grouped into intakes from: rest days (< 45 min exercise), moderate training days (45 – 90 mins exercise), high training days (90 – 210 mins exercise) or very high training days (>210 mins exercise).

Exercise Energy Expenditure:

Metabolic energy expenditure was estimated from mechanical work values recorded from the participant's crank-based power meters, using validated methodology (Haakonssen et al., 2013). Whilst acknowledging the existence of inter-individual variation in athletes, an estimated gross efficiency (GE) of 20% was used for all cyclists for the calculation of metabolic energy expenditure. This GE value was based upon the assumption that average GE would be equal to 20%, in line with reported values from similar populations (Coyle et al., 1992; Moseley et al., 2004). EEE from non-cycling sessions was estimated using the compendium of physical activity (Ainsworth et al., 2000) using the corresponding exercise type, multiplied by the session duration.

Calculation of Energy Availability:

Energy availability was calculated using the most recent definition of EA, [EA = *(Energy Intake – Net Exercise Energy Expenditure) / Fat Free Mass*] (Areta et al., 2021; Loucks et al., 1998). Net EEE (EEE_{net}) was estimated by subtracting the contribution of estimated RMR (Harris & Benedict, 1918) from calculated gross EEE (EEE_{Gross}), based upon exercise session duration. Due to limitations in access to equipment, only body mass data was accessible for anthropometric assessment of the cyclists. Participants were asked to measure their body mass at home (various weighing-scale brands, undisclosed) in the morning upon waking, after voiding, on the first day and immediately after the assessment period. FFM was calculated based upon an assumed 13% body fat for all participants. Thirteen percent body fat was selected based upon reference data from the DXA scans of five former team athletes, averaging 12.8% body fat, alongside reported body fat percentages ranging from 9 – 14% in similar populations (Campion et al., 2010; Klomsten Andersen et al., 2018).

Statistical Analysis:

Statistical analyses were conducted using IBM SPSS Statistics (v. 28.0.0.0, IBM, Armonk, NY, USA) and Graphpad Prism (v. 8.2.1, Graphpad Software Inc., San Diego, California, USA). Changes in body mass were explored using a paired t-test. Within-subject correlations (Bland & Altman, 1995) were calculated using a general linear model to assess the relationships over time between total EEE with EI, carbohydrate, fat and protein intakes. Magnitudes of correlation were classified as: r = 0.1 – 0.29 = small; 0.3 – 0.49 = moderate; 0.5 – 0.69 = large; 0.7 – 0.89 = very large; and 0.9 – 0.99 = extremely large (Hopkins et al., 2009). A sensitivity analysis was conducted on all within-subject correlations to ensure regression dilution was not unduly influencing the statistical models (Ludbrook, 1997). Furthermore, a sensitivity analysis was conducted on the within-subject correlations to ensure that a residual outlier did not unduly influence the estimates from the statistical models. Significance was set at $P < 0.05$ for all statistical tests and data are reported as means ± SDs.

4.4. Results:

Training:

Participants completed 8 ± 2 (Range: 6 - 11) training sessions during the 7-day observation period, amassing 21.5 ± 3.8 (Range: 16.0 - 26.4) hours of training with an average of 3.1 ± 0.5 hrs·day^{-1}. Most training hours (94.9% of total; 2.9 ± 0.6 hrs·day^{-1}) were road-cycling sessions, with 'other' training sessions (strength-training, yoga, walking, etc.) accounting for a minority of the total training time (5.1% of total; 0.2 ± 0.2 hrs·day^{-1}). Mean power output during cycling training sessions was 212 ± 35 W. Cycling-specific training accounted for 98.9% of EEE, with athletes expending 17199 ± 3421 kcal on the bike over the seven-day observation period during data collection. The remaining 1.1%, equating to 186 ± 259 kcal over the observation period, arose from 'other' training. Four out of the ten participants only performed cycling for their training sessions.

Energy Availability:

Daily EEE displayed a wide range from 0.0 to 99.8 kcal·kg FFM^{-1}·day^{-1}, averaging 38.4 ± 25.9 kcal·kg FFM^{-1}·day^{-1}. EI displayed a narrower range from 28.5 to 95.0 kcal·kg FFM^{-1}·day^{-1}, with average values of 57.9 ± 13.3 kcal·kg FFM^{-1}·day^{-1}. Accordingly, EA was highly variable, with values ranging from -21.9 to 76.0 kcal·kg FFM^{-1}·day^{-1}. Mean daily EA was 19.5 ± 22.0 kcal·kg FFM^{-1}·day^{-1} for all athletes and the 7-day average within athletes was 19.5 ± 9.1 kcal·kg FFM^{-1}·day^{-1}.

There was a large positive within-subjects correlation between EEE and EI (r = 0.62; 95% CI: 0.43 – 0.75; P < 0.001; Slope = 0.21, 95% CI 0.14 – 0.28. Figure 4.4.1.). This would translate into an increase in athlete EI of 210 kcal·day^{-1}, for every 1000 kcal·day^{-1} increase in EEE. Figure 4.4.2. highlights the effects of this mismatch, providing a summary graph for each participant's EI, EEE and resultant EA over the observation period.

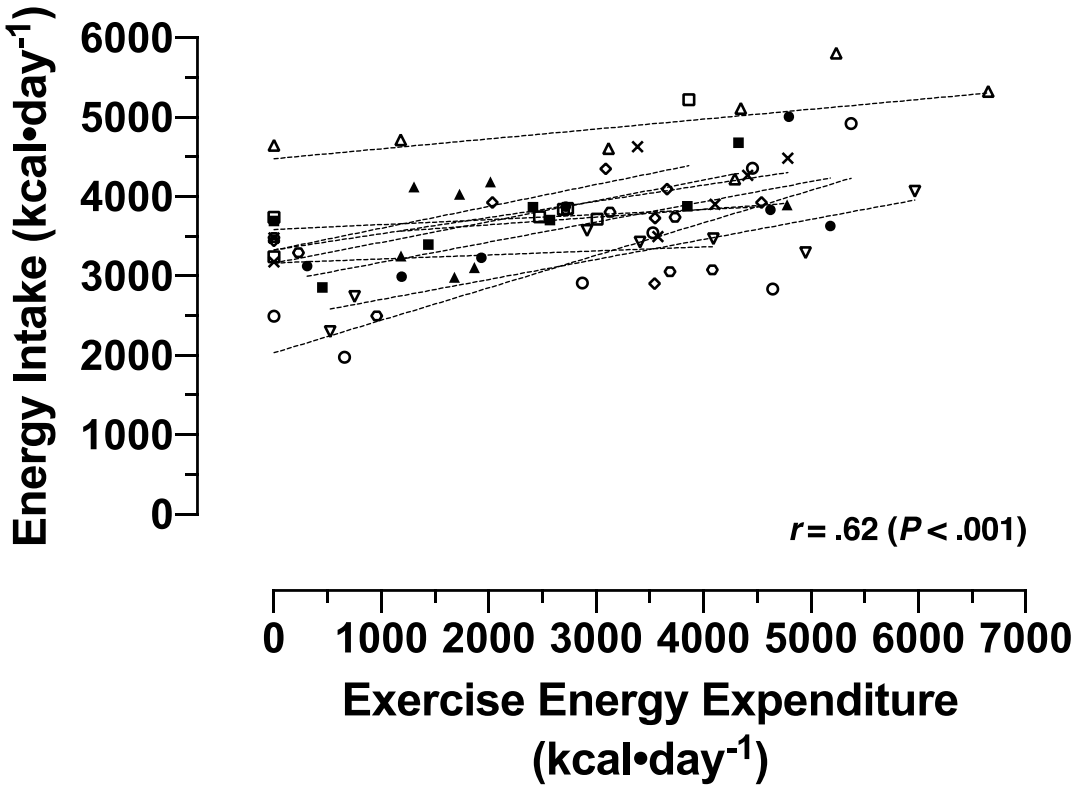


Figure 4.4.1. Within-subjects relationship between exercise energy expenditure and energy intake in elite male road-cyclists over seven days of late pre-season training. Within-subject (n = 10) correlations are represented by dashed lines and individual symbols (r = 0.62; 95% CI: 0.43 – 0.75; P < 0.001; Slope = 0.21, 95% CI 0.14 – 0.28).

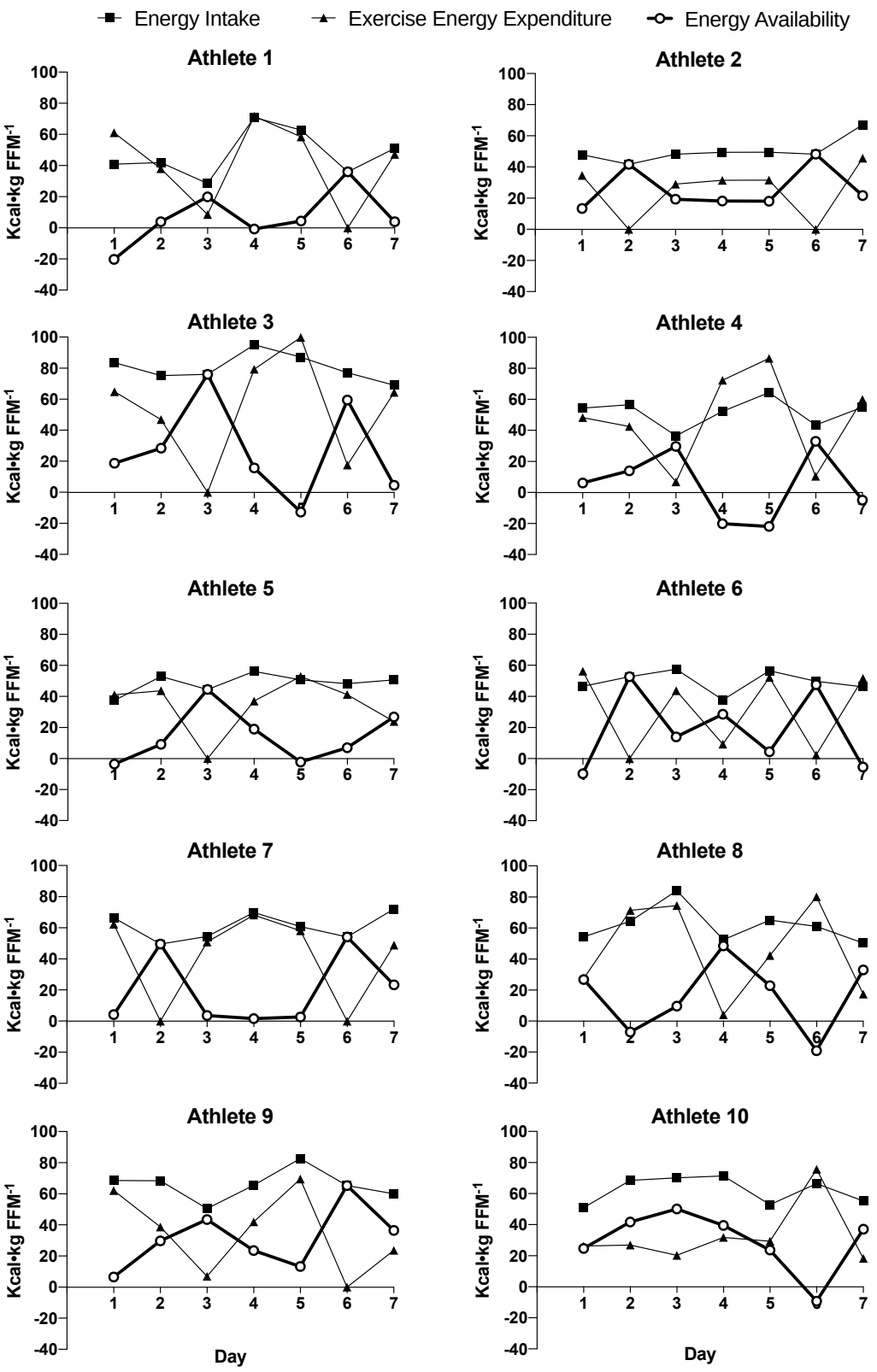


Figure 4.4.2. Individual participants' daily energy intake (thin black line, square markers), exercise energy expenditure (thin black line, triangular markers) and energy availability (thick black line, circular markers) over seven days of late pre-season training.

Macronutrient Intake:

Carbohydrate, fat, and protein accounted for 54.1 ± 8.0, 28.7 ± 7.0 and 17.2 ± 3.9 % of daily EI, respectively throughout the assessment period. In relative terms, carbohydrate, fat, and protein intakes were 6.9 ± 2.2 $g \cdot kg^{-1} \cdot day^{-1}$, 1.6 ± 0.5 $g \cdot kg^{-1} \cdot day^{-1}$ and 2.1 ± 0.6 $g \cdot kg^{-1} \cdot day^{-1}$, respectively. We observed a large positive within-subject relationship between EEE and energy intake from carbohydrates (r = 0.60; 95% CI: 0.41 – 0.74; P < 0.001; Slope = 0.16, 95% CI 0.10 – 0.21). This translates into an increased carbohydrate intake of 160 $kcal \cdot day^{-1}$, for every 1000 $kcal \cdot day^{-1}$ increase in EEE (Figure 4.4.3.A.). Conversely, only a small and not statistically significant within-subjects correlation was apparent between EEE and fat intake (r = 0.21; 95% CI: -0.04 – 0.44; P = 0.099; Slope = 0.03, 95% CI -0.01 – 0.07. Figure 4.4.3.B.), and between EEE and protein intake (r = 0.24; 95% CI: -0.01 – 0.47; P = 0.058; Slope = 0.02, 95% CI -0.00 – 0.04. Figure 4.4.3.C.).

Whilst athletes partially compensated for increasing EEE with increased EI from carbohydrate (CHO), on the majority of training days they failed to fall within the recommended CHO intake guidelines (Thomas et al., 2016) (Figure 4.4.4.). This is reflected in under-consumption of CHO on 1 out of 9 (11%) 'Rest' days, 3 out of 8 (38%) 'Moderate', 7 of 23 (30%) of 'High' and 21 of 30 (70%) 'Very High' volume training days. In contrast, the athletes consumed CHO exceeding the recommended intake (3 – 5 $g \cdot kg^{-1} \cdot day^{-1}$) on 6 out of 9 (67%) 'Rest' days.

Body mass:

Body mass reduced from 75.1 ± 8.5 kg to 74.7 ± 8.5 kg (Δ -0.4 ± 0.4 kg; P < 0.05) between the first day of the assessment and immediately upon finishing the assessment.

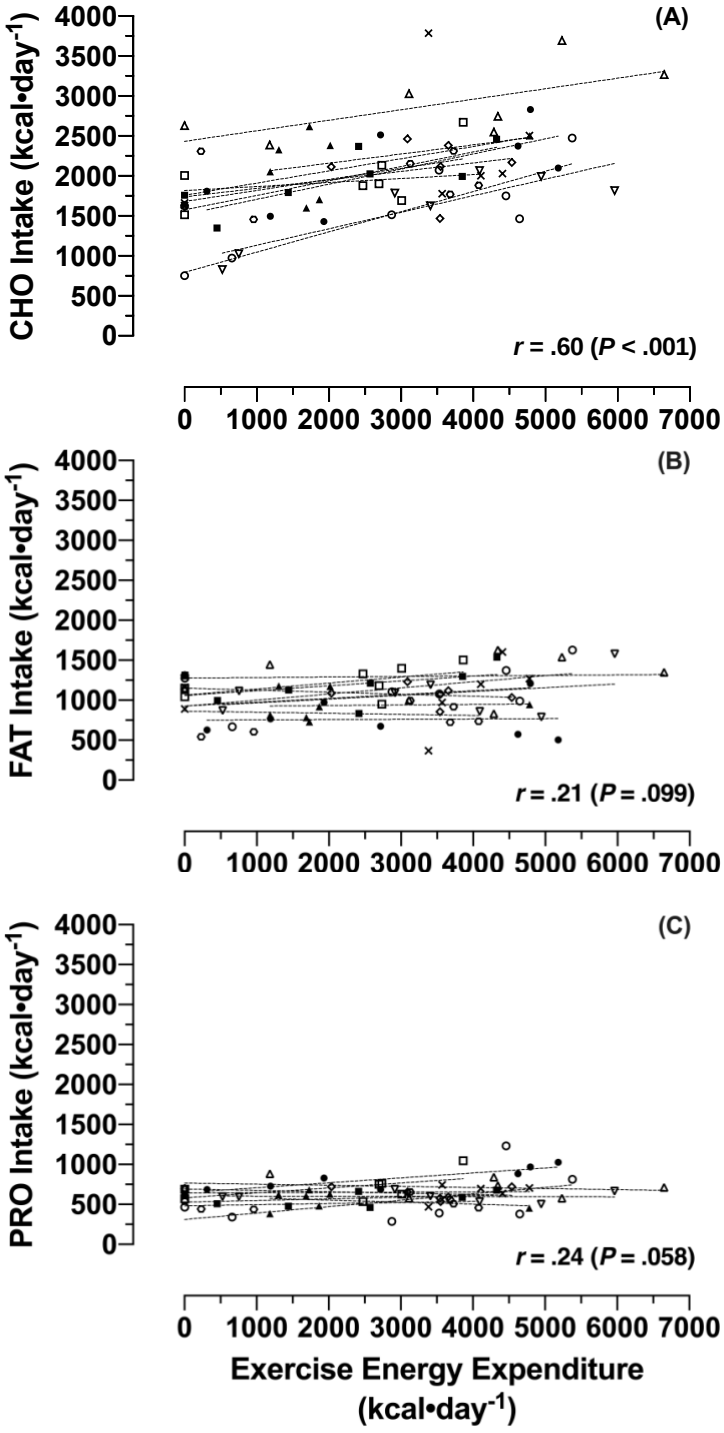


Figure 4.4.3. Within-subject relationships across the 7-day monitoring period between exercise energy expenditure and: (A) carbohydrate intake (*CHO:* r = 0.60; 95% CI: 0.41 – 0.74; P <.001; Slope = 0.16, 95% CI 0.10 – 0.21), (B) fat intake (*FAT:* r = 0.21; 95% CI: -0.04 – 0.44; P = 0.099; Slope = .03, 95% CI -.01 – .07), (C) protein intake (*PRO:* r = .24; 95% CI: -.01 - .47; P = .058; Slope = 0.02, 95% CI -0.00 – 0.04). Within-subject (n = 10) correlations are represented by dashed lines and individual symbols.

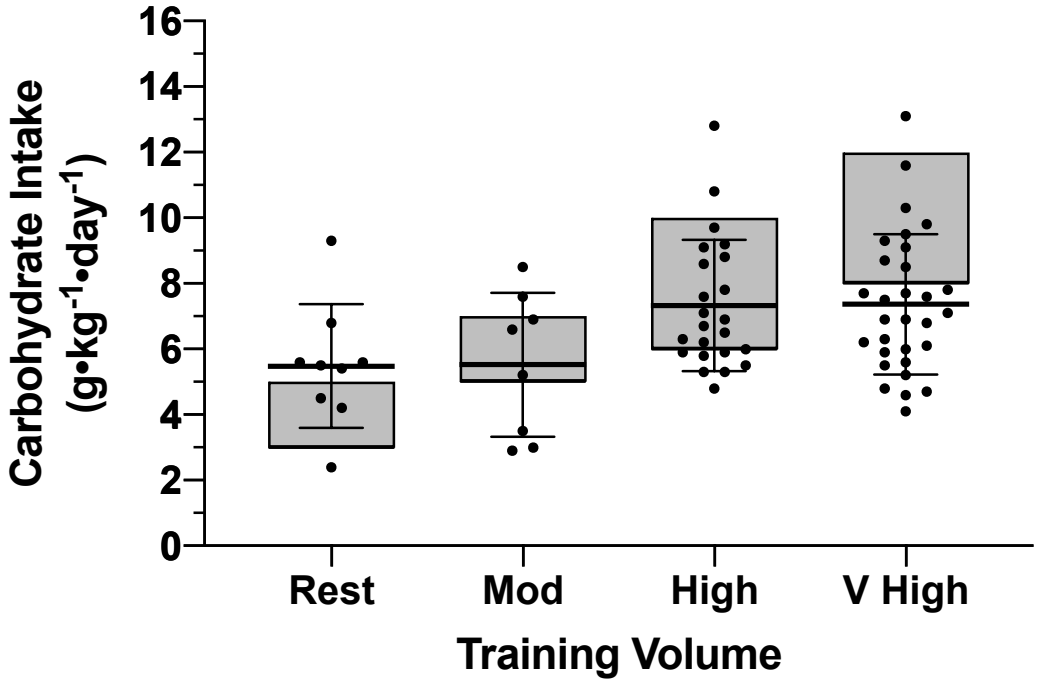


Figure 4.4.4. Relative carbohydrate intake of the cohort of elite male road cyclists, stratified by daily training volume (Rest: < 45 mins training; Mod (moderate): 45 – 90 mins training; High 90 – 210 mins training; V. High (Very High): > 210 mins training). Shaded grey boxes denote recommended carbohydrate intake for the daily training load, based upon the guidelines of Thomas et al. (2016). Individual dots represent daily values, central line average and error bars standard deviations**.**

4.5. Discussion:

The aim of this study was to assess the relationship between EEE and EI and determine daily patterns of EA in a cohort of free-living road cyclists during late pre-season training. In agreement with our hypothesis, athletes failed to compensate for increases in EEE with EI (Figure 4.4.1). This resulted in high heterogeneity of day-to-day EA, with EA values ranging from -21.9 to 76.0 kcal·kg FFM^{-1}·day^{-1} (Figure 4.4.2). Within the scope of the measurements employed in this study, these fluctuations appear to be dictated by daily changes in EEE. From a practical perspective, our data demonstrate that this cohort of athletes do not adjust daily EI in accordance with fluctuations in daily training volume. As such, these data also suggest that laboratory models of LEA are likely not representative of the patterns of LEA experienced by elite athletes. These findings highlight the need for further research regarding the effect of intermittent vs continuous LEA upon endocrine, metabolic, and physiological responses. Such research would provide insights into the ecological validity of existing laboratory-based studies on LEA.

This study provides a detailed characterisation of daily EA patterns across seven uninterrupted days of late pre-season training of elite athletes under free-living conditions using estimations of EEE and EI. The high ecological validity of the setting in which our assessments were conducted provide greater depth to our understanding of the daily EA patterns that elite athletes are likely to experience in the field. Whilst we have shown a positive relationship between EEE and EI, compensatory EI appeared insufficient to offset days of high EEE, as reflected by the heterogenous nature of recorded EA values ranging from -21.9 – 76 kcal·kg FFM^{-1}·day^{-1}. This is in agreement with the findings of previous research observing significantly lower EA values (Heikura et al., 2019; Langan-Evans et al., 2021; Moss et al., 2020), or greater energy deficits (Vogt et al., 2005), on days in which athletes had greater EEE through training and/or competition. These data, in conjunction with our findings, suggest that increasing EEE was the cause of variation in daily EA values. Athletes in the field seem to experience high variation in day-to-day EA values based on their daily training load (Figure 4.4.2). The fluctuating patterns of EA observed therefore indicate a need for laboratory-based studies investigating the relationship between LEA and physiological dysregulations to adapt future methodology to more closely reflect the patterns of EA that athletes experience daily.

The mismatch between EEE and EI may be due to a lack of compensatory increases in appetite to drive greater EI in response to increasing EEE (Loucks, 2004; Thivel et al., 2021; Westerterp & Saris, 1991). Despite a large positive within-subject correlation between EEE and EI (Figure. 4.4.1), there was still a large gap between the two parameters, with each 1000 kcal·day^{-1} increase in EEE corresponding to only a 210 kcal·day^{-1} increase in EI. In healthy adult males, acute energy deficits induced through exercise have been shown as unable to trigger compensatory increases in *ad libitum* EI over two days (King et al., 1997), three days (Cameron et al., 2016) and over seven days (Stubbs et al., 2002). This idea is also in line with the findings of (Edholm et al., 1970) who reported no discernible relationship between energy expenditure and EI across three non-consecutive weeks of initial training in army recruits. The authors also noted that days of high energy expenditure tended to lead to suppressed food intake, with energy expenditure exceeding EI on 70% of days classified as high energy expenditure. However, longer (14-day) periods have also shown increased compensation of EI of up to 30%, albeit with high variability between subjects (Whybrow et al., 2008). Nonetheless, given that the population of our study are typically highly conscious of body mass (Hoon et al., 2019) we cannot overlook the possibility that athletes may have restrained EI despite an increase in hunger. Moreover, the measurement of dietary intake may have affected EI (Stubbs et al., 2014), even if participants were instructed to continue their regular nutritional practices. Therefore, either appetite cues were insufficient to drive greater EI as EEE increased, or other factors such as body composition management, lack of nutritional knowledge, reduced opportunities to feed (Burke, Close, et al., 2018), or reporting of dietary intake (Stubbs et al., 2014) inhibited our cohort's EI.

Further analysis of the data shows that the lack of adjustment of daily EI in these athletes made them largely fall outside the nutritional guidelines for carbohydrate intake, particularly on the days classified as 'Rest' days and 'Very High' training days (Figure 4.4.4). Though there was a small increase in carbohydrate intake with increasing EEE (Figure 4.4.3), the athletes consumed excess carbohydrate on 67% of 'Rest' days and insufficient carbohydrate on 30% of 'High' and 70% of 'Very High' training volume days, relative to guideline recommendations (Thomas et al., 2016). Therefore, in the majority of the cases our cohort failed to adequately match their carbohydrate intake to their training volume in accordance with contemporary nutrition guidelines (Burke, Hawley, et al., 2018; Impey et al., 2018; Stellingwerff, 2018; Thomas et al., 2016). Collectively, these findings suggest that when elite endurance athletes are left to their own means in relation to their nutrition, they fail to ingest carbohydrates in line with current guidelines, resulting in lower energy (and carbohydrate) availability when undertaking a higher training volume. Whilst the findings of this study are based upon observations from one group of individuals, the data may be underpinned by their philosophy, eating patterns and culture. In line with the Capability, Opportunity, and Motivation Behaviour (COM-B) model of behaviour change (Michie et al., 2011), we speculate that if high carbohydrate availability and energy balance are desired, then the right fuelling culture and philosophy must be present in the team. Similarly, ease of access to carbohydrate-rich foods is important, alongside motivating athletes to increase their carbohydrate intake during heavy training load, as has been suggested previously (Burke, Lundy, et al., 2018). In support of this, Charlot et al. (2021) recently demonstrated that the provision of familiar and hyper-palatable foods, in conjunction with extra-time allowance for eating, lead to augmented caloric intake that maintained energy balance in a cohort of 12 soldiers completing a 15-day cold-weather expedition. Facilitating food access (both physical and temporal) therefore appears to positively influence energy intake, despite high daily EEE.

Whilst we consider that the current research provides clear results and new insights into the daily patterns of EA of athletes under free-living conditions, we acknowledge some limitations in the assessment of EI and EEE. Despite providing insights into a rarely accessible elite population of tier-4 athletes (McKay et al., 2022b) care should be taken in drawing conclusions from some data (e.g., changes in bodyweight) given the relatively low number of athletes recruited. Specifically, we are aware of the tendency for individuals to under-eat and under-report when dietary intake is assessed by participant dietary reports (Stubbs et al., 2014). Similarly, we acknowledge the noise introduced by the assumptions we have used to estimate body composition (we estimate this induces an uncertainty of ~3 – 6 kcal·kg FFM^{-1}·day^{-1} in the EA values, compared to the 'real' EA) and to extrapolate gross efficiency during exercise for the sake of the accuracy of measurements (Haakonssen et al., 2013). We have therefore taken care with our data so as not to over-interpret our findings, as well as not to classify specific daily EA values as 'low' or 'adequate'/'normal'. This is partly because what represents 'low' energy availability in males, or for how long this state is required to result in a physiological response,

is not yet established (Areta et al., 2021). Additionally, we also acknowledge that there may be some discrepancies from the values estimated herein and the 'actual' energy availability had this been measured under laboratory conditions as originally defined. Nonetheless, the high ecological validity of the assessments, such as the direct measurement of mechanical power output and the considered assessment of EI using the RFPM provide a solid methodological foundation. Whilst this approach may have introduced some error, this is likely to be systematic in nature. The methodology employed therefore provides an adequate framework to determine the relationship between EEE and EI, and the resultant daily patterns of EA in this cohort. Furthermore, beyond the use of weighed food intakes, we are unaware of alternative methodologies that would result in drastically improved estimates of the daily EA of athletes in free living conditions.

In conclusion, our findings provide a clear message for future laboratory-based research on energy availability as well as for applied field work with athletes. To increase ecological validity, laboratory-based studies investigating the impact of LEA upon athlete health and well-being should seek to investigate the impact of heterogenous EA patterns during periods of LEA on its endocrine, metabolic, and physiological effects. In relation to field work, athletes and support personnel should be conscious that periods with high training loads can be more susceptible to reduced energy availability. At present, the endocrine, metabolic, and physiological effects of periods of 'low' energy availability are poorly characterised in males and it is also unclear to what extent they are necessary to exert an adaptative stimulus. However, a greater understanding of the EA patterns observed during periods of increased EEE with *ad libitum* EI, as shown in this study, will facilitate the development of strategies that optimise athlete training and nutrition practices. Future studies should aim to determine the effect of intermittent 'low' energy availability on physiological responses in well-controlled settings.

Chapter 5:

Short-term low energy availability down-regulates circulating triiodothyronine and bone formation markers in healthy active males, whilst total testosterone and skeletal muscle protein synthesis is preserved

This work was presented in poster and oral format at the Department of Nutrition, Exercise, and Sports (NEXS) Grand Depart Symposium: 'Exercise Training and Performance of the Top-Class Athlete', Copenhagen, 2022, as well as at the International Sport and Exercise Nutrition Conference (ISENC), Manchester 2022, in poster format.

This study was also presented orally at the European College of Sport Sciences (ECSS) conference, Paris 2023 was awarded joint-fifth place in the ECSS 2023 Young Investigator Award and joint-fourth place in the Gatorade Sport Science Institute (GSSI) Sports Nutrition Award session.

5.1. Introduction:

Research establishing the physiological effects of low energy availability under well-controlled laboratory-based conditions has predominantly been conducted in female participants, whilst evidence to support the extension of LEA-related sequelae to male athletes is largely cross-sectional or observational in nature (Areta et al., 2021). Exercising males deemed 'at risk' of experiencing low energy availability have been reported to display signs of disruption to the HPG axis (Nattiv et al., 2021), including reduced sperm count, motility, and density (De Souza et al., 1994), acute hypogonadism, and/or the EHMC (Hackney, 2020). However, there is limited experimental data linking LEA in males to perturbations of hormones of the HPG axis and other endocrine markers associated with energy preservation, such as T_3, IGF-1, and leptin. Moreover, findings from the few male-specific studies investigating the physiological effects of acute low energy availability under controlled conditions are somewhat equivocal (See Chapter 2.8).

In controlled studies in females, exposure to ≤ 30 kcal•kg FFM^{-1}•day^{-1} EA (typically referred to as 'LEA') for 3 - 6 days shows clear disruption of the endocrine milieu (see Chapter 2.8). However, whether low energy availability can affect the male endocrine milieu and the degree of low energy availability necessary to do so is unclear. Koehler et al. (2016) assessed the physiological effect of four-days of LEA at 16 kcal•kg FFM^{-1}•day^{-1} in six healthy males, finding that LEA reduced leptin concentrations, but did not affect total testosterone, IGF-1 or free T_3. Similarly, three days of LEA at 19 kcal•kg FFM^{-1}•day^{-1} did not reduce free testosterone or IGF-1 compared to a control trial in seven healthy well-trained runners (Kojima et al., 2020). Mckay et al. (2022a) observed reduced testosterone concentrations across all three intervention groups in elite male race walkers following six days of training (~19 km•day^{-1}) with either adequate energy and carbohydrate availability (control: 40 kcal•kg FFM^{-1}•day^{-1}), an isocaloric LCHF LEA (< 50 g•day^{-1} CHO) diet, or an LEA diet (15 kcal•kg FFM^{-1}•day^{-1}). The consistent reduction in testosterone concentrations across intervention groups suggest that factors beyond energy availability, such as the stress of exercise, contribute to this effect. As summarised in the *Male Athlete Triad*, whether low energy availability elicits perturbation of the HPG axis and endocrine homeostasis in males therefore remains unclear (Nattiv et al., 2021).

Evidence of LEA-induced disruption to markers of bone health, another key aspect of both the *Male Athlete Triad* (Nattiv et al., 2021) and *REDs* (Mountjoy et al., 2023) models, is similarly inconclusive. Papageorgiou et al. (2017) reported no changes in concentrations of ß-CTx or P1NP in a cohort of 11 males exposed to five days of LEA at 15 kcal•kg FFM^{-1}•day^{-1}. Similarly, in a mixed cohort of 5 males and 2 females, no overall changes in P1NP concentrations were observed following three days of LEA (15 kcal•kg FFM^{-1}•day^{-1}) induced via dietary restriction (Murphy & Koehler, 2020). However, five days of LEA (15 kcal•kg FFM^{-1}•day^{-1}) increased ß-CTx, and reduced P1NP, IGF-1, and leptin concentrations, indicating LEA-induced alterations to endocrine and bone status in a cohort of seven

healthy, active, males (Murphy et al., 2021). More studies are therefore required to develop our understanding of the influence of LEA upon markers of bone resorption and formation in males.

Finally, there is little data characterising the influence of LEA upon skeletal muscle tissue. Two similar studies have demonstrated that acute LEA (< 20 kcal•kg FFM^{-1}•day^{-1}) reduced skeletal muscle glycogen concentrations in trained males (Ishibashi et al., 2020; Kojima et al., 2020), however this had no effect upon time to exhaustion running compared to normal energy availability (Kojima et al., 2020). Furthermore, five days of LEA (30 kcal•kg FFM^{-1}•day^{-1}) has been shown to reduce resting rates of MPS in a mixed-sex cohort, yet this was reversed by a single bout of resistance exercise and additional protein supplementation even increased MPS rates above resting values (Areta et al., 2014). Despite the importance of skeletal muscle upon health and performance (Wolfe, 2006), our understanding of the impact of LEA upon this tissue in males is limited (Pasiakos et al., 2015).

This study therefore aimed to investigate the endocrine, metabolic, and physiological effects of five days of low energy availability (10 kcal•kg FFM^{-1}•day^{-1}), compared to 'adequate' energy availability (45 kcal•kg FFM^{-1}•day^{-1}) in a cohort of healthy active males. Considering our observations in chapter 4, we aimed to utilise a non-homogenous pattern of EA to reflect the dietary practices of free-living athletes more closely. We hypothesised that five-days of LEA would reduce circulating total testosterone and other key endocrine and metabolic markers associated with energy preservation, including T_3, leptin, IGF-1, insulin, glucose, and resting metabolic rate. We also hypothesised that bone re-modelling markers ß-CTx and P1NP would increase and decrease, respectively, indicating a shift towards a negative balance in bone turnover. Body mass, fat mass, and fat free mass were expected to reduce following the LEA intervention. Finally, we hypothesised that acute low energy availability with concomitant aerobic exercise would lead to reduced muscle glycogen content and MPS rates, compared to the normal energy availability intervention.

5.2. Methods:

Participants:

Ten healthy, active males were recruited via convenience sampling from the staff and student body of Liverpool John Moores University to participate in the study. Ethical approval was granted by the NHS North-West – Liverpool Central Research Ethics Committee (Ref: 21/NW/0205) and registered on the clinical trials register (ClinicalTrials.gov ID: NCT05203133). Participant inclusion criteria are outlined in Chapter 3 (General methods).

Participant characteristics are outlined in Table 5.2.1. Based upon the criteria presented by McKay et al. (2022b), participants were classified as Tier 1-2 individuals.

Table 5.2.1. Participant descriptive characteristics

N	**Age** (yrs)	**Height** (m)	**Body Mass** (kg)	**Body Fat** (%)	$\dot{V}O_{2max}$ ($ml \cdot kg^{-1} \cdot min^{-1}$)	**Peak Power** (W)
10	25 ± 5	1.80 ± 0.06	78.8 ± 8.2	17.6 ± 3.4	51.7 ± 7.7	304 ± 62

Study Design:

Using a quasi-experimental study design, all participants completed baseline testing and assessment, followed by a study intervention-period comprising 15 consecutive days. The intervention-period consisted of three consecutive 5-day periods of, 1) Free-Living (*FREE*: Days -5 to -1); 2) Energy Balance (*EB*: Days 0 to 5); and 3) Low Energy Availability (*LEA:* Days 5 to 10). An outline of the study intervention and sample collection schedule is outlined in Figure 5.2.1. A brief description of each parameter assessed is outlined below. A full description of each protocol can be found in Chapter 3: General Methods.

Pre-participation screening:

Interested individuals were informed of the study's risks and benefits and provided written informed consent. Participants then completed an allergen screening questionnaire, readiness to exercise questionnaire, pre-biopsy medical questionnaire and the eating attitude test (*EAT-26*) (Garner et al., 1982). Provided a participant did not report any contra-indications in the screening process, they were enrolled into the study for baseline testing.

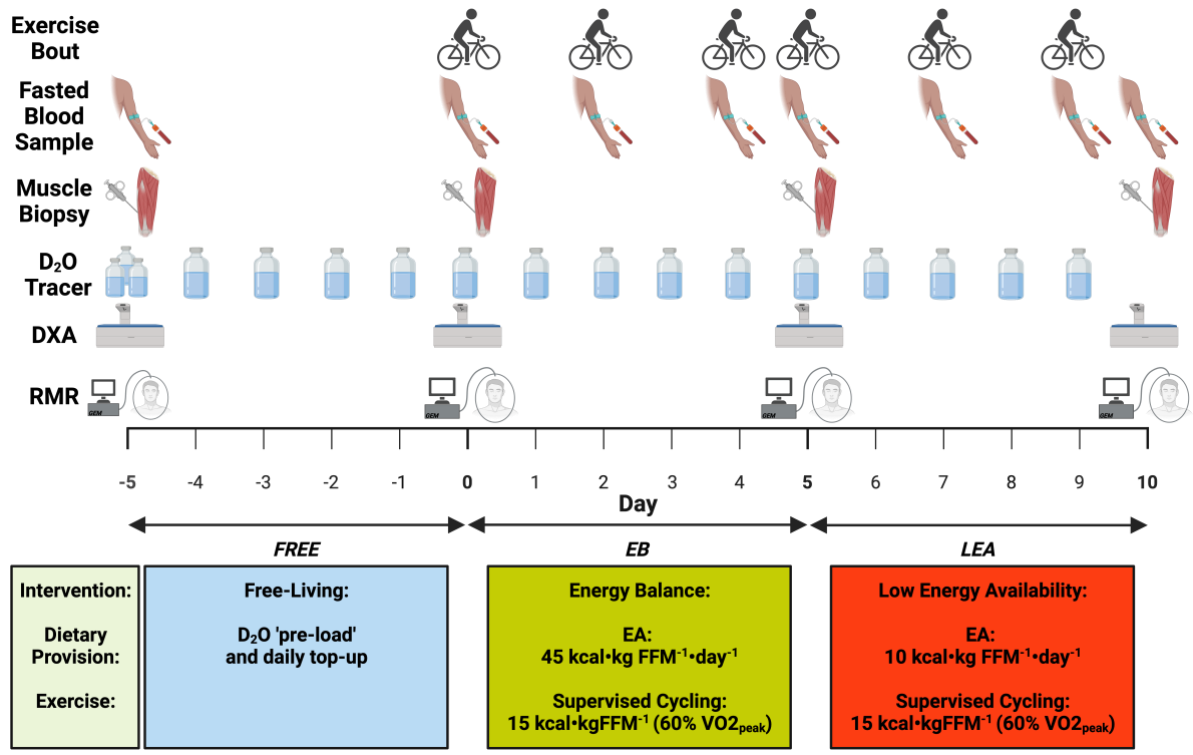


Figure 5.2.1. Schematic overview of quasi-experimental study design. Participants completed three consecutive 5-day periods of 1: Free-living (*FREE*), 2: Energy Balance (*EB*), and 3: Low Energy Availability (*LEA*). All dietary intake was provided during EB and LEA. RMR = resting metabolic rate, DXA = Dual X-ray Absorptiometry, D_2O = Deuterium oxide tracer; 3 bottles: 500 ml D_2O *pre-load*, 1 bottle: 50 ml daily D_2O *top-up* dose.

