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Impact of allostatic biomarkers on health and performance

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Woven in dreams
Spiral to meet me
You don't mind it
You kinda like it

Don't need much more
Movement is a message
Moving me to action
Chemical reaction

Leaving out emotions
Give in to rhythms
Got to value the scene
Moving me to action
Make the beat give in to you

Breathing in a moment
Breathing in a feeling
Moving into that dream
Spiral in to meet me
(Edge of the horizon)
(Dance out the feeling)

Edge of the Horizon - Groove Armada

This thesis is dedicated to my parents: Jaime and Marina

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THOUGHT:

I would say that at least 50% of who we are is given by other people, who can be close or distant, known or unknown, and by stories, memories, songs, books, or emotions.

At night, we will only remember what we did outside our comfort zone. Autobiographies are about those moments.

EVERYTHING contributes to the incessant search for balance, which, in my opinion, will never exist, and EVERYTHING has a hormonal response. This is my interpretation of Allostasis, and this thesis is based on this thought.

List of Publications

This doctoral thesis is based on the following scientific publications, referred in the text with Arabic and Roman numerals (respectively):

1. Milheiro, J. S. (2019). Impacto de biomarcadores hormonais no treino e na performance. In J. Barata & Portuguese Olympic Committee (Eds.) *Anuário do treino desportivo 2019: Esperanças Olímpicas* (pp.63-71).

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Abstract

The use of biomarkers in sports performance is essential to promote training balance, mainly because overtraining syndrome lacks standardized diagnostic criteria. The general objective of this work is to attribute a role to insulin, not only as a hormone used in the regulation of glycemia but also as a relevant and fundamental biomarker in adapting to each person's allostatic load, integrating it into the so-called allostatic biomarkers and granting a more systemic view than is usually done, considering its high impact not only on performance but also on health. For that, we evaluated the hormonal dynamics in two different conditions: (i) choosing one period of the season to assess the catabolic/anabolic relationships and the cardiovascular fitness effect on mitigate the deleterious effects of high catabolism presence; (ii) evaluating competitions that are considered as extreme in terms of fatigue and possible dysregulation of the endocrine system, such as ultramarathons and road cycling stage races. We observed that lower values of insulin were associated with higher levels of cortisol and ACTH in high-endurance male athletes, with cyclists with higher cardiovascular fitness, particularly above 70 mL/kg/min, exhibiting a higher threshold for the negative effects of cortisol on anabolic processes compared to their non-elite counterparts (lower cardiovascular fitness). That results suggested insulin's new and relevant role as a sports performance training monitoring biomarker more sensitive than testosterone, particularly elite level and that superior cardiovascular fitness may provide protection against the elevated catabolic hormonal concentrations found in road cyclist. From the competitive experimental approaches, we found a link between hormonal and RPE responses to an ultramarathon lasting an average of 24 hours, with the increase and maintenance of high cortisol and RPE perceived by the athletes and the decrease and maintenance of low insulin levels during the entire race. Also, it was demonstrated that the rest day during a twelve-days stage races enabled the body to achieve catabolic and anabolic efficiency once again. Instead of understanding how the body works, the main goal of these academic work was to understand how endurance athletes endocrine system reacts to extreme training and competition loads, developing knowledge capable of relating the

variables tested with the performance and health of athletes, thus improving current practice and research.

Keywords: cortisol; insulin; performance; health; hormonal balance.

Resumo

O uso de biomarcadores de treino no desempenho desportivo é essencial para promover o equilíbrio, principalmente porque a síndrome do overtraining carece de critérios diagnósticos padronizados. O principal objetivo deste trabalho é atribuir à insulina um papel não só como hormona utilizada na regulação da glicemia, mas também como biomarcador relevante e fundamental na adaptação à carga alostática de cada pessoa, integrando-a nos chamados biomarcadores alostáticos, e assim conferir-lhe uma visão mais sistémica do que normalmente se faz, considerando seu alto impacto não só no desempenho desportivo, mas também na saúde. Para isso, avaliamos a dinâmica hormonal em duas condições diferentes: (i) escolhendo um período da temporada para avaliar as relações catabólicas/anabólicas e o efeito da aptidão cardiovascular em mitigar os efeitos deletérios da presença de alto catabolismo; (ii) avaliando competições que são consideradas extremas em termos de fadiga e passíveis de desregulação do sistema endócrino, como ultramaratonas e voltas em bicicleta por etapas. Observamos que valores mais baixos de insulina estavam associados a níveis mais altos de cortisol e ACTH em atletas masculinos de alta resistência, com ciclistas de maior aptidão cardiovascular, particularmente acima de 70 mL/kg/min, exibindo um limiar mais elevado para os efeitos negativos do cortisol nos processos anabólicos em comparação com seus pares não-elite (menor aptidão cardiovascular). Esses resultados sugeriram um papel novo e relevante da insulina como um biomarcador de controlo do treino e do desempenho desportivo mais sensível do que a testosterona, particularmente no nível de elite, e que uma aptidão cardiovascular superior pode proporcionar proteção contra as elevadas concentrações hormonais catabólicas encontradas em ciclistas de estrada. A partir das abordagens experimentais em contexto real de competição, encontramos uma ligação entre as respostas hormonais e de RPE ao longo de uma ultramaratona com duração média de 24 horas, com o aumento e manutenção de altos níveis de cortisol e RPE percebidos pelos atletas e a diminuição e manutenção de baixos níveis de insulina durante e após a corrida. Também foi demonstrado que o dia de descanso durante uma corrida de etapas de doze dias permitiu ao corpo alcançar novamente a eficiência

catabólica e anabólica. Em vez de entender como o corpo funciona, o principal objetivo deste trabalho acadêmico foi entender como o sistema endócrino de atletas de resistência reagem a cargas de treino e competição extremas, desenvolvendo conhecimentos capazes de relacionar as variáveis testadas com o desempenho e saúde dos atletas, melhorando assim a prática e a pesquisa atuais.

Palavras-chave: cortisol; insulina; rendimento; saúde; equilíbrio hormonal

List of abbreviations and symbols

ACTH	Adrenocorticotrophic hormone / hormona adrenocorticotrofina
Akt	Protein kinase B
AM	Morning
CI	Confidence interval
CMEP	Exercise Medical Centre Laboratory
DHEA	dihidroepiandrosterona
e.g.	As example
GH	Growth hormone
HDL	high-density lipoprotein cholesterol
HPA	hypothalamic-pituitary-adrenal / hipotalâmico-pituitária-adrenal
HPA	Hypothalamic-pituitary-adrenal
HPT	Hypothalamic-pituitary-testicular
HR	Heart rate
ICC	Intraclass correlation coefficient
IGF-1	Insulin-like growth factor 1
IL-6	Interleukin 6
LEA	Low energy availability
MIUT	Madeira Island Ultra-Trail
mTOR	Mammalian target of rapamycin
NFOR	Non-Functional Overreaching
OTS	Overtraining syndrome
PI3K	Phosphoinositide 3-kinases
PM	Evening
POMS	profile of mood state questionnaire
r	Person's correlation coefficient
RBC	red blood cell
RMP	Ramp protocol until exhaustion test
RPE	Rating of perceived exertion
SD	Standard deviation
SPM	Statistical Parametric Mapping
TNF	Tumour necrosis factor
$\dot{V}O_{2max}$	maximal oxygen uptake
11 β -HSD	11 β -hydroxysteroid dehydrogenase

Chapter 1

General introduction

Everything starts with stress. The term "stress" has permeated our daily vocabulary, contributing to various physical and emotional changes. Despite its prevalence, a comprehensive understanding of its complexity and the necessary tools to manage its consequences are often lacking or misused. Dr. Hans Selye, a pioneering researcher in stress, extensively explored the enigma of stress, often considered a motivational force when managed judiciously. In his book "Stress of Life" (Selye, 1956), he wrote, "While we cannot avoid stress while living, we can learn much about reducing its harmful side effects to a minimum." He emphasized that many common diseases are mainly due to errors in our adaptive stress response rather than direct harm from germs, toxins, or life experiences, calling them adaptive diseases.

In his 1976 article "Forty Years of Stress Research," (Selye, 1976) Selye defined stress as "a nonspecific response of the body to any demand. Any stressor produces stress at any time". The concept of the general adaptation syndrome delineates the chronological development of the body's response to prolonged stressors, encompassing three phases: the alarm reaction, the stage of resistance, and the stage of exhaustion. This syndrome laid the groundwork for conceptualizing the notion of allostasis. Today, in a world entire of stimuli, it is clear that excessive stress affects physical and mental health, leading to an increased risk of premature death (Keller et al., 2012).

Allostasis

The allostasis concept proposed by Sterling & Eyer (1988) reflects the physiological response to real or perceived stressors, ensuring the organism's survival and representing the balance through change. Derived from the Greek "allo" (variable) and "stasis" (stability), allostasis emphasizes the dynamic nature of stability through variability. It was not coined "homeostasis" (remaining the

same) because internal states are frequently disturbed and corrected, rarely remaining constant (Sterling & Eyer, 1988). The term heterostasis is also sometimes used in place of allostasis.