Baseline Testing:

Participants attended the laboratory for a baseline test at least two days, and no more than seven days prior to their proposed start-date for the study intervention phase. The participant voided their bladder and body composition was assessed via BIA as outlined in Chapter 3 (General methods).

Participants were fitted with a Bluetooth heart rate (*HR*) monitor (Polar H7, Polar, Kempele, Finland) and set up on an electro-magnetically braked cycle ergometer (Lode Corival cpet: Lode, Groningen, Netherlands). Participants then performed a two-part incremental cycle test to determine sub-maximal ($\dot{V}O_2$) and maximal oxygen consumption ($\dot{V}O_{2max}$), lactate threshold and PPO followed by a brief 20-minute cycling familiarisation session, as outlined in Chapter 3 (General methods).

Study Intervention Phases (*FREE, EB, LEA*):

For all testing sessions, participants arrived between 07:00 and 08:00 hrs in a fasted state. Participants were explicitly requested to travel via public transport or car to the *RISES* laboratories. To monitor adherence, participants verbally confirmed that they had avoided active travel (e.g., walking, cycling,

etc.) to the laboratory, that they were in an overnight fasted state, and the approximate time of their last meal upon arrival.

Free-living phase (FREE: Day -5 to Day -1) - Diet and exercise monitoring:
Participants attended the laboratory on Day -5. Following the collection of all samples and completion of a deuterium oxide (*D_2O*) loading protocol (both outlined below), participants were free to leave the laboratory until Day 0. In this period, participants were 'free-living' and could continue with their routine dietary and training practices. During *FREE,* participants were asked to consume a 50 ml 'top-up' dose of D_20 daily.

EB (Day 0 to Day 5) and LEA (Day 5 to Day 10) – Energy Availability Manipulation:
Participants attended the laboratory on Day 0, following the completion of *FREE*. From day 0 to day 10, all food was provided to participants in custom-made, pre-packaged servings. Fat free mass as measured in the DXA assessment on Day -5 was used to calculate the required energy intake, exercise energy expenditure, and resultant energy availability for the intervention phase for each participant. Participants were instructed to refrain from consuming any other food or calorie containing drinks than those provided within the study intervention period. To promote compliance with the LEA phase in particular, calorie-free beverages and chewing gum of the participant's choice were permitted.

The dietary intervention and controlled exercise energy expenditure across days 0 to 10 was split into two distinct phases; Days 0 to 5 comprised the Energy Balance (*EB*) dietary provision, with days 5 – 10 providing low energy availability (*LEA*). Caloric intake was clamped at ~60% carbohydrate, 20% fat and 20% protein, across *EB* and *LEA*, with reduction in EI achieved via similar reduction in all macronutrient intakes. Other than manipulating energy availability via controlled alterations to energy intake, we aimed to keep all parameters of data collection, exercise provision, and macronutrient distribution consistent between *EB* and *LEA*.

Further details of the *EB* and *LEA* intervention phase diets are outlined in Table 5.2.2. (see next page) Briefly, the controlled dietary energy provision delivered a fixed daily energy intake of 54 kcal·kg FFM^{-1}·day^{-1} during *EB*, and 19 kcal·kg FFM^{-1}·day^{-1} during *LEA*. This dietary provision, alongside three cycling sessions to expend 15 kcal·kg FFM^{-1}·day^{-1} (outlined below) in both intervention phases, resulted in mean energy availabilities of 45- and 10 kcal·kg FFM^{-1}·day^{-1} for *EB* and *LEA*, respectively.

Table 5.2.2. Dietary provision during EB and LEA intervention periods

	EB: **(mean ± SD)**	**LEA:** **(mean ± SD)**
Energy Intake:		
kcal•day^{-1}	3502 ± 382	1232 ± 134
kcal•kg FFM^{-1}•day^{-1}	54.0 ± 0.0	19.0 ± 0.0
Carbohydrate Intake:		
kcal•day^{-1}	2105 ± 231	742 ± 82
% Energy Intake	60.1 ± 0.2	60.2 ± 0.2
g•day^{-1}	526 ± 58	185 ± 21
g•kg^{-1}•day^{-1}	6.7 ± 0.3	2.4 ± 0.1
Fat Intake:		
kcal•day^{-1}	700 ± 75	246 ± 27
% Energy Intake	20.0 ± 0.1	19.9 ± 0.1
g•day^{-1}	78 ± 8	27 ± 3
g•kg^{-1}•day^{-1}	1.0 ± 0.0	0.3 ± 0.0
Protein Intake:		
kcal•day^{-1}	695 ± 76	245 ± 26
% Energy Intake	19.9 ± 0.1	19.9 ± 0.2
g•day^{-1}	174 ± 19	61 ± 7
g•kg^{-1}•day^{-1}	2.2 ± 0.1	0.8 ± 0.0

Sample Collection Procedures:

Samples were collected in the same order each morning across *FREE*, *EB*, and *LEA*, in line with the collection schedule detailed in Figure 5.2.1. Samples were collected in the sequential order listed below as follows:

Resting Metabolic Rate Assessment:

RMR was assessed on Days -5, 0, 5, and 10 in line with the methodology provided in Chapter 3 (General methods).

Body Composition Assessments:

Assessment of body composition via BIA was performed at each lab visit, on days -5, 0, 2, 4, 5, 7, 9, and 10 as detailed previously (see Chapter 3: General methods).

Subsequently, a whole-body DXA scan for body composition and bone mineral density was conducted on days -5, 0, 5, and 10 as detailed previously (see Chapter 3: General methods). All DXA scans were conducted by the same researcher and following standardised procedure (Nana et al., 2015, 2016).

Fasting Venous Blood Sample:

Fasting venous blood samples were collected at each lab visit, on days -5, 0, 2, 4, 5, 7, 9, and 10. Samples were drawn and processed in line with the methodology outlined in Chapter 3 (General methods).

Skeletal muscle biopsy:

On days -5, 0, 5, and 10, a skeletal muscle biopsy was obtained (methodology described in Chapter 3: General methods).

Tracer (D_2O) Enrichment:

To assess biosynthetic labelling of newly synthesised proteins, following the collection of the baseline muscle sample, participants completed a 'pre-load' of 99.8 atom % deuterium oxide (D_2O; Sigma-Aldrich, Dorset, UK). On day -5, participants orally consumed 200 ml of D_2O, followed by two further 150 ml doses at 3-hr intervals, totalling 500 ml of D_2O. Participants were monitored for the first ~4 hours of the D_2O loading protocol, encompassing the first two D_2O boluses, and then provided with the final 150 ml D_2O dose to consume from home.

A daily 50 ml 'top-up' dose of D_2O was provided for the remainder of the study period, from day -4 to day 9. Home-based D_2O doses were provided in 50 ml sterile feeding bottles. Participants were reminded to consume home-based D_2O doses via a calendar reminder scheduled for 12:30 PM (Day -4 to Day 9) and prompted to confirm consumption by the researchers through their *WhatsApp* group.

Exercise protocol:

Exercise sessions were completed in the laboratory on Day 0, 2, 4, 5, 7, and 9, following the collection of all measurements and samples. 5 – 15 minutes prior to the initiation of exercise, participants were provided with a 40 g portion of instant white mash powder (*Smash*, Batchelors, Premier Foods PLC, St. Albans, Hertfordshire, United Kingdom), mixed with 160 g (/ml) of hot water and seasoned to taste with salt (139 kcal; 30 g CHO, <1 g FAT, 3 g PRO). Following consumption of the mash potato, participants completed the exercise protocol as outlined in Chapter 3 (General Methods). Cycling power output during steady state exercise was set at a power corresponding to 60% $\dot{V}O_{2max}$, attained from the baseline testing session. In the 5-minute rest periods provided following 30- and 60-minute of exercise, participants consumed a further 40 g portion of Smash instant mash potato powder, mixed with 160 g of hot water and seasoned to taste with salt. Participants exercised continuously from 60-minutes

onwards until a net exercise energy expenditure equating to 15 kcal•kg FFM^{-1}, calculated from indirect calorimetry values, had been attained.

Following completion of the exercise protocol, participants were provided with all food and D_2O doses for the intervening days prior to their next lab visit. They were then free to leave the laboratory.

Analytical Procedures:

Analysis of Blood-borne Markers:

Samples were analysed for plasma glucose, lactate, NEFA and glycerol concentrations using commercially available kits and a Randox Daytona spectrophotometer (Randox Laboratories Ltd., Crumlin, UK). Commercially available ELISA were used to measure serum total T_3, IGF-1, *GDF-15*, EPO, and plasma insulin, leptin as outlined in Chapter 3 (General methods). Blood samples were sent to a commercial laboratory (Liverpool Clinical Laboratories, Liverpool, UK) for the analysis of circulating bone turnover markers (plasma ß-CTX and P1NP), and serum total testosterone concentrations.

Skeletal Muscle Analysis:

Skeletal muscle myofibrillar, soluble, and mitochondrial protein synthetic rates, and skeletal muscle glycogen concentrations were assessed in line with the procedures outlined in Chapter 3: *General Methods.*

Statistical Analyses:

All data, except for the skeletal muscle protein synthetic rates, were collated using Microsoft Excel (v. 16.77.1, Microsoft Corp., Redmond, WA, USA) and statistical analyses conducted using IBM SPSS Statistics (v. 28.0.0.0, IBM, Armonk, NY, USA). Skeletal muscle protein synthetic rate data was processed and analysed using *R* (v. 4.2.2.*)*. Graphs were created using Graphpad Prism (v. 8.2.1, Graphpad Software Inc., San Diego, California, USA) and *R* (skeletal muscle protein synthetic rate figures only). Anthropometric, RMR, exercise indirect calorimetry, EPO, and skeletal muscle protein synthetic rate data were explored using one-way ANOVA. Where Greenhouse-Geiser epsilon values > 0.75, Huynh-Feldt *P* values correcting for aspherical data were applied (Atkinson, 2001). Changes in all blood-borne markers were explored via linear mixed models. Significance was set at $P < .05$ for all statistical tests. Data are reported as means ± SDs. Day 0 values were regarded as the 'baseline' concentration for participants prior to the administration of dietary interventions. Statistical analyses were conducted on raw data from each timepoint and are reported as the difference between raw daily values (Δ) and 95% confidence interval of the mean Δ value. Variance between differences in reported mean data values and mean Δ values are a statistical artefact, resulting from the handling of missing data within the linear mixed model analyses. Changes in all data, except for skeletal muscle protein

synthetic rates, were explored using *Hedge's g* effect sizes (Lakens, 2013) calculated manually using Microsoft Excel: < 0.20 = *Trivial*, 0.21 – 0.60 – *Small*, 0.61 – 1.20 *Moderate*, 1.21 – 1.99 = *Large*, ≥ 2.00 = *Very Large* (Hopkins et al., 2009).

5.3. Results:

Energy Availability Interventions:

An overview of the exercise data from *EB* (days 0, 2, and 4) and LEA (days 5, 7, and 9) are outlined in Table 5.3.1. Exercise sessions lasted 92.3 ± 8.8 mins during *EB*, and 92.1 ± 8.7 mins during *LEA*, resulting in net exercise energy expenditure of 15.1 ± 0.1 kcal•kg FFM^{-1} and 15.1 ± 0.0 kcal•kg FFM^{-1} for *EB* and *LEA* respectively. Following the session warm-up, mean workload as a percentage of $\dot{V}O_{2max}$ during steady state exercise was 62.8 ± 2.5 % and 63.1 ± 3.8 % during *EB* and *LEA*, respectively.

Table 5.3.1. Exercise data from the energy balance and low energy availability interventions, including total exercise time, mean $\dot{V}O_2$ during steady state exercise as a percentage of $\dot{V}O_{2max}$, and net exercise energy expenditure (EEE_{net})

	Energy Balance			Low Energy Availability		
Parameter	**Mean**	**SD**	**Range**	**Mean**	**SD**	**Range**
Total Exercise Time (mins)	92	9	78 – 104	92	9	79 – 107
Mean % $\dot{V}O_{2max}$	63	2	59 – 66	63	4	57 – 69
EEE_{net} (kcal•kg FFM^{-1})	15.1	0.1	15.0 – 15.2	15.1	0.0	15.0 – 15.2

Details of energy intake, macronutrient distribution and resultant energy availability are outlined in Table 5.2.2. (see Chapter 5.2: Methods). Briefly, the study-diets provided controlled energy intakes of 54.0 ± 0.0 kcal•kg FFM^{-1}•day^{-1} and 19.0 kcal•kg FFM^{-1}•day^{-1} during *EB* and *LEA*, respectively. Combined with the respective exercise sessions during *EB* and *LEA*, mean daily energy availability was 45.0 ± 0.0 kcal•kg FFM^{-1}•day^{-1} during *EB*, and 10.0 ± 0.0 kcal•kg FFM^{-1}•day^{-1} during *LEA*. Given that exercise was not performed daily, EA ranged from 38.9 ± 0.1 to 54.0 ± 0.0 kcal•kg FFM^{-1}•day^{-1} during *EB* and from 3.9 ± 0.1 to 19.0 ± 0.0 kcal•kg FFM^{-1}•day^{-1} during *LEA*.

Body Mass and Composition:

Body mass, fat mass and fat free mass values over *FREE*, *EB*, and *LEA* are shown in Figure 5.3.1.

Body Mass: There was a significant effect of the study intervention upon body mass ($F_{3.00, 27.00}$ = 69.82, P < 0.001). Body mass was stable across *FREE* from Day -5 (78.8 ± 8.2 kg) to Day 0 (79.2 ± 0.5 kg; mean Δ 0.4 kg, 95% CI of Δ: -0.2 to 1.0 kg, P = 0.189, g = 0.0), and across *EB* from Day 0 to Day 5 (79.1 ± 8.5 kg; mean Δ - 0.1 kg, 95% CI of Δ: -0.4 to 0.2 kg, P = 0.442, g = 0.0). Across *LEA* body mass significantly reduced from Day 5 (79.1 ± 8.5 kg) to Day 10 (76.1 ± 8.4 kg; mean Δ -3.0 kg, 95% CI of Δ: -2.4 to -3.5 kg, P < 0.001), albeit this effect was small (g = 0.3).

Fat Free Mass: Changes in FFM followed a similar pattern. There was a significant effect of the study intervention upon FFM ($F_{3.00, 27.00}$ = 42.88, P < 0.001). FFM was stable during *FREE* (Day -5 to Day 0; Mean Δ 0.5 kg, 95% CI of Δ: -0.1 to 1.2 kg, P = 0.087, g = 0.1) and *EB* (Day 0 to Day 5; Mean Δ -0.3 kg, 95% CI of Δ: -0.8 to 0.2 kg, P = 0.172, g = 0.0), but reduced from 65.1 ± 7.2 kg (Day 5) to 63.0 ± 7.2 kg (Day 10) across LEA (Mean Δ -2.1 kg, 95% CI of Δ: -2.7 to -1.7 kg, P < 0.001). Again, this effect was small (g = 0.3).

Fat Mass: Changes in fat mass again followed a similar pattern. There was a significant effect of the intervention upon fat mass ($F_{3.00, 27.00}$ = 9.64, P < 0.001). Fat Mass was stable over *FREE* (Day -5 to Day 0; Mean Δ -0.0 kg, 95% CI of Δ: -0.3 to 0.2 kg, P = 0.826, g = 0.0) and *EB* (Day 0 to Day 5; Mean Δ 0.2 kg, 95% CI of Δ: -0.2 to 0.5 kg, P = 0.259, g = 0.1). However, fat mass reduced from 14.0 ± 3.4 kg (Day 5) to 13.2 ± 3.1 kg (Day 10) across *LEA* (Mean Δ -0.8 kg, 95% CI of Δ: -1.3 to -0.4 kg, P = 0.003, g = 0.2). There was no effect of the intervention on overall body fat percentage ($F_{2.21, 19.93}$ = 2.31, P = 0.121).

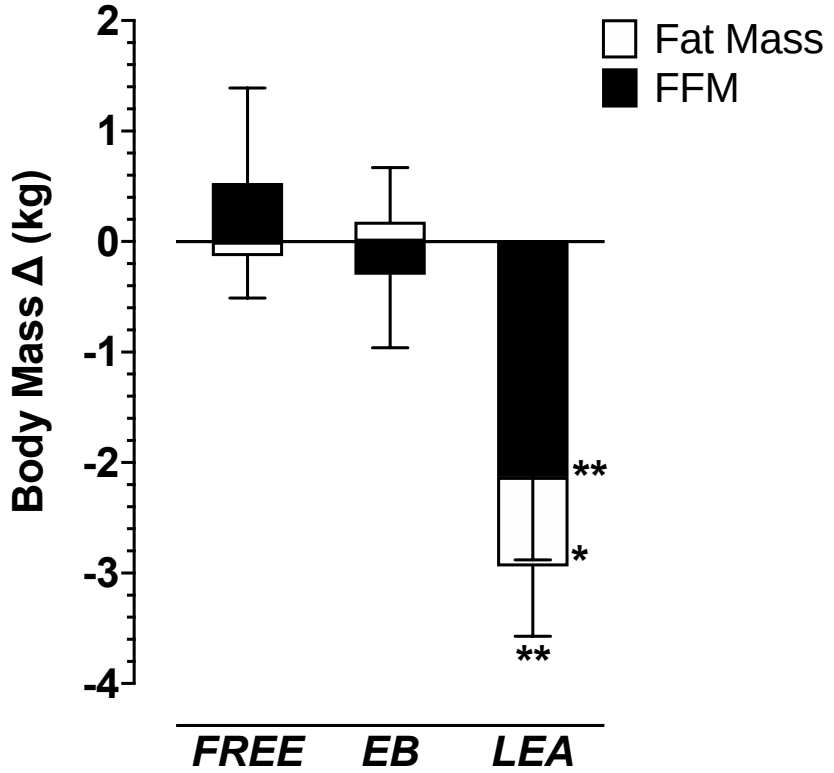


Figure 5.3.1. Change in fat mass (white boxed area), fat free mass (*FFM*; black shaded area) and resultant body mass change (net area of boxes) over *FREE*, *EB*, and *LEA* study-periods. * = P < 0.05 ** = P < 0.001, pre-post intervention phase.

Total Body Water: There was a significant effect upon Total Body Water (*TBW*) ($F_{2.04, 18.37}$ = 6.12, P = 0.009). As expected, there was no significant difference in TBW across *FREE* (Day – 5 to Day 0; Mean Δ -0.1 L, 95% CI of Δ: -0.7 to 0.6 L, P = 0.847, g = 0.0) or *EB* (Day 0 to Day 5; Mean Δ 0.2 L, 95% CI

of Δ: -0.2 to 0.5 L, P = 0.300, g = 0.0). However, TBW reduced from 48.0 L (Day 5) to 46.8 L (Day 10) across *LEA*, achieving significance (Mean Δ -1.2 L, 95% CI of Δ: -1.9 to -0.5 L, P = 0.005) but with a small effect size (g = 0.3).

Metabolic Markers:

Glucose: Plasma glucose concentrations were stable throughout the study intervention (P = 0.289, Figure 5.3.2. A), with no effect size (g) greater than 0.4 (*Trivial* to *Small*) observed relative to Day 0 concentrations.

NEFA: There was a significant effect of the intervention on plasma *NEFA* concentrations (P < 0.001). Plasma NEFA concentrations were stable across *FREE* (Day -5 to Day 0; Mean Δ 0.086 mmol•l^{-1}, 95% CI of Δ: -0.148 to 0.319 mmol•l^{-1}, P = 0.465, g = 0.1) and *EB* (Day 0 to Day 5; Mean Δ -0.111 mmol•l^{-1}, 95% CI of Δ: -0.345 to 0.122 mmol•l^{-1}, P = 0.345, g = 0.1). Following 48 hours of *LEA*, plasma NEFA concentrations were significantly greater (Day 7: 1.057 ± 0.387 mmol•l^{-1}) than baseline values (Day 0: 0.508 ± 0.201 mmol•l^{-1}, mean Δ 0.480 mmol•l^{-1}, 95% CI of Δ: 0.247 to 0.714 mmol•l^{-1}, P < 0.001, g = 1.7) and remained elevated on Day 9 (1.115 ± 0.485 mmol•l^{-1}, mean Δ 0.538 mmol•l^{-1}, 95% CI of Δ: 0.305 to 0.772 mmol•l^{-1}, P < 0.001, g = 1.5) and Day 10 (0.882 ± 0.392 mmol•l^{-1}, mean Δ 0.305 mmol•l^{-1}, 95% CI of Δ: 0.071 to 0.538 mmol•l^{-1}, P = 0.011, g = 1.1) during *LEA*, relative to Day 0 (Figure 5.3.2. B).

Glycerol: Plasma glycerol concentrations remained stable over *FREE* (Day -5 to Day 0; Mean Δ 4 µmol•l^{-1}, 95 % CI of Δ: -18 to 26 µmol•l^{-1}, P = 0.733, g = 0.4) and *EB* (Day 0 to Day 5; Mean Δ 1 µmol•l^{-1}, 95 % CI of Δ: -21 to 23 µmol•l^{-1}, P = 0.955, g = 0.2). In contrast, plasma glycerol concentrations increased relative to baseline (Day 0: 20 ± 15 µmol•l^{-1}) concentrations following 48 hours of *LEA* (Day 7: 62 ± 24 µmol•l^{-1}, mean Δ 36 µmol•l^{-1}, 95% CI of Δ: 14 to 58 µmol•l^{-1}, P = 0.002, g = 2.0), and on Days 9 (78 ± 51 µmol•l^{-1}, mean Δ 52 µmol•l^{-1}, 95% CI of Δ: 30 to 74 µmol•l^{-1}, P < 0.001, g = 1.4) and 10 (51 ± 31 µmol•l^{-1}, mean Δ 25 µmol•l^{-1}, 95% CI of Δ: 3 to 37 µmol•l^{-1}, P = 0.028, g = 1.2), during *LEA* (Figure 5.3.2 C).

Ketones: Plasma D-3-Hydroxybutyrate concentrations were stable across *FREE* (Day -5 to Day 0; Mean Δ 0.067 mmol•l^{-1}, 95% CI of Δ: -0.081 to 0.102 mmol•l^{-1}, P = 0.365, g = 0.6) and *EB* (Day 0 to Day 5; Mean Δ -0.041, 95% CI of Δ: -0.189 to 0.108, P = 0.581, g = 0.1). Plasma D-3-Hydroxybutyrate concentrations then progressively increased during *LEA*, from baseline values (Day 0: 0.112 ± 0.080 mmol•l^{-1}), to 0.400 ± 0.310 mmol•l^{-1} on Day 7 (mean Δ 0.258 mmol•l^{-1}, 95% CI of Δ: 0.109 to 0.406 mmol•l^{-1}, P = 0.001, g = 1.2), 0.504 ± 0.305 mmol•l^{-1} on Day 9 (mean Δ 0.362 mmol•l^{-1}, 95% CI of Δ: 0.213 to 0.510 mmol•l^{-1}, P < 0.001, g = 1.6), and 0.583 ± 0.362 mmol•l^{-1} on Day 10 (mean Δ 0.441 mmol•l^{-1}, 95% CI of Δ: 0.292 to 0.589 mmol•l^{-1}, P < 0.001, g = 1.7). Day 10 plasma D-3-

Hydroxybutyrate concentrations were also significantly greater than Day 7 concentrations (mean Δ 0.183 mmol•l^{-1}, 95% CI of Δ: 0.039 to 0.327 mmol•l^{-1}, P = 0.014, g = 0.5, Figure 5.3.2 D).

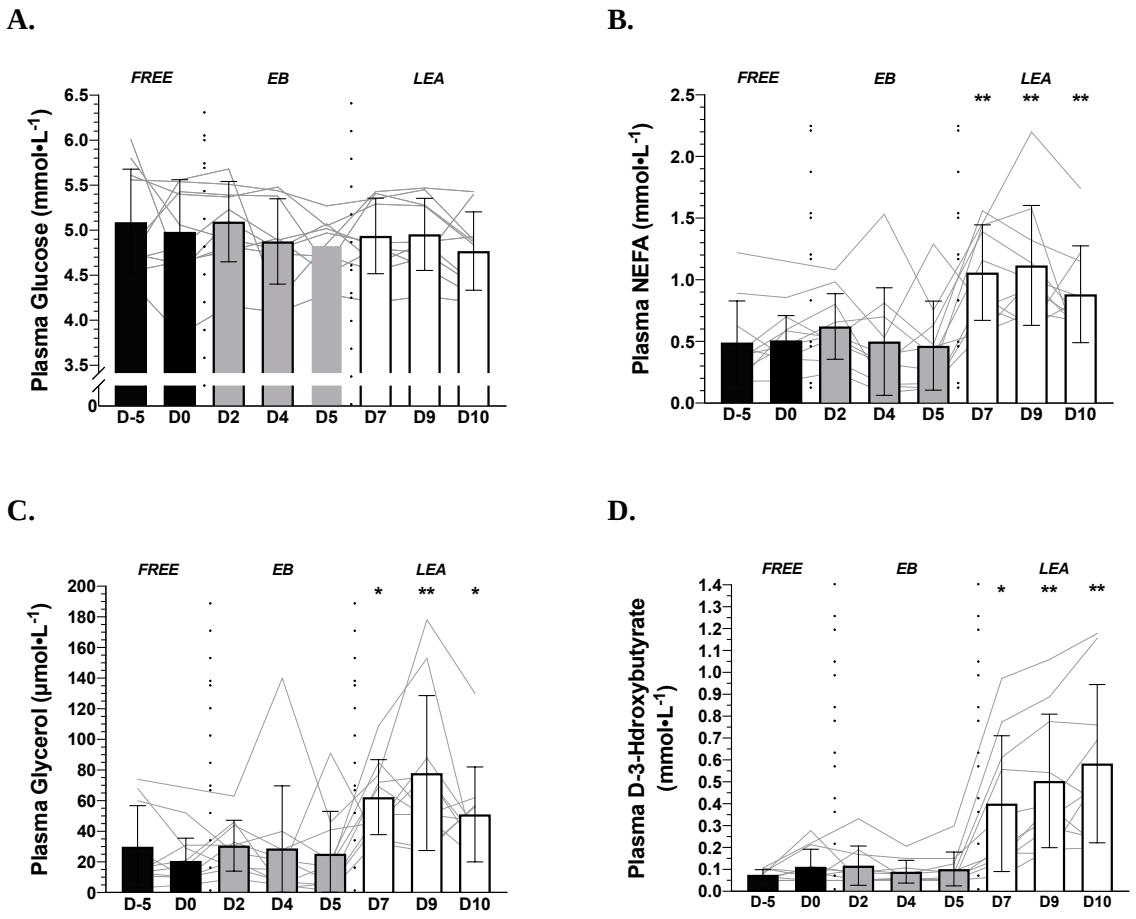


Figure 5.3.2. Concentrations of (A) plasma glucose, (B) plasma NEFA, (C) plasma glycerol, and (D) plasma D-3-Hydroxybutyrate (ketones) across the study intervention periods (Days -5 to 0; *FREE*, Days 0 – 5; *EB*, and Days 5 – 10; *LEA*). * = $P < 0.05$, ** = $P < 0.001$ relative to Day 0.

Endocrine Markers:

Testosterone: There was no significant effect of the intervention on serum testosterone concentrations (F = 1.07, P = 0.389. Figure 5.3.3. A). Relative to Day 0. No Hedge's g effect size exceeded *Small*, with all g < 0.4 for testosterone throughout the study period.

Insulin: There was no significant effect of the intervention on plasma insulin concentrations (F = 1.67, P = 0.134. Figure 5.3.3. B). Relative to Day 0 concentrations, no Hedge's g effect size greater than 0.4 (*Small*) was observed for insulin.

Triiodothyronine (T_3): There was a significant intervention effect upon serum T_3 concentrations during the study (Figure 5.3.3 C. F = 11.67, P < 0.001). Serum T_3 concentrations were stable across *FREE* (Day -5 to Day 0; Mean Δ 5.75 ng•dL^{-1}, 95% CI of Δ: -3.30 to 14.79 ng•dL^{-1}, P = 0.209, g = 0.2). On Day 4 (122.20 ± 31.37 ng•dL^{-1}), T_3 concentrations were significantly lower than on Day 0 (132.74 ± 33.40 ng•dL^{-1}; Mean Δ -10.54 ng•dL^{-1}, 95% CI of Δ: -19.57 to -1.50 ng•dL^{-1}, P = 0.023, g = 0.3), although there was no significant difference at the end of *EB* (Day 5: 123.97 ± 26.95 ng•dL^{-1}) compared to Day 0 (Mean Δ -8.76 ng•dL^{-1}, 95% CI of Δ: -17.81 to 0.28 ng•dL^{-1}, P = 0.057, g = 0.3). During *LEA*, serum T_3 concentrations decreased relative to baseline (Day 0) values after 48 hours of dietary restriction (Day 7: 108.44 ± 26.91 ng•dL^{-1}, mean Δ -24.30 ng•dL^{-1}, 95% CI of Δ: 33.34 to -15.25 ng•dL^{-1}, P < 0.001, g = 0.8) and remained significantly lower on Days 9 (104.60 ± 27.36 ng•dL^{-1}, mean Δ -28.14 ng•dL^{-1}, 95% CI of Δ: -37.18 to -19.09 ng•dL^{-1}, P < 0.001, g = 0.9) and 10 (104.62 ± 25.98 ng•dL^{-1}, mean Δ -28.11, 95% CI of Δ: -37.16 to -19.07 ng•dL^{-1}, P < 0.001, g = 0.9), during *LEA*.

Leptin: There was a significant effect of the intervention upon plasma leptin concentrations (F = 10.58, P < 0.001. Figure 5.3.3 D). There was no significant change in plasma leptin concentrations across *FREE* (Day -5 to Day 0; Mean Δ -0.22 ng•ml^{-1}, 95% CI of Δ: -0.62 to 0.18 ng•ml^{-1}, P = 0.285, g = 0.1), but 48 hours of *EB* (Day 2: 1.97 ± 1.58 ng•ml^{-1}) significantly increased plasma leptin concentrations relative to Day 0 (1.65 ± 1.73 ng•ml^{-1}; Mean Δ 0.44 ng•ml^{-1}, 95% CI of Δ: 0.04 to 0.83 ng•ml^{-1}, P = 0.033, g = 0.2). However, by the end of EB (Day 5: 1.44 ± 1.58 ng•ml^{-1}) plasma leptin concentrations were unchanged from Day 0 values (Mean Δ -0.10, 95% CI of Δ: -0.50 to 0.30 ng•ml^{-1}, P = 0.622, g = 0.1). During *LEA*, plasma leptin concentrations decreased relative to baseline (Day 0) values after 48 hours of dietary restriction (Day 7: 1.07 ± 1.60 ng•ml^{-1}, mean Δ -0.47 ng•ml^{-1}, 95% CI of Δ: -0.87 to -0.07 ng•ml^{-1}, P = 0.023, g = 0.3) and remained significantly lower on Day 9 (1.02 ± 1.62 ng•ml^{-1}, mean Δ -0.52 ng•ml^{-1}, 95% CI of Δ: -0.92 to -0.12 ng•ml^{-1}, P = 0.012, g = 0.4) and Day 10 (0.76 ± 1.66 ng•ml^{-1}, mean Δ -0.78 ng•ml^{-1}, 95% CI of Δ: -1.18 to -0.38 ng•ml^{-1}, P < 0.001, g = 0.5), during *LEA*.

IGF-1: The intervention significantly altered serum IGF-1 concentrations (F = 5.23, P < 0.001. Figure 5.3.3 E). *S*erum IGF-1 concentrations were stable across *FREE* (Day -5 to Day 0; Mean Δ -2.7 ng•ml^{-1},

95% CI of Δ: -13.5 – 8.0 ng•ml^{-1}, P = 0.614, g = 0.0), but 48 hours of *EB* (Day 2: 143.1 ± 64.6 ng•ml^{-1}) lead to a significant increase relative to Day 0 concentrations (128.7 ± 53.3 ng•ml^{-1}; Mean Δ 14.4 ng•ml^{-1}, 95% CI of Δ: 3.6 to 25.2 ng•ml^{-1}, P = 0.010, g = 0.2). However, there were no significant differences in serum IGF-1 concentrations at the end of *EB* (Day 5: 138.1 ± 53.9 ng•ml^{-1}), relative to baseline (mean Δ 9.4 ng•ml^{-1}, 95% CI of Δ -1.4 to 20.2 ng•ml^{-1}, P = 0.086, g = 0.2). During *LEA*, serum IGF-1 concentrations were only reduced on the final day of the intervention (Day 10: 115.6 ± 48.9 ng•ml^{-1}) compared to baseline values (Mean Δ 13.1 ng•ml^{-1}, 95% CI of Δ: -23.9 to -2.3 ng•ml^{-1}, P = 0.018, g = 0.2).

GDF-15: Serum GDF-15 concentrations also showed a significant intervention effect (F = 4.09, P < 0.001. Figure 5.3.3. F). There was no change in serum GDF-15 concentration across the *FREE* period (Mean Δ -8.2 pg•ml^{-1}, 95% CI of Δ: -31.6 to 15.2 pg•ml^{-1}, P = 0.487, g = 0.1). During *EB*, serum GDF-15 concentration was reduced compared to Day 0 (378.6 ± 87.6 pg•ml^{-1}) on Day 2 (349.5 ± 58.7 pg•ml^{-1}; Mean Δ -29.1 pg•ml^{-1}, 95% CI of Δ: -52.5 to -5.7 pg•ml^{-1}, P = 0.016, g = 0.4) and on Day 5 (341.9 ± 55.8 pg•ml^{-1}, mean Δ -36.7 pg•ml^{-1}, 95% CI of Δ: -60.1 to -13.3, P = 0.003, g = 0.5). There was no difference between Day 7 (373.6 ± 74.7 pg•ml^{-1}) and baseline (Day 0) serum GDF-15 concentrations (mean Δ -5.0 pg•ml^{-1}, 95% CI of Δ: -28.4 to 18.4 pg•ml^{-1}, P = 0.668, g = 0.1), indicating that 48 hours of LEA restored serum GDF-15 concentrations to baseline values. This was maintained on Day 9 (Mean Δ 9.0 pg•ml^{-1}, 95% CI of Δ: -14.4 to 32.4 pg•ml^{-1}, P = 0.445, g = 0.1) and Day 10 (Mean Δ -15.9 pg•ml^{-1}, 95% CI of Δ: -39.3 to 7.5 pg•ml^{-1}, P = 0.179, g = 0.2) during *LEA*.

Erythropoietin (EPO): There was no significant effect of the intervention upon serum EPO concentrations ($F_{3.00,\ 27.00}$ = 0.75, P = 0.535). Relative to Day 0, no Hedge's g effect size exceeded 'small', with all g < 0.5 for EPO throughout the study period.

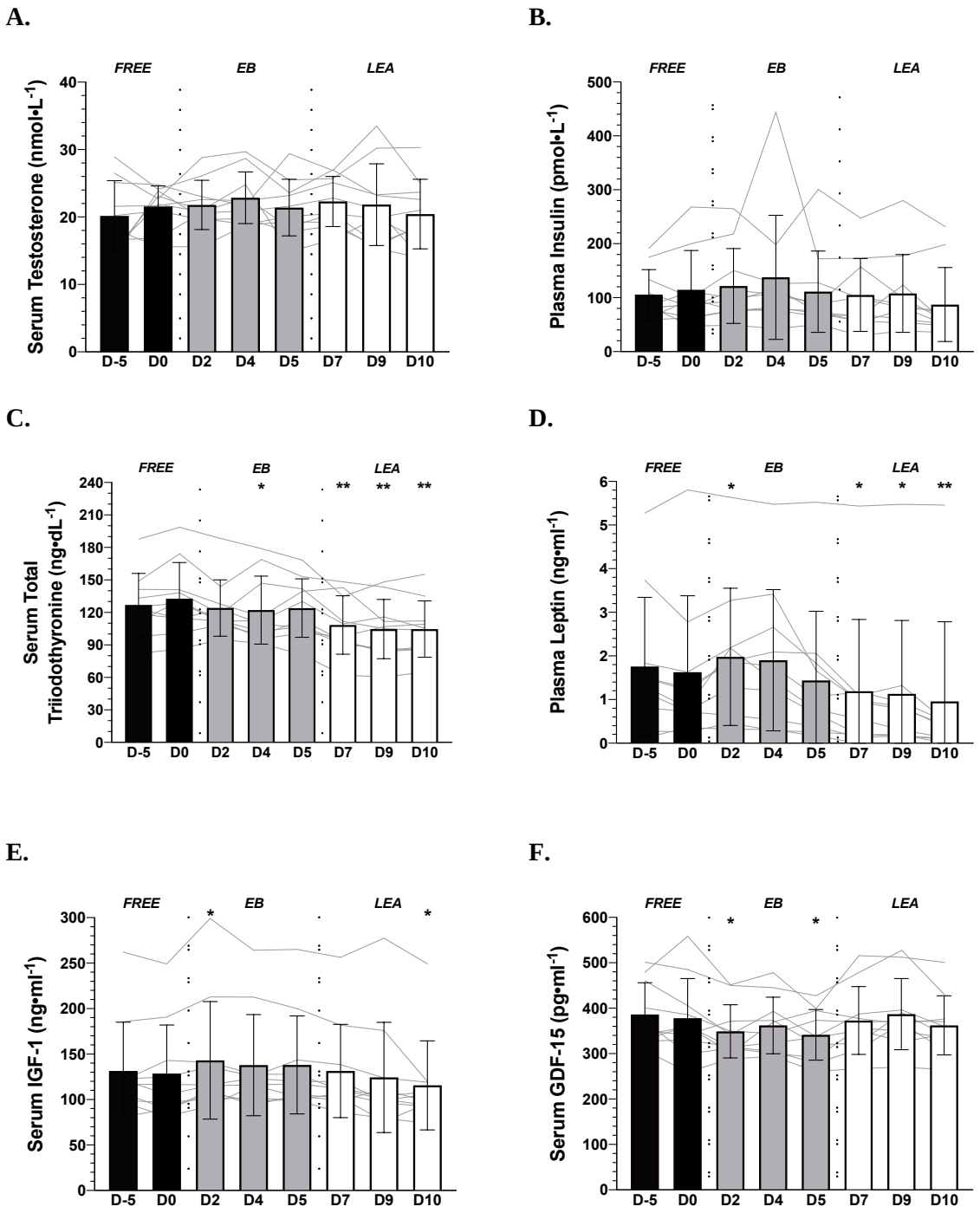


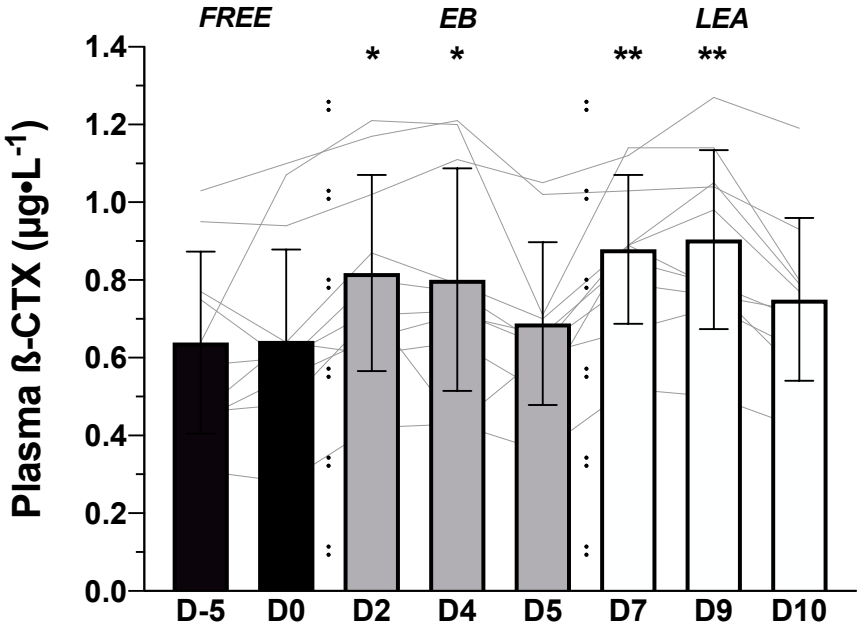
Figure 5.3.3 Concentrations of (A) plasma insulin, (B) serum total testosterone, (C) serum total triiodothyronine (T_3), (D) plasma leptin, (E) serum IGF-1, and (F) serum GDF-15 across the study intervention periods. * = $P < 0.05$, ** = $P < 0.001$ relative to Day 0.

Physiological Markers:

ß-CTX: There was a significant intervention effect upon plasma ß-CTX concentrations ($F = 10.14$, $P < 0.001$. Figure 5.3.4. A). Plasma ß-CTX concentrations remained stable over *FREE* (Day -5 to Day 0; Mean Δ 0.034 μg•l^{-1}, 95% CI of Δ: -0.054 to 0.123 μg•l^{-1}, $P = 0.441$, $g = 0.0$), but were increased relative to baseline (Day 0: 0.64 ± 0.24 μg•l^{-1}) concentrations on Day 2 (0.82 ± 0.25 μg•l^{-1}; Mean Δ 0.15 μg•l^{-1}, 95% CI of Δ: 0.06 to 0.23 μg•l^{-1}, $P = 0.002$, $g = 0.7$) and Day 4 of *EB* (0.80 ± 0.29 μg•l^{-1}, mean Δ 0.13 μg•l^{-1}, 95% CI of Δ: 0.04 to 0.22 μg•l^{-1}, $P = 0.006$, $g = 0.6$). On Day 5 (0.69 ± 0.21 μg•l^{-1}), there was no difference in plasma ß-CTX concentrations compared to baseline, following the *EB* intervention (Mean Δ 0.02 μg•l^{-1}, 95% CI of Δ: -0.07 to 0.10 μg•l^{-1}, $P = 0.744$, $g = 0.2$). A similar pattern was evident during *LEA.* Following 48 hours of *LEA*, Day 7 plasma ß-CTX (0.88 ± 0.19 μg•l^{-1}) was significantly elevated relative to Day 0 concentrations (Mean Δ 0.21 μg•l^{-1}, 95% CI of Δ: 0.18 to 0.29 μg•l^{-1}, $P < 0.001$, $g = 1.1$), as were Day 9 ß-CTX concentrations (0.90 ± 0.23 μg•l^{-1}, mean Δ 0.23 μg•l^{-1}, 95% CI of Δ: 0.14 to 0.32 μg•l^{-1}, $P < 0.001$, $g = 1.1$). However, Day 10 plasma ß-CTX concentrations (0.75 ± 0.21 μg•l^{-1}) were unchanged relative to baseline values, following five days of *LEA* (Mean Δ 0.08 μg•l^{-1}, 95% CI of Δ: -0.01 to 0.17, $P = 0.089$, $g = 0.5$).

P1NP: There was also a significant intervention effect upon plasma P1NP concentrations ($F = 8.23$, $P < 0.001$). Concentrations of plasma P1NP did not change over *FREE* (Day -5 to Day 0; Mean Δ -1 μg•l^{-1},95% CI of Δ: -7 to 5 μg•l^{-1}, $P = 0.824$, $g = 0.1$), or *EB* (Day 0 to Day 5: Mean Δ -2, 95% CI of Δ: -8 to 4 μg•l^{-1}, $P = 0.504$, $g = 0.0$). However, relative to Day 0, Day 7 (72 ± 16 μg•l^{-1}; Mean Δ -9 μg•l^{-1}, 95% CI of Δ: -15 to -3 μg•l^{-1}, $P = 0.004$, $g = 0.4$), Day 9 (69 ± 15 μg•l^{-1}; mean Δ -13, 95% CI of Δ: -19 to -7, $P < 0.001$, $g = 0.6$), and Day 10 (67 ± 18 μg•l^{-1}; mean Δ -14, 95% CI of Δ: -20 to -8 μg•l^{-1}, $P < 0.001$, $g = 0.6$) plasma P1NP concentrations were all significantly reduced (Figure 5.3.4. B).

A.



B.

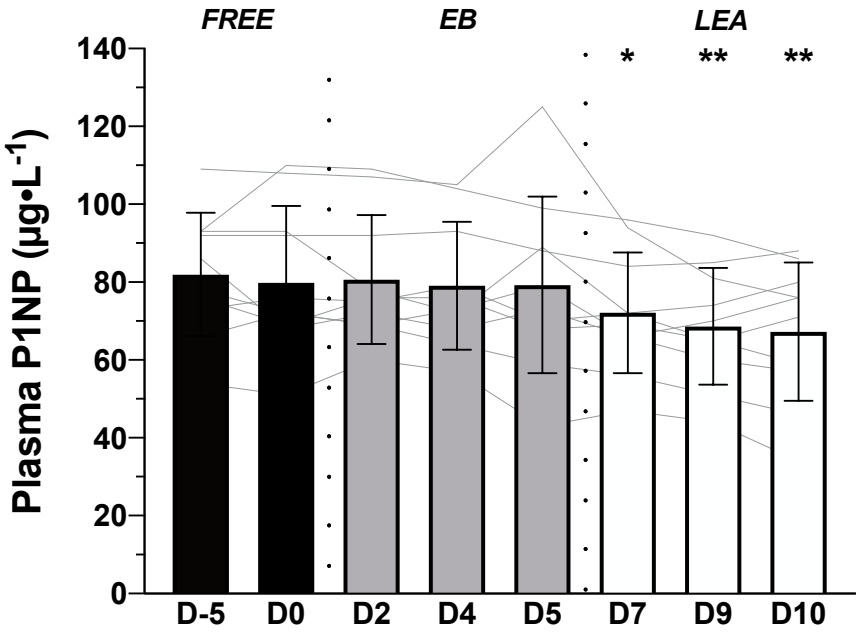


Figure 5.3.4. Time course of changes in plasma concentrations of (A) bone resorption marker ß-CTX, and (B) bone formation marker P1NP over the course of the study intervention. * = $P < 0.05$, ** = $P < 0.001$ relative to Day 0.

Resting Metabolic Rate (RMR): There was no effect of the intervention upon RMR ($F_{3.00, 27.00}$ = 1.91, P = 0.151, Figure 5.3.5. A). Relative to Day 0, no Hedge's g effect size greater than 0.4 (*Small*) was observed for changes in RMR.

Resting RER was significantly altered over the course of the study ($F_{1.71, 15.36}$ = 10.84, P = 0.002, Figure 5.3.5. B). Resting RER was stable across *FREE* (Day -5 to Day 0; Mean Δ 0.02, 95% CI of Δ: -0.00 to 0.03, P = 0.081, g = 0.2). Conversely, relative to Day 0 (0.82 ± 0.06), resting RER was increased following *EB* on Day 5 (0.87 ± 0.08; Mean Δ 0.052, 95% CI of Δ: 0.00 to 0.10, P = 0.038, g = 0.7), but decreased following *LEA* on Day 10 (0.77 ± 0.05; Mean Δ -0.05, 95% CI of Δ: -0.08 to -0.02, P = 0.006, g = 0.8).

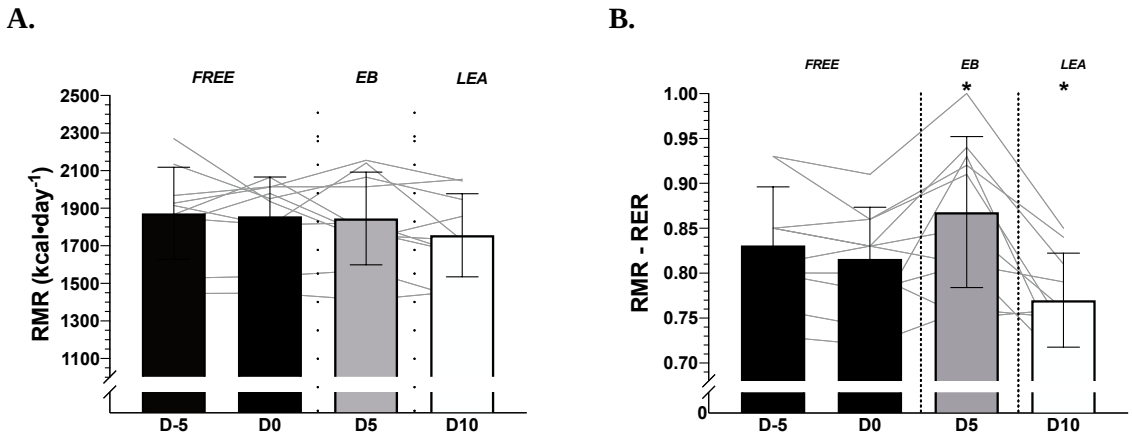


Figure 5.3.5. (A) Resting metabolic rate, and (B) respiratory exchange ratio (RER) values assessed during RMR measurements, over the time-course of the intervention period. * = P < 0.05, relative to Day 0.

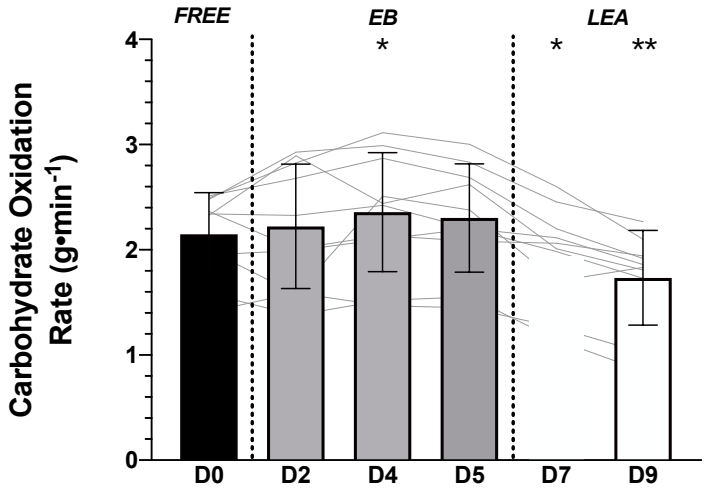
Exercise Metabolism:

Gross Efficiency: There was no effect of the intervention upon cycling gross efficiency during the warm-up stages at 50 W (F = 1.34, P = 0.267), 75 W (F = 0.81, P = 0.549), 100 W (F = 0.437, P = 0.820), 125 W (F = 1.820, P = 0.130), or for mean gross efficiency during steady state exercise (F = 0.103, P = 0.991) throughout the study period.

Carbohydrate Oxidation Rates: As a general trend, carbohydrate oxidation rates increased over the course of *EB*, and then were reduced relative to Day 0 values over the course of *LEA*. There was a significant intervention effect upon carbohydrate oxidation ($g \cdot min^{-1}$) rates for all warm-up exercise intensities (50 W: F = 12.69, P < 0.001; 75 W: F = 26.13, P < 0.001; 100 W: F = 24.99, P < 0.001; 125 W: F = 17.77, P < 0.001), as well as for mean carbohydrate oxidation rates during steady state exercise (F = 16.40, P < 0.001, Figure 5.3.6. A).

Fat Oxidation Rates: As a general trend, fat oxidation rates reduced over the course of the *EB* intervention phase, before increasing over the course of the *LEA* intervention. Similar to carbohydrate oxidation, there was a significant intervention effect upon fat oxidation ($g \bullet min^{-1}$) rates at all warm-up exercise intensities (50 W: $F = 10.88$, $P < 0.001$; 75 W: $F = 25.04$, $P < 0.001$; 100 W: $F = 21.48$, $P < 0.001$; 125 W: $F = 18.14$, $P < 0.001$), and for mean fat oxidation rate during steady state exercise ($F = 18.25$, $P < 0.001$, Figure 5.3.6. B).

A.



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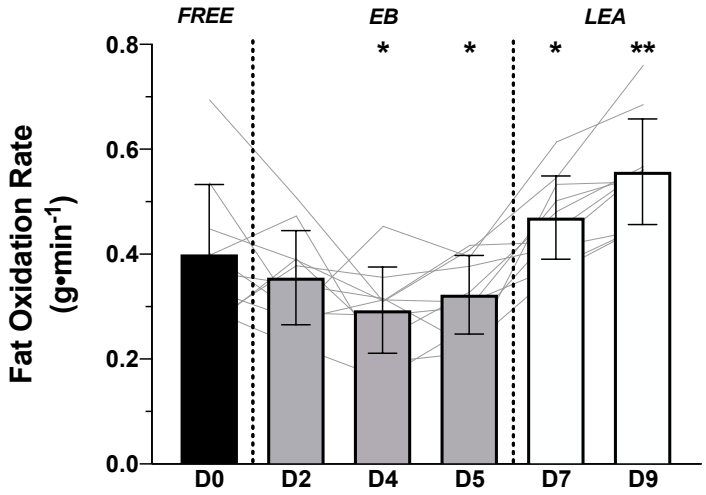


Figure 5.3.6. Rates of (A) carbohydrate, and (B) fat oxidation during steady state exercise in *EB* (Day 0, 2, and 4) and *LEA* (Day 5, 7, and 9). Calculated as the mean of the 60 s indirect calorimetry measurements collected during the steady-state (15-mins onwards) component of the controlled exercise sessions. * = $P < 0.05$, ** = $P < 0.001$ relative to Day 0.

Skeletal Muscle Responses:

Skeletal Muscle Glycogen Concentrations:

There was a significant change in skeletal muscle glycogen concentrations during the study intervention (F = 4.57, P = 0.011, Figure 5.3.7.). Skeletal muscle glycogen concentration was stable across *FREE* (Day -5 to Day 0; Mean Δ -5 mmol•kg^{-1} dw, 95% CI of Δ: -87 to 77 mmol•kg^{-1} dw, P = 0.896, g = 0.0), and *EB* (Day 0 to Day 5; Mean Δ -15 mmol•kg^{-1} dw, 95% CI of Δ: -97 to 66 mmol•kg^{-1} dw, P = 0.701, g = 0.2). However, relative to Day 0 (341 ± 74 mmol•kg^{-1} dw), skeletal muscle glycogen concentrations significantly reduced following *LEA* (Day 10: 221 ± 70 mmol•kg^{-1} dw; Mean Δ -120 mmol•kg^{-1} dw, 95% CI of Δ: -200 to -41 mmol•kg^{-1} dw, P = 0.005, g = 1.6).

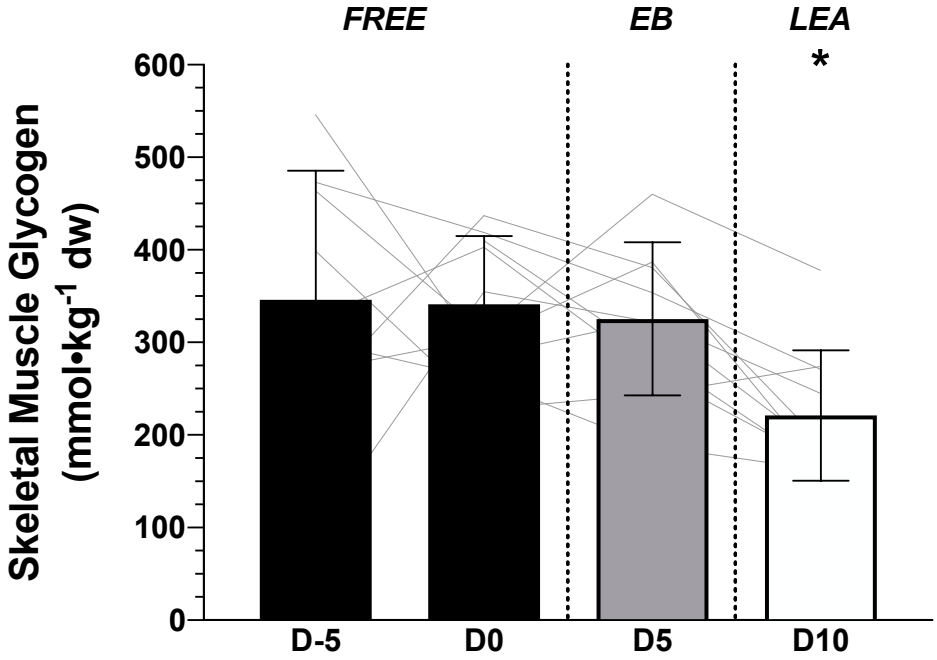


Figure 5.3.7. Skeletal muscle glycogen content over the time course of the study intervention phases. ***** = $P < 0.05$, relative to Day 0.

Muscle Protein Synthetic Rates:

Mixed-muscle (average of all individual proteins) fractional synthetic rates of the myofibrillar (P = 0.1135), and soluble fractions (P = 0.928), and mitochondrial proteins (P = 0.928) were unchanged between the *EB* and *LEA* intervention phases (Figure 5.3.8.).

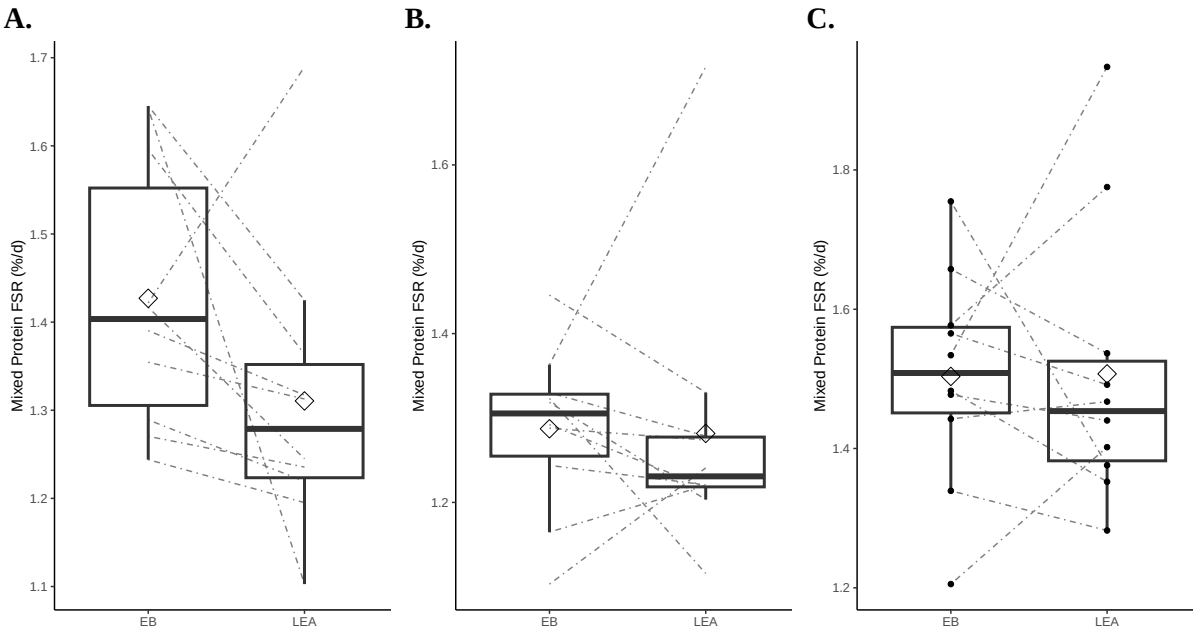


Figure 5.3.8. Changes in global fractional synthetic rates of (**A**) myofibrillar, (**B**) soluble, and (**C**) mitochondrial proteins across the *EB* (green shaded box and whisker plot) and *LEA* (red shaded box and whisker plot) intervention phases.

5.4. Discussion:

The main findings of this study were that five days of low energy availability did not affect serum total testosterone concentrations or resting metabolic rate, despite reductions in other key markers of energy preservation including plasma T_3, leptin, and serum IGF-1 concentrations in our cohort of 10 healthy, trained, males. In line with our hypothesis, bone metabolism appeared to be affected by the low energy availability intervention. Fasting plasma ß-CTx concentrations increased and plasma P1NP concentrations were reduced during *LEA*. Similarly, we observed expected reductions in body mass, fat mass, and fat free mass, whilst NEFA, glycerol, and D-3-Hydroxybutyrate concentrations increased following *LEA*. Fasting plasma glucose and insulin concentrations were unchanged throughout the study interventions, however. Despite observing an anticipated reduction in skeletal muscle glycogen concentration following *LEA*, global protein synthetic rates for the myofibrillar, soluble, and mitochondrial fractions were unaffected by *LEA*, indicating preservation of protein synthesis in the working muscle.

This study used a more pronounced reduction in energy availability than previously investigated, and provided this in a pattern of EA that is reflective of real-world scenarios (see Chapter 4). Our findings expand the limited data available investigating the effects of low energy availability upon male physiological responses. Specifically, we observed no changes in serum total testosterone concentrations. This is concurrent with the findings of Kojima et al. (2020) and Koehler et al. (2016), who reduced EA to ~15 and 19 kcal•kg FFM^{-1}•day^{-1} for three and four days, respectively, in healthy males but showed no change in total testosterone concentrations compared to 'normal' energy availability trials. A significant main effect of time was observed by Kojima et al. (2020), whereby free testosterone concentrations reduced in both the LEA and 'normal' energy availability (~53 kcal•kg FFM^{-1}•day^{-1}) trials. Mckay et al. (2022a) also reported testosterone concentrations to be reduced following six days of training with either adequate energy and carbohydrate availability (control: 40 kcal•kg FFM^{-1}•day^{-1}), an isocaloric LCHF (< 50 g•day^{-1} CHO) diet, or an LEA diet (15 kcal•kg FFM^{-1}•day^{-1}). The lack of changes observed in total testosterone concentrations therefore challenge the idea that low energy availability *per se* reduces testosterone. Other parameters were affected by low energy availability, however.

Low energy availability is also implicated in the generation of poor bone health in the *Male Athlete Triad* model (Nattiv et al., 2021). We observed a significant increase in bone resorption marker (ß-CTx) concentrations during *EB* (Day 2 and 4) and *LEA* (Day 7 and 9), with concentrations returning to baseline values at the end of both intervention stages. Bone formation marker (P1NP) concentrations were reduced at all time-points during *LEA*. These findings are not in agreement with Murphy & Koehler (2020) or Papageorgiou et al. (2017), who observed no changes in markers of bone remodelling in males following three and five days of 15 kcal•kg FFM^{-1}•day^{-1} LEA exposure, respectively. Our

findings are in agreement with those of Murphy et al. (2021), who also observed reduced P1NP and increased ß-CTx concentrations in seven males who had completed 5-days with an EA of 15 kcal•kg FFM^{-1}•day^{-1}. The differing responses of markers of bone re-modelling to low energy availability observed in the present study compared to previous research in males may relate to the more severe reduction in energy availability and its effects upon endocrinological homeostasis. In the present study, *LEA* led to significant reductions in serum IGF-1 and plasma leptin concentrations, both of which may influence bone turnover. As per numerous primary research studies (Ammann et al., 1993; Canalis et al., 1995) and reviews (De Souza, Nattiv, et al., 2014; Yakar & Rosen, 2003), IGF-1 has been shown to be bone-trophic. Plasma leptin concentrations changed alongside alterations to bone remodelling markers and may have mediated these responses. Leptin influences bone metabolism directly and indirectly, with a review from Upadhyay et al. (2015) outlining that its effects are generally thought to be protective of BMD.

More broadly, we observed changes in the endocrine milieu typically associated with energy preservation, as indicated by the drop in leptin concentrations. Limited research has suggested that males may be resilient to perturbation of the hypothalamic-pituitary-thyroid axis (Koehler et al., 2016; Papageorgiou et al., 2017). However, the more stringent reduction in energy availability to 10 kcal•kg FFM^{-1}•day^{-1} in this study led to a significant reduction in total T_3 concentrations after 48 hours of *LEA*, that was maintained across the remainder of the intervention phase. The primary function of thyroid hormones T_3 and T_4 is to set the general metabolic rate of an organism, as well as play a key mediating role in growth and reproduction (Martin et al., 2008). Concentrations of thyroid hormones are typically stable, yet as reviewed by Feldt-Rasmussen (2007), they undergo major physiological regulation in response to caloric deficit/energy expenditure and altered substrate storage. In humans, reductions in circulating T_3 concentrations are believed to contribute to adaptive thermogenesis (Feldt-Rasmussen, 2007; Müller & Bosy-Westphal, 2013). Despite reductions in plasma T_3 concentrations, however, we did not observe changes in RMR throughout the study period. This may have been due to a 'lag' effect between endocrine adaptations - observed within the first days of caloric restriction - and whole-body metabolic responses which develop over multiple weeks (Müller & Bosy-Westphal, 2013). Long-term controlled experiments may therefore observe these effects following LEA in males.

Previous observations in females (Oxfeldt et al., 2023) and males (Areta et al., 2014) have demonstrated that LEA is associated with reductions in myofibrillar protein synthetic rates. We therefore investigated the global fractional synthetic rates for proteins in the myofibrillar, soluble, and mitochondrial fractions and observed that these were not affected by the *LEA* intervention. Areta et al. (2014), demonstrated that a single bout of resistance exercise rescues LEA-related declines in acute fractional synthetic rate. Our findings suggest that the moderate intensity endurance exercise stimulus we employed (~90 mins cycling at 60% $\dot{V}O_{2max}$) also provided sufficient stimulus to preserve skeletal muscle protein synthesis.

These effects may be limited to the working muscle, however, as we analysed muscle samples from the *vastus lateralis*, a knee extensor recruited during cycling exercise (Jorge & Hull, 1986). In support of this, following four-days of severe caloric restriction with exercise (8-hrs of walking and 45-min of unilateral arm-crank exercise), Calbet et al. (2017) observed significantly greater reduction in the FFM of a non-exercised control arm, compared to the three limbs that were exercised. Despite the preservation of muscle protein fractional synthetic rates, we observed a significant reduction in FFM of following LEA. This may be explained by the corresponding and significant reduction in skeletal muscle glycogen concentrations following *LEA*, and associated losses in water associated with glycogen storage (Areta & Hopkins, 2018; Olsson & Saltin, 1970). In agreement with this, assessment of body composition via bioelectrical impedance analysis revealed a 1.2 L reduction in TBW across *LEA*.

Metabolic responses were broadly in line with our hypotheses, with expected increases in plasma NEFA, glycerol, ketone concentrations, as well as increased rates of fat oxidation at rest and during exercise. Plasma glucose or insulin concentrations did not change, which does not align with previous observations reported from LEA studies in females (Loucks, 2006; Loucks & Thuma, 2003; Loucks & Verdun, 1998, 1998; Oxfeldt et al., 2023). In males, Koehler et al. (2016) observed that four days of LEA (15 kcal•kg FFM^{-1}•day^{-1}) reduced fasting glucose concentrations. Our data therefore support the findings of Kojima et al. (2020) and Mckay et al. (2022a), who showed no effect of short-term low energy availability on glucose or insulin concentrations, suggesting that fasting glycaemic responses in males are relatively robust to conditions of short-term low energy availability.

Based upon concerns of residual effects of the *LEA* intervention upon physiological responses, we opted not to randomise participant treatment order within our experimental design. Owing to the cost and quantity of the deuterium oxide tracer required to 'pre-load' and sustain D_2O enrichment, we were unable to perform this on multiple occasions per participant. We therefore could not use a repeated-measures cross-over study design with a washout period between trials. However, we believe that the stringent participant inclusion criteria, in conjunction with the controlled dietary provision and standardisation of sample collections, mitigate this limitation and promote confidence that we are observing treatment effects. Finally, we reduced dietary macronutrient contribution equally from *EB* to *LEA*. Therefore, by reducing EA we also elicited a state of low carbohydrate availability during *LEA*. Given that limited data is emerging to suggest that facets of the *Male Athlete Triad* and *REDs* models (Mountjoy et al., 2023; Nattiv et al., 2021) may in fact be carbohydrate mediated (Fensham et al., 2022; Heikura et al., 2020; Mckay et al., 2022a), the effects of low carbohydrate availability upon these same parameters are investigated in Chapter 6 of this thesis.

In conclusion, five days of low energy availability providing an EA of 10 kcal•kg FFM^{-1}•day^{-1} resulted in expected reductions in body mass, fat mass, and fat free mass in healthy active males. This led to

significant alterations to markers of bone resorption and formation and the endocrine milieu, indicators consistent with early stages of adaptations to preserve energy. However, we did not observe any changes in circulating total testosterone concentrations, RMR, or skeletal muscle protein synthesis. Taken together, these findings suggest that males may be susceptible to certain physiological dysregulations typically associated with LEA, such as impaired bone metabolism and endocrine status.

Chapter 6:

Four days of low carbohydrate availability increases markers of bone resorption and reduces insulin-like growth factor-1 but does not affect other endocrine and physiological markers of low energy availability in active males

6.1. Introduction:

Low carbohydrate availability is a major confounding factor in much of the laboratory-based research investigating the physiological effects of low energy availability. As reviewed by Areta et al. (2021), most experimental low energy availability research studies have reduced EA whilst maintaining a constant relative contribution of each macronutrient. As a representative example, in chapter 5 of this thesis we reduced mean energy availability from 45 (*EB*) to 10 (*LEA*) kcal•kg FFM^{-1}•day^{-1} for five days whilst preserving a macronutrient distribution of 60% carbohydrate, 20% fat, and 20% protein. Mean carbohydrate intake was consequently reduced from 6.7 to 2.4 g•kg^{-1}•day^{-1}. Corresponding reductions in dietary fat, protein, and micronutrient intake might also contribute to the alterations in physiological state observed in low energy availability research to-date. For example, infusion of ionised calcium to has been shown to attenuate exercise-induced increases in ß-CTx and parathyroid hormone during and after cycling exercise (60 minutes at 80% of maximum heart rate), with no effect on P1NP concentrations in healthy, training, males (Kohrt et al., 2018). Altering dietary carbohydrate content is of particular interest though, as this has been shown to be a major factor influencing metabolism and training adaptation (for detailed reviews, see Gonzalez et al., 2016, and Hearris et al., 2018).

Low carbohydrate availability has also been shown to influence factors related to low energy availability independently from an energy deficit, such as markers of bone resorption and formation (Fensham et al., 2022; Hammond et al., 2019; Heikura et al., 2020). It is therefore unclear as to what extent the physiological effects typically associated with low energy availability are due to an energy deficit, *per se,* or a corresponding reduction in carbohydrate availability. In practice, contemporary sports nutrition guidelines typically recommend athletes increase carbohydrate intake relative to greater exercise load (Thomas et al., 2016). However, considering the discrepancy between nutrition guidelines for training and competition, and athlete's nutrition knowledge, attitudes, and practices (McHaffie et al., 2022; Sampson et al., 2023), it is possible that low carbohydrate availability is at least a contributing factor in the physiological responses typically attributed to low energy availability in the wider athlete population. Indeed, in chapter four of this thesis, we have shown that elite male road cyclists only consumed an extra ~160 kcal•day^{-1} of carbohydrate per 1000 kcal•day^{-1} increase in exercise energy expenditure (Taylor et al., 2022). Expanding our knowledge of the effects of low carbohydrate availability, in the absence of low energy availability, to parameters more commonly associated with the *Triad* (De Souza, Nattiv, et al., 2014; Nattiv et al., 2021) and *REDs* (Mountjoy et al., 2023) models is therefore warranted.

Research into the effects of low carbohydrate diets on substrate use and physical performance have been conducted for over a century and provide a cornerstone of sports nutrition guidelines (Bergström et al., 1967; Krogh & Lindhard, 1920; Thomas et al., 2016). However, little is known about the effect of low carbohydrate availability on physiological parameters typically thought to be mediated by low

energy availability. Most of the available evidence appears to relate to carbohydrate availability and bone health. Providing a mixed-macronutrient meal pre- (Scott et al., 2012), or carbohydrate feeding during- (Sale et al., 2015), and post- (Townsend et al., 2017) a single bout of exercise, as well as around twice-daily exercise sessions (Hammond et al., 2019) has been shown to attenuate ß-CTx concentrations in males, whilst P1NP responses appear mixed. Similarly, Fensham et al. (2022) and Heikura et al. (2020) have shown that following a ketogenic diet providing < 50 g•day^{-1} of carbohydrate over 6 days and 3.5 weeks, respectively, increased ß-CTx and reduced P1NP concentrations in elite race-walkers. Despite the possibly widespread underconsumption of carbohydrates in athletic populations (Chapter 4), little is known about the effects of reduced carbohydrate availability upon the parameters typically associated with low energy availability.

As such, this study aimed to investigate the physiological impact of four days of low carbohydrate availability under conditions of energy balance, compared to an isoenergetic 'normal' carbohydrate control trial, in healthy active males. We hypothesised that four days of low carbohydrate availability would reduce fat free mass, bone formation marker P1NP, T_3, leptin, IGF-1, glucose, insulin, and muscle glycogen concentrations. We also hypothesised that concentrations of bone resorption marker ß-CTx, plasma glycerol, NEFA, and ketone concentrations would increase during the low carbohydrate availability intervention, but that total testosterone and resting metabolic rate would not change.

6.2. Methods:

Participants:

Eight healthy, active males were recruited via convenience sampling from the staff and student body of Liverpool John Moores University to participate in the study. Inclusion criteria for participants is outlined in Chapter 3 (General methods). Participant characteristics are outlined in Table 6.2.1. Based upon the criteria presented by McKay et al. (2022b), all participants were classified as Tier 1 – 2 individuals.

Table 6.2.1. Participant descriptive characteristics

N	Age (yrs)	Height (m)	Body Mass (kg)	Body Fat (%)	$\dot{V}O_{2max}$ ($ml \cdot kg^{-1} \cdot min^{-1}$)	Peak Power Output (W)
8	27 ± 4	1.79 ± 0.06	79.0 ± 11.5	16.8 ± 4.2	54 ± 6	323 ± 43

Study Design:

Using a randomised, cross-over study design, all participants completed baseline screening and testing, followed by two separate study intervention-periods of four consecutive days of either normal (*NORM*), or low (*LOW*) carbohydrate availability (Figure 6.2.1). A washout period ≥ 7 days was used between trials. Ethical approval was granted by Liverpool John Moores University's research ethics committee (Ref: 22/SPS/026) and registered on the clinical trials register (ClinicalTrials.gov ID: NCT05551455).

Pre-participation screening:

Participants provided written informed consent and then completed an allergen screening questionnaire, readiness to exercise questionnaire and pre-biopsy medical questionnaire. Participants were also screened for possible eating disorders using the eating attitude test (*EAT-26*) (Garner et al., 1982). Provided that a participant did not report any contra-indications in the screening process, they were enrolled into the study for baseline testing.

Baseline Testing:

Participants attended the laboratory in an overnight fasted state for a baseline test at least two days, and no more than seven days prior to their proposed start-date for the study intervention phase. The participant voided their bladder and body composition was assessed in light clothing via bio-electrical impedance analysis (See Chapter 3: General methods).

Participants were fitted with a Bluetooth heart rate (*HR*) monitor (Polar H7, Polar, Kempele, Finland) and set up on an electro-magnetically braked cycle ergometer (Lode Corival cpet: Lode, Groningen, Netherlands) to an appropriate saddle height and handlebar position, with this recorded for future lab

visits. Participants then performed a two-part incremental cycle test to determine sub-maximal ($\dot{V}O_2$) and maximal oxygen consumption ($\dot{V}O_{2max}$), lactate threshold and PPO, as outlined in Chapter 3 (General methods). Following a rest period of ≥ 20 mins, participants completed 20 minutes of cycling to serve as a brief familiarisation. Participants completed three minutes of cycling at a 125 W, followed by a further 17 minutes at 95%. lactate turn-point 1 (*LTP 1*). LTP 1 was defined as the exercise intensity corresponding to an increase of ≥ 1 mmol•L^{-1} between stages in the submaximal exercise test (Newell et al., 2015). Expired gases were collected continuously for 15 minutes and then from 17.5 – 20 mins. HR and RPE were also recorded at 3-minute intervals from 0 – 15 mins and for minute 20.

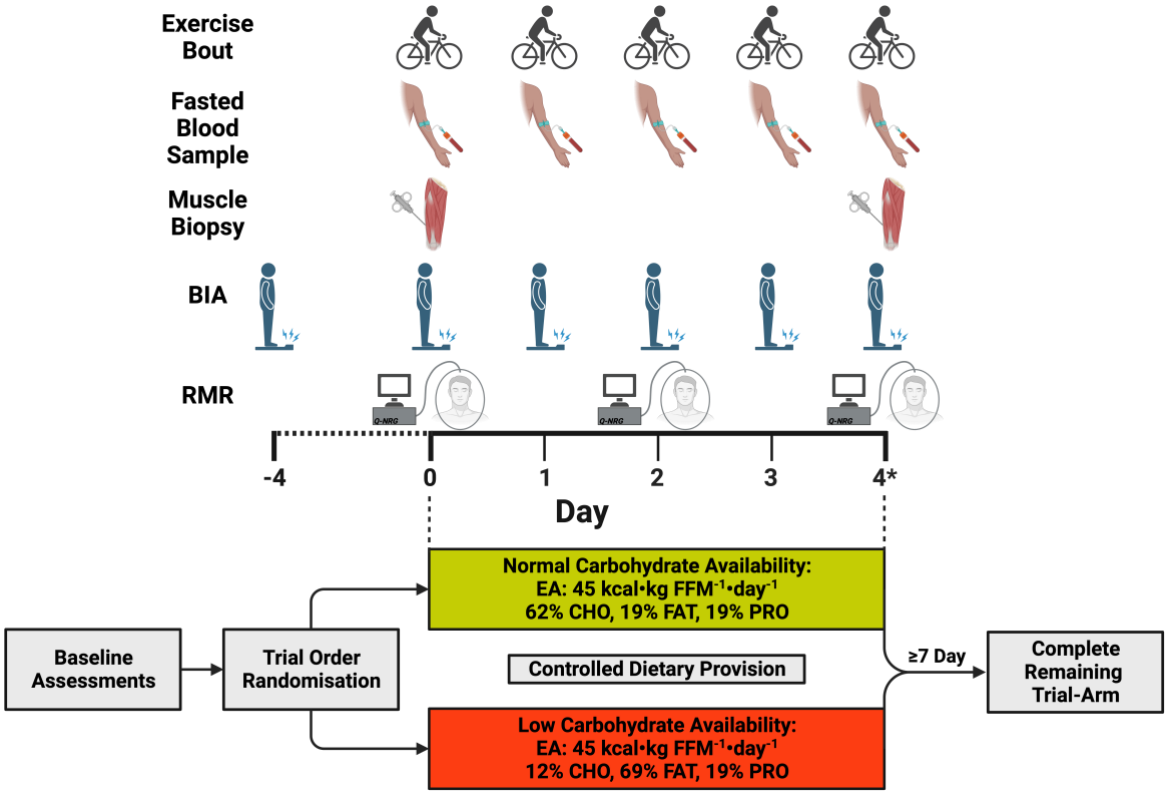


Figure 6.2.1. Overview of study design and sample collection schedule. Participants completed two 4-day intervention periods in randomised order, with a ≥7-day washout period between trials. *On Day 4, participants performed only 30 mins of exercise and were then free to return to their normal diet. Exercise bout = cycle ergometry at 95% lactate turn-point 1 to expend 15 kcal•kg FFM^{-1}, BIA = bio-electrical impedance analysis of body composition, RMR = resting metabolic rate assessment.

Study Phases:

For all testing sessions participants attended the *RISES* laboratories on Day -4 and daily from Day 0 to Day 4, arriving between 07:00 – 08:00 hrs in a fasted state and having minimised active transport to arrive at campus. To monitor adherence, participants verbally confirmed that they had avoided exercise to travel to the laboratory, that they were in an overnight fasted and reported the approximate time of

their last dietary intake upon arrival. An outline of the study intervention and sample collection schedule is outlined in Figure 6.2.1. A full description of each protocol can be found in Chapter 3 (General methods).

Free-living monitoring phase:

Four-days prior to each intervention phase (Day -4), participants were asked to attend the laboratory to for a baseline body composition assessment. Upon arrival, participants were asked to void their bladder and then completed a body composition assessment via bio-electrical impedance analysis (SECA mBCA 515: SECA GMBH, Hamburg, Germany), as previously described. The FFM recorded on Day -4 of a participant's first trial served as the reference FFM for dietary provision and EEE throughout the rest of the study.

Study Intervention Phases:

Participants attended the *RISES* laboratories between 07:00 – 08:00 hrs on Day 0 to Day 4 for both *NORM* and *LOW*. A brief description of each assessment parameter is outlined below, a full description of each protocol can be found in Chapter 3: General Methods. Each assessment is listed below in the chronological order that the parameter was collected in on corresponding data collection mornings:

Resting metabolic rate assessment:

RMR was assessed on Days 0, 2, and 4 of *NORM* and *LOW*, as outlined previously (See Chapter 3: General methods).