Prolonged exposure to stress can lead to wear and tear, culminating in functional impairments known as allostatic load, representing an insufficiently adaptive function. The human body is adaptable but cannot sustain allostatic overload for an extended period without consequences, resulting in a "disease of adaptation." as Selye suggested. The balancing process is achieved through fluctuating neuroendocrine responses to physical and psychological stressors, summing up to meet the challenge presented (Sterling & Eyer, 1988; Schulkin, 2003). This intricate interplay between homeostasis and allostasis underscores their roles in preserving internal stability.

Current times – “never offline”

In contemporary society, individuals grapple with relentless stress arising from various sources, including emotional, financial, occupational, and physical stressors like sleep deprivation, excessive caffeine intake, exposure to pain, or engaging in extreme exercise without proper recovery. Chronic exposure to stressors leads to increased production of hormones related to the hypothalamic-pituitary-adrenal (HPA) axis, namely, ACTH and cortisol, leading to disorders ranging from anxiety and memory loss to weight gain and infertility (Chrousos, 1992, 2009).

The prevailing "never offline" culture, characterized by continuous Internet connectivity, exacerbates this stress, disrupting the circadian rhythm and hormonal function, especially the catabolic-anabolic balance. While many individuals effectively cope with stress with proper hormonal responses, others overly strain their adrenal glands. In these cases, an initial phase of hyperproduction is followed by pauses, as hormonal glands have finite responses despite infinite demands. The resulting hypocortisolism manifests in symptoms

like chronic fatigue, pain, and stress intolerance, significantly impairing functionality and inducing morbidity.

Cortisol

The pivotal role of adrenal glands in human health and disease was initially elucidated by Thomas Addison (1855), giving rise to the term Addison's disease. In the early 20th century, Dr. Charles Sajous furthered this understanding, coining hypoadrenia to characterize a state of adrenal "exhaustion" where symptoms like fatigue and susceptibility to infection stem from prolonged stress (Sajous, 1903). In the 1920s and 30s, Dr. Walter Cannon made substantial contributions to stress analysis, being the first to describe the "fight or flight" response to an immediate threat and recognize the physical and mental nature of stressors (Cannon, 1929).

Cortisol is the principal glucocorticoid in the body involved in many functions of stress response mediation, such as metabolism regulation, inflammatory response, and immune function. It profoundly influences the human skeletal muscle in coordination with anabolic hormones (Oakley and Cidlowski 2013; Ando et al. 2021; Katsu et al. 2015). Its release in the adrenal's fasciculate zone is stimulated by adrenocorticotrophic hormone (ACTH) and controlled through negative feedback by the hypothalamus and pituitary, forming the hypothalamic-pituitary-adrenal (HPA) axis (Gagner and Drouin 1985; Stocco and Clark 1996; Malkoski and Dorin 1999). In a physiologically balanced state, cortisol exhibits a circadian rhythm, peaking in the morning, decreasing throughout the day, and reaching its lowest values at night (Oster et al., 2017), promoting adequate concentration, memory, and performance during the day, transitioning to relaxation and sleep at night.

It assumes in skeletal muscle during exercise a pivotal role in energy homeostasis and metabolism, enhancing metabolic substrates' availability and increasing circulation in response to heightened endurance training (Alves et al., 2020; Daly & Hackney, 2005). However, high cortisol levels resulting from chronic

endurance training have been reported to negatively affect anabolic signaling, namely by reducing testosterone production (Daly & Hackney, 2005; Lane et al., 2015), compromising the recovery and performance of athletes due to its role in protein synthesis and erythropoiesis (Shahani et al., 2009). Additionally, hypercortisolism resulting from energy deficiency states (Elliott-Sale et al., 2018) can inhibit local anabolic stimulation in the muscle, mainly by reducing insulin, IGF1, mammalian rapamycin (mTOR), and amplifying myostatin signaling, a catabolic muscle growth factor. This anti-anabolic effect potentially compromises cellular nutrition and regenerative protein synthesis, contributing to muscle atrophy (Liu et al., 2004; Patel et al., 2014; Schakman et al., 2013), which, in addition to physical and mental impairment, can eventually lead to illness.

Conversely, lower cortisol levels are associated with fatigue, melancholy, anhedonia, slow recovery, and inability to respond to training stimuli, leading to significant declines in performance found in chronic fatigue syndrome, common burnout, or overtraining syndrome in athletes (Armstrong et al., 2021; Juster et al., 2011; Kreher & Schwartz, 2012; Schmikli et al., 2012).

Insulin

Insulin is the primary anabolic hormone responsible for entering glucose into cells. It increases cellular permeability predominantly of muscle cells, reducing circulating plasma glucose and contributing to the deposition of glycogen in the liver and muscles, playing a central role in muscle growth and protein renewal for muscle recovery (O'Neill et al., 2015; Urhausen et al., 1995), with its interactions with exercise being fundamental for metabolic regulation and homeostasis (Horton et al., 2006).

Produced in the beta cells of the islets of Langerhans, insulin is one of the few hormones that increase with age due to increased resistance to its function, triggering the appearance of metabolic diseases typical of contemporary society, being this resistance enhanced by prolonged and excessive production of

cortisol. However, in young and lean athletes with high training volumes and caloric deficits, the opposite phenomenon may occur, hypoinsulinism, already described above as the anti-anabolic effect of cortisol (Liu et al., 2004; Patel et al., 2014; Schakman et al., 2013),

Conversely, one of the positive effects of cardiovascular fitness is to promote greater insulin sensitivity and optimize lipid oxidation (Horton et al., 2006). This increased sensitivity may be attributed to training-induced adaptations in the vasculature, skeletal muscle, and adipose tissue, justifying lower basal insulin levels in well-trained athletes (Goodpaster et al., 2001). Nevertheless, a nuanced analysis is warranted, as excessively low values may eventually jeopardize its efficacy (Borghouts & Keizer, 2000).

High Performance

Discerning between constructive fatigue and overtraining is crucial in elite sports, necessitating heightened physiological evaluations, especially in individual and endurance sports. Relying solely on the athlete's subjective sensations risks severe misjudgement. Monitoring athletes comprehensively - physically, mentally, and biochemically - is imperative for the early detection of training maladaptation and sub-states of overtraining syndrome (OTS) when prolonged (Budget, 1990; Kreher & Schwartz, 2012; Le Meur et al., 2013, Cadegiani et al., 2020). Cortisol release during exercise plays a pivotal role in performance, and the "cortisol blunted response" can signal an athlete's inability to overcompensate for exercise-induced stress, typically exhibiting compromised physical performance, being slower, weaker, or more unfocused than usual (Schmikli et al., 2011; Schmikli et al., 2012). The concept of allostatic load introduces a new evaluation method for the maladaptation process, representing visible markers of chronic exposure to stress.

The current thesis aims as overall objective to attribute a role to insulin, not only as for glycaemic regulation, but also as a relevant and fundamental biomarker in

adapting to the allostatic load, integrating it into the so-called allostatic biomarkers. It comprises 10 chapters, with the **Chapter 1** serving as the General Introduction where it is provided context for the training process and emphasized the importance and inclusion of hormonal assessment and control. Additionally, it is presented and described all the chapters of this dissertation, particularly **Chapters 2** through **6** where it is featured a perspective study and four experimental studies. In **Chapter 7**, the General Discussion compare the results obtained in our experimental studies with relevant literature and in **Chapters 8, 9** and **10** are contained the main conclusions of this dissertation, suggestions for future research and the respective bibliographic references. Also included in this work is the **Appendix I**, which present a supplementary perspective study that complement our primary research.

Based on our clinical experience, research, and reflection over the years, we have observed that much of the symptomatology associated with delayed recovery and performance decrease in athletes is linked to cortisol dysregulation. In more pronounced situations, additional hypoinsulinism scenarios may appear. This insight, derived from our clinical experience, is currently little mentioned in the scientific literature since the topic of insulin is almost exclusively used in pathological conditions, particularly those related to diabetes. Additionally, we noticed that the catabolic risk is more prevalent in endurance modalities, particularly those requiring a lot of training volume, such as road cycling and ultra-marathons where caloric deficits are frequent due to the struggle to meet energy requirements, risking chronic low energy availability (LEA), and also the high-energy demand, and mandatory leanness (Heikura et al., 2019; Hohl et al., 2019; Ramos-Campo et al., 2016; Schofield et al., 2021).

As such, we designed this thesis on catabolic/anabolic dynamics in athletes subject to endurance training volumes and prone to caloric deficiencies. In **Chapter 2**, is presented a perspective study on this topic highlighting the importance of using sports performance and allostatic biomarkers to better understand and promote the balance of training. As a complement to the ongoing

discussion of that theme, in **Appendix I** is presented another perspective study based on a consensus statement on the topic of anabolic-androgenic steroids use in sports, health, and society (Bhasin et al., 2021). The aging-athlete is debated here since, one of the current training topics, particularly in high-level sports, is how to cope with age and maintain optimal health and performance (Martín et al., 2018; Menard & Stanish, 1989).