Body Composition Assessments:

Participants completed a body composition assessment using bio-electrical impedance analysis at each lab visit, on Day -4 and Days 0 – 4 during *NORM* and *LOW* as outlined in Chapter 3 (General methods).

Fasting venous blood sample:

A fasting blood sample was drawn and processed in line with the methodology outlined in Chapter 3 (General methods) on Days 0 – 4 of both the *NORM* and *LOW* trials.

Skeletal muscle biopsy:

A skeletal muscle biopsy was obtained from the lateral portion of the VL muscle on Day 0 and Day 4 of *NORM* and *LOW*, in line with the procedures detailed previously (See Chapter 3: General methods).

Exercise protocol:

Following the collection of all measurements and samples, participants completed cycling exercise at 95% of LTP 1, in line with the methodologies detailed in (Chapter 3: General methods). Following 30-

, 60-, and (if necessary) 90-mins of exercise, participants were provided with a 5-minute rest period where they were allowed to dismount the cycle ergometer. At the 30-minute rest point, participants were provided with a single *SIS GO* carbohydrate gel, providing 22 g CHO, 0 g PRO, 0 g FAT, 87 kcal (Science in Sport, Nelson, UK). Following each rest period, exercise was resumed at the same power output and sessions were continued until a net exercise energy expenditure equating to 15 kcal·kg FFM^{-1}, calculated from indirect calorimetry values, had been attained. Total exercise time and a final HR and RPE were recorded at the cessation of exercise.

Exercise sessions were completed on days 0, 1, 2, and 3. On day 4, following the final collection of all samples, only the first 30 minutes of the exercise session was performed as described previously.

Dietary Interventions:

Following completion of the exercise protocol during *NORM* and *LOW*, participants were provided with all dietary intake for the remainder of the day until their next lab visit. They were then free to leave the laboratory.

Other than the manipulation of carbohydrate availability, all parameters of data collection and exercise were kept consistent between interventions. Details of the dietary intake provided during *NORM* and *LOW* interventions are outlined in Table 6.2.2. Briefly, dietary intake during both trial arms provided an EI of 60 kcal·kg FFM^{-1}·day^{-1}. In conjunction with a daily exercise energy expenditure of 15 kcal·kg FFM^{-1}·day^{-1}, resultant energy availability was fixed at 45 kcal·kg FFM^{-1}·day^{-1} in both trials. The *NORM* diet provided a 62% carbohydrate, 19% protein, and 19% fat macronutrient distribution, whilst *LOW* provided 12% carbohydrate, 19% protein, and 69% fat. Dietary micronutrient content associated with the *NORM* and *LOW* diets is reported in Table 6.2.3. The *Nutrient Reference Value* column of Table 6.3.2. refers to the minimum daily reference intake for adults for vitamins and minerals, as specified in *Regulation (EU) No 1169/2011 of The European Parliament and of the Council of 25 October 2011*.

Table 6.2.2. Dietary provision during the *NORM* and *LOW* carbohydrate availability interventions

	NORM **(mean ± SD)**	***LOW*** **(mean ± SD)**
Energy Intake:		
kcal•day^{-1}	3927 ± 460	3924 ± 458
kcal•kg FFM^{-1}•day^{-1}	60.0 ± 0.0	60.0 ± 0.1
Carbohydrate Intake:		
kcal•day^{-1}	2427 ± 292	466 ± 59
% Energy Intake	61.8 ± 0.5	11.9 ± 0.4
g•day^{-1}	607 ± 73	117 ± 15
g•kg^{-1}•day^{-1}	7.7 ± 0.4	1.5 ± 0.0
Fat Intake:		
kcal•day^{-1}	759 ± 74	2713 ± 311
% Energy Intake	19.4 ± 0.6	69.2 ± 1.0
g•day^{-1}	84 ± 8	301 ± 35
g•kg^{-1}•day^{-1}	1.1 ± 0.1	3.8 ± 0.2
Protein Intake:		
kcal•day^{-1}	741 ± 99	744 ± 96
% Energy Intake	18.9 ± 0.6	18.9 ± 0.6
g•day^{-1}	185 ± 25	186 ± 24
g•kg^{-1}•day^{-1}	2.4 ± 0.1	2.4 ± 0.1

Sample Analyses:

Plasma glucose, lactate, NEFA, and glycerol concentrations were analysed using commercially available kits and a Randox Daytona spectrophotometer (Randox Laboratories Ltd., Crumlin, UK). Commercially available ELISA were used to measure plasma total T_3 and leptin, and serum IGF-1, EPO, and GDF-15. Plasma bone turnover markers (ß-CTX and P1NP), serum insulin, and serum total testosterone concentrations were analysed by a commercial laboratory (for details on all methods, please see Chapter 3: General methods).

Statistical Analyses:

Data were collated using Microsoft (v. 16.77.1, Microsoft Corp., Redmond, WA, USA). Statistical analyses were conducted using IBM SPSS Statistics (v. 28.0.0.0, IBM, Armonk, NY, USA). Graphs were created using Graphpad Prism (v. 8.2.1, Graphpad Software Inc., San Diego, California, USA). Data were explored via a 2-way repeated measures ANOVA. Sphericity was checked and the

Greenhouse-Geisser epsilon value used when this value was ≤ 0.75. Where the Greenhouse-Geisser value was > 0.75, the Huynh-Feldt corrected value was used (Atkinson, 2001). Data collected during exercise were explored using linear mixed models. Post-hoc analyses were conducted using a least significant difference correction in the event of a significant ANOVA model. Statistical significance was set at the $P < 0.05$ threshold. Changes in all data were explored using *Hedge's g* effect sizes (Lakens, 2013) calculated manually using Microsoft Excel: < 0.20 = *Trivial*, 0.21 – 0.60 – *Small*, 0.61 – 1.20 *Moderate*, 1.21 – 1.99 = *Large*, ≥ 2.00 = *Very Large* (Hopkins et al., 2009).

Table 6.2.3. Micronutrient provision during the *NORM* and *LOW* carbohydrate availability interventions

	NORM **(mean ± SD)**	***LOW*** **(mean ± SD)**	***Nutrient Reference Values***
Minerals and Trace Elements:			
Sodium (mg)	3849 ± 504	3918 ± 524	
Potassium (mg)	416 ± 65	504 ± 39	2000
Chloride (mg)	6497 ± 893	6114 ± 838	800
Calcium (mg)	1071 ± 191	1667 ± 245	800
Phosphorous (mg)	833 ± 137	1451 ± 174	700
Magnesium (mg)	175 ± 41	90 ± 10	375
Iron (mg)	14 ± 1	4 ± 0	14
Zinc (mg)	6 ± 1	11 ± 1	10
Copper (mg)	1 ± 0	0 ± 0	1
Manganese (mg)	2 ± 0	0 ± 0	2
Selenium (µg)	17 ± 2	25 ± 1	55
Iodine (µg)	127 ± 15	190 ± 13	150
Vitamins:			
Vitamin A (µg)	547 ± 80	1228 ± 143	800
Vitamin D (µg)	10 ± 1	4 ± 0	5
Vitamin E (mg)	3 ± 1	16 ± 5	12
Vitamin K1 (µg)	2 ± 1	29 ± 3	75
Thiamin (Vitamin *B1*) (mg)	2 ± 0	0 ± 0	1.1
Riboflavin (Vitamin B2) (mg)	2 ± 0	1 ± 0	1.4
Total Niacin (Vitamin B3) (mg)	16 ± 3	21 ± 2	16
Pantothenic Acid (Vitamin B5) (mg)	4 ± 1	3 ± 0	6
Vitamin B6 (mg)	3 ± 1	1 ± 0	1.4
Total Folates (Vitamin B9) (µg)	317 ± 40	139 ± 13	200
Vitamin B12 (µg)	7 ± 1	9 ± 1	2.5
Biotin (B7) (µg)	41 ± 5	38 ± 1	50
Vitamin C (mg)	189 ± 59	38 ± 1	80

6.3. Results:

Exercise energy expenditure and energy intake:

An overview of the exercise data for all days of the *NORM* and *LOW* interventions are outlined in Table 6.3.1. Exercise sessions lasted 89 ± 12 mins during *NORM* and 86 ± 12 mins during *LOW*, resulting in mean net exercise energy expenditures of 15.0 ± 0.1 kcal•kg FFM^{-1} for both *NORM* and *LOW*, CHO interventions. After the standardised warm-up, mean workload as a percentage of $\dot{V}O_{2max}$ during steady state exercise was 62.5 ± 3.6 % and 63.8 ± 4.3 % during *NORM* and *LOW*, respectively.

Table 6.3.1. Mean total exercise time, relative workload (as a percentage of lactate turn point 1 and of $\dot{V}O_{2max}$) during steady state exercise, and net exercise energy expenditure during *NORM* and *LOW* carbohydrate availability trials

Parameter	*NORM*			*LOW*		
	Mean	**SD**	**Range**	**Mean**	**SD**	**Range**
Total Exercise Time (mins)	89	12	67 – 108	87	13	62 – 108
% Lactate Turn Point 1	95	3	90 – 99	95	3	90 – 99
% $\dot{V}O_{2max}$	63	4	56 – 66	64	4	56 – 68
EEE_{net} (kcal/kg FFM)	15.0	0.1	14.8 – 15.2	15.0	0.1	14.9 – 15.1

A breakdown of the energy provision, macronutrient intake and resultant energy availability are outlined in Table 6.2.2 (see Chapter 6.2: Methods). In short, the controlled diets provided 60 ± 0 kcal•kg FFM^{-1}•day^{-1} in *NORM* and 60 ± 0 kcal•kg FFM^{-1}•day^{-1} during *LOW*. In conjunction with the exercise sessions, resultant mean energy availabilities were 45 ± 0 kcal•kg FFM^{-1}•day^{-1} during both *NORM* and *LOW*. Daily dietary provision and prescribed exercise energy expenditure were kept consistent throughout both interventions.

Body Mass and Body Composition:

Body Mass: There was a significant main effect of treatment ($F_{1, 7}$ = 11.73, P = 0.011), and time x treatment interaction effect ($F_{1.68, 11.75}$ = 17.76, P < 0.001) for CHO availability, but no main effect of time ($F_{1.31, 9.16}$ = 3.25, P = 0.098). Body mass was significantly greater from Day 2 onwards in *NORM*, relative to *LOW* **(**Figure 6.3.1.A, Table 6.3.2), yet the magnitude of the effect was *Trivial* ($g \leq 0.1$) at all these time-points.

Fat Free Mass: There was a significant main effect of treatment ($F_{1, 7}$ = 18.63, P = 0.003), and time x treatment interaction effect ($F_{2.17, 15.17}$ = 6.04, P = 0.010) for CHO availability upon fat free mass, but no main effect of time ($F_{2.08, 14.59}$ = 3.50, P = 0.056). From Day 1 onwards fat free mass was greater in *NORM* relative to *LOW* (Figure 6.3.1.B, Table 6.3.2), although the magnitude of effect was *Trivial* or *Small* ($g \leq 0.2$) at all time points.

Fat Mass: Absolute fat mass was unaffected within or between interventions (Figure 6.3.1.C. Main effect of treatment: $F_{1, 7} = 1.73$, $P = 0.230$; time: $F_{2.85, 19.93} = 1.55$, $P = 0.235$; time x treatment interaction: $F_{1.86, 13.0} = 1.55$, $P = 0.248$) and the magnitude of effect was *Trivial* ($g \leq 0.1$) at all time points.

Fat mass percentage was also unchanged within or between interventions (main effect: $F_{1, 7} = 3.41$, $P = 0.107$; effect of time: $F_{2.72, 19.05} = 1.92$, $P = 0.164$; time x treatment interaction effect: $F_{1.97, 13.80} = 1.68$, $P = 0.222$). The magnitude of effect was *Trivial* or *Small* ($g \leq 0.2$) at all time points.

Total Body Water: There was a significant main effect of treatment ($F_{1, 7} = 20.39$, $P = 0.003$), and time x treatment interaction effect ($F_{2.29, 16.00} = 5.88$, $P = 0.010$), but no main effect of time ($F_{2.15, 15.15} = 3.46$, $P = 0.055$). TBW was significantly greater from Day 2 onwards in *NORM*, compared to *LOW* (Figure 6.3.1.D, Table 6.3.2), yet the magnitude of effect was *Trivial* or *Small* ($g \leq 0.2$) at all timepoints.

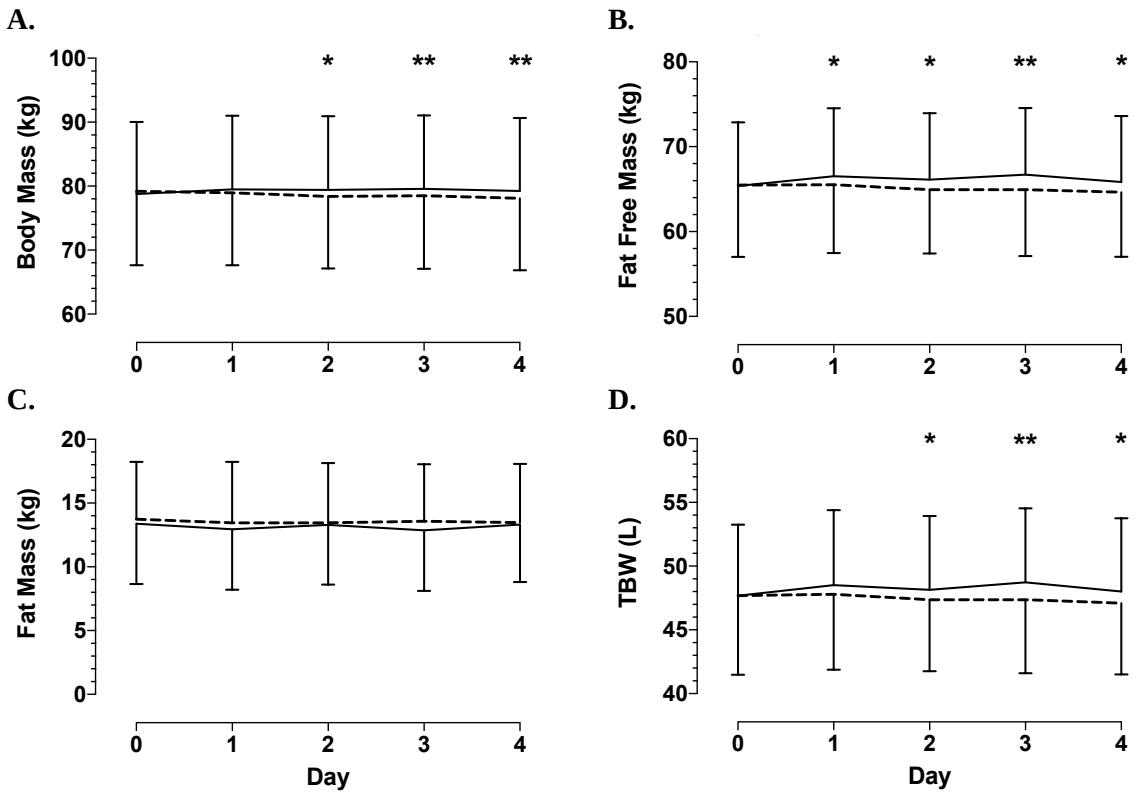


Figure 6.3.1. (A) Body Mass, (B) Fat Free Mass, (C) Fat Mass, and (D) Total Body Water (*TBW*) values over the *NORM* (solid line) and *LOW* (dashed line) CHO availability interventions. Significance markers represent a difference between respective time-points in *NORM* compared to *LOW* intervention; $^{*} = P < 0.05$, $^{**} = P < 0.001$.

Table 6.3.2. Body mass, fat free mass, and total body water (*TBW*) values for *NORM* and *LOW* interventions, post-hoc assessment of differences and magnitudes of effect between body composition parameters where a significant time x treatment interaction effect (P < 0.05) was observed

	Day 0		**Day 1**		**Day 2**		**Day 3**		**Day 4**	
	NORM	***LOW***	***NORM***	***LOW***	***NORM***	***LOW***	***NORM***	***LOW***	***NORM***	***LOW***
Body Mass (kg):	78.8 ± 11.3	79.2 ± 11.6	79.5 ± 11.5	79.0 ± 11.3	79.4 ± 11.5	78.4 ± 11.3	79.6 ± 11.5	78.5 ± 11.4	79.3 ± 11.4	78.1 ± 11.2
Mean Δ (95% CI)	0.4 (-0.4 to 1.3)		0.5 (-0.0 to 1.1)		1.0 (0.5 to 1.5)		1.1 (0.8 to 1.4)		1.2 (0.7 to 1.6)	
P	0.246		0.062		**0.003**		**< 0.001**		**< 0.001**	
g	0.0		0.0		0.1		0.1		0.1	
Fat Free Mass (kg):	65.4 ± 7.5	65.5 ± 8.5	66.5 ± 8.0	65.5 ± 8.1	66.1 ± 7.8	64.9 ± 7.5	66.7 ± 7.9	64.9 ± 7.8	65.8 ± 7.8	64.6 ± 7.6
Mean Δ (95% CI)	0.1 (-1.1 to 1.3)		1.0 (0.1 to 1.9)		1.2 (0.7 to 1.7)		1.8 (1.1 to 2.5)		1.2 (0.6 to 1.8)	
P	0.858		**0.033**		**0.001**		**< 0.001**		**0.002**	
g	0.0		0.1		0.1		0.2		0.1	
TBW (L)	47.7 ± 5.6	47.7 ± 6.2	48.5 ± 5.9	47.8 ± 5.9	48.1 ± 5.8	47.4 ± 5.6	48.7 ± 5.8	47.4 ± 5.8	48.0 ± 5.8	47.1 ± 5.6
Mean Δ (95% CI)	0.0 (-0.8 to 0.8)		0.7 (-0.0 to 1.5)		0.8 (0.4 to 1.1)		1.4 (0.9 to 1.9)		0.9 (0.5 to 1.3)	
P	0.997		0.054		**0.001**		**< 0.001**		**0.001**	
g	0.0		0.1		0.1		0.2		0.2	

Post-hoc statistical changes assessed via least significant difference (*LSD*) following observation of a significant time x treatment interaction effect. Significant changes (*P* < 0.05) denoted in bold. *P* value represents difference at respective time-points between *NORM* and *LOW* trial intervention arms. Effect sizes (Hedge's *g*) represent difference between *NORM* and *LOW* respective time-points: < 0.20 = *Trivial*, 0.21 – 0.60 – *Small*, 0.61 – 1.20 *Moderate*, 1.21 – 1.99 = *Large*, ≥ 2.00 = *Very Large*.

Bloodborne Metabolic Markers:

Glucose: Plasma glucose concentrations were unchanged within or between interventions, with no significant main effect of treatment ($F_{1, 7}$ = 1.40, P = 0.275), time ($F_{2.51, 17.53}$ = 1.33, P = 0.295), or time x treatment interaction ($F_{2.94, 20.57}$ = 0.99, P = 0.418) apparent (Figure 6.3.2.A). A *small* magnitude of effect (g = 0.3) was shown on Day 1 and a *moderate* magnitude of effect was shown on Day 3 (g = 0.6) between *NORM* and *LOW*. Magnitudes of effect were trivial (g < 0.2) for all other time-points.

Ketones: Plasma D-3-Hydroxybutyrate concentrations displayed a significant main effect of treatment ($F_{1, 7}$ = 8.08, P = 0.025), time ($F_{1.60, 11.20}$ = 6.49, P = 0.017), and time x treatment interaction effect ($F_{1.85, 12.98}$ = 16.10, P < 0.001). Ketone concentrations were increased on Days 3 and 4 of *LOW*, compared to *NORM* (Figure 6.3.2.B, Table 6.3.3). The magnitude of effect for the differences in ketone concentrations between *NORM* and *LOW* were *large* on Day 3 (g = 1.2) and *very large* on Day 4 (g = 2.4).

NEFA: Fasting plasma NEFA concentrations showed a significant time x treatment interaction effect for the CHO intervention ($F_{2.34, 16.36}$ = 5.05, P = 0.016), but no significant main effect of treatment ($F_{1, 7}$ = 0.01, P = 0.910) or time ($F_{2.70, 18.89}$ = 0.45, P = 0.702). Plasma NEFA concentrations increased on Day 4 of *LOW*, compared to *NORM* (Figure 6.3.2.C; Table 6.3.3), with a *very large* magnitude of effect (g = 2.3) observed between interventions at this time-point.

Glycerol: Fasting plasma glycerol concentrations exhibited a significant time x treatment interaction effect ($F_{2.52, 17.60}$ = 5.13, P = 0.013), but no main effect of treatment ($F_{1, 7}$ = 2.70, P = 0.145) or time ($F_{2.71, 18.94}$ = 0.71, P = 0.544). Concentrations of plasma glycerol were elevated on Days 3 and 4 of *LOW*, compared to *NORM* (Figure 6.3.2.D, Table 6.3.3). Magnitudes of effect were *trivial* or *small* ($g \leq 0.4$) from Day 0 to Day 2. A *large* magnitude of effect (g = 1.8) observed on both Day 3 and Day 4 between *NORM* and *LOW*.

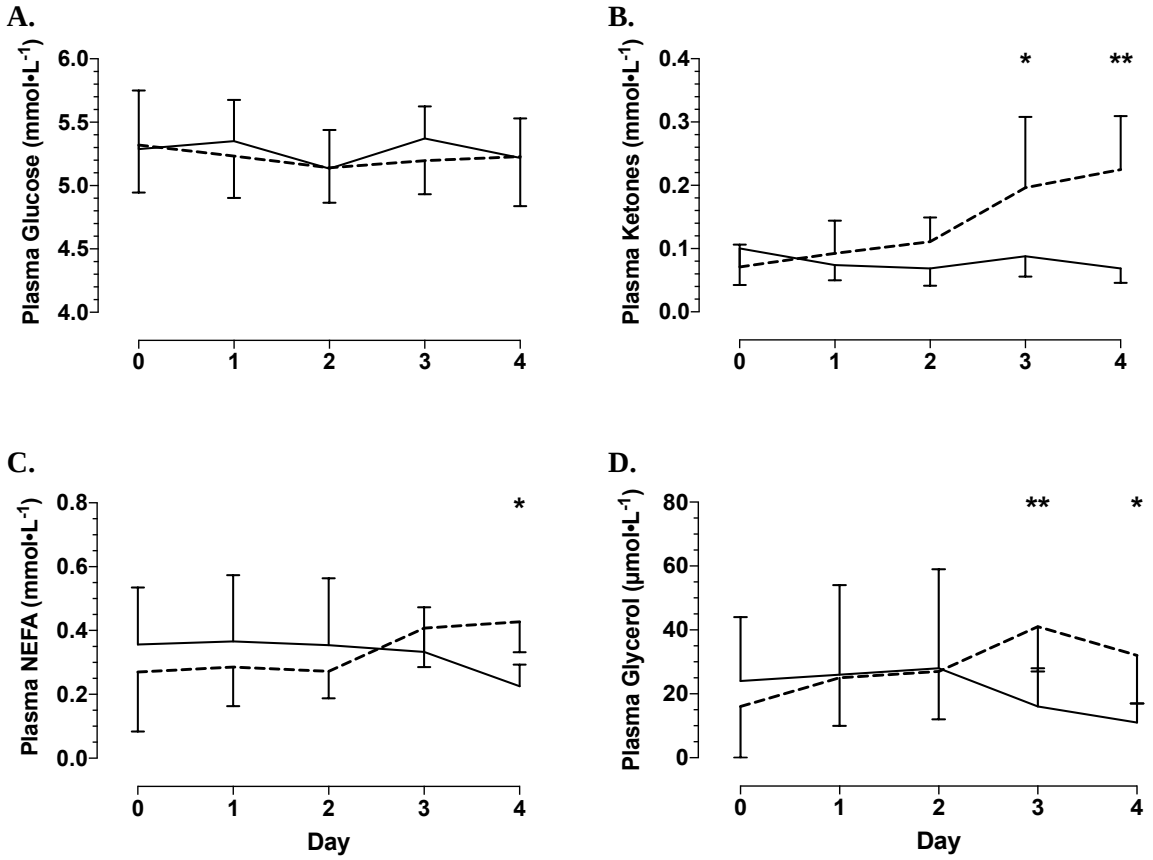


Figure 6.3.2. Concentrations of (A) plasma glucose, (B) plasma ketone (D-3-Hydroxybutyrate), (C) plasma NEFA, and (D) plasma glycerol concentrations over the course of *NORM* (solid line) and *LOW* (dashed line) interventions. $^{*} = P < 0.05$, $^{**} = P < 0.001$ between respective trial days in *NORM* and *LOW*.

Table 6.3.3. Concentrations of circulating ketones (D-3-Hydroxybutyrate), NEFA, and glycerol, post-hoc differences, and magnitudes of effect between *NORM* and *LOW* concentrations following the observation of a significant time x treatment interaction effect ($P < 0.05$)

	Day 0		**Day 1**		**Day 2**		**Day 3**		**Day 4**	
	NORM	***LOW***	***NORM***	***LOW***	***NORM***	***LOW***	***NORM***	***LOW***	***NORM***	***LOW***
D-3-Hydroxybutyrate (mmol•L^{-1})	0.10 ± 0.06	0.07 ± 0.04	0.07 ± 0.02	0.09 ± 0.05	0.07 ± 0.03	0.11 ± 0.04	0.09 ± 0.03	0.20 ± 0.11	0.07 ± 0.02	0.22 ± 0.08
Mean Δ (95% CI)	0.03 (-0.01 to 0.07)		0.02 (-0.03 to 0.07)		0.04 (-0.00 to 0.08)		0.11 (0.01 to 0.21)		0.16 (0.09 to 0.22)	
P	0.100		0.389		0.050		**0.036**		**< 0.001**	
g	0.6		0.4		1.2		1.2		2.4	
NEFA (mmol•L^{-1})	0.36 ± 0.18	0.27 ± 0.19	0.37 ± 0.21	0.29 ± 0.12	0.35 ± 0.21	0.27 ± 0.08	0.33 ± 0.14	0.41 ± 0.12	0.23 ± 0.07	0.43 ± 0.10
Mean Δ (95% CI)	0.09 (-0.08 to 0.25)		0.08 (-0.14 to 0.30)		0.08 (-0.10 to 0.27)		0.07 (-0.19 to 0.04)		0.20 (0.09 to 0.32)	
P	0.255		0.410		0.325		0.182		**0.004**	
g	0.4		0.5		0.5		0.5		2.3	
Glycerol (µmol•L^{-1})	24 ± 20	16 ± 17	26 ± 28	25 ± 15	28 ± 31	27 ± 15	16 ± 12	41 ± 14	11 ± 6	32 ± 15
Mean Δ (95% CI)	8 (-9 to 24)		1 (-23 to 26)		1 (-19 to 21)		25 (16 to 34)		22 (8 to 35)	
P	0.325		0.916		0.896		**< 0.001**		**0.008**	
g	0.4		0.0		0.0		1.8		1.8	

Post-hoc statistical changes assessed via least significant difference (*LSD*) following observation of a significant time x treatment interaction effect. Significant changes ($P < 0.05$) denoted in bold. *P* value and effect sizes (Hedge's *g*) represents difference at respective time-points between *NORM* and *LOW* trial intervention arms. Effect size (*g*): < 0.20 = *Trivial*, 0.21 – 0.60 = *Small*, 0.61 – 1.20 = *Moderate*, 1.21 – 1.99 = *Large*, ≥ 2.00 = *Very Large*.

Endocrine Markers:

Insulin: Serum insulin concentrations were unaffected by the CHO availability intervention, with no main effect of treatment ($F_{1, 7}$ = 2.57, P = 0.153), time ($F_{2.43, 17.04}$ = 2.59, P = 0.096), or time x treatment interaction effect ($F_{2.15, 15.06}$ = 1.74, P = 0.208) observed (Figure 6.3.3.A). There was a *moderate* magnitude of effect on Day 3 (g = 0.7) of the intervention, however.

Testosterone: Serum total testosterone concentrations were similarly unaffected by the CHO availability intervention (main effect of treatment: $F_{1, 7}$ = 3.80, P = 0.092; time: $F_{1.95, 13.66}$ = 2.51, P = 0.118; time x treatment interaction: $F_{2.21, 15.45}$ = 0.317, P = 0.753; Figure 6.3.3.B). Magnitudes of effect were ≤ 0.4 and therefore *trivial* or *small* at all timepoints.

Triiodothyronine (T_3): Plasma T_3 concentrations were stable throughout the study (main effect of treatment: $F_{1, 7}$ = 0.61, P = 0.461; time: $F_{2.11, 14.76}$ = 0.99, P = 0.398; time x treatment interaction: $F_{1.77, 12.39}$ = 0.15, P = 0.840; Figure 6.3.3.C). Magnitudes of effect were ≤ 0.2 and therefore either *trivial* or *small* at all timepoints.

Leptin: Similarly, plasma leptin concentrations exhibited no significant main effect of treatment ($F_{1, 7}$ = 4.53, P = 0.071) or time x treatment interaction effect ($F_{1.56, 10.90}$ = 0.396, P = 0.632) of CHO availability intervention upon plasma leptin concentrations (Figure 6.3.3.D). However, a significant time effect was observed ($F_{2.69, 18.84}$ = 5.03, P = 0.012). Magnitudes of effect were ≤ 0.4 and therefore either *trivial* or *small* at all timepoints.

IGF-1: Serum IGF-1 concentrations displayed a significant time x treatment effect ($F_{4.00, 28.00}$ = 5.52, P = 0.002), but no main effect of treatment ($F_{1, 7}$ = 1.21, P = 0.309), or time (Huynh-Feldt corrected: $F_{2.17, 15.16}$ = 0.575, P = 0.587). Serum IGF-1 concentrations were significantly reduced on Day 4 of *LOW*, compared to *NORM* (Figure 6.3.3.E; Table 6.3.4). Magnitudes of effect were ≤ 0.4 and therefore either *trivial* or *small* from Day 0 to Day 2. A *moderate* magnitude of effect was observed on Day 3 (g = 0.6). Despite a significantly reduced serum IGF-1 concentrations on Day 4 of *LOW* compared to *NORM*, the magnitude of effect was *small* (g = 0.5) at this time-point.

Growth/Differentiation Factor-15 (GDF-15): Plasma GDF-15 concentrations displayed a significant main effect of treatment ($F_{1, 7}$ = 19.00, P = 0.003), but no main effect of time ($F_{1.53, 10.71}$ = 1.73, P = 0.223), or time x treatment interaction ($F_{1.86, 12.98}$ = 2.73, P = 0.105; Figure 6.3.3.F). A *moderate* magnitude of effect was observed between *NORM* and *LOW* on Day 0 (g = 0.6) but was either *trivial* or *small* for all other timepoints.

Erythropoietin (EPO): Plasma EPO concentrations were unaffected within or between interventions (main effect of treatment: $F_{1, 7}$ = 2.19, P = 0.183; time: $F_{1, 7}$ = 2.56, P = 0.153; time x treatment interaction: $F_{1, 7}$ = 0.414, P = 0.603). Magnitude of effects were ≤ 0.3 at all timepoints and therefore either *trivial* or *small*.

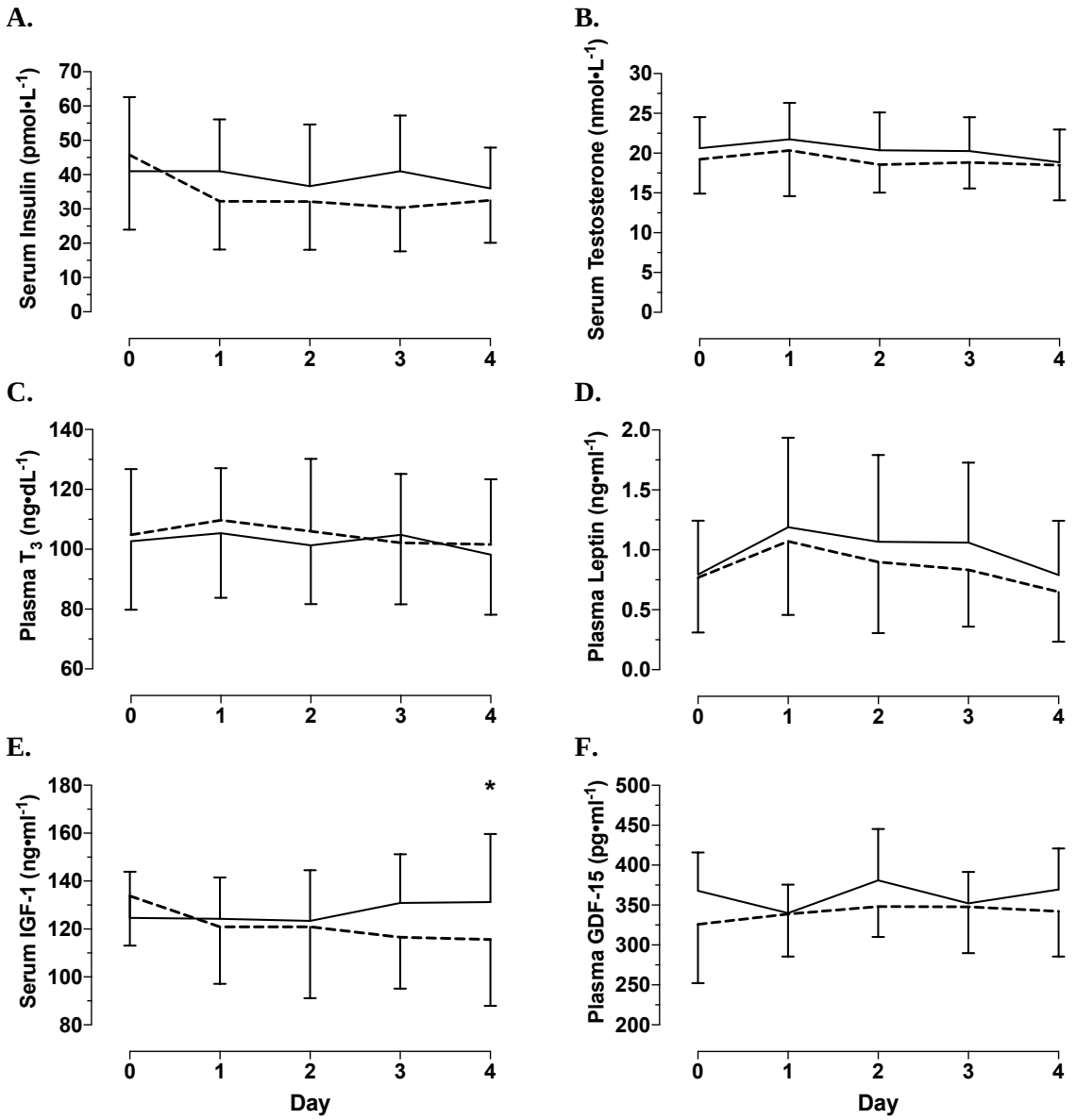


Figure 6.3.3. Concentrations of (A) serum insulin, (B) serum testosterone, (C) plasma triiodothyronine (T_3), (D) plasma leptin, (E) serum IGF-1, and (F) plasma GDF-15 over the course of *NORM* (solid line) and *LOW* (dashed line) interventions. * = P < 0.05 between respective trial days in *NORM* and *LOW*.

Physiological Markers:

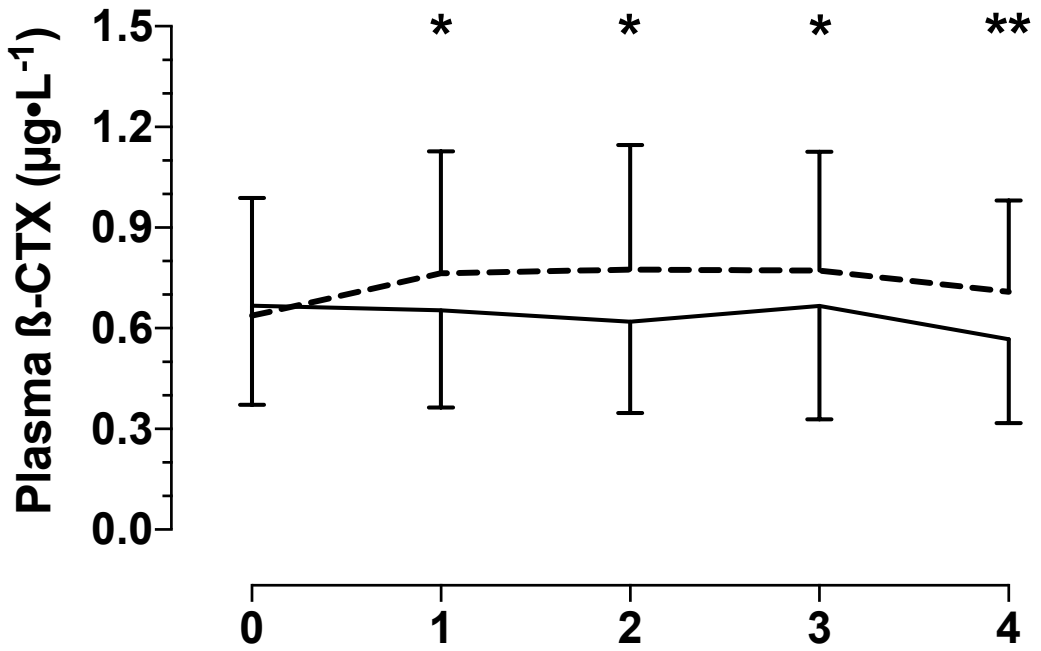
ß-CTX: Plasma ß-CTx concentrations displayed a significant main effect of treatment ($F_{1, 7}$ = 19.13, P = 0.003), time ($F_{2.32, 16.25}$ = 5.07, P = 0.016), and time x treatment interaction ($F_{1.97, 13.82}$ = 5.56, P = 0.017). Concentrations of plasma ß-CTx were greater from Day 1 onwards during *LOW* compared to *NORM* (Figure 6.3.4 A, Table 6.3.4.). Magnitudes of effect were *trivial* (g = 0.1) on Day 0, and *small* from Day 1 to Day 4 (Table 6.3.4.).

P1NP: In contrast, plasma P1NP concentrations were unaffected within or between interventions, displaying no significant main effect of treatment ($F_{1, 7}$ = 0.11, P = 0.755), time ($F_{1.69, 11.79}$ = 1.94, P = 0.190), or time x treatment interaction ($F_{1.24, 8.66}$ = 1.20, P = 0.317; Figure 6.3.4.B). Furthermore, magnitudes of effect were ≤ 0.1 and therefore *trivial* at all timepoints.

Resting Metabolic Rate: RMR was unchanged throughout the study, with no main effect of treatment ($F_{1, 7}$ = 0.58, P = 0.470), time ($F_{1.30, 9.09}$ = 4.58, P = 0.054), or time x treatment interaction ($F_{2.00, 14.00}$ = 1.70, P = 0.470) apparent (Figure 6.3.5.A). Magnitudes of effect were < 0.2 and therefore *trivial* at all timepoints between *NORM* and *LOW*.

However, resting RER exhibited a significant main effect of treatment ($F_{1, 7}$ = 26.24, P = 0.001) and a significant time x treatment interaction effect ($F_{1.84, 12.90}$ = 17.90, P < 0. 001), but no main effect of time ($F_{1.53, 10.68}$ = 1.04, P = 0.366). Resting RER was reduced on Day 2 (g = 2.9) and Day 4 (g = 2.1) of *LOW*, compared to *NORM* (Figure 6.3.5.B).

A.



B.

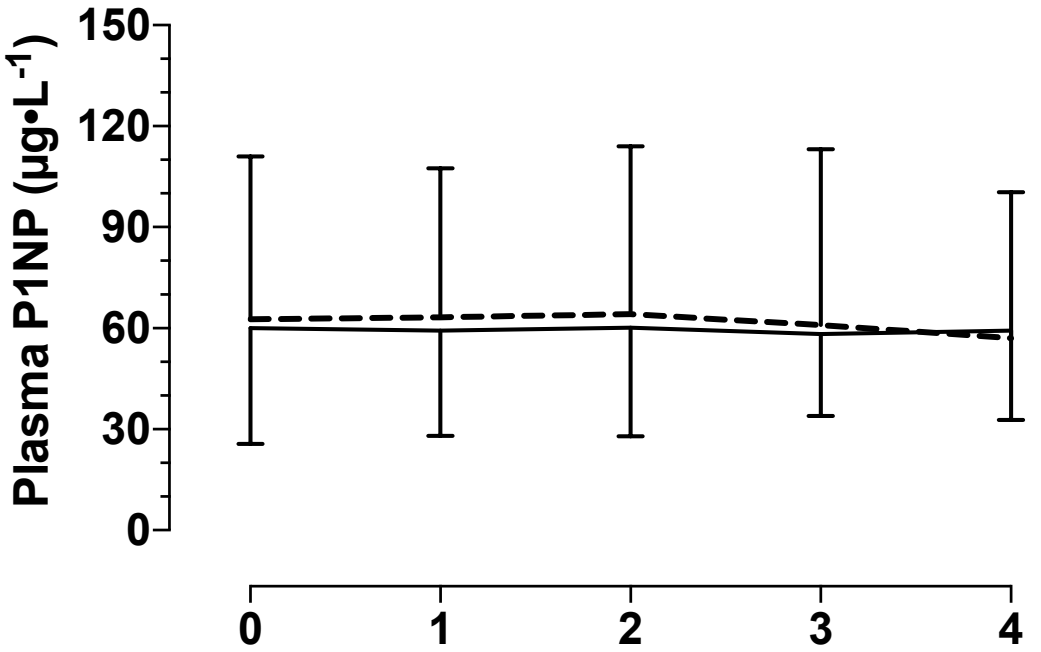


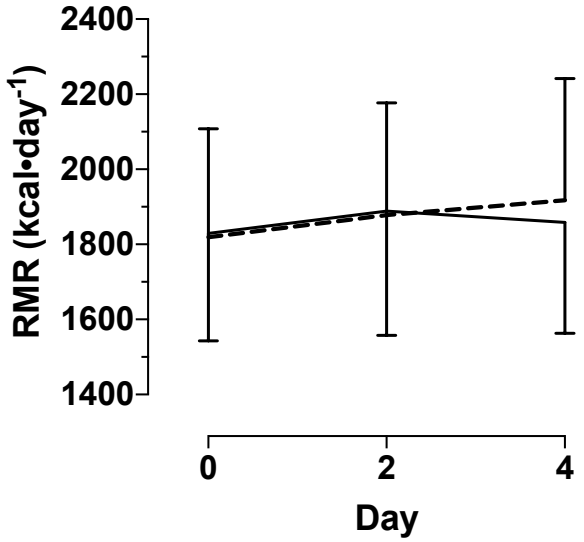
Figure 6.3.4. Concentrations of (A) plasma ß-CTX, and (B) plasma P1NP over the course of the *NORM* (solid line) and *LOW* (dashed line) CHO availability interventions. * = $P < 0.05$, ** = $P < 0.001$ between respective trial days in *NORM* and *LOW*.

Table 6.3.4. Concentrations of circulating serum IGF-1, and plasma ß-CTx, post-hoc differences and magnitude of effect between *NORM* and *LOW* concentrations following the observation of a significant time x treatment interaction effect ($P < 0.05$)

	Day 0		**Day 1**		**Day 2**		**Day 3**		**Day 4**	
	NORM	***LOW***	***NORM***	***LOW***	***NORM***	***LOW***	***NORM***	***LOW***	***NORM***	***LOW***
IGF-1 (ng•ml^{-1})	124.7 ± 19.3	133.8 ± 20.7	124.2 ± 7.3	120.9 ± 23.8	123.4 ± 21.1	120.9 ± 29.8	130.9 ± 20.3	116.6 ± 21.5	131.3 ± 28.3	115.6 ± 27.7
Mean Δ (95% CI)	9.2 (-6.3 to 24.6)		3.3 (-4.6 to 11.2)		2.5 (-11.4 to 16.4)		14.3 (-4.4 to 32.9)		15.7 (0.5 to 30.8)	
P	0.205		0.355		0.681		0.113		**0.045**	
g	0.4		0.2		0.1		0.6		0.5	
ß-CTx (µg•L^{-1})	0.67 ± 0.30	0.64 ± 0.35	0.65 ± 0.29	0.76 ± 0.36	0.62 ± 0.27	0.78 ± 0.37	0.67 ± 0.34	0.77 ± 0.35	0.57 ± 0.25	0.71 ± 0.27
Mean Δ (95% CI)	0.03 (-0.09 to 0.15)		0.11 (0.04 to 0.18)		0.16 (0.07 to 0.24)		0.11 (0.03 to 0.18)		0.14 (0.09 to 0.19)	
P	0.573		**0.008**		**0.004**		**0.013**		**< 0.001**	
g	0.1		0.3		0.5		0.3		0.5	

Post-hoc statistical changes assessed via least significant difference (*LSD*) following observation of a significant time x treatment interaction effect. Significant changes ($P < 0.05$) denoted in bold. *P* value represents difference at respective time-points between *NORM* and *LOW* trial intervention arms. Effect sizes (Hedge's *g*) represent difference between *NORM* and *LOW* respective time-points: < 0.20 = *Trivial*, 0.21 – 0.60 – *Small*, 0.61 – 1.20 *Moderate*, 1.21 – 1.99 = *Large*, ≥ 2.00 = *Very Large*.

A.



B.

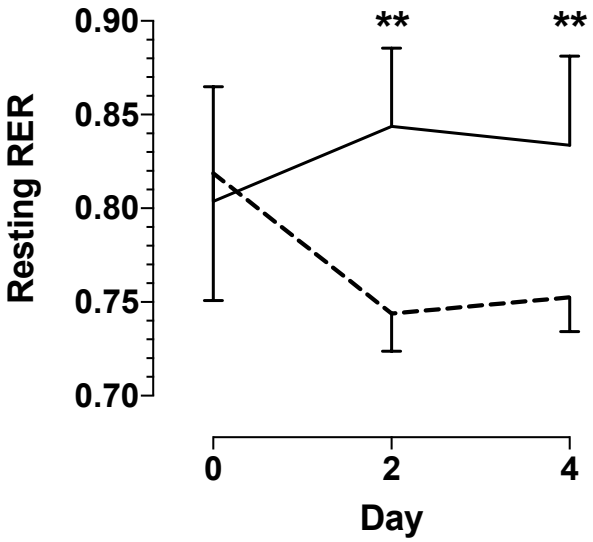


Figure 6.3.5. (A) Resting metabolic rate (*RMR*), and (B) resting respiratory exchange ratio (*RER*) across the *NORM* (solid line) and LOW (dashed line) CHO availability interventions. ** = $P < 0.001$ between respective trial days in *NORM* and *LOW*.

Exercise Metabolism:

Data and statistical analysis for the metabolic parameters assessed during exercise sessions are outlined fully in Table 6.3.5. Briefly, as a general trend, during exercise heart rate, fat oxidation, and gross exercise energy expenditure were elevated during the *LOW* intervention, compared to *NORM*. There was therefore a corresponding reduction in exercising RER, carbohydrate oxidation rates and gross efficiency during *LOW*, as well as a reduction in plasma glucose concentration on the final day of *LOW*, compared to *NORM*.

Table 6.3.5. Daily exercise data for 3-minute warm-up stages (50 – 125 W) and mean data during steady state (*SS*) exercise at 95% lactate turn-point

				NORM						***LOW***			***P***	
Parameter	**Power**	**D 0**	**D 1**	**D 2**	**D 3**	**D 4**	**D 0**	**D 1**	**D 2**	**D 3**	**D 4**	**Time**	**Treatment**	**Interaction**
HR	**50 W**	88 ± 10	91 ± 10	91 ± 10	91 ± 13	91 ± 9	90 ± 7	93 ± 7	94 ± 7	98 ± 10	99 ± 7	**0.030**	**< 0.001**	0.432
(Beats •	**75 W**	98 ± 12	99 ± 10	100 ± 11	102 ± 12	98 ± 9	99 ± 7	100 ± 10	104 ± 8	106 ± 11	107 ± 7	**0.010**	**< 0.001**	0.105
min^{-1})	**100 W**	111 ± 11	108 ± 10	111 ± 10	**111 ± 14***	**108 ± 11****	109 ± 8	111 ± 12	114 ± 9	**118 ± 12***	**118 ± 11****	**0.006**	**< 0.001**	**0.005**
	125 W	120 ± 13	121 ± 12	122 ± 14	**121 ± 16***	**119 ± 12****	120 ± 11	123 ± 13	125 ± 14	**127 ± 14***	**128 ± 13****	0.076	**< 0.001**	**0.041**
	SS	142 ± 8	142 ± 10	**143 ± 10***	**139 ± 9***	**133 ± 10****	142 ± 11	144 ± 12	**147 ± 10***	**145 ± 9***	**141 ± 11****	**< 0.001**	**< 0.001**	**0.032**
Lactate	**50 W**	1.2 ± 0.5	1.0 ± 0.3	0.9 ± 0.2	1.3 ± 0.9	1.2 ± 0.3	1.1 ± 0.2	1.0 ± 0.4	1.1 ± 0.3	0.9 ± 0.2	0.9 ± 0.3	0.677	0.084	0.352
(mmol•	**75 W**	1.2 ± 0.4	0.8 ± 0.3	1.0 ± 0.3	0.9 ± 0.3	0.9 ± 0.1	1.2 ± 0.3	0.9 ± 0.5	1.0 ± 0.3	0.8 ± 0.1	0.9 ± 0.3	**0.004**	0.862	0.900
L^{-1})	**100 W**	1.4 ± 0.8	1.2 ± 0.4	1.1 ± 0.3	1.1 ± 0.5	1.0 ± 0.3	1.3 ± 0.4	1.0 ± 0.5	1.1 ± 0.2	0.9 ± 0.2	0.9 ± 0.4	**0.004**	**0.026**	0.630
	125 W	1.9 ± 1.2	1.3 ± 0.6	1.6 ± 0.5	1.3 ± 0.5	1.3 ± 0.5	1.7 ± 0.7	1.3 ± 0.7	1.1 ± 0.4	1.1 ± 0.4	0.9 ± 0.3	**< 0.001**	**0.002**	0.628
	SS	2.8 ± 1.1	2.5 ± 0.6	2.4 ± 0.5	2.4 ± 0.5	1.9 ± 0.5	2.5 ± 0.7	1.7 ± 0.3	1.7 ± 0.5	1.6 ± 0.3	1.4 ± 0.3	**< 0.001**	**< 0.001**	0.454
Glucose	**50 W**	4.37 ± 0.35	4.64 ± 0.30	4.60 ± 0.24	4.33 ± 0.47	4.45 ± 0.16	4.18 ± 0.68	4.32 ± 0.41	4.33 ± 0.16	4.42 ± 0.35	4.04 ± 0.74	0.124	**0.003**	0.229
(mmol•	**75 W**	4.43 ± 0.24	4.50 ± 0.42	4.46 ± 0.31	4.35 ± 0.55	4.30 ± 0.39	4.42 ± 034	4.21 ± 0.24	4.16 ± 0.63	4.33 ± 0.39	4.03 ± 0.75	0.349	**0.027**	0.604
L^{-1})	**100 W**	4.33 ± 0.48	4.41 ± 0.36	4.08 ± 0.66	4.14 ± 0.29	4.24 ± 0.38	4.47 ± 0.52	4.20 ± 0.27	4.25 ± 0.32	4.36 ± 0.25	4.01 ± 0.68	0.149	0.820	0.160
	125 W	4.49 ± 0.22	4.27 ± 0.39	4.27 ± 0.45	4.14 ± 0.40	4.29 ± 0.41	4.53 ± 0.33	4.17 ± 0.39	4.12 ± 0.43	4.33 ± 0.27	3.84 ± 0.68	**0.005**	0.197	0.076
	SS	4.31 ± 0.34	4.17 ± 0.35	4.09 ± 0.52	3.76 ± 0.52	**4.19 ± 0.33***	4.46 ± 0.27	4.01 ± 0.37	3.95 ± 0.43	3.98 ± 0.40	**3.63 ± 0.53***	**< 0.001**	0.220	**0.016**
EEE$_{Gross}$	**50 W**	6.3 ± 0.6	6.1 ± 0.7	6.1 ± 0.5	6.0 ± 0.4	6.2 ± 1.1	6.5 ± 0.8	6.1 ± 0.6	6.4 ± 0.6	6.3 ± 0.5	6.0 ± 0.9	0.635	0.291	0.775
(kcal•	**75 W**	7.5 ± 0.5	7.4 ± 0.6	7.2 ± 0.5	7.3 ± 0.4	7.5 ± 1.1	7.5 ± 0.6	7.5 ± 0.7	7.7 ± 0.6	7.6 ± 0.5	7.5 ± 1.2	0.998	0.225	0.726
min^{-1})	**100 W**	8.9 ± 0.6	9.0 ± 0.7	8.7 ± 0.6	8.7 ± 0.6	8.9 ± 1.2	9.0 ± 0.6	9.2 ± 0.7	9.2 ± 0.6	9.4 ± 0.6	8.8 ± 1.5	0.860	0.148	0.533
	125 W	10.6 ± 0.6	10.5 ± 0.7	10.4 ± 0.8	10.4 ± 0.5	10.4 ± 1.1	10.5 ± 0.4	10.8 ± 0.5	10.9 ± 0.7	10.9 ± 0.6	10.3 ± 1.3	0.695	0.077	0.370
	SS	13.2 ± 2.2	13.4 ± 2.3	**13.1 ± 2.2***	**13.0 ± 2.3***	**12.6 ± 2.3***	13.1 ± 2.1	13.5 ± 2.3	**13.5 ± 2.3***	**13.5 ± 2.4***	**13.2 ± 2.4***	**0.001**	**< 0.001**	**0.021**
CHO Ox	**50 W**	1.2 ± 1.0	**1.4 ± 1.1***	**1.8 ± 1.2****	**2.0 ± 0.9****	**1.6 ± 0.6****	1.5 ± 1.2	**0.3 ± 0.6***	**0.0 ± 1.2****	**0.4 ± 0.6****	**0.0 ± 0.9****	0.062	**< 0.001**	**< 0.001**
(kcal•	**75 W**	3.6 ± 1.6	**3.6 ± 0.7***	**3.7 ± 1.3****	**4.0 ± 1.0****	**3.9 ± 0.3****	3.4 ± 1.5	**2.3 ± 1.0***	**1.7 ± 1.1****	**1.5 ± 0.8****	**1.2 ± 0.8****	**0.011**	**< 0.001**	**< 0.001**

min^{-1})	**100 W**	5.4 ± 2.3	**5.6 ± 1.4****	**5.7 ± 1.4****	**5.9 ± 1.5****	**5.7 ± 0.6****	5.3 ± 1.8	**4.2 ± 1.3****	**3.4 ± 1.0****	**2.8 ± 1.0****	**2.1 ± 1.0****	**< 0.001**	**< 0.001**	**< 0.001**
	125 W	7.4 ± 2.7	**7.8 ± 1.6****	**8.0 ± 1.7****	**8.0 ± 1.6****	**7.9 ± 0.8****	7.6 ± 2.2	**5.7 ± 2.0****	**4.9 ± 1.5****	**4.1 ± 2.3****	**3.4 ± 1.3****	**< 0.001**	**< 0.001**	**< 0.001**
	SS	8.5 ± 1.8	**9.1 ± 1.5****	**9.3 ± 1.9****	**8.9 ± 1.9****	**10.0 ± 2.4****	8.9 ± 2.3	**6.7 ± 1.5****	**6.3 ± 1.3****	**5.5 ± 1.2****	**5.5 ± 1.1****	**< 0.001**	**< 0.001**	**< 0.001**
Fat Ox	**50 W**	5.0 ± 1.2	**4.7 ± 1.2***	**4.3 ± 1.1****	**4.1 ± 0.9****	**4.5 ± 1.3****	5.0 ± 1.4	**5.8 ± 0.5***	**6.4 ± 1.1****	**5.9 ± 0.8****	**6.2 ± 1.4****	0.488	**< 0.001**	**0.004**
(kcal•	**75 W**	4.0 ± 1.5	**3.8 ± 0.7****	**3.5 ± 1.4****	**3.3 ± 1.1****	**3.6 ± 1.0****	4.0 ± 1.5	**5.2 ± 1.2****	**5.9 ± 1.2****	**6.1 ± 1.1****	**6.4 ± 1.6****	**0.021**	**< 0.001**	**< 0.001**
min^{-1})	**100 W**	3.6 ± 2.3	**3.4 ± 1.3****	**3.0 ± 1.4****	**2.9 ± 1.3****	**3.2 ± 1.5****	3.6 ± 2.0	**5.1 ± 1.3****	**5.8 ± 1.1****	**6.6 ± 1.3****	**6.7 ± 1.8****	**0.002**	**< 0.001**	**< 0.001**
	125 W	3.2 ± 2.6	**2.6 ± 1.6****	**2.4 ± 1.8****	**2.4 ± 1.3****	**2.5 ± 1.3****	2.9 ± 2.4	**5.0 ± 2.1****	**6.0 ± 1.5****	**6.8 ± 2.4****	**7.0 ± 1.9****	**< 0.001**	**< 0.001**	**< 0.001**
	SS	4.8 ± 1.9	**4.3 ± 1.2****	**3.8 ± 1.0****	**4.1 ± 1.0****	**2.6 ± 0.9****	4.2 ± 2.2	**6.9 ± 2.2****	**7.2 ± 1.3****	**8.1 ± 1.6****	**7.7 ± 2.0****	**< 0.001**	**< 0.001**	**< 0.001**
RER	**50 W**	0.76 ± 0.04	**0.77 ± 0.05****	**0.79 ± 0.05****	**0.80 ± 0.04****	**0.78 ± 0.03****	0.77 ± 0.06	**0.72 ± 0.03****	**0.70 ± 0.05****	**0.72 ± 0.03****	**0.70 ± 0.04****	0.112	**< 0.001**	**< 0.001**
	75 W	0.84 ± 0.06	**0.84 ± 0.02****	**0.85 ± 0.05****	**0.86 ± 0.04****	**0.86 ± 0.02****	0.84 ± 0.05	**0.79 ± 0.04****	**0.77 ± 0.04****	**0.76 ± 0.03****	**0.75 ± 0.03****	**0.008**	**< 0.001**	**< 0.001**
	100 W	0.88 ± 0.08	**0.88 ± 0.04****	**0.89 ± 0.04****	**0.90 ± 0.05****	**0.89 ± 0.03****	0.88 ± 0.06	**0.83 ± 0.04****	**0.81 ± 0.03****	**0.79 ± 0.03****	**0.77 ± 0.04****	**< 0.001**	**< 0.001**	**< 0.001**
	125 W	0.90 ± 0.07	**0.92 ± 0.05****	**0.93 ± 0.05****	**0.93 ± 0.04****	**0.93 ± 0.03****	0.92 ± 0.07	**0.86 ± 0.05****	**0.83 ± 0.04****	**0.81 ± 0.06****	**0.80 ± 0.04****	**< 0.001**	**< 0.001**	**< 0.001**
	SS	0.89 ± 0.03	**0.90 ± 0.02****	**0.91 ± 0.02****	**0.90 ± 0.02****	**0.93 ± 0.02****	0.90 ± 0.04	**0.85 ± 0.03****	**0.84 ± 0.01****	**0.82 ± 0.01****	**0.83 ± 0.02****	**< 0.001**	**< 0.001**	**< 0.001**
Gross	**50 W**	11.5 ± 1.2	11.9 ± 1.4	11.9 ± 0.9	12.0 ± 0.9	11.9 ± 1.7	11.3 ± 1.5	11.8 ± 1.2	11.3 ± 1.2	11.4 ± 0.9	11.5 ± 1.3	0.629	0.087	0.901
Efficiency	**75 W**	14.3 ± 1.0	14.6 ± 1.2	15.0 ± 1.1	14.9 ± 0.9	14.6 ± 1.8	14.5 ± 1.2	14.4 ± 1.4	14.1 ± 1.1	14.3 ± 1.0	13.7 ± 1.3	0.647	**0.024**	0.374
(%)	**100 W**	16.1 ± 1.1	16.1 ± 1.3	16.5 ± 1.1	16.5 ± 1.2	16.3 ± 2.0	16.1 ± 1.1	15.6 ± 1.2	15.6 ± 1.1	15.3 ± 1.1	15.5 ± 1.1	0.889	**0.004**	0.489
	125 W	17.0 ± 1.0	17.2 ± 1.1	17.3 ± 1.2	17.4 ± 0.9	17.4 ± 1.6	17.1 ± 0.7	16.7 ± 0.8	16.5 ± 1.0	16.6 ± 1.0	16.7 ± 0.9	0.999	**0.003**	0.465
	SS	16.4 ± 1.8	16.3 ± 1.9	**16.6 ± 1.8***	**16.7 ± 1.6***	**17.4 ± 1.9***	16.6 ± 1.5	16.1 ± 1.7	**16.1 ± 1.6***	**16.0 ± 1.6***	**16.6 ± 1.7***	**< 0.001**	**< 0.001**	**0.042**

Mean ± SD values for exercise parameters during *NORM* and *LOW* at 50 W, 75 W, 100 W, and 125 W (warm-up stages) and mean values during steady state (*SS*) exercise at 95% lactate turn-point 1 from (Note: Day 4 exercise was terminated at 30 minutes). *P* columns display Time, Treatment, and Time x Treatment Interaction effects observed from linear mixed model, bold values highlight significantly changed ($P < 0.05$) parameters. *NORM* and *LOW* values in bold highlight differences between a parameter on corresponding trial days (i.e., Day 4 *NORM*, compared to Day 4 *LOW*) from *least significant difference* (*LSD*) post-hoc analyses, where Time x Treatment Interaction effect was significant. * = $P < 0.05$, ** = $P < 0.001$ between interventions (*NORM* vs *LOW*) on the same respective days. HR = Heart Rate, EEE_{Gross} = Gross Exercise Energy Expenditure, CHO Ox = Carbohydrate Oxidation Rate, FAT Ox = Fat Oxidation Rate, RER = Respiratory Exchange Ratio.

6.4. Discussion:

The main findings of this study were that four-days of low carbohydrate availability, under conditions of energy balance, increased fasting plasma ß-CTx and reduced serum IGF-1 concentrations in healthy active males, compared to a normal carbohydrate availability trial. However, we observed no change in concentrations of serum total testosterone, plasma T_3, P1NP, leptin, insulin, glucose, or RMR between *NORM* and *LOW*. Taken together, these data suggest that acute periods of low carbohydrate availability have limited effect upon endocrine and physiological markers typically associated with low energy availability.

This study used a low-carbohydrate, high fat diet (*LOW*) to investigate the physiological effects of reduced carbohydrate availability independently from the influence of low energy availability. Using a similar methodological approach to previous LEA research, we investigated the effect of this reduction in carbohydrate availability upon physiological parameters typically associated with low energy availability (Areta et al., 2021), including markers of the HPG axis, HPT axis, and of bone resorption and formation. *LOW* elicited major metabolic shifts with significant increases in concentrations of plasma NEFA, glycerol, ketones, and corresponding increases in fat oxidation rates at rest and during exercise observed. Cycling gross efficiency also reduced during *LOW*, as expected for a LCHF diet (Burke et al., 2017; Krogh & Lindhard, 1920). Despite a clear metabolic and physiological shift triggered by altered substrate availability, relevant endocrine and physiological markers, including T_3, leptin, insulin, total testosterone, and RMR were unchanged by the low carbohydrate availability intervention. IGF-1 concentrations were reduced, however. Moreover, in line with recent observations, markers of bone remodelling appear to be sensitive to reductions in carbohydrate availability.

Reducing carbohydrate intake from 7.7 $g \cdot kg^{-1} \cdot day^{-1}$ to 1.5 $g \cdot kg^{-1} \cdot day^{-1}$ (117 ± 15 $g \cdot day^{-1}$) during *LOW* increased plasma ß-CTx concentrations compared to during *NORM* and concentrations of bone formation marker P1NP were unchanged. In agreement with these data, Hammond et al. (2019) reported that restricting carbohydrate intake to ~3 $g \cdot kg^{-1} \cdot day^{-1}$ around repeated bouts of high-intensity running led to greater ß-CTx concentrations compared to a high carbohydrate control group in a cohort of healthy male runners, although exercise suppressed overall concentrations in all trials (see graph 7.B. in Hammond et al., 2019). Heikura et al. (2020) also observed that 3.5 weeks of a ketogenic diet (< 50 $g \cdot day^{-1}$ of carbohydrate) increased fasting ß-CTx concentrations in a cohort of elite race walkers, compared to an isocaloric high carbohydrate control group. However, our findings do not align with Fensham et al. (2022), who showed that six-days following a ketogenic diet (< 50 $g \cdot day^{-1}$ of carbohydrate) did not alter fasting ß-CTx concentrations in a cohort of elite race walkers.

Regarding P1NP responses, our data differ from similar studies. Hammond et al. (2019) observed that P1NP concentrations were reduced in the LCHF, LEA and high carbohydrate control trials, with no

differences between groups. Fasting P1NP concentrations were reduced in Heikura et al. (2020), however it is noteworthy that participants appeared to lose ~1.8 kg body mass across the intervention phase (Burke et al., 2017). Fensham et al. (2022) observed a significant decrease in P1NP concentrations following six-days of LCHF diet, compared to a high carbohydrate control group. This difference may be due to the difference in study intervention durations, Fensham et al. (2022) used a 6-day protocol, compared to four days in the present study. In agreement with this, bone resorption has been shown to increase without concomitant increases in bone formation for up to four days, albeit with running rather than cycling exercise (Scott et al., 2010). Based upon the data from the present study, markers of bone resorption appear sensitive to carbohydrate availability, but markers of bone formation were unchanged.

It is possible that the observed changes in markers of bone resorption were due to differences in macronutrient (protein or fat) or micronutrient availability between *NORM* and *LOW*. A difference in protein intake would be of particular note to these findings regarding macronutrient difference. A recent review from Dolan et al. (2020) summarised protein to generally be a 'bone-protective nutrient'. Yet protein intake was matched at 2.4 ± 0.1 $g \bullet kg^{-1} \bullet day^{-1}$ across the *NORM* and *LOW* interventions. Regarding micronutrients, the influence of calcium and Vitamin D ingestion with corresponding exercise bouts on bone metabolism are best understood and calcium supplementation may be protective of athlete bone health, again as reviewed by Dolan et al. (2020). Infusion of ionised calcium 15 minutes prior to and during 60 minutes of cycling exercise has been shown to attenuate increases in ß-CTx and parathyroid hormone in healthy, training, males. P1NP concentrations were not affected by the infusion of ionised calcium (Kohrt et al., 2018). This study provided neat mechanistic data to suggest that disruption of serum calcium concentrations during exercise can regulate concentrations of ß-CTx. Perhaps a small limitation, however, was that trial order was not randomised, with calcium always administered in trial one. In the present study, the *NORM* and *LOW* intervention diets provided more than the recommended daily intake of 800 mg for adults for all participants (see Table 6.2.3. for mean values). Calcium deficiency was therefore unlikely to contribute to these findings, and all exercise sessions were performed under the same dietary conditions (overnight fasted for 30 mins, followed by the consumption of a single carbohydrate gel). Calcium intake was 1071 ± 191 during *NORM* and 1667 ± 245 during *LOW*, yet ß-CTx concentrations were significantly elevated during the *LOW* intervention. Future research may seek to establish this relationship further.

T_3 has consistently been shown to respond to low energy availability interventions in females (See Chapter 2.8) and is therefore viewed as a reliable marker of LEA and associated disruptions of the hypothalamic-pituitary-thyroid axis. Although, this response is less established in males, we have shown that five days of low energy availability (10 $kcal \bullet kg\ FFM^{-1} \bullet day^{-1}$) reduced plasma T_3 concentrations in healthy active males (see Chapter 5). Limited data suggests that this response may be carbohydrate mediated. For example, in males with overweight/obesity a four-week ketogenic diet

elicited a reduction in fasting plasma free T_4 and T_3, as well as total T_3 concentrations. Although these findings are confounded by an apparent energy deficit throughout the intervention (Hall et al., 2016). We therefore speculated that low carbohydrate availability might reduce total T_3 concentrations based upon its putative role in the deiodination of T_4 to T_3 (Gavin et al., 1981; Loucks et al., 1992). Despite the reduction of carbohydrate consumption to 1.5 g•kg^{-1} for four days, we did not observe significant alterations to plasma total T_3 concentrations in *LOW* compared to *NORM*. As a key energy-sensing hormone (Blüher & Mantzoros, 2009), leptin has also consistently been shown to reduce in response to controlled low energy availability interventions in females (see Chapter 2.8). In males, the leptin responses to low energy availability are less clear. Leptin concentrations reduce dramatically in response to even small reductions in adipose tissue (Dubuc et al., 1998), but also fall in response to fasting, independently from changes in fat mass (Kolaczynski et al., 1996). Both fat mass and plasma leptin concentrations were unchanged in the present study. This suggests that leptin is unaffected by acute periods of low carbohydrate availability under conditions of energy balance.

As hypothesised, we did not observe any changes in total testosterone concentrations. This agrees with the findings of a meta-analysis by Whittaker & Harris (2022), who concluded that moderate-protein low carbohydrate diets were not associated with reductions in fasting total testosterone. From the limited data available, a recent systematic review from Kang et al. (2020) also concluded that total testosterone concentrations were unchanged in studies investigating the effects of ≥ 3 weeks following a low carbohydrate, high fat diet. Mckay et al. (2022a) observed reductions in total testosterone concentrations in elite race walkers following six-days of a training camp (performing ~18 – 19 km•day^{-1} of training). However, this effect was ubiquitous across control, LCHF (< 50 g•day^{-1} of carbohydrate), and LEA (15 kcal•kg FFM^{-1}•day^{-1}) intervention groups. Therefore, factors outside of (or in addition to) the dietary manipulation, such as the stress of exercise may explain the reduction in testosterone. A recent study of bodybuilders adhering to an eight-week ketogenic diet (< 50 g•day^{-1} of carbohydrate) observed that the low carbohydrate intake reduced total testosterone concentrations, whilst no changes occurred in an isocaloric western diet control group (Paoli et al., 2021). The lack of change in total testosterone concentrations following only four days of low carbohydrate availability was expected, and more studies of longer duration may be required to elucidate the relationship between carbohydrate availability and the HPG axis further.

We observed a reduction in the anabolic hormone IGF-1 on the final day of *LOW*. In males, four to five days of low energy availability (~15 kcal•kg FFM^{-1}•day^{-1}) did not affect IGF-1 concentrations in Papageorgiou et al. (2017), Koehler et al. (2016), or Murphy et al. (2021). A shorter three-day LEA protocol (< 20 kcal•kg FFM^{-1}•day^{-1}) reduced IGF-1 concentrations in trained male runners, however (Kojima et al., 2020). Three days of LEA (15 kcal•kg FFM^{-1}•day^{-1}) achieved through dietary restriction also led to GH resistance, indicated by increased GH and reduced IGF-1 concentrations, in a mixed

cohort of five males and two females (Murphy & Koehler, 2020). Our findings suggest that IGF-1 regulation is also sensitive to carbohydrate availability.

In conclusion, four days of low carbohydrate availability elicited an increase in concentrations of the bone resorption marker ß-CTx. The low carbohydrate intervention also led to significant reductions in plasma IGF-1, possibly indicating disruption of the GH-IGF-1 axis. Markers of bone metabolism and IGF-1 therefore appear to be sensitive to carbohydrate availability as well as energy availability. In contrast, further markers of energy preservation, such as RMR, or fasting testosterone, T_3, leptin, glucose, and insulin concentrations were unchanged by the reduction in carbohydrate availability. Taken together, these data suggest that the body can detect overall energy availability irrespective of the dietary macronutrient content, preserving physiological functioning.

Chapter 7:

Synthesis of Findings

This chapter critically appraises the experimental findings of this thesis in relation to the achievement of its original aims and objectives. A critical discussion of the findings then follows, in which specific attention is given to how the data presented advance our understanding of energy availability patterns in free-living athletes and the physiological effects of low energy and carbohydrate availability in males. A discussion of the practical implications with consideration of the respective study limitations is then presented. Finally, the thesis closes by outlining directions and recommendations for future research.

7.1. Achievement of aims and objectives:

The primary aims of this thesis were, 1) To assess the free-living patterns of energy availability in a cohort of athletes typically deemed 'at risk' of low energy availability by monitoring their nutrition and training habits, and 2) To investigate the endocrine, metabolic, and physiological responses of healthy adult males to acute (< 1 week) periods of low energy availability under controlled laboratory conditions, using an ecologically valid pattern of energy availability.

Objective 1: To examine the habitual exercise and dietary patterns of a cohort of elite (professional) male cyclists under free-living conditions during a 7-day pre-season training period. This will allow us to characterise the relationship between energy intake, exercise energy expenditure, and subsequent energy availability in a cohort of athletes that are commonly identified as being at particularly high-risk of LEA (Study 1, Chapter 4).

The data presented in Study 1 demonstrated that free-living elite male endurance athletes (cyclists) experience high variability in energy availability on a day-to-day basis. The heterogenous nature of daily energy availability – which ranged from -22 to 76 kcal·kg FFM^{-1}·day^{-1} – was attributed to relative stability in daily energy intake despite fluctuating daily exercise energy expenditure. As such, whilst a significant within-participant correlation between energy intake and exercise energy expenditure was observed, this relationship only equated to an increase in energy intake of 210 kcal•day^{-1} per 1000 kcal•day^{-1} increase in exercise energy expenditure. Consequently, using fluctuating patterns of energy availability in future laboratory-based studies investigating the endocrine, metabolic, and physiological effects of LEA would appear to be more ecologically valid. Undertaking this study therefore provided crucial methodological considerations for the subsequent energy availability manipulation used in Chapter 5.

Objective 2: To investigate the effects of short-term low energy availability, elicited through caloric restriction with concomitant aerobic exercise, on markers of the hypothalamic-pituitary-gonadal and hypothalamic-pituitary-thyroid axes, markers of bone resorption and formation, and further key endocrine, metabolic, and physiological responses (Study 2, Chapter 5).

Data from Study 1 established that free-living elite male road cyclists experience heterogenous daily energy availability. To enhance ecological validity, Study 2 therefore used a fluctuating pattern of low energy availability to investigate its endocrine, metabolic, and physiological effects. The data presented in this study demonstrate that when energy availability is reduced to levels lower than previously investigated, males also display similar responses to those of females, which are consistent with a physiological state of energy preservation. Plasma Total T_3, leptin, IGF-1, and bone formation marker P1NP were all suppressed during LEA, whilst bone resorption marker ß-CTx, plasma D-3-

hydroxybutyrate, NEFA, and glycerol increased. However, serum total testosterone, RMR, fasting plasma glucose and insulin, and protein synthetic rates of the myofibrillar, soluble, and mitochondrial fractions were all unchanged despite five days of severely low energy availability. Short-term low energy availability therefore led to shifts in bone remodelling markers and the hypothalamic-pituitary-thyroid axis that, from the perspective of the *Male Athlete Triad* and *REDs,* would classically be interpreted as 'negative'. Yet, these data also suggest that transient bouts of low energy availability with concomitant exercise do not perturb testosterone concentrations, RMR, or muscle protein synthetic rates in the working muscle (Figure 7.1.).

Objective 3: To investigate the physiological effects of short-term low carbohydrate availability, under conditions of energy balance, upon the same endocrinological, metabolic, and physiological parameters related to the regulation of bone health, the hypothalamic-pituitary-thyroid and hypothalamic-pituitary-gonadal axes in healthy adult males. The objective of this study was to provide insight into the mediating role of carbohydrate availability in the physiological responses to low energy availability in males (Study 3, Chapter 6).

Study 2 established that males experience alterations in endocrine, metabolic, and physiological regulation in response to acute low energy availability. However, it is impossible to create an energy deficit without a corresponding deficit in at least one of the major macronutrients. Carbohydrate deficits have been identified as a potential contributor to physiological responses typically attributed to low energy availability. In Study 2, it was opted to maintain a constant relative macronutrient distribution of 60 % carbohydrate, 20 % protein, and 20 % fat in normal and low energy availability treatments. However, as the physiological role of endogenous and exogenous carbohydrate upon metabolism, training adaptation, and possibly markers of bone resorption and formation is particularly noteworthy, the potential for the findings of Study 2 to be confounded by a concomitant reduction in carbohydrate availability was acknowledged.

The data from Study 3 presented herein demonstrate that at least acutely, low carbohydrate availability under conditions of energy balance had no effect upon key endocrine parameters typically associated with LEA, such as total testosterone, T_3, leptin, or insulin. Resting metabolic rate was similarly unchanged. Markers of bone resorption (ß-CTx) were increased by low carbohydrate availability, indicating a regulatory role of carbohydrates upon bone turnover independent of energy availability. Additionally, serum IGF-1 concentrations were reduced following the low carbohydrate availability intervention, suggesting altered function of the GH-IGF-1 axis. Taken together, low carbohydrate availability may contribute to the regulation of certain endocrine and physiological processes typically associated with LEA, such as bone metabolism and the GH-IGF-1 axis. However, considering the lack of changes in further key parameters associated with energy preservation, such as T_3 or leptin, total

energy availability appears to be the major factor underpinning endocrine, metabolic, and physiological responses that serve to preserve energy (Figure 7.1.).

7.2. General discussion of findings:

The main findings of this thesis indicate that male endocrinology, metabolism, and physiology are responsive to acute periods of low energy availability. These responses mainly appear to be sensitive to a reduction in energy *per se*, rather carbohydrate availability. Our free-living observational study (Study 1) and controlled laboratory experiments (Study 2 and Study 3) were of short duration. There is therefore complexity in defining the responses we observed as 'positive' or 'negative'. Through the lens of the *Male Athlete Triad* and *REDs* models, increased bone resorption and reduced bone formation would typically be regarded as 'negative'. Yet from a *Life History Theory* perspective, short-term energetic trade-offs are regarded as expected and represent essential survival mechanisms for periods of energy scarcity (Oliveira-Junior et al., 2022; Shirley et al., 2022). As such, it is difficult to define whether the responses observed in the studies herein might help or hinder the health, training adaptation, and ultimate performance capabilities of exercising males.

The seminal work of Professor Anne Loucks established that short-term low energy availability disrupts the hypothalamic-pituitary-thyroid axis (Loucks & Callister, 1993; Loucks & Heath, 1994b), the hypothalamic-pituitary-gonadal axis (Loucks et al., 1998; Loucks & Heath, 1994a; Loucks & Thuma, 2003), and markers of bone metabolism (Ihle & Loucks, 2004) in sedentary females. Subsequently, the *Female Athlete Triad* position stand adopted low energy availability as the key aetiological factor underpinning the disruption of reproductive function and bone health in exercising females (De Souza, Nattiv, et al., 2014; Nattiv et al., 2007). Both the *Male Athlete Triad* (Fredericson et al., 2021; Nattiv et al., 2021) and *REDs* (Mountjoy et al., 2014, 2018, 2023) models have since stipulated that exercising males experience equivalent physiological dysregulations to females. The findings of this thesis provide some of the first evidence of a causal role of low energy availability upon parameters typically associated with low energy availability. The longer-term effects of low energy availability in males remain unclear, however.

Patterns of energy availability in free-living athletes

Experimental research investigating the endocrine, metabolic, and physiological effects of low energy availability has typically clamped daily energy intake and exercise energy expenditure at a fixed level throughout the controlled intervention periods. The resultant patterns of daily energy availability in these studies are therefore homogenous, with no variation in EA values between days (Areta et al., 2021; Loucks, 2020). However, there is limited data available characterising the relationship between energy intake, exercise energy expenditure, and resultant energy availability in athletes training under free-living conditions. Limited field-based research in elite athlete populations suggest that daily energy

availability levels fluctuate based upon training or competition load (Heikura et al., 2019; Langan-Evans et al., 2021; Moss et al., 2020). However further research is required from athletes under completely free-living conditions to support the notion that athletes with a high training load experience reduced energy availability. As such, Study 1 (Chapter 4) characterised the energy intake, exercise energy expenditure, and resultant variability in daily energy availability in a cohort of elite male road cyclists across a week of pre-season training. Data was collected whilst athletes followed their regular pre-season training schedule and uncontrolled dietary assessments were conducted in real-world conditions, providing high ecological validity to our findings.