Furthermore, it is acknowledged that professional endurance athletes, specifically those engaged in road cycling and ultramarathon events, demonstrate an extended career span compared to their counterparts in resistance training (Hohl et al., 2019; Barranco-Gil et al., 2024). Lastly, emphasis is placed on the post-career phase, which is crucial for understanding the challenges that athletes may confront during this transition (Menard & Stanish, 1989; Siekańska & Jan, 2019; Tinley, 2015).

In **Chapter 3** is presented the assessment of the hormonal balance between cortisol and insulin values in a large sample of well-trained road cyclists and how they relate to each other. In **Chapter 4**, as a complementary study to Chapter 3, is presented the evaluation of the anti-anabolic effect of cortisol in professional cyclists, namely in the commitment of testosterone and insulin release, depending on their previous cardiovascular fitness, since the adaptations created by endurance training may create hormonal resistance or tolerances, eventually mitigating deleterious effects. Insulin assessment and monitoring may gain particular importance in these well-trained athletes because, of the several positive hormonal adaptations, insulin sensitivity is heightened with this type of training and competitions compared to regular athletes (Hohl et al., 2019; Barranco-Gil et al., 2024).

Following the examination of the correlation between cortisol and insulin fluctuations based on the athlete's cardiovascular fitness, the subsequent phase involves delineating and investigating the dynamics of this hormonal equilibrium during real competitive exercise scenarios. The first aim is to comprehend the

reaction to prolonged and continuous endurance exertion within a singular race, manifesting in the acute release of catabolic hormones to sustain energy levels throughout the entire event. The second aim involves understanding the response to the repetitive daily occurrence of the same catabolic process over several days, incorporating the requisite post-exertion anabolic compensation to uphold the body's essential equilibrium and energy availability throughout every single day (Lucía, Hoyos, et al., 2001).

To address these objectives, two distinct studies were devised to elucidate and interpret endocrine dynamics in exercises involving extreme endurance efforts—one encompassing nearly 24 hours continuously and the other spanning multiple days of competitive events. The ultramarathon serves as an ideal paradigm for the first situation, offering a unique opportunity to explore the limits of the adaptive response of the human body (Millet & Millet, 2012). Consequently, in **Chapter 5** is presented a study evaluating cortisol and insulin responses throughout a trail marathon lasting approximately 24 hours, probing their association with the rate of perceived exertion.

On the other hand, cycling tours prove optimal for the second scenario, representing the epitome of strenuous endurance exercise and often regarded as the pinnacle of sustained energy expenditure for humans (Barranco-Gil et al., 2024; Lucía, Díaz, et al., 2001). Thus, in **Chapter 6** is presented the final experimental study, the most comprehensive one, aiming to assess the entire catabolic-anabolic dynamism throughout a 12-day cycling race—the "*Volta a Portugal*" in road cycling.

Impacto de biomarcadores hormonais no treino e na performance

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Resumo

Resultante do incremento da prática desportiva em idades cada vez mais precoces, associada ao possível estrelato que a competição possa proporcionar na geração milenar (considerada a mais narcisística da história), a prática desportiva inadequada pode perder as suas características anabólicas e construtivas, levando, entre várias coisas, ao dano físico e mental. Isso implica uma evidente necessidade crescente do apoio médico-fisiológico e psicológico ao atleta de alto rendimento, nomeadamente nos desportos de perfil individual e de *endurance* (mas não exclusivamente). Considerando que a linha que separa a fadiga construtiva do *overtraining* é ténue, a avaliação baseada na interpretação das sensações do atleta corre o risco de falhar redondamente. Nos dias de hoje é fundamental fazer um seguimento físico, mental e bioquímico, indicando o caminho a que o treino nos está a conduzir. É essencial a deteção precoce do denominado *NFOR – Non-Functional Over Reaching*, evitando o declínio físico e mental do atleta. Esta situação para além de provocar muita sintomatologia, é precedida de alterações hormonais, nomeadamente com a incapacidade de libertar hormonas necessárias para o esforço (resposta travada de cortisol) e hormonas regeneradoras após o esforço (diminuição de insulina e o seu factor de crescimento), não havendo assim a necessária reação orgânica para a obtenção dos resultados, potenciando quadros depressivos, fadigas acentuadas, frenadoras da capacidade funcional desportiva e cognitiva, promovendo múltiplos distúrbios orgânicos e abandono precoce da competição. O diagnóstico precoce e objetivo desta condição é determinante para a evolução da performance desportiva, necessitando de uma vigilância forte da equipa multidisciplinar de apoio ao atleta.

Palavras-chave: *Overtraining*, Cortisol, Insulina, Prevenção, Alto rendimento

Introdução

Quando pensamos num atleta de alto rendimento e na conquista dos objetivos, por vezes achamos que os resultados desportivos são diretamente proporcionais ao volume e intensidade dos treinos e à aplicação do atleta (assiduidade, dedicação, capacidade de sofrimento, entre outras características). O objetivo do múltiplo efeito agudo do exercício consiste em agitar o organismo, de modo a que ele consiga adaptar-se a um patamar superior (seguindo uma das leis da física), ou seja, criar instabilidade para obter um patamar superior de estabilidade, para tal, stressa-se o organismo. Contudo, nem sempre conseguimos atingir os objetivos (patamares superiores de performance). Muitas justificações serão de colocar. Neste artigo irei tocar numa delas, a parte hormonal.

Stress/treino – resposta

A palavra stress tornou-se um lugar-comum do vocabulário diário. Creditado por causar múltiplas alterações físicas e emocionais, hoje em dia aprendemos a considerá-lo parte normal das nossas vidas, apesar de ainda não entendermos verdadeiramente a sua complexidade nem estarmos adaptados para verdadeiramente lidar com as suas consequências. Contudo a palavra normalmente sugere interpretações erradas. Stress não é um verbo mas sim uma resposta, ou seja, para todo o estímulo sentido ou percebido existe sempre uma resposta stressora orgânica com respectiva tradução hormonal.

Allostasis

O conceito de *Allostasis*, proposto por Sterling e Eyer (1988), será a resposta fisiológica aos reais ou percebidos estímulos stressores, a fim de melhorar a capacidade de sobrevivência do organismo: será o saldo da mudança. Respondendo à denominada carga alostática, o organismo poderá sofrer desgaste numa exposição prolongada ao stress e apresentar falhas na

capacidade adaptativa, apresentando estragos funcionais. *Allostasis*, do grego *allo*, significa “variável”, enquanto *stasis* significa “estabilidade”. “Variando para manter a estabilidade”, será a tentativa de manter a homeostasia. A longo prazo, as funções alostáticas poderão não ser suficientemente adaptativas porque a excessiva exposição ao estímulo cria alterações que se acumulam: será a chamada carga alostática.

Se um organismo desidratado, por exemplo, for compensado, mas simultaneamente continue em excessiva carga e não restabeleça as funções normais, os sistemas orgânicos do indivíduo irão comprometer-se, nomeadamente com uma massiva rhabdomiólise (destruição muscular), levando a uma insuficiência pré-renal, com consequente risco de vida, podendo criar o saldo mais negativo de todos: a morte. O corpo humano é adaptável, mas não consegue manter a sobrecarga alostática por muito tempo sem as devidas consequências, ou seja, “uma doença de adaptação” (Selye, 1956; Selye, 1976).

Tempos atuais – “never offline”

Os jovens na sociedade de hoje em dia são submetidos a estilos de vida frenéticos, fatigantes e altamente desequilibradores. Atualmente encontram-se muitos atletas que experimentam, ao longo de uma época, períodos de stress contínuo, proveniente de distúrbios emocionais (necessidade de resultados desportivos e escolares) e de agressões físicas, como a privação do sono, excesso de estimulantes ou cargas de treino extremas sem a devida recuperação.

A exposição crónica ao treino fora do conforto e a pressão emocional provocam aumento da produção de hormonas libertadas pelas glândulas suprarrenais (ou adrenais), nomeadamente do cortisol (a mais conhecida hormona produzida com o stress) e da dihidroepiandrosterona (DHEA), conduzindo a vários distúrbios nomeadamente ansiedade, aumento de peso, perda de massa muscular, entre outros (Chrousos, 1992; Chrousos, 2009).

O mundo global criou a postura '*never offline*' ao longo das 24 horas do dia. A contínua ligação à rede (net), de permanente exposição e demanda, com as concomitantes hiperestimulações hormonais crónicas, faz com que não se cumpra o ritmo circadiano do dia e do sol, levando a que o atleta desequilibre a produção da maestrina das hormonas: o cortisol, com evidente compromisso do descanso e da reparação hormonal noturna, período em que produzimos hormonas regenerativas como é o caso da hormona de crescimento e da melatonina.