The data presented in Study 1 demonstrate that under free-living conditions, elite male road cyclists only partially compensated on days of higher exercise energy expenditure with a corresponding increase in energy intake. Day-to-day energy intake was broadly consistent, irrespective of training load. We observed significant within-participant correlations between daily exercise energy expenditure and energy intake. Yet this only translated to an increase in energy intake of 210 kcal•day^{-1} per 1000 kcal•day^{-1} increase in exercise energy expenditure. Resultant energy availability was therefore typically lower on days of high training load, and higher on rest days. Heikura et al. (2019) also assessed the dietary practices of a cohort of professional male road cyclists, albeit over eight-days of competition. They observed that energy and carbohydrate intake increased on competition days where exercise energy expenditure was greater, but that this was not sufficient to preserve energy availability. Observed daily energy availability values therefore fluctuated between race- and rest-days (Heikura et al., 2019). However, participants were competing in a multiple-day competition in this study, where adequate fuelling was likely of high priority (Impey et al., 2018; Thomas et al., 2016) and the participants had most food prepared for them by a team chef, removing a proposed potential barrier to meeting fuelling needs (Burke, Lundy, et al., 2018). Our data therefore extend these findings to elite male road cyclists under entirely free-living conditions and outside of the competition phase.

In further agreement with the findings of Study 1, Moss et al. (2020) showed that a cohort of female professional footballers did not vary their energy intake from rest days to light training days, heavy training days, or matchdays. Consequently, energy availability in the cohort was greater on rest days relative to training and match days, with more players becoming classified as in a state of low energy availability (< 30 kcal•kg FFM^{-1}•day^{-1}) as exercise energy expenditure increased. Case-studies from Louis et al. (2020) and Langan-Evans et al. (2021) have also agreed with the findings of Study 1, showing that energy availability reduced with respect to greater training load. Taken together, free-living athletes appear to experience greater variability in daily exercise energy expenditure than energy intake values.

Current evidence therefore suggests that resultant energy availability appears to be reduced in response to days/periods of high exercise energy expenditure, possibly intermittently exposing athletes to periods of LEA. At present, it is unclear whether such intermittent exposures to low energy availability have a positive or negative influence on athlete health, training responses, and ultimately performance. Moving forwards, it would be interesting to investigate whether matching energy intake with exercise energy expenditure might enhance training adaptation and performance levels. Furthermore, most of the existing controlled research studies investigating the physiological impact of low energy availability have utilised a homogenous daily pattern of energy availability. As such, the physiological impact of transient increases in energy availability akin to those observed on rest days is currently unclear. To enhance ecological validity, future controlled LEA research would therefore benefit from structuring energy availability manipulations in a manner that more closely reflects the dietary practices of free-living athletes around their training.

The endocrine, metabolic, and physiological effects of low energy availability in males

The endocrine, metabolic, and physiological responses of males to periods of controlled low energy availability have been poorly characterised to-date. Yet cross-sectional and observational studies summarised across a variety of reviews have suggested that regularly exercising males display disruption of the hypothalamic-pituitary-gonadal axis, impaired bone health (Fredericson et al., 2021; Hackney, 2020; Nattiv et al., 2021) and possibly even further physiological dysregulations (Mountjoy et al., 2023). Limited evidence available thus far has suggested that male participants exposed to acute low energy availability only experience mild disruption of endocrine, metabolic, and physiological parameters. Recent reviews have therefore suggested that males are more robust to physiological perturbations associated with low energy availability than females (Areta et al., 2021). At present, it is unclear whether these responses are simply insensitive to LEA in males, or whether the reductions in energy availability used so far have not been severe enough to elicit perturbations to physiological parameters of interest. Study 2 therefore investigated the endocrine, metabolic, and physiological effects of five days of low energy availability, using a more severe reduction in EA than previously used to 10 kcal•kg FFM^{-1}•day^{-1}, in a cohort of ten healthy active males. LEA elicited changes in endocrine and metabolic markers typically associated with energy preservation, including T_3, leptin, IGF-1, glycerol, NEFA and ketones. Bone metabolism also appeared to be affected, displaying elevated concentrations of markers of resorption and reduced formation. However, testosterone, RMR, and muscle protein synthetic rates were unchanged by low energy availability.

Disruption of the hypothalamic-pituitary-gonadal axis manifesting in reductions of circulating testosterone concentrations has typically been associated with low energy availability in exercising males in recent reviews and position stands (Hackney, 2020; Mountjoy et al., 2023; Nattiv et al., 2021). However, no controlled low energy availability studies have yet shown sufficient disruption of the

hypothalamic-pituitary-gonadal axis to perturb circulating testosterone concentrations in males. A four-day low energy availability intervention providing 15 kcal•kg FFM^{-1}•day^{-1} has been shown not to effect testosterone concentrations in males (Koehler et al., 2016). Kojima et al. (2020) showed that free testosterone was reduced following both three days of LEA (~19 kcal•kg FFM^{-1}•day^{-1}) and normal EA (~53 kcal•kg FFM^{-1}•day^{-1}) in a cohort of seven healthy well-trained male runners. Mckay et al. (2022a) observed reductions in total testosterone concentrations in elite male race walkers after a six-day period of LEA (15 kcal•kg FFM^{-1}•day^{-1}). Yet LCHF and control intervention groups (both 40 kcal•kg FFM^{-1}•day^{-1}) also had reduced testosterone concentrations. The reduction in testosterone concentrations in all groups across both of these studies suggest that factors other than energy availability, such as the training load (~19 km•day^{-1}), contributed to this effect. The findings of Study 2 therefore agree with the limited data available investigating the effects of low energy availability upon the hypothalamic-pituitary-gonadal axis in males. To better characterise male responses, future research investigating the acute physiological effects of LEA in males may seek to examine 'up-stream' markers of the HPG axis instead, such as LH pulsatility. For example, controlled low energy availability research in females has repeatedly demonstrated disruption of LH release (Loucks, 2006; Loucks et al., 1998; Loucks & Heath, 1994a; Loucks & Verdun, 1998). Whilst logistically challenging, longer-term controlled studies of LEA in males are also required to elucidate the impact of LEA upon testosterone regulation further.

The response of markers of bone remodelling to low energy availability in males are also of interest. Both the *Male Athlete Triad* and *REDs* models purport that exercising males are at risk of low BMD and stress fractures (Mountjoy et al., 2023; Nattiv et al., 2021). However, only a few studies have investigated the effects of controlled low energy availability upon markers of bone resorption and formation in males. The studies available have produced mixed findings in bone turnover marker responses. In Study 2, reducing energy availability to 10 kcal•kg FFM^{-1}•day^{-1} for five days increased concentrations of ß-CTx and reduced P1NP concentrations. Papageorgiou et al. (2017) observed that five days of low energy availability (15 kcal•kg FFM^{-1}•day^{-1}) led to increased bone resorption marker and reduced bone formation marker concentrations in female participants, but that males were not affected by the LEA intervention. Similarly, Murphy & Koehler (2020) also observed no changes in bone turnover markers following three days of low energy availability (15 kcal•kg FFM^{-1}•day^{-1}) elicited through dietary restriction in a small mixed-sex cohort (n = 5 M, n = 2 F). However, Murphy et al. (2021), showed that an LEA of 15 kcal•kg FFM^{-1}•day^{-1} EA for five days increased ß-CTx and reduced P1NP concentrations in healthy males. Fensham et al. (2022) observed that six days of low energy availability (15 kcal•kg FFM^{-1}•day^{-1}) reduced fasting P1NP concentrations in a cohort of elite male race walkers, compared to a control trial. However, a LCHF group also showed reduced fasting P1NP concentrations and were the only intervention to display reduced P1NP AUC in response to a 25-km race-walking test. Low carbohydrate availability may therefore be a mediating factor in the responses of markers of bone resorption and formation to exercise and energy availability. Collectively, these data

suggest that markers of bone metabolism may be affected by low energy availability in males. These findings make sense, considering that male military recruits and athletes have been observed to experience stress fractures, albeit at a lower prevalence rate than females (Wentz et al., 2011). The disruption of markers of bone remodelling in males in response to very low energy availability agrees with these epidemiological findings. However, the magnitude and duration of low energy availability required to elicit perturbations in bone metabolism and the long-term implications for athlete bone health require further investigation. As does the prospect that carbohydrate availability may mediate bone turnover marker responses in males.

Disruptions observed in the endocrine and metabolic milieu were consistent with energy preserving mechanisms, characterised by reduced plasma leptin and T_3 concentrations, as well as increased circulating glycerol, NEFA, and ketone concentrations. The degree of energy availability reduction within Study 2 was more severe than previous controlled LEA experiments in males. The more marked reduction in energy availability in this study may explain the difference in findings compared to those of Papageorgiou et al. (2017) and Koehler et al. (2016), who both showed no change in T_3 concentrations in male cohorts following LEA. The short duration of the intervention in Study 2 may have been a reason why no changes in resting metabolic rate were observed, however.

Low energy availability has also been shown to reduce skeletal muscle protein synthetic rates in both females (Oxfeldt et al., 2023) and males (Areta et al., 2014). Interestingly, global protein fractional synthetic rates of the myofibrillar, soluble, and mitochondrial fraction were all unchanged in Study 2. This possibly can be attributed to the endurance exercise bouts performed throughout the intervention phases preserving skeletal muscle protein synthesis in the working muscle, extending the findings of Areta et al. (2014) who demonstrated that a single bout of resistance exercise can rescue LEA-related declines in MPS.

Taken together, these data indicate that in males, severe short-term low energy availability elicited changes in some endocrine, metabolic, and physiological markers associated with the *Male Athlete Triad* (Nattiv et al., 2021) and *REDs* models (Mountjoy et al., 2023). However, this acute period of low energy availability appeared not to influence the HPG axis or muscle protein synthetic rates in males. Whether these responses would lead to positive or negative health and performance outcomes in exercising males remains to be determined.

The endocrine, metabolic, and physiological effects of low carbohydrate availability in males

Reducing energy availability necessarily leads to a concomitant reduction in at least one of the major macronutrients. In Study 2, we reduced energy availability from 45- to 10 kcal•kg FFM^{-1}•day^{-1} but maintained the same relative contribution of each macronutrient between interventions. Carbohydrate

availability was therefore reduced from 6.7 to 2.4 g•kg^{-1}•day^{-1} during LEA. Reduced endogenous and exogenous carbohydrate availability is of particular interest considering its putative role in enhancing insulin sensitivity, regulating metabolic substrate utilisation, and increasing cellular signalling responses to exercise and muscle protein degradation, amongst other factors (see Bartlett et al., 2015, Gonzalez et al., 2016, and Hearris et al., 2018 for detailed reviews). Much of the low energy availability literature is therefore confounded by a concomitant state of low carbohydrate availability (Areta et al., 2021). However, the influence of low carbohydrate availability on physiological parameters typically associated with low energy availability is less well understood. Study 3 therefore sought to determine the endocrine, metabolic, and physiological effects of low carbohydrate availability under conditions of energy balance.

Four days of low carbohydrate availability (1.5 g•kg^{-1}•day^{-1}) with concomitant 'adequate' energy availability (45 kcal•kg FFM^{-1}•day^{-1}) achieved through high fat feeding led to greater concentrations of plasma ß-CTx, glycerol, NEFA, and ketones, whilst serum IGF-1 concentrations were reduced. Resting and exercise metabolism shifted towards greater fat oxidation rates, with a corresponding reduction in exercise gross efficiency. However, endocrine, metabolic, and physiological markers including RMR, total testosterone, T_3, leptin, insulin, and glucose were unaffected by the low carbohydrate intervention.

The findings of Study 3 add to a growing body of data suggesting that markers of bone resorption and formation is sensitive to carbohydrate availability. Over four days, Study 3 showed that fasted ß-CTx concentrations were greater in the low carbohydrate trial, compared to control, but that P1NP concentrations were unchanged. In agreement with these findings, Hammond et al. (2019) observed that providing LCHF meals around two glycogen depleting exercise bouts led to greater ß-CTx concentrations over the course of the intervention, compared to a high carbohydrate control trial. There were no differences in P1NP concentrations between LCHF and LEA interventions (Hammond et al., 2019). The findings of Study 3 partially agree with Heikura et al. (2020). 3.5 weeks following a ketogenic diet increased fasting ß-CTx concentrations, but also reduced P1NP in a cohort of elite race walkers. Conversely, in the fasted rested state, Fensham et al. (2022) showed that six days following a ketogenic diet did not increase ß-CTx concentrations, but P1NP concentrations were reduced in both the LCHF and an LEA trial, compared to control. Taken together, carbohydrate availability therefore appears to influence bone turnover markers independently from energetic status.

There is less evidence available regarding the role of carbohydrate availability upon the HPG axes. Recent meta-analyses and systematic reviews from Whittaker & Harris (2022) and Kang et al. (2020), respectively, concluded that consuming low carbohydrate intake has limited influence upon testosterone concentrations in males. However, Paoli et al. (2021) have observed that eight weeks following a ketogenic diet reduced total testosterone concentrations in male bodybuilders, with no changes observed

in a control group consuming a western diet. In agreement with Whittaker & Harris (2022) and Kang et al. (2020), Study 3 showed that four-days consuming a low carbohydrate, high fat diet that provided energy balance did not affect fasting total testosterone concentrations. Considering that five days of LEA with slightly greater carbohydrate availability (2.4 $g \bullet kg^{-1} \bullet day^{-1}$) also did not affect this parameter, this was expected. In contrast, the observed reduction in total testosterone by Mckay et al. (2022a) is likely attributable to exercise stress, considering that only a significant main effect of time was observed in this study. Short-term experimental research has not shown reductions in total testosterone, irrespective of whether energy or carbohydrate availability is reduced. HPG axis function, reflected in testosterone status, therefore appears robust to transient reductions in metabolic fuel availability in males.

The same is true for the role of carbohydrate availability upon the HPT axis. Study 3 showed that four days of low carbohydrate availability had no effect upon plasma total T_3 concentrations. This does not align with the findings of Hall et al. (2016) in a longer-term study, which observed that 4-weeks adherence to a ketogenic diet reduced plasma total T_3 concentrations in males with overweight/obesity. However, this study was confounded by an unintentional energy deficit. We have shown that even acute LEA can disrupt the HPT axis; in Study 2, we observed significant reductions in fasting plasma total T_3 concentrations after only 48 hours of LEA. Naturally, the findings of Study 2 compared to Study 3 imply that regulation of thyroid hormones is energy-, rather than carbohydrate-mediated. However, under conditions of energy deficit, even small increases in carbohydrate intake have been shown to mitigate reductions in T_3 concentrations in both rodent and human experiments (Gavin et al., 1981; Mathieson et al., 1986; Spaulding et al., 1976). Taken together, there is a surprising lack of literature regarding the influence of low carbohydrate, high fat diets upon the hypothalamic-pituitary-thyroid axis and even less that is not confounded by a concomitant energy deficit. Given the apparently key influence of carbohydrate availability on thyroid hormones under conditions of energy deficit, future research should seek to understand this interaction further.

Overall, Study 3 presents novel data regarding the broad physiological effects of low carbohydrate availability upon the physiological parameters that are typically thought to be affected by low energy availability. At least acutely, low carbohydrate appears to have minimal impact on the sequelae typically associated with low energy availability. Future research may seek to replicate Study 3 over longer durations and investigate the responses of males and females under the same experimental conditions.

7.3. Practical applications:

Practically, Study 1 has added to the limited knowledge of the patterns of energy availability experienced by free-living athletes. The findings highlight that energy intake is relatively stable on a day-to-day basis, despite highly variable exercise energy expenditure. It is currently unknown how the

resultant fluctuating pattern of daily energy availability might affect the health, training adaptation, and performance of athletes. Carbohydrate intake was also low relative to contemporary nutrition guidelines (Thomas et al., 2016) on days of *High* and *Very High* training volume. Should athletes want to achieve high carbohydrate availability levels in line with these guidelines, they should target increasing carbohydrate intake on days of high training volume. This might be achieved by promoting the availability of carbohydrate-rich, familiar, and hyper-palatable foods, encouraging/motivating athletes to consume such products, and protecting time for feeding opportunities during periods of heavy training load (Burke, Lundy, et al., 2018; Charlot et al., 2021).

Study 2 has demonstrated that males are also susceptible to LEA-related physiological dysregulations, displaying signs of energy preservation characterised by reduced T_3, leptin, and IGF-1 concentrations, disruption of bone turnover markers, and a shift towards fat oxidation at rest and during exercise. However, these effects did not extend to disruptions in RMR, or skeletal muscle protein synthetic rates. Whether these responses are 'positive' or 'negative' for athlete health and performance remains unclear at present.

At least acutely, the findings of Study 3 suggest that changes in physiological markers typically associated with LEA largely appear to be mediated by energy itself, rather than carbohydrate availability (Figure 7.1.). These findings therefore suggest that achieving 'adequate' energy availability through high-fat intake preserves most physiological functions when carbohydrate availability is reduced. The findings of Study 3 show that carbohydrate availability influences bone resorption markers and IGF-1 concentrations, however. Therefore, it may be prudent for athletes to still be encouraged to maintain a high carbohydrate intake during periods of heavy training volume/load, in line with contemporary sports nutrition guidelines (Thomas et al., 2016). On days of high training volume, athletes may not be able achieve the recommended carbohydrate intake, or doing so may still lead to a state of low energy availability. Under these circumstances, the findings of Study 3 suggest that increasing fat intake to maintain adequate energy availability could help to preserve physiological homeostasis for most markers of low energy availability. However, as high-fat diets are well-documented to impair carbohydrate metabolism and performance capacity (Burke et al., 2017; Stellingwerff et al., 2006), carbohydrate intake should still be strongly prioritised in line with sports nutrition guidelines (Thomas et al., 2016), and elevated dietary fat intake should be viewed as supplementary.

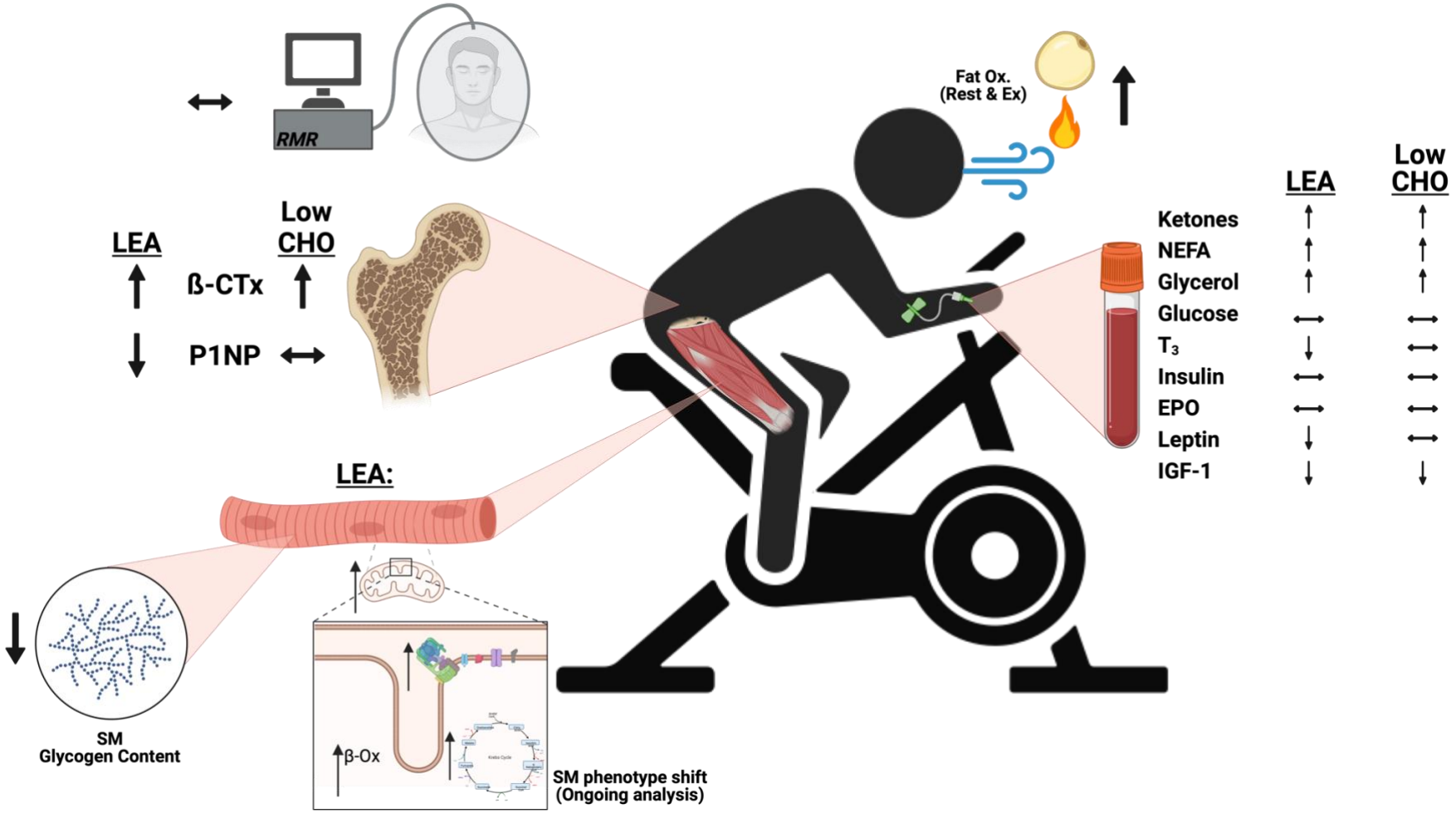


Figure 7.1. Illustrative summary of the endocrine, metabolic, and physiological effects of acute (≤ 5 days) low energy (Study 2, Chapter 5: ***LEA***) and carbohydrate (Study 3, Chapter 6: ***Low CHO***) availability observed in healthy, exercising, adult males within this thesis. RMR = resting metabolic rate, SM = skeletal muscle, NEFA = non-esterified fatty acids, T_3 = triiodothyronine, EPO = erythropoietin, IGF-1 = insulin-like growth factor 1, ß-CTx = β-Carboxyl-terminal cross-Linked telopeptide of Type I collagen, P1NP = procollagen type 1 N-terminal propeptide.

7.4. Experimental limitations:

Study protocol design:

Study 1 (Chapter 4): Assessment of dietary intake in Study 1 was conducted using the remote food photography method (Martin et al., 2009). Currently there is no gold standard for assessing energy intake and contemporary methods are prone to significant error (Burke, Lundy, et al., 2018; Capling et al., 2017). Participants may have unintentionally or intentionally under-reported their dietary intake, leading to a weaker relationship between energy intake and exercise energy expenditure. We were therefore correspondingly cautious about over-interpreting the energy availability data attained and classifying the 'status' of participant energy availability. However, these limiting factors were sources of systematic error within individual participant data, which was partly overcome through the use of within-participant correlation statistical analyses (Bland & Altman, 1995). As such, we were able to examine the intra-participant relationship between energy/macronutrient intake and exercise energy expenditure with confidence, in line with the study aims. Furthermore, as all data collection was conducted remotely, we were unable to collect any biological samples from participants to assess relationships between energy availability and physiological markers. Similarly, as we could not conduct body composition analyses, we had to estimate participant fat free mass for the calculation of fat free mass from historical team data and reference data reported for our target population. We also could not assess gross efficiency during exercise prior to the data collection period. A mean reference value providing an approximation of cycling gross efficiency was therefore applied to all participants. Participants were likely to have a degree of over- or under-estimation of their cycling exercise energy expenditure, although this again would be a source of systematic error within-participant.

Study 2 (Chapter 5): Owing to the cost and quantity of the deuterium oxide tracer required to 'pre-load' and sustain D_2O enrichment, we were unable to perform this on multiple occasions per participant. We therefore could not use a true-experimental, repeated-measures study design with a washout period between trials. Based upon concerns of a residual effect of the *LEA* intervention upon physiological responses, we also opted not to randomise participant treatment order within the experimental design. Our statistical interpretation therefore cannot technically delineate between time and treatment effects. However, this was mitigated by the stringent participant inclusion criteria, in conjunction with the controlled dietary provision and standardisation of sample collections, increasing confidence that we are observing *true* treatment effects.

A potential further step may have been to use a short washout period between the *EB* and *LEA* interventions, where participants returned to their free-living diet and exercise routines. This would not have provided a 'true-experimental' study design due to a lack of randomisation in trial order. But such a washout period would have allowed a more robust statistical approach by permitting the comparison of pre-post changes between interventions. Future studies may seek to replicate the design of Study 2,

but also randomise participants to a separate control group that remain on the EB protocol throughout the intervention phases. This would isolate time effects from treatment effects in future research, providing a more robust study design.

Study 3 (Chapter 6): Limited evidence suggesting that low carbohydrate availability may be a confounding variable in energy availability research provided the central rationale underpinning Study 3 (Chapter 6). This premise has been reinforced by the most recent *REDs* position stand (Mountjoy et al., 2023). Yet most experimental low energy availability research studies have elicited a state of LEA whilst maintaining a constant relative contribution of each macronutrient, as reviewed by Areta et al. (2021). It is therefore possible that corresponding reductions in fat, protein, micronutrient intake, or a combination of these factors may also have contributed to the observations of previous low energy availability research studies. Future research may therefore seek to investigate whether the independent restriction of dietary protein, fat, or micronutrient intake contributes to the physiological effects of low energy availability such as those observed in Study 2 (Chapter 5) of this thesis.

Studies 2 & 3 (Chapters 5 & 6): We selected an intervention duration that reflected the seminal work of Professor Anne Loucks (Loucks, 2020) investigating the effects of low energy availability upon a range of physiological responses in sedentary females. Consequently, whilst novel, the findings of the studies outlined in Chapters 5 & 6 are limited to short-term periods of low energy and carbohydrate availability. In Study 1, reductions in energy availability were predominantly caused by increases in exercise energy expenditure. In Study 2 & Study 3, the cycling protocol to expend 15 kcal•kg FFM^{-1}•day^{-1} took approximately 90 minutes to complete in both interventions and for both studies. Owing to time and further logistical limitations, alongside the calibre of fitness of the participants, we were unable to reduce energy availability through further increases in exercise energy expenditure. We therefore reduced energy availability through restrictions in energy intake. A question therefore remains on whether large increases in exercise energy expenditure would elicit similar physiological responses in males to those observed in Study 2 and Study 3. Previous research has shown that it is the energy available, not the stress of exercise that elicits perturbations in physiological responses (Loucks et al., 1998). However future research should seek to investigate the physiological effects of varying combinations of energy intake and exercise energy expenditure using different exercise modalities in males.

Furthermore, due to the short-term nature of Study 2 and Study 3's interventions, we did not expect to observe changes in bone mineral content or density (Bauer et al., 2012). We therefore assessed changes in the concentration of blood-borne markers of bone resorption and formation, ß-CTx and P1NP, respectively. Thus, our findings relating to 'bone health' are subject to the following limitations; Firstly, as reviewed by Dolan et al. (2020), these markers are products of collagen metabolism, the key

structural protein of numerous connective tissues. The ß-CTx and P1NP data are therefore not specific to bone only. Secondly, by assessing such markers in the blood, we assessed systemic changes in concentrations of ß-CTx and P1NP, yet bone responses appear highly sensitive to site-specific loading (Kannus et al., 1994). By analysing changes in bloodborne markers of bone remodelling, it is therefore not possible to assess changes in bone resorption and formation from any one site of particular interest (i.e. loaded sites). Finally, as reviewed by Dolan et al. (2020), bone remodelling markers are subject to variation due to sex, age, circadian rhythms, prior exercise and injury, and sample handling, amongst other factors. To mitigate these factors, we analysed ß-CTx and P1NP concentrations, aligning with the recommendations of *The National Bone Health Alliance* and *International Osteoporosis Foundation*. These markers of resorption and formation, respectively, are identified to be subject to smaller biological variability, possess greater specificity to bone metabolism, and are responsive to acute interventions (Bauer et al., 2012; Chubb et al., 2017; Dolan et al., 2020). Similarly, there is some evidence that these markers can serve as predictors of long-term changes in bone mineral density (Villareal et al., 2016).

Finally, due to budget and time constraints, we were not able to establish internal quality control and reliability data for the ELISAs conducted internally. This represents a limitation to the biochemistry data presented herein. With greater resources, we would have sought to establish internal ELISA quality control and reliability standards to increase confidence in the data derived from these assays. Despite this, we adhered to manufacturer guidelines diligently to ensure assays met acceptable standards. In instances where ELISA standards were not met, assays were either re-run or outsourced when affordable. With further resources, it would also have been interesting to investigate some of the endocrine parameters studied in greater detail. For example, both testosterone and T_3 assays were conducted on the total concentrations of these hormones. Future research might also examine the free concentrations of testosterone, T_3, and the ratio of total:free concentrations of these hormones. Whilst beyond the scope of this thesis, such data would provide greater understanding of the influence of low energy and/or carbohydrate availability on the regulation of such endocrine markers.

Participants:

Based upon the criteria presented by (McKay et al., 2022b), the participants in Study 2 & Study 3 were classified as *Tier 1 - 2* individuals and were not specifically trained cyclists. Participants inclusion criteria specified that individuals were completing ≥ 3 aerobic sessions per week, but not that they were specifically a cyclist, endurance trained, or a competitive athlete. In contrast, the participants in Study 1 were *Tier 4* athletes and completed 21.5 ± 3.8 hours of training across the 7-day observation period. As such, the endocrine, metabolic, and physiological responses of participants in Study 2 & Study 3 may be different to participants such as those in Study 1, considering the adaptations associated with increased training status. The concept of low energy availability is typically applied to athletic

populations. As such, future research may seek to replicate these studies in more-highly trained (*Tiers 3 – 5*) individuals. In the present studies, our recruitment strategy was designed to promote 'proof-of-concept' evidence that males are affected by low energy availability in a similar manner to females. As such, we were concerned that prospective participants with an exceptionally high training load might already be in a state of low energy availability, thus increasing the risk of type-2 error. We therefore advertised to recruit 'exercising', but not specifically 'endurance-trained' individuals.

7.5. Future research directions:

Recommendation 1:

The data presented within Study 1 highlight the need for future research to investigate the effects of low energy availability using heterogenous patterns of daily EA manipulation. Furthermore, considering the *Tier 4* status of participants recruited in this study, it could be argued that these athletes were in a state of optimal functioning. It remains to be determined if alternating days of low energy availability with adequate energy availability might therefore be a means of enhancing training adaptations. Future research should therefore investigate the physiological and performance effects of alternating days of low energy availability (with high exercise energy expenditure) with days of adequate energy intake, similar to the protocol used in Study 2.

Recommendation 2:

Controlled studies of longer duration are still required to better understand the physiological effects of low energy availability in males further. Males undertaking long-term exercise training have been identified in recent reviews and position stands to display reduced testosterone concentrations, impaired bone health (Fredericson et al., 2021; Hackney, 2020; Nattiv et al., 2021) and further proposed dysregulations (Mountjoy et al., 2023). However, data from controlled, laboratory-based studies are typically limited to intervention periods of less than one week and experimental findings are currently equivocal. Longer-term controlled studies, whilst logistically challenging, are therefore required to further characterise these effects. More direct markers of health and performance would be of interest in these studies.

Recommendation 3:

Low energy availability studies in males are yet to observe alterations in the hypothalamic-pituitary-gonadal axis, as currently assessed through circulating testosterone concentrations. However, no studies appear to have investigated the effects of low energy availability on upstream markers of the HPG axis, such as the release of LH and FSH, in males. Whilst potentially labour intensive, such a study seems essential to determine if the male HPG axis is sensitive to short-term low energy availability.

Recommendation 4:

The field of low energy availability research represents one of the few areas of scientific knowledge that is better developed in females than males (Areta et al., 2021; Cowley et al., 2021). Recommendations 1 and 2 are equally applicable to females as they are males. Moreover, apparent sexual dimorphism exists in the responses of males and females to low energy availability. To continue expanding our knowledge of the differing effects of low energy availability in both sexes, future research should therefore seek to do so in both male and female participants.

7.6. Closing thoughts:

In summary, the data presented within this thesis show that free-living athletes experience variability in daily availability that has not typically been reflected in laboratory-based experiments investigating the physiological effects of low energy availability. Athletes may therefore experience low energy availability and/or low carbohydrate availability intermittently, particularly during periods of high training load. The findings of Study 2 demonstrate that male individuals are susceptible to physiological alterations associated with low energy availability, such as reductions in T_3, leptin, IGF-1, and markers of bone formation, as well as increased markers of bone resorption. However, whether these effects are 'positive' or 'negative' for health and performance remains unclear. Study 3 showed that four days of low carbohydrate availability increased markers of bone resorption and reduced serum IGF-1 concentrations. Plasma NEFA, glycerol, and D-3-Hydroxybutyrate were increased by the low carbohydrate availability intervention, yet further markers of energy preservation typically associated with LEA, such as T_3, leptin, IGF-1, and insulin were unchanged. Taken together, most endocrine responses typically associated with low energy availability therefore appear to be sensitive to the energy itself, rather than reductions in carbohydrate availability that often occur simultaneously. Interpreting whether the apparent energy preserving mechanism we observed in response to LEA in Study 2 are 'positive' or 'negative' requires caution. The effects of low energy availability are complex, multi-faceted and significantly more research is required before we can draw such conclusions with any confidence.

Chapter 8:

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Chapter 9:

Appendix

a) Remaining samples for future analyses:

Study:	Sample type remaining:
Study 2 (Chapter 5):	Skeletal muscle processed for trans-electron microscopy (*TEM*) imaging Skeletal muscle processed for dynamic proteomic profiling (data analyses) Saliva Plasma/serum
Study 3 (Chapter 6):	Skeletal muscle processed for trans-electron microscopy (*TEM*) imaging Skeletal muscle processed/mounted ready for immunohistochemistry Snap-frozen skeletal muscle for glycogen assay Saliva Urine Plasma/serum

b) Liverpool Clinical Laboratories Roche Diagnostic ECLIA kit datasheets:

i. ß-CTx

07026960500V5.0

Elecsys β-CrossLaps/serum

cobas®

REF		Σ	SYSTEM
07026960190	07026960500	100	**cobas e** 801

English

System information

Short name	ACN (application code number)
CROSSL	10062

Intended use

Immunoassay for the in vitro quantitative determination of degradation products of type I collagen in human serum and plasma as an aid in assessing bone resorption. The test may be used as an aid in monitoring antiresorptive therapies in osteoporotic patients.

The **e**lectro**c**hemi**l**uminescence **i**mmuno**a**ssay "ECLIA" is intended for use on the **cobas e** 801 immunoassay analyzer.

Summary

Type I collagen is an important component of the bone matrix and its degradation products are the most commonly used bone resorption markers.[1]

During normal bone metabolism, mature type I collagen is degraded and small fragments pass into the circulation and are excreted via the kidneys.

In physiologically or pathologically elevated bone resorption (e.g. in old age or as a result of osteoporosis), type I collagen is degraded to an increased extent, and there is a commensurate rise in the level of collagen fragments in the blood.

Especially relevant fragments are the β-isomerized C (carboxy)-terminal cross-linking telopeptides (β-CTx), produced by osteoclastic hydrolysis of type I collagen.[1,2,3]

Elevated serum levels of isomerized C-terminal telopeptides of type I collagen have been reported for patients with increased bone resorption. The serum levels return to normal during anti-resorptive therapy.[4,5,6,7]

Determination of the C-terminal telopeptides in serum is recommended for monitoring the efficacy of antiresorptive therapy (e.g. bisphosphonates or hormone replacement therapy - HRT) in osteoporosis or other bone diseases. By these means, therapy-induced changes can be demonstrated after just a few months.[8,9]

Serum CTx has been selected by the IOF-IFCC Bone Marker Standards Working Group as marker for bone resorption, mainly based on the following criteria:

- It has been evaluated both for fracture prediction and monitoring osteoporosis therapies.
- The assay is widely available, for serum or plasma samples, with well documented requirements for sample handling and stability.
- The analyte is well characterized and allows the development of clearly defined reference standard.[1]

The Elecsys β-CrossLaps/serum assay is specific for crosslinked β-isomerized type I collagen fragments, independent of the nature of the crosslink (e.g. pyrrole, pyridinolines, etc.).[10] The assay specificity is guaranteed through the use of two monoclonal antibodies each recognizing linear β-8AA octapeptides (EKAHD-β-GGR). The Elecsys β-CrossLaps/serum assay therefore quantifies all type I collagen degradation fragments that contain the isomerized octapeptide β-8AA twice (β-CTx).[6,7]

Test principle

Sandwich principle. Total duration of assay: 18 minutes.

- 1st incubation: 30 µL of sample and a biotinylated monoclonal anti-β-CrossLaps antibody are incubated together; the antigen in the sample is liberated from the serum components.
- 2nd incubation: Following addition of streptavidin-coated microparticles and a monoclonal β-CrossLaps-specific antibody labeled with a ruthenium complex[a)], a sandwich complex is formed which becomes bound to the solid phase via interaction of biotin and streptavidin.
- The reaction mixture is aspirated into the measuring cell where the microparticles are magnetically captured onto the surface of the electrode. Unbound substances are then removed with ProCell II M. Application of a voltage to the electrode then induces chemiluminescent emission which is measured by a photomultiplier.
- Results are determined via a calibration curve which is instrument-specifically generated by 2-point calibration and a master curve provided via the **cobas** link.

a) Tris(2,2'-bipyridyl)ruthenium(**II**)-complex ($Ru(bpy)_3^{2+}$)

Reagents - working solutions

The **cobas e** pack is labeled as CROSSL.

M Streptavidin-coated microparticles, 1 bottle, 5.8 mL:
Streptavidin-coated microparticles 0.72 mg/mL; preservative.

R1 Anti-β-CrossLaps-Ab~biotin, 1 bottle, 7.6 mL:
Biotinylated monoclonal anti-β-CrossLaps antibody (mouse) 2.5 mg/L; phosphate buffer 100 mmol/L, pH 7.2; preservative.

R2 Anti-β-CrossLaps-Ab~$Ru(bpy)_3^{2+}$, 1 bottle, 6.8 mL:
Monoclonal anti-β-CrossLaps antibody (mouse) labeled with ruthenium complex 2.4 mg/L; phosphate buffer 100 mmol/L, pH 7.2; preservative.

Precautions and warnings

For in vitro diagnostic use for health care professionals. Exercise the normal precautions required for handling all laboratory reagents.

Infectious or microbial waste:
Warning: handle waste as potentially biohazardous material. Dispose of waste according to accepted laboratory instructions and procedures.

Environmental hazards:
Apply all relevant local disposal regulations to determine the safe disposal.

Safety data sheet available for professional user on request.

This kit contains components classified as follows in accordance with the Regulation (EC) No. 1272/2008:

Warning

H317 May cause an allergic skin reaction.

Prevention:

P261 Avoid breathing dust/fume/gas/mist/vapours/spray.

P272 Contaminated work clothing should not be allowed out of the workplace.

P280 Wear protective gloves.

Response:

P333 + P313 If skin irritation or rash occurs: Get medical advice/attention.

P362 + P364 Take off contaminated clothing and wash it before reuse.

Disposal:

P501 Dispose of contents/container to an approved waste disposal plant.

Product safety labeling follows EU GHS guidance.

Contact phone: all countries: +49-621-7590

Avoid foam formation in all reagents and sample types (specimens, calibrators and controls).

Elecsys β-CrossLaps/serum

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Reagent handling

The reagents in the kit have been assembled into a ready-for-use unit that cannot be separated.

All information required for correct operation is available via the **cobas** link.

Storage and stability

Store at 2-8 °C.

Do not freeze.

Store the **cobas e** pack **upright** in order to ensure complete availability of the microparticles during automatic mixing prior to use.

Stability:	
unopened at 2-8 °C	up to the stated expiration date
on the **cobas e** 801 analyzer	16 weeks

Specimen collection and preparation

Only the specimens listed below were tested and found acceptable.

Serum collected using standard sampling tubes or tubes containing separating gel.

Li-heparin, K_2-EDTA and K_3-EDTA plasma.

Li-heparin plasma tubes containing separating gel can be used.

Criterion: Slope 0.9-1.1 + intercept within ≤ ± 0.02 ng/mL + coefficient of correlation ≥ 0.95.

It is recommended to draw blood as fasting, morning samples. For long-term investigations, the samples should always be taken under same conditions as the baseline sample, as the serum β-CTx concentration is to some extent subject to a circadian rhythm.

Preference should be given to K_2- or K_3-EDTA plasma, as it is stable longer than serum.

Stability of serum: 6 hours at 20-25 °C, 8 hours at 2-8 °C.

Stability of Li-heparin plasma: 4 hours at 20-25 °C, 8 hours at 2-8 °C.

Stability of K_2- and K_3-EDTA plasma: 24 hours at 20-25 °C, 8 days at 2-8 °C.

Serum, heparinized and EDTA plasma are stable for 3 months at -20 °C (± 5 °C). For longer periods, store at -80 °C (± 10 °C). Freeze only once.

Hemolyzed samples (Hb > 0.5 g/dL) elicit a decrease in the β-CTx concentration.

The sample types listed were tested with a selection of sample collection tubes that were commercially available at the time of testing, i.e. not all available tubes of all manufacturers were tested. Sample collection systems from various manufacturers may contain differing materials which could affect the test results in some cases. When processing samples in primary tubes (sample collection systems), follow the instructions of the tube manufacturer.

Centrifuge samples containing precipitates before performing the assay.

Do not use heat-inactivated samples.

Do not use samples and controls stabilized with azide.

Ensure the samples and calibrators are at 20-25 °C prior to measurement.

Due to possible evaporation effects, samples and calibrators on the analyzers should be analyzed/measured within 2 hours.

Materials provided

See "Reagents – working solutions" section for reagents.

Materials required (but not provided)

- REF 11972316122, β-CrossLaps CalSet, 4 x 1.0 mL
- REF 05618860190, PreciControl Varia, for 4 x 3.0 mL
- General laboratory equipment
- **cobas e** 801 analyzer

Additional materials for the **cobas e** 801 analyzer:

- REF 06908799190, ProCell II M, 2 x 2 L system solution
- REF 04880293190, CleanCell M, 2 x 2 L measuring cell cleaning solution
- REF 07485409001, Reservoir Cup, 8 cups to supply ProCell II M and CleanCell M
- REF 06908853190, PreClean II M, 2 x 2 L wash solution
- REF 05694302001, Assay Tip/Assay Cup tray, 6 magazines x 6 magazine stacks x 105 assay tips and 105 assay cups, 3 wasteliners
- REF 07485425001, Liquid Flow Cleaning Cup, 2 adaptor cups to supply ISE Cleaning Solution/Elecsys SysClean for Liquid Flow Cleaning Detection Unit
- REF 07485433001, PreWash Liquid Flow Cleaning Cup, 1 adaptor cup to supply ISE Cleaning Solution/Elecsys SysClean for Liquid Flow Cleaning PreWash Unit
- REF 11298500316, ISE Cleaning Solution/Elecsys SysClean, 5 x 100 mL system cleaning solution

Assay

For optimum performance of the assay follow the directions given in this document for the analyzer concerned. Refer to the appropriate operator's manual for analyzer-specific assay instructions.

Resuspension of the microparticles takes place automatically prior to use.

Place the cooled (stored at 2-8 °C) **cobas e** pack on the reagent manager. Avoid foam formation. The system automatically regulates the temperature of the reagents and the opening/closing of the **cobas e** pack.

Calibration

Traceability: This method has been standardized against reference standards precisely defined by weighing out synthetic peptide.

The predefined master curve is adapted to the analyzer using the relevant CalSet.

Calibration frequency: Calibration must be performed once per reagent lot using fresh reagent (i.e. not more than 24 hours since the **cobas e** pack was registered on the analyzer).

Calibration interval may be extended based on acceptable verification of calibration by the laboratory.

Renewed calibration is recommended as follows:

- after 12 weeks when using the same reagent lot
- after 28 days when using the same **cobas e** pack on the analyzer
- as required: e.g. quality control findings outside the defined limits

Quality control

For quality control, use PreciControl Varia.

In addition, other suitable control material can be used.

Controls for the various concentration ranges should be run individually at least once every 24 hours when the test is in use, once per **cobas e** pack, and following each calibration.

The control intervals and limits should be adapted to each laboratory's individual requirements. Values obtained should fall within the defined limits. Each laboratory should establish corrective measures to be taken if values fall outside the defined limits.

If necessary, repeat the measurement of the samples concerned.

Follow the applicable government regulations and local guidelines for quality control.

Calculation

The analyzer automatically calculates the analyte concentration of each sample either in ng/mL or pg/mL.

Limitations - interference

The effect of the following endogenous substances and pharmaceutical compounds on assay performance was tested. Interferences were tested up to the listed concentrations and no impact on results was observed.

Endogenous substances

Compound	Concentration tested
Bilirubin	≤ 1112 µmol/L or ≤ 65 mg/dL
Hemoglobin	≤ 0.3 mmol/L or ≤ 500 mg/dL
Intralipid	≤ 1500 mg/dL
Biotin	≤ 123 nmol/L or ≤ 30 ng/mL
Rheumatoid factors	≤ 1000 IU/mL

Criterion: For concentrations ≤ 0.50 ng/mL the deviation is ≤ 0.05 ng/mL. For concentrations > 0.50 ng/mL the deviation is ≤ ± 10 %.

Samples should not be taken from patients receiving therapy with high biotin doses (i.e. > 5 mg/day) until at least 8 hours following the last biotin administration.

There is no high-dose hook effect at β-CTx concentrations up to 150 ng/mL (150000 pg/mL).

Pharmaceutical substances
In vitro tests were performed on 16 commonly used pharmaceuticals. No interference with the assay was found.

In addition, the following special drugs were tested. No interference with the assay was found.

Special drugs

Drug	Concentration tested mg/L
Ibandronate	6
Actonel (Risedronate)	150
Vitamin D3	0.075
Calcium Carbonate	2500
Vitamin D (25-OH)	1
17-β-Estradiol	2.5
β-Estradiol-17-valerate	2.5
β-Estradiol-3-sulfate	2.5

In rare cases, interference due to extremely high titers of antibodies to analyte-specific antibodies, streptavidin or ruthenium can occur. These effects are minimized by suitable test design.

Results may be confounded by clinical conditions known to affect bone resorption, e.g. hyperparathyroidism or hyperthyroidism.

Caution should be exercised when measuring serum β-CTx levels in patients with reduced renal function as this may lead to reduced excretion of serum β-CTx and a consequent increase in the apparent serum β-CTx levels is seen.[11]

There is evidence that β-CTx can predict loss of bone density.[12] However, a correlation with increased fracture risk has not yet been demonstrated. The properties of β-CTx in case of hyperparathyroidism or hyperthyroidism have not yet been unequivocally described, either.

For diagnostic purposes, the results should always be assessed in conjunction with the patient's medical history, clinical examination and other findings.

They should not be used as a sole determinant for deciding on or modifying an existing treatment regimen.

Limits and ranges

Measuring range

0.01-6.00 ng/mL or 10-6000 pg/mL (defined by the Limit of Detection and the maximum of the master curve). Values below the Limit of Detection are reported as < 0.010 ng/mL (< 10 pg/mL). Values above the measuring range are reported as > 6.00 ng/mL (> 6000 pg/mL).

Lower limits of measurement

Limit of Blank, Limit of Detection and Limit of Quantitation

Limit of Blank = 0.008 ng/mL

Limit of Detection = 0.01 ng/mL

Limit of Quantitation = 0.05 ng/mL

The Limit of Blank, Limit of Detection and Limit of Quantitation were determined in accordance with the CLSI (Clinical and Laboratory Standards Institute) EP17-A2 requirements.

The Limit of Blank is the 95th percentile value from n ≥ 60 measurements of analyte-free samples over several independent series. The Limit of Blank corresponds to the concentration below which analyte-free samples are found with a probability of 95 %.

The Limit of Detection is determined based on the Limit of Blank and the standard deviation of low concentration samples. The Limit of Detection corresponds to the lowest analyte concentration which can be detected (value above the Limit of Blank with a probability of 95 %).

The Limit of Quantitation is the lowest analyte concentration that can be reproducibly measured with an intermediate precision CV of ≤ 20 %.

Dilution

Not necessary due to the broad measuring range.

Expected values

The following 95 % reference intervals (RI) for serum βCTx in 1039 healthy men, and in 1029 healthy premenopausal and postmenopausal women (age range 24-76 years) were obtained from a Danish study with the Elecsys β-CrossLaps/serum assay. Subjects included were characterized by their history of osteoporosis and lifestyle, and women by their menopausal state and by taking no anti-osteoporotic medication. Based on the patterns in the sex-, age-, and menopause-stratified 95 % RIs, subjects were grouped into age intervals for each gender, and into pre-menopausal and postmenopausal.[13] Other studies have demonstrated, that ranges can differ between ethnicities and geographical locations.[14,15] Thus, measurements should be compared to reference intervals established on material from similar geographical regions and should reflect the same gender, age, and pre-/post-menopausal status.

The table shows the geometric means and 95% reference intervals of healthy male and female subjects after stratification for age, and for menopause of female subjects from a Danish study.[13]

1. Healthy subjects

Age range (years)	Men			Women		
	N	GM (pg/mL)	95 % RI (pg/mL)	N	GM (pg/mL)	95 % RI (pg/mL)
< 29.9	39	492	238-1019	58	378	148-967
30-39.9	80	459	225-936	111	308	150-635
40-49.9	234	382	182-801	257	296	131-670
50-59.9	248	345	161-737	281	440	183-1060
60-69.9	303	316	132-752	234	408	171-970
>70	135	302	118-776	88	362	152-858
Pre-menopause	-	-	-	449	306	136-689
Post-menopause	-	-	-	578	424	177-1015

Intra-individual variance and least significant change (LSC)

The intra-individual variance of βCTx was determined in a subset of 18 healthy postmenopausal women (mean βCTx at baseline 0.516 ± 0.217 ng/mL) at 5 time points over 3 months. The median intra-individual variability as expressed by the mean CVi (intra-individual coefficient of variation) for serum βCTx values was 9.4 % (range, 4.1-27 %). On the basis of this CVi, the least significant change (LSC) was determined to be 27 %, meaning that an individual should display a ≥ 27 % decrease of serum βCTx concentrations when receiving antiresorptive therapy to have a < 5 % chance ($p < 0.05$) of the decrease being the result of random variation in marker concentration.[16]

Monitoring during antiresorptive therapy

Detecting changes of serum βCTx concentrations is valuable in the monitoring of antiresorptive therapies with bisphosphonates and in the assessment of therapy adherence of patients.[17] Bisphosphonates including alendronate, risedronate, ibandronate and zoledronic acid are commonly used medications to treat osteoporosis. They reduce bone resorption by inhibiting osteoclasts and thereby increasing bone mineral density (BMD). BMD is widely used to monitor response to treatment; however, treatment-induced increments in BMD are modest (typically 2 % per year). Taking a repeat error of 1-2 % into account true changes in BMD are observed only several years after starting treatment. Treatment-induced changes in bone turnover markers are much more rapid and occur at 3-6 months[18] or earlier.

a. Ibandronate therapy

The DIVA (Dosing IntraVenous Administration) study enrolled 1395 women aged 55-80 years, who were > 5 years menopausal, with osteoporosis diagnosed by lumbar spine [L2-L4] bone mineral density T score less than -2.5. Participants received a daily calcium dose of 500 mg and 400 IU vitamin D. A dosing scheme of oral 2.5 mg ibandronate daily, which has

Elecsys β-CrossLaps/serum

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proven antifracture efficacy was compared with an i.v. 3 mg every 3 months dosing scheme and investigated for non-inferiority.

The table shows the median (%) change from baseline in serum βCTx levels after 2, 3, 4, 6 and 12 months[9] and after 24 months.[19]

The following values have been obtained from studies with the Elecsys β-CrossLaps/serum assay in healthy test subjects:

	Oral ibandronate 2.5mg/daily		I.v. ibandronate 3 mg every 3 months	
Month	N	Median (95 % CI)	N	Median (95 % CI)
2	181	-45.0 (-48.7, -40.5)	-	-
3	192	-54.1 (-57.8, -48.7)	356	-43.2 (-45.9, -40.8)
4	180	-57.6 (-66.7, -50.0)	-	-
6	372	-62.5 (-65.3, -60.0)	353	-58.4 (-61.5, -55.2)
12	368	-62.6 (-66,0, -58.9)	352	-58.6 (-61.5, -55.4)
24	310	-59.9 (no CI available)	298	-53.4 (no CI available)

b. Other anti-osteoporotic medications

Studies with different anti-osteoporotic medications (alendronate, risedronate, zoledronic acid and other drugs) at licensed doses revealed that βCTx reductions from baseline varied between the treatments, but serum βCTx was clinically useful in monitoring all anti-resorptive therapies. In a placebo-controlled clinical study with healthy postmenopausal women comparing the changes of different bone turnover markers, serum βCTx levels showed the highest decrease with 63.7 % in the alendronate group (N = 75) and 21.6 % in the placebo group (N = 73) after 12 months. Serum βCTx showed the highest correlation ($r = 0.60$, $p < 0.0001$) with changes in lumbar spine bone mineral density after 12 months.[20] In a clinical study performed in 54 study centers worldwide, the effectiveness of 5 mg i.v. zoledronic acid on the increase of lumbar spine bone mineral density was compared with 5 mg oral risedronate and monitored with bone turnover markers, e.g. serum βCTx.[21] The strong decrease of βCTx levels occurring within 9-11 days after onset of both treatments was maintained during the 12 months of the study. The reduction of βCTx levels after 6 and 12 months reflected the efficacy of both medications, i.e. the changes in lumbar bone mineral density.

Comparison of the least significant change (LSC) with the observed change in serum βCTx is a commonly proposed approach to determine its physiological relevance. A reduction in serum βCTx of smaller than the LSC of 27 % in a treated patient after 3 months from treatment initiation can thus be used as indicator of poor adherence or poor response of the patient to the anti-osteoporotic therapy.[17]

Each laboratory should investigate the transferability of the expected values to its own patient population and if necessary determine its own reference ranges.

Specific performance data

Representative performance data on the analyzer is given below. Results obtained in individual laboratories may differ.

Precision

Precision was determined using Elecsys reagents, samples and controls in a protocol (EP05-A3) of the CLSI (Clinical and Laboratory Standards Institute): 2 runs per day in duplicate each for 21 days (n = 84). The following results were obtained:

cobas e 801 analyzer					
		Repeatability		**Intermediate precision**	
Sample	Mean ng/mL	SD ng/mL	CV %	SD ng/mL	CV %
Human serum 1	0.074	0.003	4.5	0.004	5.8
Human serum 2	0.444	0.013	3.5	0.020	5.5
Human serum 3	0.630	0.013	2.5	0.015	2.8
Human serum 4	3.28	0.054	2.0	0.074	2.7
Human serum 5	5.59	0.081	1.8	0.100	2.1

cobas e 801 analyzer					
		Repeatability		**Intermediate precision**	
Sample	Mean ng/mL	SD ng/mL	CV %	SD ng/mL	CV %
PC[b)] Varia1	0.295	0.007	3.1	0.009	3.4
PC Varia2	0.814	0.018	2.7	0.021	2.9

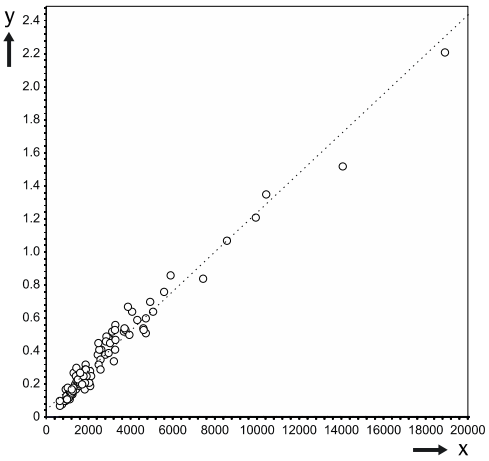
b) PC = PreciControl

Method comparison

A comparison of the Elecsys β-CrossLaps/serum assay (y) - ng/mL - with the Serum CrossLaps™ One Step ELISA test from Osteometer Bio Tech A/S (x) - pmol/L - using human serum is shown in the diagram below (linear regression):

Number of samples measured: 96

The sample concentrations were between approximately 0.07 and 2.2 ng/mL for the Elecsys β-CrossLaps/serum assay and between approximately 620 and 18900 pmol/L for the comparison test.



x: β-CrossLaps comparison β-CTx (pmol/L)

y: Elecsys β-CrossLaps/serum assay (ng/mL)

$y = 0.0001x + 0.048$

$r = 0.983$

The differing magnitudes of the concentrations is mainly due to the different forms of standardization used. Recalculation of the units is not possible.

A comparison of the Elecsys β-CrossLaps/serum assay, REF 07026960190 (**cobas e** 801 analyzer; y) with the β-CrossLaps/serum assay, REF 11972308122 (**cobas e** 601 analyzer; x) gave the following correlations (ng/mL):

Number of samples measured: 146

Passing/Bablok[22]	Linear regression
$y = 0.913x - 0.002$	$y = 0.894x + 0.026$
$r = 0.968$	$r = 0.996$

The sample concentrations were between 0.048 and 5.69 ng/mL.

Analytical specificity

The monoclonal antibodies used in the Elecsys β-CrossLaps/serum assay recognize all fragments of type I collagen containing the β-8AA octapeptide twice. No cross-reactivity detectable with osteocalcin, parathyroid hormone (PTH) or bone Alkaline phosphatase (ALP).

Elecsys β-CrossLaps/serum

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For further information, please refer to the appropriate operator's manual for the analyzer concerned, the respective application sheets, the product information and the Method Sheets of all necessary components (if available in your country).

A point (period/stop) is always used in this Method Sheet as the decimal separator to mark the border between the integral and the fractional parts of a decimal numeral. Separators for thousands are not used.

Any serious incident that has occurred in relation to the device shall be reported to the manufacturer and the competent authority of the Member State in which the user and/or the patient is established.

Symbols

Roche Diagnostics uses the following symbols and signs in addition to those listed in the ISO 15223-1 standard (for USA: see dialog.roche.com for definition of symbols used):

CONTENT	Contents of kit
SYSTEM	Analyzers/Instruments on which reagents can be used
REAGENT	Reagent
CALIBRATOR	Calibrator
→	Volume after reconstitution or mixing
GTIN	Global Trade Item Number

CE 0123

Roche Diagnostics GmbH, Sandhofer Strasse 116, D-68305 Mannheim
www.roche.com
+800 5505 6606

Roche

ii. P1NP

07027940500V6.0

Elecsys total P1NP

cobas®

REF		Σ	SYSTEM
07027940190	07027940500	100	**cobas e** 402 **cobas e** 801

English

System information

Short name	ACN (application code number)
TP1NP	10119

Intended use

Immunoassay for the in vitro quantitative determination of total P1NP in human serum and plasma.

This assay is intended for use in monitoring therapy following the diagnosis of osteoporosis[1,2,3] in post-menopausal women and in patients diagnosed with Paget's disease of the bone.[4,5,6]

The **e**lectro**c**hemi**l**uminescence **i**mmuno**a**ssay "ECLIA" is intended for use on **cobas e** immunoassay analyzers.

Summary

Type I collagen is an important component of bone matrix and osteoblasts secrete its precursor procollagen molecule during bone formation.[7]

Type 1 procollagen contains both N-(amino) and C-(carboxy) terminal extensions. These extensions (propeptides) are cleaved by enzymes during bone matrix formation and released into the circulation. The propeptide measured by Elecsys total P1NP assay is from the amino terminal, hence P1NP procollagen-type 1 N-terminal-propeptide. P1NP is released during type 1 collagen formation and its subsequent incorporation into the bone matrix, and thus may be defined as a true bone formation marker.[8,9] P1NP appears to be released as a trimeric structure (derived from the trimeric collagen structure) but is rapidly broken down to a monomeric form by thermal degradation effects.[10,11] This Elecsys P1NP assay detects both fractions present in blood and is therefore called total P1NP.

Circulating levels of total P1NP demonstrate a significant change upon anti-resorptive[12,13] as well as anabolic[13] therapy within few months from treatment start. Suboptimal response to treatment may indicate non-compliance or the presence of secondary causes of osteoporosis which may need addressing.

Serum P1NP has been selected by the IOF-IFCC Bone Marker Standards Working Group as marker for bone formation, mainly based on the following criteria:

- it has been evaluated both for fracture prediction and monitoring osteoporosis therapies
- the assay is widely available, suitable for serum or plasma samples, with well documented sample handling and stability.[7]

Test principle

Sandwich principle. Total duration of assay: 18 minutes.

- 1st incubation: 12 µL of sample and a biotinylated monoclonal P1NP-specific antibody are incubated together.
- 2nd incubation: After addition of streptavidin labeled microparticles and a monoclonal P1NP-specific antibody labeled with a ruthenium complex[a)], a sandwich complex is formed which becomes bound to the solid phase via interaction of biotin and streptavidin.
- The reaction mixture is aspirated into the measuring cell where the microparticles are magnetically captured onto the surface of the electrode. Unbound substances are then removed with ProCell II M. Application of a voltage to the electrode then induces chemiluminescent emission which is measured by a photomultiplier.
- Results are determined via a calibration curve which is instrument-specifically generated by 2-point calibration and a master curve provided via the **cobas** link.

a) Tris(2,2'-bipyridyl)ruthenium(II)-complex ($Ru(bpy)_3^{2+}$)

Reagents - working solutions

The **cobas e** pack is labeled as TP1NP.

M Streptavidin-coated microparticles, 1 bottle, 6.4 mL:
Streptavidin-coated microparticles 0.72 mg/mL; preservative.

R1 Anti-P1NP-Ab~biotin, 1 bottle, 10.3 mL:
Biotinylated monoclonal anti-P1NP antibody (mouse) 2.5 mg/L; phosphate buffer 100 mmol/L, pH 7.2; preservative.

R2 Anti-P1NP-Ab~$Ru(bpy)_3^{2+}$, 1 bottle, 7.2 mL:
Monoclonal anti-P1NP antibody (mouse) labeled with ruthenium complex 2.5 mg/L; phosphate buffer 100 mmol/L, pH 7.2; preservative.

Precautions and warnings

For in vitro diagnostic use for health care professionals. Exercise the normal precautions required for handling all laboratory reagents.

Infectious or microbial waste:
Warning: handle waste as potentially biohazardous material. Dispose of waste according to accepted laboratory instructions and procedures.

Environmental hazards:
Apply all relevant local disposal regulations to determine the safe disposal.

Safety data sheet available for professional user on request.

This kit contains components classified as follows in accordance with the Regulation (EC) No. 1272/2008:

Warning

H317	May cause an allergic skin reaction.
Prevention:	
P261	Avoid breathing dust/fume/gas/mist/vapours/spray.
P272	Contaminated work clothing should not be allowed out of the workplace.
P280	Wear protective gloves.
Response:	
P333 + P313	If skin irritation or rash occurs: Get medical advice/attention.
P362 + P364	Take off contaminated clothing and wash it before reuse.
Disposal:	
P501	Dispose of contents/container to an approved waste disposal plant.

Product safety labeling follows EU GHS guidance.

Contact phone: all countries: +49-621-7590

Avoid foam formation in all reagents and sample types (specimens, calibrators and controls).

Reagent handling

The reagents in the kit have been assembled into a ready-for-use unit that cannot be separated.

All information required for correct operation is available via the **cobas** link.

Storage and stability

Store at 2-8 °C.

Do not freeze.

Store the **cobas e** pack **upright** in order to ensure complete availability of the microparticles during automatic mixing prior to use.

Elecsys total P1NP

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Stability:	
unopened at 2-8 °C	up to the stated expiration date
on the analyzers	16 weeks

Specimen collection and preparation

Only the specimens listed below were tested and found acceptable.

Serum collected using standard sampling tubes or tubes containing separating gel.

Li-heparin, K_2-EDTA and K_3-EDTA plasma.

Li-heparin plasma tubes containing separating gel can be used.

Criterion: Slope 0.9-1.1 + intercept within ≤ ± 2x Limit of Blank + coefficient of correlation ≥ 0.95.

Stable for 24 hours at 15-25 °C, 5 days at 2-8 °C, 6 months at -20 °C (± 5 °C). Samples may be frozen and thawed up to 5 times without adverse effects.

The sample types listed were tested with a selection of sample collection tubes that were commercially available at the time of testing, i.e. not all available tubes of all manufacturers were tested. Sample collection systems from various manufacturers may contain differing materials which could affect the test results in some cases. When processing samples in primary tubes (sample collection systems), follow the instructions of the tube manufacturer.

Centrifuge samples containing precipitates before performing the assay.

Do not use heat-inactivated samples.

Do not use samples and controls stabilized with azide.

Ensure the samples and calibrators are at 20-25 °C prior to measurement.

Due to possible evaporation effects, samples and calibrators on the analyzers should be analyzed/measured within 2 hours.

Note: Avoid hemolysis! Samples showing visible signs of hemolysis may cause interference.

Materials provided

See "Reagents – working solutions" section for reagents.

Materials required (but not provided)

- REF 03141080190, total P1NP CalSet, for 4 x 1.0 mL
- REF 05618860190, PreciControl Varia, for 4 x 3.0 mL
- REF 07299001190, Diluent Universal, 45.2 mL sample diluent
- General laboratory equipment
- **cobas e** analyzer

Additional materials for **cobas e** 402 and **cobas e** 801 analyzers:

- REF 06908799190, ProCell II M, 2 x 2 L system solution
- REF 04880293190, CleanCell M, 2 x 2 L measuring cell cleaning solution
- REF 07485409001, Reservoir Cup, 8 cups to supply ProCell II M and CleanCell M
- REF 06908853190, PreClean II M, 2 x 2 L wash solution
- REF 05694302001, Assay Tip/Assay Cup tray, 6 magazines x 6 magazine stacks x 105 assay tips and 105 assay cups, 3 wasteliners
- REF 07485425001, Liquid Flow Cleaning Cup, 2 adaptor cups to supply ISE Cleaning Solution/Elecsys SysClean for Liquid Flow Cleaning Detection Unit
- REF 07485433001, PreWash Liquid Flow Cleaning Cup, 1 adaptor cup to supply ISE Cleaning Solution/Elecsys SysClean for Liquid Flow Cleaning PreWash Unit
- REF 11298500316, ISE Cleaning Solution/Elecsys SysClean, 5 x 100 mL system cleaning solution

Assay

For optimum performance of the assay follow the directions given in this document for the analyzer concerned. Refer to the appropriate operator's manual for analyzer-specific assay instructions.

Resuspension of the microparticles takes place automatically prior to use.

Place the cooled (stored at 2-8 °C) **cobas e** pack on the reagent manager. Avoid foam formation. The system automatically regulates the temperature of the reagents and the opening/closing of the **cobas e** pack.

Calibration

Traceability: This method has been standardized against reference standards precisely defined by weighing native P1NP into an analyte-free human serum matrix.

The predefined master curve is adapted to the analyzer using the relevant CalSet.

Calibration frequency: Calibration must be performed once per reagent lot using fresh reagent (i.e. not more than 24 hours since the **cobas e** pack was registered on the analyzer).

Calibration interval may be extended based on acceptable verification of calibration by the laboratory.

Renewed calibration is recommended as follows:

- after 12 weeks when using the same reagent lot
- after 28 days when using the same **cobas e** pack on the analyzer
- as required: e.g. quality control findings outside the defined limits

Quality control

For quality control, use PreciControl Varia.

In addition, other suitable control material can be used.

Controls for the various concentration ranges should be run individually at least once every 24 hours when the test is in use, once per **cobas e** pack, and following each calibration.

The control intervals and limits should be adapted to each laboratory's individual requirements. Values obtained should fall within the defined limits. Each laboratory should establish corrective measures to be taken if values fall outside the defined limits.

If necessary, repeat the measurement of the samples concerned.

Follow the applicable government regulations and local guidelines for quality control.

Calculation

The analyzer automatically calculates the analyte concentration of each sample (either in µg/L or ng/mL).

Limitations - interference

The effect of the following endogenous substances and pharmaceutical compounds on assay performance was tested. Interferences were tested up to the listed concentrations and no impact on results was observed.

Endogenous substances

Compound	Concentration tested
Bilirubin	≤ 1112 µmol/L or ≤ 65 mg/dL
Hemoglobin	≤ 0.062 mmol/L or ≤ 100 mg/dL
Intralipid	≤ 2000 mg/dL
Biotin	≤ 205 nmol/L or ≤ 50 ng/mL
Rheumatoid factors	≤ 1000 IU/mL

Criterion: For concentrations of ≤ 15 ng/mL the deviation is ≤ 1.5 ng/mL. For concentrations > 15 ng/mL the deviation is ≤ 10 %.

Samples should not be taken from patients receiving therapy with high biotin doses (i.e. > 5 mg/day) until at least 8 hours following the last biotin administration.

There is no high-dose hook effect at P1NP concentrations up to 3900 µg/L (ng/mL).

Pharmaceutical substances

In vitro tests were performed on 16 commonly used pharmaceuticals. No interference with the assay was found.

In addition, the following special drugs were tested. No interference with the assay was found.

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Special drugs

Drug	Concentration tested mg/L
Ibandronate	6
Actonel (Risedronat)	150
Fosamax (Alendronate)	350
β-Estradiol	2.5
β-Estradiol-17-Valerate	2.5
β-Estradiol-3-Sulfate	2.5
Calciumcarbonate	2500
Vitamin D3	0.075

In rare cases, interference due to extremely high titers of antibodies to analyte-specific antibodies, streptavidin or ruthenium can occur. These effects are minimized by suitable test design.

Bone metabolism may be affected by the use of cytotoxic agents. Results obtained from patients treated with such therapies should be interpreted with caution.

For diagnostic purposes, the results should always be assessed in conjunction with the patient's medical history, clinical examination and other findings.

Limits and ranges

Measuring range

5-1200 ng/mL (defined by the Limit of Detection and the maximum of the master curve). Values below the Limit of Detection are reported as < 5 ng/mL. Values above the measuring range are reported as > 1200 ng/mL (or up to 2400 ng/mL for 2-fold diluted samples).

Lower limits of measurement

Limit of Blank, Limit of Detection and Limit of Quantitation

Limit of Blank = 2.5 ng/mL

Limit of Detection = 5 ng/mL

Limit of Quantitation = 10 ng/mL

The Limit of Blank, Limit of Detection and Limit of Quantitation were determined in accordance with the CLSI (Clinical and Laboratory Standards Institute) EP17-A2 requirements.

The Limit of Blank is the 95th percentile value from n ≥ 60 measurements of analyte-free samples over several independent series. The Limit of Blank corresponds to the concentration below which analyte-free samples are found with a probability of 95 %.

The Limit of Detection is determined based on the Limit of Blank and the standard deviation of low concentration samples. The Limit of Detection corresponds to the lowest analyte concentration which can be detected (value above the Limit of Blank with a probability of 95 %).

The Limit of Quantitation is the lowest analyte concentration that can be reproducibly measured with an intermediate precision CV of ≤ 20 %.

Dilution

Samples with P1NP concentrations above the measuring range can be diluted with Diluent Universal. The recommended dilution is 1:2 (either automatically by the analyzers or manually). The concentration of the diluted sample must be ≥ 100 µg/L (ng/mL).

After manual dilution, multiply the result by the dilution factor.

After dilution by the analyzers, the software automatically takes the dilution into account when calculating the sample concentration.

Non-linear dilution behaviour may be seen when using sera from patients diagnosed with renal insufficiency.

Expected values

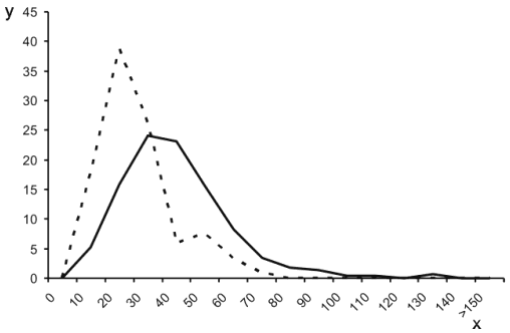
Sera taken from 573 healthy female volunteers who had been enrolled in a study of determinants of bone loss (OFELY[14,15]) were measured for total P1NP levels. The following results were obtained (µg/L or ng/mL):[16]

	Post-menopausal			Pre-menopausal
	All	HRT[b)] yes	HRT no	All
N	444	154	290	129
5th percentile	16.27	14.28	20.25	15.13
Median	37.09	28.48	42.94	27.80
Mean	40.43	31.74	45.05	30.10
95th percentile	73.87	58.92	76.31	58.59

b) HRT = patients receiving hormone replacement therapy

Below are frequency plots showing total P1NP concentration ranges with normal, untreated pre- versus untreated post-menopausal women (figure 1) and below (figure 2) P1NP ranges in post-menopausal women receiving HRT therapy versus those not receiving therapy.

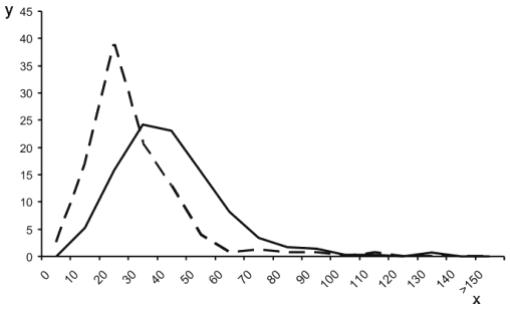
Figure 1: Frequency of total P1NP concentrations (µg/L or ng/mL) observed in normal, untreated, pre- (n = 129) and post- (n = 290) menopausal women



x: total P1NP (µg/L or ng/mL) - -: pre-menopausal

y: Frequency (%) —: post-menopausal

Figure 2: The effect of hormone replacement therapy on total P1NP (µg/L or ng/mL) concentration frequency distribution in treated ("HRT yes"; n = 154) and untreated ("HRT no"; n = 290) post-menopausal women



x: total P1NP (µg/L or ng/mL) – –: HRT yes

y: Frequency (%) —: HRT no

The measurement of total P1NP shows minimal circadian or seasonal variation (approximately 6 %)[17,18] and food intake or diet show no detectable influence upon serum levels.[19,20]

Significantly elevated serum total P1NP levels are associated with the presence of metastatic bone disease and may also be seen in patients with renal insufficiency.[21,22,23] Diseases associated with secondary bone disease may have an effect upon levels of total P1NP.[24,25]

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Each laboratory should investigate the transferability of the expected values to its own patient population and if necessary determine its own reference ranges.

Specific performance data

Representative performance data on the analyzers are given below. Results obtained in individual laboratories may differ.

Precision

Precision was determined using Elecsys reagents, samples and controls in a protocol (EP05-A3) of the CLSI (Clinical and Laboratory Standards Institute): 2 runs per day in duplicate each for 21 days (n = 84). The following results were obtained:

cobas e 402 and **cobas e** 801 analyzers					
		Repeatability		**Intermediate precision**	
Sample	Mean µg/L (ng/mL)	SD µg/L (ng/mL)	CV %	SD µg/L (ng/mL)	CV %
Human serum 1	6.24	0.137	2.2	0.144	2.3
Human serum 2	15.6	0.246	1.6	0.313	2.0
Human serum 3	577	10.2	1.8	13.8	2.4
Human serum 4	596	11.8	2.0	13.8	2.3
Human serum 5	1163	41.2	3.5	44.5	3.8
PC[c)] Varia1	29.7	0.480	1.6	0.599	2.0
PC Varia2	211	3.78	1.8	4.48	2.1

c) PC = PreciControl

Method comparison

a) A comparison of the Elecsys total P1NP assay, REF 07027940190 (**cobas e** 801 analyzer; y) with the Elecsys total P1NP assay, REF 03141071190 (**cobas e** 601 analyzer; x) gave the following correlations (ng/mL):

Number of samples measured: 139

Passing/Bablok[26]	Linear regression
y = 0.986x - 1.73	y = 0.980x - 2.30
τ = 0.975	r = 0.999

The sample concentrations were between 7.24 and 1161 ng/mL.

b) A comparison of the Elecsys total P1NP assay, REF 07027940190 (**cobas e** 402 analyzer; y) with the Elecsys total P1NP assay, REF 07027940190 (**cobas e** 801 analyzer; x) gave the following correlations (ng/mL):

Number of samples measured: 135

Passing/Bablok[26]	Linear regression
y = 0.978x - 1.18	y = 1.03x -5.81
τ = 0.983	r = 0.998

The sample concentrations were between 6.09 and 1076 ng/mL.

Analytical specificity

No cross-reactivities were seen with the following analytes: β-CrossLaps, N-MID Osteocalcin, parathyroid hormone (PTH), and 25-hydroxy vitamin D.

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For further information, please refer to the appropriate operator's manual for the analyzer concerned, the respective application sheets, the product information and the Method Sheets of all necessary components (if available in your country).

A point (period/stop) is always used in this Method Sheet as the decimal separator to mark the border between the integral and the fractional parts of a decimal numeral. Separators for thousands are not used.

Any serious incident that has occurred in relation to the device shall be reported to the manufacturer and the competent authority of the Member State in which the user and/or the patient is established.

Symbols

Roche Diagnostics uses the following symbols and signs in addition to those listed in the ISO 15223-1 standard (for USA: see dialog.roche.com for definition of symbols used):

CONTENT	Contents of kit
SYSTEM	Analyzers/Instruments on which reagents can be used
REAGENT	Reagent
CALIBRATOR	Calibrator
→	Volume after reconstitution or mixing
GTIN	Global Trade Item Number

CE 0123

Roche Diagnostics GmbH, Sandhofer Strasse 116, D-68305 Mannheim
www.roche.com
+800 5505 6606

Roche

iii. Insulin

07027559500V7.0

Elecsys Insulin

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REF		Σ	SYSTEM
07027559190*	07027559500	100	**cobas e** 402
07027559214*			**cobas e** 801

* Some kits shown may not be available in all countries.

English

System information

Short name	ACN (application code number)
INSULIN	10059

Intended use

Immunoassay for the in vitro quantitative determination of human insulin in human serum and plasma. The determination of insulin is utilized in the diagnosis and therapy of various disorders of carbohydrate metabolism, including diabetes mellitus and hypoglycemia.

The **e**lectro**c**hemi**l**uminescence **i**mmuno**a**ssay "ECLIA" is intended for use on **cobas e** immunoassay analyzers.

Summary

Insulin is a 51-residue peptide hormone with a molecular weight of 5808 Da. It is secreted by the β-cells of the islets of Langerhans in the pancreas, and passes into circulation via the portal vein and the liver. Insulin is generally released in pulses.[1,2]

The biologically active insulin molecule is monomeric and consists of two polypeptide chains, the 21 amino acid α-chain and the 30 amino acid β-chain joined by disulphide bridges. Insulin is the biosynthetic product of the single-chain precursor preproinsulin, which is subsequently cleaved to give proinsulin.[2,3,4,5] Specific proteases further cleave proinsulin to produce insulin and the connecting (C)-peptide which pass into the bloodstream simultaneously in equimolar concentrations. Circulating insulin has a half-life of 3-5 minutes and is preferentially retained and degraded in the liver. Therefore only about half of the insulin reaches the systemic circulation. Inactivation or excretion of proinsulin and C-peptide mainly takes place in the kidney and virtually none of the C-peptide is retained in the liver. As a result, C-peptide has a higher plasma concentration than insulin.[6]

The amino acid sequence of insulin is extremely well conserved, with the result that prior to the development of genetically engineered human insulin it was possible to successfully use porcine or bovine insulin in the therapy of diabetes mellitus.[7]

The action of insulin is mediated by specific receptors and primarily consists of facilitation of glucose uptake by the cells of the liver, fatty tissue and musculature; this is the basis of its hypoglycemic action.[2,8]

Serum insulin determinations are mainly performed on patients with symptoms of hypoglycemia and may be useful in classifying the different types of diabetes.[9,10] They are used to ascertain the glucose/insulin quotients and for clarification of questions concerning insulin secretion and β-cell function, e.g. in the evaluation of oral glucose tolerance tests or hunger provocation tests.[11]

A disorder in insulin metabolism can have a significant impact on a number of metabolic processes. Low concentrations of free, biologically active insulin can lead to the development of diabetes mellitus. Possible causes of this include destruction of the β-cells (type I diabetes), reduced activity of insulin or reduced pancreatic synthesis (type II), circulating antibodies to insulin, delayed release of insulin or the absence (or inadequacy) of insulin receptors.[12]

Conversely, autonomous, non-regulated insulin secretion is generally the cause of hypoglycemia. This condition is brought about by inhibition of gluconeogenesis, e.g. as a result of severe hepatic or renal failure, islet cell adenoma, or carcinoma. Hypoglycemia can, however, also be facilitated intentionally or unintentionally (factitious hypoglycemia).[10,13]

In certain individuals with reduced glucose tolerance, the metabolic state deteriorates towards diabetes mellitus over a period of time. Reduced glucose tolerance during pregnancy always requires treatment. The clearly elevated risk of mortality for the fetus necessitates intensive monitoring.[12]

The Elecsys Insulin assay employs two monoclonal antibodies which are specific for human insulin.