Enquanto muitas atletas são capazes de lidar com o stress propiciando libertações adequadas de cortisol perante os estímulos recebidos, numa correta resposta adaptativa, outras porém, solicitam em demasia as adrenais, gerando respostas inadequadas. Nestes casos, a uma fase inicial de hiperprodução segue-se uma redução da produção, porque todas as glândulas são finitas apesar de expostas a estímulos infinitos: '*never offline*'. A resultante insuficiência adrenal, também conhecida como "esgotamento ou fadiga adrenal", pode ocasionar uma constelação de sintomas desde a fadiga crónica, dor crónica e intolerância ao stress, levando muitas vezes ao abandono precoce da competição.

Sintomas de disfunção adrenal

Sob a direção do hipotálamo e da hipófise (pituitária), e controlada por um circuito de *feedback* negativo, a suprarrenal é estimulada pela hormona adrenocorticotrofina (ACTH) para produzir cortisol em resposta ao stress. Este circuito fechado de *feedback* é vulgarmente referido como o eixo hipotalâmico-pituitária-adrenal (HPA). De ritmo circadiano, o cortisol apresenta maiores produções matinais e decréscimo progressivo vespertino, permitindo que haja adequados níveis de concentração, memória e desempenho durante o dia e, com a sua progressiva diminuição, o relaxamento e o sono à noite.

O cortisol tem uma ampla gama de efeitos sobre a mente e o corpo. Interage, entre outras incidências, com a função pancreática, tiroideia e imunológica. Fazendo parte da resposta a qualquer estímulo sentido ou percebido, ao preparar o corpo para a “luta ou fuga”, o cortisol suprime a produção de outras hormonas, reduzindo temporariamente os processos que desviariam os recursos corporais das exigências mais imediatas, para responder ao referido estímulo, incluindo funções do sistema imunológico (Chrousos, 1992; Chrousos, 2009).

Neste sentido, níveis cronicamente elevados de cortisol podem resultar em aumento da suscetibilidade à infeção, compromisso da função tiroideia e do metabolismo, aumento de mediadores inflamatórios (IL-6, TNF), promovendo a progressão da inflamação orgânica, diminuição da sensibilidade à insulina, alterações de comportamentos alimentares, nomeadamente a compulsão por hidratos de carbono, aumento de peso, alteração do humor por menor produção de serotonina e, entre muitos outros sintomas, diminuição do anabolismo (Chrousos, 1992; Chrousos, 2009).

Níveis de cortisol mais baixos que os normais estão associados à fadiga, melancolia, falta de motivação, lentificação da recuperação muscular e terapêutica, incapacidade de resposta a estímulos do treino, implicando massivas diminuições da performance num quadro que interseta sintomatologia do síndrome da fadiga crónica (quadro ininterrupto de fadiga com mais de seis meses de duração, não relacionada com qualquer outra doença, sem alívio com repouso e marcada redução da capacidade cognitiva e funcional; Cunha, 2006; Tak et al., 2011), o *burnout* do comum dos cidadãos (Juster et al., 2011) ou o *overtraining* dos atletas (quadro que se reflete por uma fadiga prolongada e redução da performance, de duração superior a um mês, consequente a um período longo de treino forte ou competição, apresentando marcas alterações comportamentais e uma maior suscetibilidade à infeção; Budget, 1990; Kreher e Schwartz, 2012; Le Meur et al., 2013).

Qualquer distúrbio, neste (des)equilíbrio, quer por híper quer por hipo produção, irá potenciar descompensações hormonais, sendo promotor de inúmera sintomatologia.

Resumidamente:

O cortisol, fisiologicamente, apresenta de base uma função anti-inflamatória e protetora, mas cuja produção excessiva estimula o catabolismo (consumo), podendo desencadear desequilíbrios orgânicos por deterioração da parte anabólica (construção) do sistema. Produzir-se-á então, em tal situação, o denominado efeito antianabólico, com compromisso na alimentação celular, atuando principalmente na produção da insulina e do fator de crescimento insulínico (IFG1), hormonas com responsabilidade anabólica marcante. Será por isso fácil entender que a regulação da produção de cortisol é fundamental para os atletas de alto rendimento, considerando a sua repercussão nas atividades físicas e mentais.

Alto rendimento

Entrando no alto rendimento desportivo, sabemos que a linha que divide o esforço construtivo do destrutivo é muito ténue e leva a necessidades crescentes do apoio medicofisiológico e psicológico, nomeadamente nos desportos de perfil individual e de endurance, onde a linha que separa a fadiga construtiva do *overtraining* dificilmente se visualiza. Uma avaliação que, apenas assente na interpretação subjetiva das sensações por parte do atleta, corre o risco de falhar severamente. É fundamental, hoje, fazer um seguimento físico, mental e bioquímico do atleta, indicando o caminho a que o treino possa conduzir. Será essencial a deteção precoce do denominado *NFOR – Non-Functional Over Reaching*, evitando a decadência física e mental do atleta.

O termo em si dá de imediato uma descrição aproximada do que realmente significa, ou seja, o atleta literalmente ultrapassa o ponto de equilíbrio entre a

carga de treino e a capacidade de recuperação orgânica, levando a uma diminuição da performance acompanhada por distúrbios do humor, contudo, ao contrário do quadro de *overtraining*, se a periodização da recuperação for corretamente estabelecida (*taper*), os atletas posteriormente irão beneficiar da fase de supercompensação e aumentar significativamente o seu desempenho (Budget, 1990; Kreher e Schwartz, 2012; Le Meur et al., 2013). Uma das principais variáveis limitadoras da performance é a diminuição da libertação de cortisol ao longo do esforço (Schmikli et al., 2011; Schmikli et al., 2012).

Denominado “resposta travada do cortisol”, tal quadro clínico corresponde a uma incapacidade de as glândulas suprarrenais produzirem quantidades suficientes de cortisol para saltar da zona de conforto, de modo a obter a sobrecompensação do exercício. Normalmente, os atletas disso portadores apresentam compromissos do seu desempenho físico, encontrando-se geralmente mais lentos, mais fracos ou mais desconcentrados do que o habitual. Tal quadro precede por norma a sintomatologia típica de *overtraining*, sendo que, se detetada precocemente, a sua tentativa de reversão será muito mais eficiente.

Schmikli et al. publicaram no *British Journal of Sports Medicine*, em 2011, a relação entre a diminuição da performance e a menor produção de cortisol. Demonstraram que, no grupo com diminuição da performance com mais de um mês de duração, os baixos níveis de cortisol estavam dissociados dos níveis da ACTH comparativamente ao grupo controlo, apresentando igualmente sintomatologia típica de *Non Functional Over Reaching*, nomeadamente depressão e raiva (Schmikli et al., 2011). O conceito de carga alostática traz um novo método de avaliação à resposta desregulada desse stress, representando um marcador visível da exposição crónica a esse mesmo stress.

Biomarcadores alostáticos

Vários biomarcadores alostáticos têm sido utilizados na literatura recente como indicadores de compromisso entre a saúde e a performance. Os mais utilizados

são, sem dúvida, as hormonas libertadas pela suprarrenal, nomeadamente o cortisol e o DHEA, bem como a hormona responsável pela sua estimulação da hipófise: a ACTH (Aizaka et al., 2006). Pelo facto de ambas derivarem da denominada cascata do colesterol, igualmente o colesterol total e o colesterol HDL têm sido considerados para esse fim (Bahreinian et al., 2013). Bahreinian et al., em 2013, demonstraram, num estudo prospetivo, o aumento do risco de asma em adolescentes sujeitos a elevada carga alostática, tendo sugerido como biomarcadores os valores de glicose em jejum, o colesterol total, o colesterol HDL, o sulfato de DHEA e o cortisol (Bahreinian et al., 2013).

Insulina

Na nossa prática clínica decorrente dos últimos 8-10 anos, temos vindo a analisar os efeitos da exposição prolongada ao cortisol nos atletas, com as repercussões catabólicas que daí derivam e que promovem a inflamação e a depleção de nutrientes, por um processo de consumo acelerado, à maneira de um “motor desafinado” de elevado consumo.

Como é do conhecimento geral, a insulina é uma hormona anabólica, responsável pela entrada da glicose para as células. Aumenta a permeabilidade celular (exceto nas células nervosas), predominantemente nas células musculares, as quais, pouco permeáveis à glicose em condições de repouso, utilizam principalmente ácidos gordos livres para produção de energia e reduzem desse modo a quantidade disponível circulante do plasma, mantendo os valores de glicemia controlados. Contribui igualmente para a criação de depósitos de glicogénio no fígado e no músculo, desempenhando um papel essencial no consumo dos hidratos de carbono, na síntese proteica e no armazenamento lipídico.

A insulina, produzida nas células beta dos ilhéus de Langerhans do pâncreas, é uma das poucas hormonas que aumenta com a idade, devido a um acréscimo da resistência à sua função, levando a que o pâncreas, progressivamente, vá

aumentando a sua produção até à desregulação do sistema, desencadeando assim o início da doença ou síndrome metabólico. Dos vários efeitos deletérios referidos na literatura da longa e excessiva produção de cortisol, um deles é o aumento do risco da doença metabólica exatamente pelo processo de aumento de resistência à insulina. Contudo, nas populações mais jovens e com elevadas cargas alostáticas, temos vindo a observar exatamente o oposto deste fenómeno (Milheiro, 2016, resultados não publicados).