Test principle

Sandwich principle. Total duration of assay: 18 minutes.

- 1st incubation: Insulin from 12 µL sample, a biotinylated monoclonal insulin-specific antibody, and a monoclonal insulin-specific antibody labeled with a ruthenium complex[a)] form a sandwich complex.
- 2nd incubation: After addition of streptavidin-coated microparticles, the complex becomes bound to the solid phase via interaction of biotin and streptavidin.
- The reaction mixture is aspirated into the measuring cell where the microparticles are magnetically captured onto the surface of the electrode. Unbound substances are then removed with ProCell II M. Application of a voltage to the electrode then induces chemiluminescent emission which is measured by a photomultiplier.
- Results are determined via a calibration curve which is instrument-specifically generated by 2-point calibration and a master curve provided via the **cobas** link.

a) Tris(2,2'-bipyridyl)ruthenium(II)-complex ($Ru(bpy)_3^{2+}$)

Reagents - working solutions

The **cobas e** pack is labeled as INSULIN.

M Streptavidin-coated microparticles, 1 bottle, 5.8 mL:
Streptavidin-coated microparticles 0.72 mg/mL; preservative.

R1 Anti-insulin-Ab~biotin, 1 bottle, 10.3 mL:
Biotinylated monoclonal anti-insulin antibody (mouse) 1 mg/L; MES[b)] buffer 50 mmol/L, pH 6.0; preservative.

R2 Anti-insulin-Ab~$Ru(bpy)_3^{2+}$, 1 bottle, 9.5 mL:
Monoclonal anti-insulin antibody (mouse) labeled with ruthenium complex 1.75 mg/L; MES buffer 50 mmol/L, pH 6.0; preservative.

b) MES = 2-morpholino-ethane sulfonic acid

Precautions and warnings

For in vitro diagnostic use for health care professionals. Exercise the normal precautions required for handling all laboratory reagents.

Infectious or microbial waste:
Warning: handle waste as potentially biohazardous material. Dispose of waste according to accepted laboratory instructions and procedures.

Environmental hazards:
Apply all relevant local disposal regulations to determine the safe disposal.

Safety data sheet available for professional user on request.

This kit contains components classified as follows in accordance with the Regulation (EC) No. 1272/2008:

Warning

H317	May cause an allergic skin reaction.
Prevention:	
P261	Avoid breathing dust/fume/gas/mist/vapours/spray.
P272	Contaminated work clothing should not be allowed out of the workplace.
P280	Wear protective gloves.
Response:	
P333 + P313	If skin irritation or rash occurs: Get medical advice/attention.
P362 + P364	Take off contaminated clothing and wash it before reuse.

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Disposal:

P501 Dispose of contents/container to an approved waste disposal plant.

Product safety labeling follows EU GHS guidance.

Contact phone: all countries: +49-621-7590

Avoid foam formation in all reagents and sample types (specimens, calibrators and controls).

Reagent handling

The reagents in the kit have been assembled into a ready-for-use unit that cannot be separated.

All information required for correct operation is available via the **cobas** link.

Storage and stability

Store at 2-8 °C.

Do not freeze.

Store the **cobas e** pack **upright** in order to ensure complete availability of the microparticles during automatic mixing prior to use.

Stability:	
unopened at 2-8 °C	up to the stated expiration date
on the analyzers	16 weeks

Specimen collection and preparation

Only the specimens listed below were tested and found acceptable.

Serum collected using standard sampling tubes or tubes containing separating gel.

Li-heparin, K_2-EDTA and K_3-EDTA plasma.

Criterion: Slope 0.9-1.1 + intercept within ≤ ± 0.8 µU/mL + coefficient of correlation ≥ 0.95.

Stable for 4 hours at 20-25 °C, 2 days at 2-8 °C, 6 months at -20 °C (± 5 °C). Freeze only once.

The sample types listed were tested with a selection of sample collection tubes that were commercially available at the time of testing, i.e. not all available tubes of all manufacturers were tested. Sample collection systems from various manufacturers may contain differing materials which could affect the test results in some cases. When processing samples in primary tubes (sample collection systems), follow the instructions of the tube manufacturer.

Centrifuge samples containing precipitates before performing the assay.

Do not use heat-inactivated samples.

Do not use samples and controls stabilized with azide.

Ensure the samples and calibrators are at 20-25 °C prior to measurement.

Due to possible evaporation effects, samples and calibrators on the analyzers should be analyzed/measured within 2 hours.

Materials provided

See "Reagents – working solutions" section for reagents.

Materials required (but not provided)

- REF 12017504122, Insulin CalSet, for 4 x 1.0 mL
- REF 05341787190, PreciControl Multimarker, for 6 x 2.0 mL or REF 11731416190, PreciControl Universal, for 4 x 3.0 mL
- General laboratory equipment
- **cobas e** analyzer

Additional materials for **cobas e** 402 and **cobas e** 801 analyzers:

- REF 06908799190, ProCell II M, 2 x 2 L system solution
- REF 04880293190, CleanCell M, 2 x 2 L measuring cell cleaning solution
- REF 07485409001, Reservoir Cup, 8 cups to supply ProCell II M and CleanCell M
- REF 06908853190, PreClean II M, 2 x 2 L wash solution
- REF 05694302001, Assay Tip/Assay Cup tray, 6 magazines x 6 magazine stacks x 105 assay tips and 105 assay cups, 3 wasteliners
- REF 07485425001, Liquid Flow Cleaning Cup, 2 adaptor cups to supply ISE Cleaning Solution/Elecsys SysClean for Liquid Flow Cleaning Detection Unit
- REF 07485433001, PreWash Liquid Flow Cleaning Cup, 1 adaptor cup to supply ISE Cleaning Solution/Elecsys SysClean for Liquid Flow Cleaning PreWash Unit
- REF 11298500316, ISE Cleaning Solution/Elecsys SysClean, 5 x 100 mL system cleaning solution

Assay

For optimum performance of the assay follow the directions given in this document for the analyzer concerned. Refer to the appropriate operator's manual for analyzer-specific assay instructions.

Resuspension of the microparticles takes place automatically prior to use.

Place the cooled (stored at 2-8 °C) **cobas e** pack on the reagent manager. Avoid foam formation. The system automatically regulates the temperature of the reagents and the opening/closing of the **cobas e** pack.

Calibration

Traceability: This method has been standardized using the 1st IRP WHO Reference Standard 66/304 (NIBSC).

The predefined master curve is adapted to the analyzer using the relevant CalSet.

Calibration frequency: Calibration must be performed once per reagent lot using fresh reagent (i.e. not more than 24 hours since the **cobas e** pack was registered on the analyzer).

Calibration interval may be extended based on acceptable verification of calibration by the laboratory.

Renewed calibration is recommended as follows:

- after 12 weeks when using the same reagent lot
- after 28 days when using the same **cobas e** pack on the analyzer
- as required: e.g. quality control findings outside the defined limits

Quality control

For quality control, use PreciControl Multimarker or PreciControl Universal.

In addition, other suitable control material can be used.

Controls for the various concentration ranges should be run individually at least once every 24 hours when the test is in use, once per **cobas e** pack, and following each calibration.

The control intervals and limits should be adapted to each laboratory's individual requirements. Values obtained should fall within the defined limits. Each laboratory should establish corrective measures to be taken if values fall outside the defined limits.

If necessary, repeat the measurement of the samples concerned.

Follow the applicable government regulations and local guidelines for quality control.

Please note: Commercial controls may contain insulin of animal origin. When assessing results, the corresponding cross-reactivity of this test must be taken into account; see under "Analytical specificity".

Calculation

The analyzer automatically calculates the analyte concentration of each sample (either in µU/mL or pmol/L).

Conversion factors: µU/mL x 6.945 = pmol/L

pmol/L x 0.144 = µU/mL

Limitations - interference

The effect of the following endogenous substances and pharmaceutical compounds on assay performance was tested. Interferences were tested up to the listed concentrations and no impact on results was observed.

Endogenous substances

Compound	Concentration tested
Bilirubin	≤ 1539 µmol/L or ≤ 90 mg/dL
Intralipid	≤ 1800 mg/dL
Biotin	≤ 246 nmol/L or ≤ 60 ng/mL

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Compound	Concentration tested
Rheumatoid factors	≤ 1200 IU/mL

Criterion: For concentrations of 0.4-2 µU/mL the deviation is ≤ 0.5 µU/mL. For concentrations > 2 µU/mL the deviation is ≤ 10 %.

Hemolysis interferes, as insulin-degrading peptidases are released from erythrocytes.[14]

Samples should not be taken from patients receiving therapy with high biotin doses (i.e. > 5 mg/day) until at least 8 hours following the last biotin administration.

There is no high-dose hook effect at insulin concentrations up to 20000 µU/mL or 138900 pmol/L.

Pharmaceutical substances
In vitro tests were performed on 16 commonly used pharmaceuticals. Of these, only acetylcysteine at therapeutic dosage levels showed interference with the assay (insulin values depressed).

In addition, the following special drugs were tested. No interference with the assay was found.

Special drugs

Drug	Concentration tested mg/L
Euglucon	10.5
Tolbutamide	3

Samples from patients treated with bovine, porcine or human insulin sometimes contain anti-insulin antibodies.[15,9] Insulin bound to these antibodies is at least partially recognized by the antibodies used in the Elecsys Insulin assay.[16]

In rare cases, interference due to extremely high titers of antibodies to analyte-specific antibodies, streptavidin or ruthenium can occur. These effects are minimized by suitable test design.

For diagnostic purposes, the results should always be assessed in conjunction with the patient's medical history, clinical examination and other findings.

Limits and ranges

Measuring range

0.4-1000 µU/mL or 2.78-6945 pmol/L (defined by the Limit of Detection and the maximum of the master curve). Values below the Limit of Detection are reported as < 0.4 µU/mL (< 2.78 pmol/L). Values above the measuring range are reported as > 1000 µU/mL (> 6945 pmol/L).

Lower limits of measurement

Limit of Blank, Limit of Detection and Limit of Quantitation

Limit of Blank = 0.2 µU/mL (1.39 pmol/L)

Limit of Detection = 0.4 µU/mL (2.78 pmol/L)

Limit of Quantitation = 1 µU/mL (6.95 pmol/L)

The Limit of Blank, Limit of Detection and Limit of Quantitation were determined in accordance with the CLSI (Clinical and Laboratory Standards Institute) EP17-A2 requirements.

The Limit of Blank is the 95th percentile value from n ≥ 60 measurements of analyte-free samples over several independent series. The Limit of Blank corresponds to the concentration below which analyte-free samples are found with a probability of 95 %.

The Limit of Detection is determined based on the Limit of Blank and the standard deviation of low concentration samples. The Limit of Detection corresponds to the lowest analyte concentration which can be detected (value above the Limit of Blank with a probability of 95 %).

The Limit of Quantitation is the lowest analyte concentration that can be reproducibly measured with an intermediate precision CV of ≤ 20 %.

Dilution

Not necessary due to the broad measuring range.

Expected values

Studies with the Elecsys Insulin assay conducted in a clinical center in Germany with samples from 57 healthy, fasting individuals gave the following results (5th-95th percentile range):

2.6-24.9 µU/mL (17.8-173 pmol/L)

Status: Elecsys Insulin MCE, study No.: B99P027 of 29 March 2001.

Each laboratory should investigate the transferability of the expected values to its own patient population and if necessary determine its own reference ranges.

Specific performance data

Representative performance data on the analyzers are given below. Results obtained in individual laboratories may differ.

Precision

Precision was determined using Elecsys reagents, pooled human sera and controls in a protocol (EP05-A3) of the CLSI (Clinical and Laboratory Standards Institute): 2 runs per day in duplicate each for 21 days (n = 84). The following results were obtained:

cobas e 402 and **cobas e** 801 analyzers					
		Repeatability		**Intermediate precision**	
Sample	Mean µU/mL	SD µU/mL	CV %	SD µU/mL	CV %
Human serum 1	22.1	0.310	1.4	0.466	2.1
Human serum 2	3.25	0.141	4.3	0.172	5.3
Human serum 3	49.7	0.403	0.8	0.719	1.4
Human serum 4	505	5.49	1.1	8.19	1.6
Human serum 5	973	10.4	1.1	14.2	1.5
PC[c)] Multimarker 1	20.2	0.280	1.4	0.400	2.0
PC Multimarker 2	66.8	0.692	1.0	1.06	1.6

c) PC = PreciControl

cobas e 402 and **cobas e** 801 analyzers					
		Repeatability		**Intermediate precision**	
Sample	Mean pmol/L	SD pmol/L	CV %	SD pmol/L	CV %
Human serum 1	153	2.15	1.4	3.24	2.1
Human serum 2	22.6	0.979	4.3	1.19	5.3
Human serum 3	345	2.80	0.8	4.99	1.4
Human serum 4	3507	38.1	1.1	56.9	1.6
Human serum 5	6757	72.2	1.1	98.6	1.5
PC Multimarker 1	140	1.94	1.4	2.78	2.0
PC Multimarker 2	464	4.81	1.0	7.36	1.6

Method comparison

a) A comparison of the Elecsys Insulin assay, REF 07027559190 (**cobas e** 801 analyzer; y) with the Elecsys Insulin assay, REF 12017547122 (**cobas e** 601 analyzer; x) gave the following correlations (µIU/mL):

Number of serum samples measured: 164

Passing/Bablok[17]	Linear regression
y = 0.988x - 0.048	y = 0.973x + 1.09
τ = 0.993	r = 1.00

The sample concentrations were between 0.924 and 989 µIU/mL.

b) A comparison of the Elecsys Insulin assay, REF 07027559190 (**cobas e** 402 analyzer; y) with the Elecsys Insulin assay, REF 07027559190 (**cobas e** 801 analyzer; x) gave the following correlations (µU/mL):

Number of serum samples measured: 198

Passing/Bablok[17]	Linear regression
y = 0.996x + 0.133	y = 0.993x + 0.173

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τ = 0.984 r = 1.00

The sample concentrations were between 0.694 and 957 µU/mL.

Analytical specificity

For the monoclonal antibodies used, the following cross-reactivities were found:

Cross-reactant	Concentration tested	Cross-reactivity %
Bovine insulin	20000 pmol/L	9.2
Porcine insulin	10000 pmol/L	22.2
Human proinsulin	111083 pmol/L	0.36
C-peptide	33109 pmol/L	n. d.[d)]
Glucagon	288 pmol/L	n. d.
Somatostatin	60 pmol/L	n. d.
Insulin-like growth factor I	10000 pmol/L	n. d.

d) n. d. = not detectable

Results for cross-reactivity with recombinant insulin analogs in a number of insulin methods have been published for example by two groups in France and the USA.[16,18,19] The following results were published by Owen et al.[18] for the Elecsys Insulin assay:

Insulin lispro, insulin aspart, and insulin glargine were each tested in concentrations of 30, 100, 300, and 1000 mIU/L in the absence of insulin. The results obtained were below the detection limit of the Elecsys Insulin assay (< 0.4 µU/mL or < 2.78 pmol/L) at all the concentrations tested.

Moreover, these results also correlate with those published earlier by Sapin et al. for insulin lispro.[16]

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For further information, please refer to the appropriate operator's manual for the analyzer concerned, the respective application sheets and the Method Sheets of all necessary components (if available in your country).

A point (period/stop) is always used in this Method Sheet as the decimal separator to mark the border between the integral and the fractional parts of a decimal numeral. Separators for thousands are not used.

Any serious incident that has occurred in relation to the device shall be reported to the manufacturer and the competent authority of the Member State in which the user and/or the patient is established.

Symbols

Roche Diagnostics uses the following symbols and signs in addition to those listed in the ISO 15223-1 standard (for USA: see dialog.roche.com for definition of symbols used):

CONTENT	Contents of kit
SYSTEM	Analyzers/Instruments on which reagents can be used
REAGENT	Reagent
CALIBRATOR	Calibrator
→	Volume for reconstitution
GTIN	Global Trade Item Number

CE 0123

Roche Diagnostics GmbH, Sandhofer Strasse 116, D-68305 Mannheim
www.roche.com
+800 5505 6606

Roche

iv. Testosterone

08946370500V2.0

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REF		Σ	SYSTEM
08946370190	08946370500	300	**cobas e** 402 **cobas e** 801

English

System information

Short name	ACN (application code number)
TESTO 2	10020

Intended use

Immunoassay for the in vitro quantitative determination of testosterone in human serum and plasma.

The **e**lectro**c**hemi**l**uminescence **i**mmuno**a**ssay "ECLIA" is intended for use on **cobas e** immunoassay analyzers.

Summary

Testosterone is regarded as one of the key androgen steroids. It is a steroid secreted from the testis and the adrenal cortex in men and from the adrenal cortex and the ovary in women. It is also produced by the peripheral tissues from androstenedione.

In men, testosterone is synthesized almost exclusively by the Leydig cells of the testes. The secretion of testosterone is regulated by luteinizing hormone (LH) and testosterone promotes the development of the secondary sex characteristics, such as the growth of pubic, facial, and axillary hair, or the accessory sex organs. Most of the circulating testosterone is bound to carrier proteins (SHBG = sex hormone-binding globulin).[1,2,3]

In women, small quantities of testosterone are formed in the ovaries, adrenal gland, and peripheral fatty tissues, and it has a serum concentration that is approximately 10 times less than in males. In physiological concentrations, androgens have no specific effects in women. Increased production of testosterone in women can cause virilization (depending on the increase).[2,3]

The Elecsys Testosterone II assay is based on a competitive test principle using a high affinity monoclonal antibody (sheep) specifically directed against testosterone. Endogenous testosterone released from the sample by 2-bromoestradiol competes with the added testosterone derivative labeled with a ruthenium complex[a)] for the binding sites on the biotinylated antibody.

a) Tris(2,2'-bipyridyl)ruthenium(II)-complex ($Ru(bpy)_3^{2+}$)

Test principle

Competition principle. Total duration of assay: 18 minutes.

- 1st incubation: 12 µL of sample are incubated with a biotinylated monoclonal testosterone-specific antibody. The binding sites of the labeled antibody become occupied by the sample analyte (depending on its concentration).
- 2nd incubation: After addition of streptavidin-coated microparticles and a testosterone derivate labeled with a ruthenium complex, the complex becomes bound to the solid phase via interaction of biotin and streptavidin.
- The reaction mixture is aspirated into the measuring cell where the microparticles are magnetically captured onto the surface of the electrode. Unbound substances are then removed with ProCell II M. Application of a voltage to the electrode then induces chemiluminescent emission which is measured by a photomultiplier.
- Results are determined via a calibration curve which is instrument-specifically generated by 2-point calibration and a master curve provided via the **cobas** link.

Reagents - working solutions

The **cobas e** pack is labeled as TESTO 2.

M Streptavidin-coated microparticles, 1 bottle, 12.4 mL:
Streptavidin-coated microparticles 0.72 mg/mL, preservative.

R1 Anti-testosterone-Ab~biotin, 1 bottle, 21.0 mL:
Biotinylated monoclonal anti-testosterone antibody (sheep) 40 ng/mL; releasing reagent 2-bromoestradiol; MES[b)] buffer 50 mmol/L, pH 6.0; preservative.

R2 Testosterone-peptide~$Ru(bpy)_3^{2+}$, 1 bottle, 18.8 mL:
Testosterone derivative, labeled with ruthenium complex 1.5 ng/mL; MES buffer 50 mmol/L, pH 6.0; preservative.

b) MES = 2-morpholino-ethane sulfonic acid

Precautions and warnings

For in vitro diagnostic use for health care professionals. Exercise the normal precautions required for handling all laboratory reagents.

Infectious or microbial waste:
Warning: handle waste as potentially biohazardous material. Dispose of waste according to accepted laboratory instructions and procedures.

Environmental hazards:
Apply all relevant local disposal regulations to determine the safe disposal.

Safety data sheet available for professional user on request.

This kit contains components classified as follows in accordance with the Regulation (EC) No. 1272/2008:

Warning

H317	May cause an allergic skin reaction.
Prevention:	
P261	Avoid breathing mist or vapours.
P272	Contaminated work clothing should not be allowed out of the workplace.
P280	Wear protective gloves.
Response:	
P333 + P313	If skin irritation or rash occurs: Get medical advice/attention.
P362 + P364	Take off contaminated clothing and wash it before reuse.
Disposal:	
P501	Dispose of contents/container to an approved waste disposal plant.

Product safety labeling follows EU GHS guidance.

Contact phone: all countries: +49-621-7590

Avoid foam formation in all reagents and sample types (specimens, calibrators and controls).

Reagent handling

The reagents in the kit have been assembled into a ready-for-use unit that cannot be separated.

All information required for correct operation is available via the **cobas** link.

Storage and stability

Store at 2-8 °C.

Do not freeze.

Store the **cobas e** pack **upright** in order to ensure complete availability of the microparticles during automatic mixing prior to use.

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Stability:	
unopened at 2-8 °C	up to the stated expiration date
on the analyzers	16 weeks

Specimen collection and preparation

Only the specimens listed below were tested and found acceptable.

Serum collected using standard sampling tubes or tubes containing separating gel.

Li-heparin, K_2-EDTA and K_3-EDTA plasma.

Criterion: Recovery within 80-120 % of serum value > 1 ng/mL, recovery of ± 0.2 ng/mL of serum value ≤ 1 ng/mL and slope 0.9-1.1 + bias at 0.5 ng/mL and 3.0 ng/mL ≤ 10 % + coefficient of correlation ≥ 0.95.

Stable for 14 days at 2-8 °C, 5 days at 20-25 °C, 6 months at -20 °C (± 5 °C). Freeze only once.

The sample types listed were tested with a selection of sample collection tubes that were commercially available at the time of testing, i.e. not all available tubes of all manufacturers were tested. Sample collection systems from various manufacturers may contain differing materials which could affect the test results in some cases. When processing samples in primary tubes (sample collection systems), follow the instructions of the tube manufacturer.

Centrifuge samples containing precipitates before performing the assay.

Do not use heat-inactivated samples.

Do not use samples and controls stabilized with azide.

Ensure the samples and calibrators are at 20-25 °C prior to measurement.

Due to possible evaporation effects, samples and calibrators on the analyzers should be analyzed/measured within 2 hours.

Materials provided

See "Reagents – working solutions" section for reagents.

Materials required (but not provided)

- REF 05202230190, Testosterone II CalSet II, for 4 x 1.0 mL
- REF 11731416190, PreciControl Universal, for 4 x 3.0 mL
- General laboratory equipment
- **cobas e** analyzer

Additional materials for **cobas e** 402 and **cobas e** 801 analyzers:

- REF 06908799190, ProCell II M, 2 x 2 L system solution
- REF 04880293190, CleanCell M, 2 x 2 L measuring cell cleaning solution
- REF 07485409001, Reservoir Cup, 8 cups to supply ProCell II M and CleanCell M
- REF 06908853190, PreClean II M, 2 x 2 L wash solution
- REF 05694302001, Assay Tip/Assay Cup tray, 6 magazines x 6 magazine stacks x 105 assay tips and 105 assay cups, 3 wasteliners
- REF 07485425001, Liquid Flow Cleaning Cup, 2 adaptor cups to supply ISE Cleaning Solution/Elecsys SysClean for Liquid Flow Cleaning Detection Unit
- REF 07485433001, PreWash Liquid Flow Cleaning Cup, 1 adaptor cup to supply ISE Cleaning Solution/Elecsys SysClean for Liquid Flow Cleaning PreWash Unit
- REF 11298500316, ISE Cleaning Solution/Elecsys SysClean, 5 x 100 mL system cleaning solution

Assay

For optimum performance of the assay follow the directions given in this document for the analyzer concerned. Refer to the appropriate operator's manual for analyzer-specific assay instructions.

Resuspension of the microparticles takes place automatically prior to use.

Place the cooled (stored at 2-8 °C) **cobas e** pack on the reagent manager. Avoid foam formation. The system automatically regulates the temperature of the reagents and the opening/closing of the **cobas e** pack.

Calibration

Traceability: This method is traceable to highly purified testosterone by weight via ID-GC/MS ("Isotope Dilution - Gas Chromatography/Mass Spectrometry").[4]

The predefined master curve is adapted to the analyzer using the relevant CalSet.

Calibration frequency: Calibration must be performed once per reagent lot using fresh reagent (i.e. not more than 24 hours since the **cobas e** pack was registered on the analyzer).

Calibration interval may be extended based on acceptable verification of calibration by the laboratory.

Renewed calibration is recommended as follows:

- after 12 weeks when using the same reagent lot
- after 28 days when using the same **cobas e** pack on the analyzer
- as required: e.g. quality control findings outside the defined limits

Quality control

For quality control, use PreciControl Universal.

In addition, other suitable control material can be used.

Controls for the various concentration ranges should be run individually at least once every 24 hours when the test is in use, once per **cobas e** pack, and following each calibration.

The control intervals and limits should be adapted to each laboratory's individual requirements. Values obtained should fall within the defined limits. Each laboratory should establish corrective measures to be taken if values fall outside the defined limits.

If necessary, repeat the measurement of the samples concerned.

Follow the applicable government regulations and local guidelines for quality control.

Calculation

The analyzer automatically calculates the analyte concentration of each sample (either in ng/mL, ng/dL or nmol/L).

Conversion factors:	ng/mL x 3.47 = nmol/L
	ng/mL x 100 = ng/dL
	nmol/L x 0.288 = ng/mL

Limitations - interference

The effect of the following endogenous substances and pharmaceutical compounds on assay performance was tested. Interferences were tested up to the listed concentrations and no impact on results was observed.

Endogenous substances

Compound	Concentration tested
Bilirubin	≤ 513 µmol/L or ≤ 30 mg/dL
Hemoglobin	≤ 0.373 mmol/L or ≤ 600 mg/dL
Intralipid	≤ 800 mg/dL
Biotin	≤ 3600 ng/mL
Rheumatoid factors	≤ 1000 IU/mL

Criterion: Recovery within ± 10 % of initial value (concentration range > 1-15 ng/mL), recovery within ± 15 % of initial value (concentration range > 0.5-1 ng/mL) and recovery of ± 0.075 ng/mL (concentration range of 0.025-0.500 ng/mL).

Pharmaceutical substances

In vitro tests were performed on 17 commonly used pharmaceuticals and 2 special pharmaceuticals. Of these, only phenylbutazone at therapeutic dosage levels showed interference with the assay (testosterone values increased).

A strong interaction with Nandrolone (INN international nonproprietary name, WHO) was found. Do not use samples from patients under Nandrolone treatment.

Testosterone undecanoate (INN international nonproprietary name, WHO) is metabolized to testosterone after administration. The Elecsys Testosterone II assay does not differentiate between endogenous

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testosterone and exogenous testosterone resulting from metabolized testosterone under testosterone supplementation therapy.

In isolated cases, elevated testosterone levels can be seen in samples from female patients with end stage renal disease (ESRD).

Implausible elevated testosterone values in women should be verified by an extraction method or a validated LC-MS/MS tandem method.[5]

In rare cases, interference due to extremely high titers of antibodies to analyte-specific antibodies, streptavidin or ruthenium can occur. These effects are minimized by suitable test design.

For diagnostic purposes, the results should always be assessed in conjunction with the patient's medical history, clinical examination and other findings.

Limits and ranges

Measuring range

0.025-15.0 ng/mL or 0.087-52.0 nmol/L (defined by the Limit of Detection and the maximum of the master curve). Values below the Limit of Detection are reported as < 0.025 ng/mL or < 0.087 nmol/L. Values above the measuring range are reported as > 15.0 ng/mL or > 52.0 nmol/L.

Lower limits of measurement

Limit of Blank, Limit of Detection and Limit of Quantitation

Limit of Blank = 0.015 ng/mL (0.052 nmol/L)

Limit of Detection = 0.025 ng/mL (0.087 nmol/L)

Limit of Quantitation = 0.120 ng/mL (0.416 nmol/L)

The Limit of Blank, Limit of Detection and Limit of Quantitation were determined in accordance with the CLSI (Clinical and Laboratory Standards Institute) EP17-A2 requirements.

The Limit of Blank is the 95th percentile value from n ≥ 60 measurements of analyte-free samples over several independent series. The Limit of Blank corresponds to the concentration below which analyte-free samples are found with a probability of 95 %.

The Limit of Detection is determined based on the Limit of Blank and the standard deviation of low concentration samples. The Limit of Detection corresponds to the lowest analyte concentration which can be detected (value above the Limit of Blank with a probability of 95 %).

The Limit of Quantitation is defined as the lowest amount of analyte in a sample that can be accurately quantitated with a total allowable relative error of ≤ 20 %.

Dilution

Not necessary due to the broad measuring range.

Expected values

The following tables show the results obtained using the Elecsys Testosterone II assay in a reference population of 95 males (7-18 years) and 100 females (8-18 years), who were in good endocrinological health. Subjects were clinically characterized according to their Tanner Stage. Tanner Stage was characterized according to the method of Marshall and Tanner.[6,7]

Reference values for males (7-18 years) characterized by Tanner Stage

Tanner Stage	N	Median	5-95th percentiles (ng/mL)
1	26	< 0.025	< 0.025
2	18	0.597	< 0.025-4.32
3	15	2.45	0.649-7.78
4	16	3.44	1.80-7.63
5	20	4.46	1.88-8.82

Reference values for females (8-18 years) characterized by Tanner Stage

Tanner Stage	N	Median	5-95th percentiles (ng/mL)
1	37	< 0.025	< 0.025-0.061
2	12	< 0.025	< 0.025-0.104
3	12	0.079	< 0.025-0.237
4	12	0.122	< 0.025-0.268
5	27	0.197	0.046-0.383

The following table shows the results obtained with the Elecsys Testosterone II assay in an apparently healthy group of 214 males and 160 females without intake of contraceptives and prescription drugs (study number CIM 000669). Blood samples were taken between 6.30 am and 1.00 pm. This clinical study with focus on the Elecsys Testosterone II assay included measurements in parallel with the Elecsys SHBG assay. The results were evaluated for the Elecsys Testosterone II and Elecsys SHBG assays and commonly used parameters derived from different calculation procedures, including albumin as an important parameter involved.[8]

- Free testosterone index (% FTI) or free androgen index (% FAI) as calculated on a molar/molar basis:

  FTI (%) = (testosterone in nmol/L divided by SHBG in nmol/L) x 100
- Free testosterone calculated (FTc) in nmol/L and %
- Bioavailable testosterone calculated (BATc) in nmol/L and %

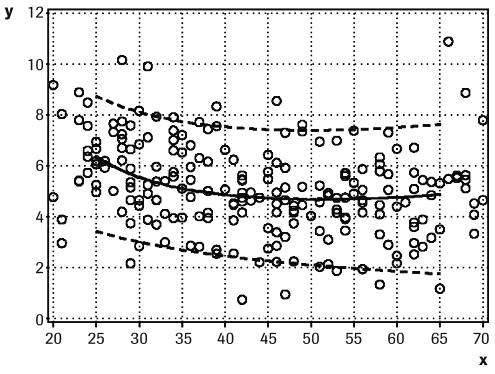
FTc and BATc were calculated by means of individual concentrations for total testosterone, SHBG, and albumin and via the association constant of albumin to testosterone. A detailed description of the calculation procedure is available on request. Refer also to the homepage of www.issam.ch/freetesto.htm.

The following results were obtained:

Testosterone

Test subjects	N	Percentiles			
		Median	5-95th	Median	5-95th
		ng/mL		nmol/L	
Males 20-49 years	136	5.36	2.49-8.36	18.6	8.64-29.0
Males ≥ 50 years	78	4.76	1.93-7.40	16.5	6.68-25.7
Females 20-49 years	89	0.271	0.084-0.481	0.941	0.290-1.67
Females ≥ 50 years	71	0.162	0.029-0.408	0.563	0.101-1.42

Distribution of testosterone values in the apparently healthy male group based on age (n = 214). Solid line: 50 % percentile, upper line: 95 % percentile, lower line: 5 % percentile.



x: Age (years)

y: Testosterone (ng/mL) - male group

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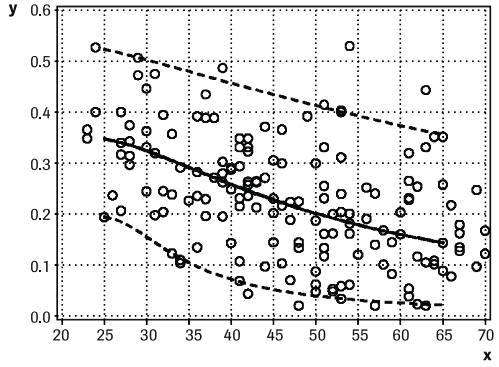
Distribution of testosterone values in the apparently healthy female group based on age (n = 160). Solid line: 50 % percentile, upper line: 95 % percentile, lower line: 5 % percentile.



x: Age (years)

y: Testosterone (ng/mL) - female group

SHBG

Test subjects	N	Median	5-95th percentiles
		nmol/L	
Males 20-49 years	136	33.5	16.5-55.9
Males ≥ 50 years	78	40.8	19.3-76.4
Females 20-49 years	89	64.3	24.6-122
Females ≥ 50 years	71	57.4	17.3-125

Free testosterone index or free androgen index

Test subjects	N	Median	5-95th percentiles
		FTI or FAI (%)	
Males 20-49 years	136	57.2	35.0-92.6
Males ≥ 50 years	78	38.2	24.3-72.1
Females 20-49 years	89	1.53	0.297-5.62
Females ≥ 50 years	71	1.15	0.187-3.63

Free testosterone, calculated

Test subjects	N	Percentiles			
		Median	5-95th percentiles	Median	5-95th percentiles
		FTc (nmol/L)		FTc (%)	
Males 20-49 years	136	0.379	0.198-0.619	2.10	1.53-2.88
Males ≥ 50 years	78	0.304	0.163-0.473	1.91	1.23-2.59
Females 20-49 years	89	0.011	0.003-0.033	1.19	0.701-2.19
Females ≥ 50 years	71	0.008	0.001-0.020	1.26	0.685-2.64

Bioavailable testosterone, calculated

Test subjects	N	Percentiles			
		Median	5-95th percentiles	Median	5-95th percentiles
		BATc (nmol/L)		BATc (%)	
Males 20-49 years	136	9.10	4.36-14.3	49.8	35.0-66.3
Males ≥ 50 years	78	6.63	3.59-11.0	42.1	27.5-60.7
Females 20-49 years	89	0.246	0.059-0.756	25.7	15.3-47.7
Females ≥ 50 years	71	0.168	0.030-0.430	28.0	15.1-55.2

Each laboratory should investigate the transferability of the expected values to its own patient population and if necessary determine its own reference ranges.

Specific performance data

Representative performance data on the analyzers are given below. Results obtained in individual laboratories may differ.

Precision

Precision was determined using Elecsys reagents, pooled human sera and controls in a protocol (EP05-A3) of the CLSI (Clinical and Laboratory Standards Institute): 2 runs per day in duplicate each for 21 days (n = 84). The following results were obtained:

cobas e 402 and **cobas e** 801 analyzers					
			Repeatability		
Sample	Mean		SD		CV
	ng/mL	nmol/L	ng/mL	nmol/L	%
Human serum 1	0.100	0.347	0.006	0.020	5.7
Human serum 2	0.315	1.09	0.009	0.032	3.0
Human serum 3	0.765	2.65	0.012	0.043	1.6
Human serum 4	2.09	7.25	0.032	0.111	1.5
Human serum 5	13.9	48.2	0.235	0.815	1.7
PC[c)] Universal 1	5.58	19.4	0.071	0.246	1.3
PC Universal 2	2.57	8.92	0.035	0.120	1.3

c) PC = PreciControl

cobas e 402 and **cobas e** 801 analyzers					
			Intermediate precision		
Sample	Mean		SD		CV
	ng/mL	nmol/L	ng/mL	nmol/L	%
Human serum 1	0.100	0.347	0.012	0.040	11.5
Human serum 2	0.315	1.09	0.015	0.051	4.7
Human serum 3	0.765	2.65	0.025	0.086	3.2
Human serum 4	2.09	7.25	0.046	0.161	2.2
Human serum 5	13.9	48.2	0.306	1.06	2.2
PC Universal 1	5.58	19.4	0.112	0.389	2.0
PC Universal 2	2.57	8.92	0.051	0.177	2.0

Method comparison

a) A comparison of the Elecsys Testosterone II assay, REF 08946370190 (**cobas e** 402 analyzer; y) with the Elecsys Testosterone II assay, REF 08946370190 (**cobas e** 801 analyzer; x) gave the following correlations (ng/mL):

Elecsys Testosterone II

cobas®

Number of samples measured: 166

Passing/Bablok[9]	Linear regression
y = 1.02x - 0.006	y = 1.02x - 0.003
τ = 0.984	r = 0.999

The sample concentrations were between 0.029 and 14.2 ng/mL.

b) A comparison of the Elecsys Testosterone II assay, REF 08946370190 (**cobas e** 801 analyzer; y) with the Elecsys Testosterone II assay, REF 07027915190 (**cobas e** 801 analyzer; x) gave the following correlations (ng/mL):

Number of samples measured: 169

Passing/Bablok[9]	Linear regression
y = 1.02x - 0.027	y = 1.02x - 0.060
τ = 0.980	r = 0.999

The sample concentrations were between 0.046 and 14.0 ng/mL.

c) A comparison of the Elecsys Testosterone II assay, REF 08946370190 (**cobas e** 801 analyzer; y) with the Elecsys Testosterone II assay, REF 08946353190 (**cobas e** 601 analyzer; x) gave the following correlations (ng/mL):

Number of samples measured: 167

Passing/Bablok[9]	Linear regression
y = 0.999x - 0.039	y = 0.978x + 0.040
τ = 0.979	r = 0.998

The sample concentrations were between 0.040 and 14.4 ng/mL.

Analytical specificity

For the antibody derivative used, the following cross-reactivities were found (in %):

	Concentration ng/mL	Cross-reactivity %
Androstendione	100	2.66
Cortisol	1000	0.016
Cortisone	2000	0.002
Danazol	1000	0.442
Dexamethasone	2000	0.0004
DHEA	1000	0.007
DHEA-S	50000	0.001
D-5-Androstene-3β,17β-diol	1000	0.186
Estradiol	1000	0.148
Estrone	1000	n.d.[d)]
Ethisterone	1000	2.78
Norgestrel	1000	0.461
Testosterone propionate	100	3.73
5-α-Androstane-3β,17β-diol	1000	3.65
5-α-Dihydro-testosterone	500	1.84
11-β-Hydroxy-testosterone	100	20.4
11-Keto-testosterone	1000	3.79
19-Norethisterone	40	3.44
Prednisone	1000	0.004
Prednisolone	1000	0.016
Progesterone	1000	0.023

d) n.d. = not detectable

References

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7 Marshall WA, Tanner JM. Variations in the pattern of pubertal changes in girls. Arch Dis Childh 1969;44:291-303.

8 Vermeulen A, Verdonck L, Kaufman JM. A critical evaluation of simple methods for the estimation of free testosterone in serum. J Clin Endocrinol Metab 1999;84:3666-3672.

9 Bablok W, Passing H, Bender R, et al. A general regression procedure for method transformation. Application of linear regression procedures for method comparison studies in clinical chemistry, Part III. J Clin Chem Clin Biochem 1988 Nov;26(11):783-790.

For further information, please refer to the appropriate operator's manual for the analyzer concerned, the respective application sheets and the Method Sheets of all necessary components (if available in your country).

A point (period/stop) is always used in this Method Sheet as the decimal separator to mark the border between the integral and the fractional parts of a decimal numeral. Separators for thousands are not used.

Any serious incident that has occurred in relation to the device shall be reported to the manufacturer and the competent authority of the Member State in which the user and/or the patient is established.

Symbols

Roche Diagnostics uses the following symbols and signs in addition to those listed in the ISO 15223-1 standard (for USA: see dialog.roche.com for definition of symbols used):

CONTENT	Contents of kit
SYSTEM	Analyzers/Instruments on which reagents can be used
REAGENT	Reagent
CALIBRATOR	Calibrator
→	Volume for reconstitution
GTIN	Global Trade Item Number

CE 0123

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