Segundo a nossa experiência ao longo dos últimos anos, temos vindo a reparar que muita da sintomatologia associada ao atraso da recuperação dos atletas está relacionada com quadros de hipocortisolismo. Pior ainda, em situações mais acentuadas, a quadros adicionais de hipoinsulinismo consequentes. É fácil entender que, se não houver insulina biodisponível, a recuperação muscular estará comprometida.

Considerações finais

Um dos efeitos mais deletérios da produção prolongada do cortisol associada ao alto rendimento é o seu efeito antianabólico, comprometendo, entre outras, a função de uma das principais hormonas anabólicas do organismo: a insulina. Uma das principais considerações deste artigo é sensibilizar os treinadores e fisiologistas que acompanham os atletas de alto rendimento para a importância da avaliação hormonal, nomeadamente do cortisol e da insulina, atribuindo-lhes um papel de biomarcadores relevantes e fundamentais para a análise da adaptação à carga alostática de cada um, integrando-as nos denominados biomarcadores alostáticos e concedendo-lhes uma visão mais sistémica do que habitualmente se faz, considerando a sua elevada repercussão não só na performance mas também na saúde.

Tracking performance based on anabolic and catabolic hormones. Is insulin a “new” overload marker?

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Abstract

Purpose: The use of biomarkers in sports performance is essential to promote the balance of training, notably since overtraining syndrome lacks standardized diagnostic criteria. This study aimed to explore the use of insulin as an indicator of training maladaptation by evaluating its relationship with cortisol and adrenocorticotrophic hormone (ACTH) concentrations.

Methods: A sample of 143 cyclists (age 31 ± 7 years and maximal oxygen uptake $60.2 \pm 8.9 \text{ mL}\cdot\text{kg}^{-1}\cdot\text{min}^{-1}$) volunteered to participate in this study. The subjects reached the laboratory at 08.00 am, and a venous blood sample was withdrawn in a fasting state for insulin, glucose, and ACTH concentration analysis. Additionally, the subjects delivered a 24-hour urine collection collected the previous day for the total free Cortisol secretion analysis. Pearson correlation coefficients were calculated between insulin, cortisol, and ACTH concentrations, and a significance level was set at 5%.

Results: The insulin concentration is negatively affected by the rises of ACTH ($r = -0.23$, -0.38 to -0.07 95% CI; $p = 0.005$) and cortisol ($r = -0.21$, -0.36 to -0.05 95% CI; $p = 0.011$) concentrations. The hypothesis that in high-endurance athletes, high cortisol production could impair insulin release was confirmed.

Conclusion: These findings suggested insulin's new and relevant role as a sports performance training monitoring biomarker. We should consider hormonal imbalances such as hypoinsulinism, if confirmed as chronic, as a possible cause of compromising the athlete's physical and mental health, which could lead to disease.

Keywords: Hormonal balance, Performance, Health, Cycling, Endurance

Introduction

The use of biomarkers in sports performance is of increasing interest to the medical and scientific community to understand better and promote the balance of training, decreasing the risk of overtraining. Overtraining syndrome (OTS), an emerging condition without standardized diagnostic criteria (Cadegiani et al., 2020; Cadegiani & Kater, 2019), can be defined as an extreme state of persistent maladaptation of physiology during excessive training load without adequate rest and recovery. This condition leads to a decline in athlete's performance by impairing the neurological, psychological, neuroendocrine, and immunological functions (Kreher & Schwartz, 2012; Meeusen et al., 2013).

This consequent state of overload will potentiate the injury and illness risk, compromising the health and well-being of the athletes (Herring et al., 2019; Meeusen et al., 2013). Therefore, tracking biomarkers related to OTS condition is not only of particular importance for optimal performance but especially as a prevention strategy (Herring et al., 2019; Meeusen et al., 2013). Also, non-exercise-related triggers like inadequate nutrition (with chronic low energy availability [LEA]), illness, psychological stressors, or sleep disorders may potentiate the overload (Cadegiani et al., 2020; Meeusen et al., 2013; Schofield et al., 2021).

The hormonal catabolic-anabolic balance supports the load and recovery equilibrium (Cadegiani et al., 2020; Meeusen et al., 2013; Urhausen et al., 1995), where cortisol uniquely influences the catabolic response to exercise. Produced in the cortex of the adrenal glands and controlled through negative feedback on the hypothalamic-pituitary-adrenal (HPA) (Gagner & Drouin, 1985; Malkoski & Dorin, 1999; Stocco & Clark, 1996), cortisol is the principal glucocorticoid involved in many functions of the stress response mediation of the human body, such as metabolism regulation, inflammatory response and immune function (Ando et al., 2021; Katsu et al., 2015; Oakley & Cidlowski, 2013). Cortisol levels also represent the physiological response to the demands of exercise (Duclos & Tabarin, 2011; Duclos & Tabarin, 2016) and play a critical role in integrating adaptive responses

to stress and training (Gouarne et al., 2005; Smith & Vale, 2022), increasing significantly during prolonged endurance exercise to maintain appropriate energy levels (Viru & Viru, 2004).

Regarding the hormonal role in the post-exercise regenerative anabolic process, several hormones have been held responsible for this effect, namely testosterone, growth hormone (GH), and insulin-like growth factor 1 (IGF-1) (Kraemer et al., 2017). In addition, insulin (as an anabolic hormone) is also involved in this process, as it is responsible for glucose homeostasis and plays a central role in muscle growth and protein turnover (O'Neill et al., 2015; Urhausen et al., 1995), promoting muscle recovery. Its concentrations decrease during prolonged exercise, responding to the increase in catecholamines, inhibiting insulin secretion (Hartley et al., 1972; Kindermann, 1982; Richter et al., 2021) in order to maintain glucose available for the central nervous system (Richter et al., 2021; Weicker, 1985). Conversely, insulin concentration rises during the post-exercise phase to enhance glycogen replacement, decreasing carbohydrate oxidation and increasing lipid oxidation (Horton et al., 2006).

Although lower insulin baseline levels and higher sensitivity are assumed to be adaptations in aerobic endurance athletes to optimize lipid oxidation (Goodpaster et al., 2001; Richter et al., 2021), it is essential to be aware that the broad spectrum of cortisol tasks for metabolic processes control, includes an anti-anabolic action (Viru & Viru, 2004). From a pathological point of view, insulin dysfunction, as seen in hypoinsulinemia (e.g., in type I diabetes) or insulin resistance (e.g., due to obesity, aging, and type II diabetes), can lead to muscle wasting and atrophy (D'Souza et al., 2013; Krause et al., 2011). Therefore, controlling insulin levels becomes relevant in athletes exposed to prolonged endurance training.

As OTS is characterised by an imbalance in the body's responses and adaptations to exercise-induced stress and recovery, in which the endocrine system plays a key role, cycling, as a sport that combines prolonged endurance

training with LEA, has a higher risk of this type of imbalances (Heikura et al., 2019; Lucía et al., 2001; Schofield et al., 2021). The current study aimed to evaluate the relationship between cortisol and insulin in elite cyclists since it is still scarce in the literature. We hypothesized that in high-endurance athletes, elevated cortisol production could impair insulin release. Complementary, as cortisol production is controlled by the adrenocorticotrophic hormone (ACTH), we also aimed to explore the relationship between ACTH and insulin concentrations.

Methods

Participants

A sample of 143 male cyclists (age 30.5 ± 6.5 years; height 176.6 ± 6.1 cm, weight 74.1 ± 7.8 kg, % body fat $11.6 \pm 4.1\%$, maximal oxygen uptake 60.2 ± 8.9 mL·kg⁻¹·min⁻¹), regularly involved in competitive cycling events in the past five years, volunteered to participate in this study. They gave their written informed consent after being informed of all experimental procedures and possible risks associated with the experiments. Before the study, all cyclists underwent a medical questionnaire to ensure that no exogenous anabolic-androgenic steroids, drugs, or other supplements that might affect physical performance or hormonal balance were taken during the study period. Ethical approval was obtained by the University of Porto Ethics Committee (CEFADE 20_2023), and all procedures complied with the Declaration of Helsinki Ethics Code and the Standards for Ethics in Sports and Exercise Science Research. All data were collected between March and April to minimize the seasonal cortisol rhythm and ensure similar phases of the training season.

Study design

All experimental procedures were carried out in the Exercise Medical Centre Laboratory (CMEP), Porto, Portugal, on two occasions (separated each by at least 48h intervals), and subjects did not complete experimental sessions of familiarization, as they were already accustomed to this type of test. Figure 1 presents the scheme of the timeline of periodization and testing for this study.

Subjects were instructed to arrive at the laboratory rested (no strenuous exercise performed in the preceding 48 h), refrain from alcohol and caffeine-containing drinks for 24 h and abstain from food in the three hours before testing. In the first visit, several body parameters were measured, and a venous blood sample was always withdrawn at 08.00 am, always by the same technician, in a fasting state for insulin, glucose, and ACTH analysis. Additionally, the subjects delivered a 24-hour urine collection collected on the previous day for the total free cortisol secretion analysis. In the second visit (around noon), participants performed an incremental ramp protocol until the exhaustion test (RMP) to assess their maximal oxygen uptake, critical for characterizing their competitive level during hormonal analysis.

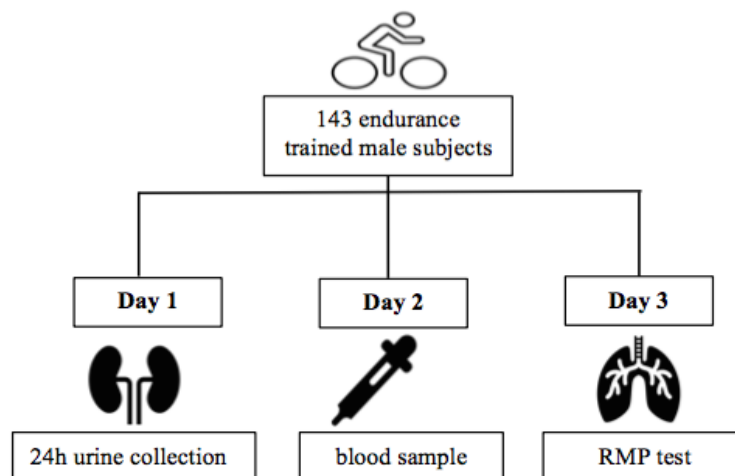


Figure 1. Schematic representation of the testing timeline. Abbreviations: RMP, ramp protocol until exhaustion.

RMP

The cyclist performed on an electromagnetically braked cycle ergometer (Lode Excalibur Sport, Groningen, The Netherlands) a ramp test consisting of a 9-12 min incremental protocol, starting at 0 W with increments of 35-45 W (0.58 – 0.75 W/s) every minute, with a self-selected cadence between 70 and 90 rpm (Lucia et al., 1998; Sousa et al., 2015). The test started with a standard 3-minute rest period followed by an unloaded baseline pedalling (0 W). Every subject chose his cadence and was requested to keep it constant for the duration of the test with the aid of a visual revolution counter. The power increments were set according to the subject's fitness level, and during the tests, the power was controlled by

visual feedback. The subjects used their pedals and racing shoes and adjusted the seat height and handlebar positions to the individual morphological characteristics and habitual racing position. The test ended when the cadence could no longer be maintained within 10 rpm of the preferred cadence for more than 5 seconds. Standard room temperature (18-22° C) and air humidity (40-50%) were established. Verbal encouragement was given to motivate the subjects to perform their best effort for as long as possible. All tests were performed in the conventional seated posture.

Experimental measurements

After the venous blood samples were withdrawn, plasma was immediately separated by centrifugation (10 min at 3,500 revolution/min; 4° C), deposited in aliquots, and immediately stored at -80° C for subsequent analysis. Insulin, glucose, and ACTH were assessed in venous blood collection using the immuno-chemiluminescence method (Kricka, 2003). The 24-hour urinary-free cortisol was analysed after collecting the entire urine volume for an entire day, not counting the first urine of the day, and ended after collecting the first urine of the following day. The sample was then centrifuged (5 min at 2,500 revolution/min; 15° C), with a prior preparation with Dichloromethane, and then analysed in Cobas (cobas® 6800 System, Roche, Switzerland). Cortisol was then detected using the immuno-chemiluminescence method (Kricka, 2003). After assessing all the concentrations, the participants were classified by presenting hypo-, normal- or hypercortisolism/insulinemia based on the ranges from the laboratory that performed the analysis (Daly & Hackney, 2005; Tohidi et al., 2014).

Respiratory and pulmonary gas-exchange variables were continuously measured during the ramp test (Metalyzer 3B, Cortex, Germany). The gas analyser was calibrated before each test with gases of known concentration (15% oxygen and 5% carbon dioxide). According to the manufacturer's instructions, the turbine volume transducer was calibrated with a 3-L syringe. Collected ventilatory data were processed to exclude errant breaths (e.g., caused by swallowing, coughing, and signal interruptions). For the analysis, only data between the mean $\pm$ 3 SD

were considered, which were subsequently smoothed with a moving and time average of three breaths and 10 s, respectively (Sousa et al., 2015). Maximal oxygen uptake (measured over the last 30 s) was defined when (despite an increase in power) occurred an occurrence of an oxygen uptake plateau (< 2.1 mL/kg/min), respiratory exchange ratio ≥ 1.0 , heart rate $> 90\%$ of (220 minus age) and volitional exhaustion controlled visually (Howley et al., 1995).

Statistical analysis

The normality and homogeneity of distribution were checked using the Kolmogorov–Smirnov test for all the assessed variables, and mean values $\pm$ SD were calculated. Pearson correlation coefficients were calculated between insulin, cortisol, and ACTH concentrations. The threshold used to assess the correlation values qualitatively was based on the following criteria (Hopkins et al., 2009): < 0.1 (trivial), 0.1 – 0.3 (small), 0.3 – 0.5 (moderate), 0.5 – 0.7 (large), 0.7 – 0.9 (very large) and > 0.9 (nearly perfect). Magnitude-based inference and precision of estimation were calculated to assess practical significance with 95% confidence intervals (CI; Hopkins et al., 2009), and correlations are presented as $r \pm 95\%$ CI. Linear regression modelling assessed the insulin concentration variance determined by cortisol and ACTH. All statistical procedures were conducted with SPSS 27.0, and the significance level was set at 5%.

Results

The concentration values of ACTH, 24-hour urinary cortisol, and fasting morning plasmatic insulin and the correlations between ACTH and cortisol with insulin are presented in Table 1. Of the 143 cyclists, 28 presented hypoinsulinemia and 17 hypercortisolism. Of the 17 cyclists with hypercortisolism, eight also showed hypoinsulinemia. Both ACTH and cortisol presented a clear, small negative correlations with insulin (see Table 1). In addition, as expected, ACTH and cortisol presented a clear, moderate positive correlation ($r = 0.36$, $0.20 - 0.49$ 95% CI; $p < 0.001$).

Table 1. ACTH, 24-hour urinary cortisol and fasting morning plasmatic insulin concentration values, and correlations with insulin.

Hormones	Mean $\pm$ SD	Minimum	Maximum	Pearson r (95% CI)	p
ACTH (pg/mL)	26.0 $\pm$ 12.1	5.0	58.8	-0.23 (-0.38; -0.07)	0.005
Cortisol (μ g/24h)	73.9 $\pm$ 38.6	15.2	212.5	-0.21 (-0.36; -.05)	0.011
Insulin (μ IU/mL)	4.6 $\pm$ 1.7	0.5	9.3	-	-

Abbreviations: ACTH, Adrenocorticotrophic hormone; SD, standard deviation.

Figure 2 shows the dispersion graphics of the pairs ACTH – cortisol, cortisol – insulin, and ACTH – insulin concentrations with the respective linear regression lines. ACTH and cortisol can explain approximately 5% of the variance observed in the insulin blood concentration, with a lower dispersion in the higher ACTH and cortisol concentrations. The insulin concentration is negatively affected by the rise of ACTH and cortisol concentrations.

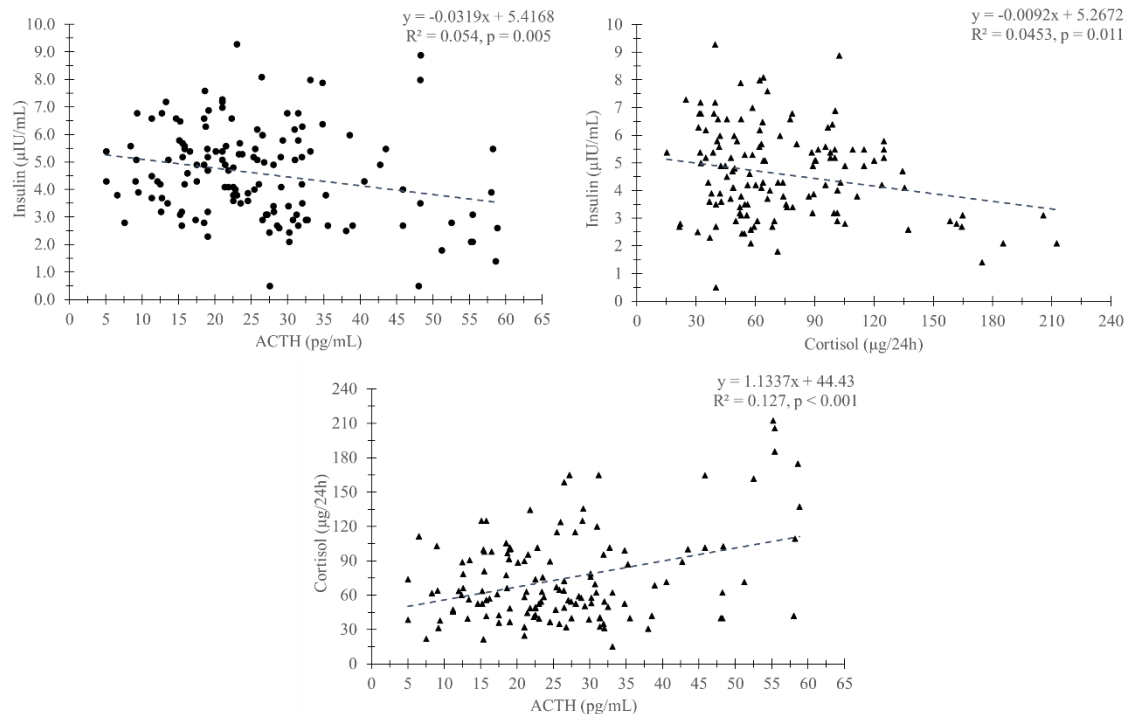


Figure 2. Dispersion (n = 143) of the concentrations pairs: ACTH – cortisol (bottom panel), cortisol – insulin (upper right panel) and ACTH – insulin (upper left panel) with the respective linear regression lines, equations, coefficient of determination (r^2) and p value. Abbreviations: ACTH, Adrenocorticotrophic hormone.

Discussion

Even knowing that no single marker indicates impending OTS (Meeusen et al., 2013), we characterized a significant sample of well-trained cyclists to understand their cortisol-insulin balance. In this study, we hypothesized that high cortisol production in high-endurance athletes could impair insulin release. That hypothesis was confirmed, suggesting a new and relevant role of insulin as a biomarker of sports performance.

Our results revealed an inverse relationship between 24-hour urinary free cortisol and plasmatic fasting morning insulin, with maximal cortisol values corresponding to insulin values below the standard lower reference threshold of 3 μ IU/mL (Tohidi et al., 2014). In contrast, plasmatic glucose levels showed no alterations (data not published). These low levels of fasting morning insulin, which has been reported as an adaptation process through a greater sensitivity in aerobic endurance athletes to optimize lipid oxidation (Goodpaster et al., 2001), should be further analysed since it might eventually compromise its efficacy (Borghouts & Keizer, 2000).

The 24-hour urine-free cortisol collection was chosen to represent an integrated measure of 24-hour cortisol secretion. This analysis corrects the normal daily circadian rhythm of the HPA axis since resting cortisol is not a helpful measure to assess daily cortisol release accurately (Duclos et al., 1997; Duclos et al., 2007). Furthermore, the data collection was performed in the spring period to avoid possible alterations associated with the seasonal rhythmicity of cortisol release (Gouarne et al., 2005).

During prolonged training of endurance athletes, glycogen stores approach total depletion, decreasing glycogenolysis and glucose transport in muscle and liver, IGF-1 production in the liver, and catabolism is induced (Steinacker et al., 2004). Increases in cortisol circulation have been observed during prolonged endurance

exercise (Daly & Hackney, 2005) as a response to the organic stress associated with the increased training load along the macrocycle.

Also, in conditions of energy deficiency, cortisol concentrations are usually elevated during severe caloric restriction or fasting periods (Elliott-Sale et al., 2018). The resulting hyperproduction of cortisol can inhibit anabolic stimulation locally in the muscle, mainly by inhibiting the production of insulin and IGF-1 and decreasing the signalling mediated by amino acids involved in regulating muscle protein synthesis. This anabolic reduction is accompanied by inhibition of the mammalian target of rapamycin (mTOR) (Liu et al., 2004). Several studies also suggest the inhibition of the Phosphoinositide 3-kinases / Protein kinase B (PI3K/Akt) pathway by glucocorticoids, responsible for mediating the anabolic actions of insulin/IGF-1 (Braun & Marks, 2015; Hu et al., 2009; Nakao et al., 2009), and thus compromising the regenerative synthesis of proteins (Liu et al., 2004). Other studies have reported an inhibitory effect of high cortisol levels on testosterone production (Daly & Hackney, 2005; Lane et al., 2015), possibly compromising the recovery and performance of athletes due to its role in protein synthesis and erythropoiesis (Shahani et al., 2009).

Reduced insulin levels have also been observed during prolonged exercise (catecholamines inhibition effect) and in energy-deficient athletes (Elliott-Sale et al., 2018), adding the anabolic commitment mentioned above to the cortisol inhibitory effect on testosterone. More recently, a longitudinal study during the sports season in high-level endurance runners demonstrated a decrease in basal insulin values compared to initial values throughout the season, being more visible in periods of higher training volume (Alves et al., 2020). Other studies also reported a negative correlation between insulin and training volume in elite rowers (Jürimäe et al., 2006), a significant reduction in exercise-induced insulin level (Elliott-Sale et al., 2018), and an increase in insulin resistance in periods of overtraining (Petibois & Déléris, 2004). Moreover, it can negatively influence bone health by decreasing bone mineral density, increasing the risk of stress

fracture so common in long-distance runners (Korpelainen et al., 2001), fertility (Hackney, 2008), and cardiovascular function.

Cycling is considered a significant risk factor for LEA, even in male practitioners, due to the inherent characteristics of this sport: low impact, high energy demands and a common perception that leanness is fundamental (Schofield et al., 2021), with constant energy deficits throughout the season as well as low bone mineral density due to the unloaded bearing nature of the sport correlated with LEA (Barry & Kohrt, 2008; Olmedillas et al., 2012). It is also commonly characterized by an endocrine system dysfunction with elevated cortisol and reduced testosterone (Torstveit et al., 2018; Torstveit et al., 2019) and IGF-1 (Heikura et al., 2019; Torstveit et al., 2019). However, LEA markers are not mandatory for the OTS condition. Therefore, the absence of intake control of the participants could not be considered a limitation of this study.

Complementary, the analysis of ACTH concentrations revealed a direct relationship between 24-hour cortisol release, as expected by the normal function of the HPA axis. Additionally, we observed an inverse relationship between ACTH and insulin, corroborating that these hormones are released under feedback control, some controlled by their peripheral concentration and others by other hormones or circulating cytokines, metabolites, osmolality, or other factors. This feedback relationship demonstrates why hormone status should be assessed in context rather than by isolated hormone assessments. Consideration should also be given to the fact that physiological processes related to endocrine balance are influenced by more than a single hormone in an integrated manner and at multiple levels (Barat et al., 2008; Duclos & Tabarin, 2011).

Furthermore, the adaptation of the HPA axis to chronic training is characterized by a rise in the ACTH/cortisol ratio only during the recovery process due to increased pituitary resistance to cortisol (Duclos et al., 1997; Lehmann et al., 1993), as well as variation in tissue sensitivity to glucocorticoids (Duclos et al., 2003), and, it should be noted that in high-endurance athletes, 24-hour urinary

cortisol production on non-training days should be normalized (Duclos et al., 2003). Thus, theoretically, morning plasma and 24-hour urinary cortisol production in high-endurance athletes at rest would be similar to sedentary individuals of the same age (Duclos et al., 1997; Gouarne et al., 2005; Kern, 1995).

In our study, on the day of 24-hour urinary free cortisol collection (48 hours before the cardiopulmonary exercise test), although the subjects performed short and low-intensity training, therefore abstaining from relevant cortisol production, 17 of the 143 evaluated cyclists presented high cortisol levels within 24 hours (115 µg/dL, based on the ranges given by the local laboratory). This high value might indicate that cortisol production may come from other stimuli rather than physical effort, namely mental stress associated with the competition process, representing the sum of multiple life stressors (Anderson & Wideman, 2017). Since our research is a transversal approach, we must conduct longitudinal studies based on the concepts debated in the current discussion to confirm our assumptions.

Another possible explanation for hormonal imbalances could be related to the long-term planning of the athlete's training, based on macrocycles, developed to a singular competitive objective (Anderson & Wideman, 2017). In this case, daily cortisol values may not return to the sedentary levels, which could be due to various factors such as inadequate recovery from acute training, imbalances in caloric intake with LEA, training monotony (one of the triggers for OTS), sleep disturbances, exposure to environmental stressors (such as heat, high humidity, cold, high altitude) or increased tissue or pituitary resistance to cortisol.

Hence, hormonal imbalances in the catabolic/anabolic context can be interpreted as a maladaptation process as described by Selye's General Adaptation Syndrome, representing the chronological development of the response to stressors, when their activation is prolonged, consisting of three phases: "the alarm reaction, the resistance state, and the exhaustion state" (Selye, 1976).

Although analytical changes can precede physical symptoms, adaptation is not always guaranteed (Meeusen et al., 2013). Prevention is critical, and even with training periodization, maladaptation can still occur, increasing the risk of sports injuries and relapse (Meeusen et al., 2013). Recovery is essential for prevention, and an excessive amount of catabolism or insufficient anabolism can negatively affect it, leading to increased proteolysis, decreased protein synthesis, and myocyte proliferation inhibition, thereby inhibiting muscle recovery (Martín et al., 2018).

Conclusion

So far, only a few studies have pointed out insulin as a biomarker of sports performance, and when used, it is almost exclusively linked to pathological conditions. However, our analysis detected a significant decrease in the insulin anabolic component in high-endurance male athletes when presenting high levels of cortisol and ACTH. These findings suggested insulin's new and relevant role as a sports performance training monitoring biomarker. The remaining question we need to ask ourselves is, does these athlete's imbalance in their catabolic/anabolic hormonal profile be prejudicial for their performance and impact their health, or is it just an adaptation to training? We should consider hormonal imbalances such as hypoinsulinism, if confirmed as chronic, as a possible cause of compromising the athlete's physical and mental health, which could lead to disease.

Tracking hormonal balance in endurance athletes. Does cardiovascular fitness mitigate cortisol anti-anabolic actions?

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Abstract

We aimed to evaluate the cardiovascular fitness effect on the anti-anabolic action of hypercortisolism in male road cyclists. Thirty cyclists (11 elite, 19 non-elite; age 29.4 ± 5.9 years) underwent fasting blood sample collection for insulin, testosterone, and ACTH analysis, and completed a maximal oxygen uptake test (separated by ≥ 48 h). Also, a 24-hour urine collection was analysed for cortisol. Comparisons between elite and non-elite cyclists and Pearson correlation coefficients among hormone levels were assessed at a 5% significance level. Cortisol exhibited negative correlations with insulin in both elite ($r = -0.94$, $p < 0.001$) and non-elite ($r = -0.51$, $p = 0.027$) groups and negative correlation with testosterone only in the non-elite group ($r = -0.70$, $p < 0.001$). Also, in the non-elite group, insulin and testosterone displayed a positive correlation ($r = 0.58$, $p = 0.009$). A clear negative correlation between ACTH and insulin was solely seen in the elite group ($r = -0.94$, $p < 0.001$). Among cyclists with normal insulin, the elite cyclists presented higher cortisol (118.7 ± 8.6 - 107.4 ± 11.7 $\mu\text{g}/24$ h, $p = 0.046$, $\text{ES} = 1.1$) but similar insulin (5.2 ± 0.5 - 5.2 ± 1.6 $\mu\text{IU}/\text{mL}$, $p = 0.968$, $\text{ES} = 0.0$) concentration values than the non-elite ones. Elite cyclists with superior cardiovascular fitness (> 70 $\text{mL}/\text{Kg}/\text{min}$) exhibited a threshold of cortisol anti-anabolic effect higher than the non-elite. Insulin emerged as a more sensitive biomarker than testosterone in controlling the catabolic/anabolic balance in elite male cyclists.

Keywords: Hormonal balance, Performance, Health, Cycling, Endurance

Introduction

Physical endurance training has been shown to decrease the risk of cardiovascular diseases and provide direct endogenous cardiovascular protection (Kaminsky et al., 2019; Li et al., 2020). It enhances metabolic and cardiovascular functions, positively influencing overall fitness and reducing the risk of coronary artery disease (Li et al., 2020). Endurance exercise induces cardiovascular adaptations, including a decrease in resting heart rate, an increase in stroke volume, elevated maximal cardiac output, and increased maximal oxygen uptake ($\dot{V}O_{2\max}$) (Braun, 1991; Burtcher et al., 2022; Kaminsky et al., 2019), thus lowering the risk of maladaptation to training (Meeusen et al., 2013).

However, endurance training induces stress in the organism, leading to significant changes in the endocrine system to recover the initial homeostasis (Hackney & Walz, 2013). Prolonged periods of endurance training, characteristic of sports like road cycling, may result in maladaptation due to an inability to cope with excessive load with inadequate rest and recovery. This compromise, among other things, can affect the neuroendocrine function (Faria et al., 2005; Kreher & Schwartz, 2012; Meeusen et al., 2013). Furthermore, external stressors unrelated to training, such as inadequate nutrition (leading to chronic low energy availability [LEA]), potentiate the risk for these imbalances (Cadegiani et al., 2020; Meeusen et al., 2013; Schofield et al., 2021).

Central to this context is the balance between catabolic and anabolic hormones (Cadegiani et al., 2020; Meeusen et al., 2013). Cortisol, the primary glucocorticoid known as the stress hormone, plays a crucial role in the integrated adaptive response to stress and training (Gouarne et al., 2005; Smith & Vale, 2022), regulating metabolic processes, including catabolic and anti-anabolic actions, inflammation and immune modulation (Ando et al., 2021; Katsu et al., 2015). Produced in the adrenal gland cortex and controlled by negative feedback in the hypothalamic-pituitary-adrenal (HPA) axis (Malkoski & Dorin, 1999; Stocco &

Clark, 1996), cortisol significantly increases during prolonged endurance exercise, and with its catabolic action, facilitates the rise of blood glucose levels to maintain adequate energy supplies (Viru & Viru, 2004).

In contrast, anabolic hormones like testosterone and insulin contribute to post-exercise regenerative muscle recovery. With its ergogenic and anabolic functions, testosterone promotes protein synthesis, red blood cell production, and glycogen replenishment. A decrease in testosterone levels may signal an overload situation and lead to a performance decline during a training season (Alves et al., 2020; Lee et al., 2017). Typically, cortisol antagonizes testosterone, inhibiting protein synthesis and interfering with anabolic signalling (Kraemer et al., 2020; Lee et al., 2017). Additionally, insulin, the primary anabolic hormone, plays a central role in muscle growth and protein turnover for muscle recovery by promoting the deposition of energy substrates from food into the form of glycogen (O'Neill et al., 2015; Urhausen et al., 1995), with its interactions with exercise being fundamental for metabolic regulation and homeostasis (Horton et al., 2006).

Road cycling is a modality that involves prolonged endurance training in athletes with a major risk for LEA, which poses a greater risk of hormonal imbalances (Heikura et al., 2019; Lucía et al., 2001; Schofield et al., 2021). The impact of cardiovascular fitness on cortisol hormonal ranges for assessing catabolic/anabolic balance is a topic that requires further investigation. Therefore, the present study aimed to evaluate the anti-anabolic effects of cortisol in elite male cyclists and how cardiovascular fitness copes with that response. We hypothesize that hypercortisolism could impair insulin and testosterone release and that the adaptations promoted by high cardiovascular fitness could mitigate this detrimental effect.

Methods

Participants

Thirty male cyclists (age 29.4 ± 5.9 years), regularly involved in competitive cycling events over the last five years, volunteered to participate in this study. The inclusion criterion for participation was cortisol concentrations ≥ 100 ($\mu\text{g}/24$ h) since the objective was to analyse cardiovascular fitness's possible protective effect on hypercortisolism's anti-anabolic effects (UCLA-Health, 2023). The cyclists were then divided into two groups (elite and non-elite) based on their maximum oxygen uptake, using > 70 ($\text{mL}/\text{Kg}/\text{min}$) as a cut-off threshold (Lucía et al., 2002). They gave their written informed consent after being informed of all experimental procedures and possible risks associated with the experiments. Before the study, all cyclists underwent a medical questionnaire to ensure that no exogenous anabolic-androgenic steroids, drugs, or other supplements that might affect physical performance or hormonal balance were taken during the study period. Ethical approval was obtained by the with the local research Ethics Committee (CEFADE 20_2023), and all procedures complied with the Declaration of Helsinki Ethics Code. All data were collected between March and April to minimize the seasonal cortisol rhythm and ensure similar phases of the training season.

Study design

All experimental procedures were carried out in the Exercise Medical Centre Laboratory (CMEP), Porto, Portugal, on two occasions (separated each by at least 48 h). Familiarisation was unnecessary since cyclists were regularly tested in this setting during the past two competitive seasons. The subjects were instructed to arrive at the laboratory rested (no strenuous exercise performed in the preceding 48 h) and refrain from alcohol and caffeine-containing drinks for the last 24 h. In the first visit, body composition was assessed, and a venous blood sample was collected (always withdrawn at 08.00 am by the same technician) in a fasting state for insulin, testosterone, and ACTH analysis. Additionally, the subjects delivered a 24-hour urine collection collected on the

previous day for the total free cortisol secretion analysis. In the second visit (around noon), participants performed an incremental ramp protocol test until exhaustion (RMP) to assess their $\dot{V}O_2\text{max}$, assumed as cardiovascular fitness, and to allocate the cyclists to the elite or non-elite group.

Ramp protocol test

The cyclists performed on an electromagnetically braked cycle ergometer (Lode Excalibur Sport, Groningen, The Netherlands) a ramp test consisting of a 9-12 min incremental protocol, starting at 0 W with increments of 35-45 W (0.58 – 0.75 W/s) every minute, with a self-selected cadence between 70 and 90 rpm (Sousa et al., 2015). The test started with a standard 3-minute rest period followed by an unloaded baseline pedalling (0 W). Every subject chose his cadence and was requested to keep it constant for the duration of the test with the aid of a visual revolution counter. The power increments were set according to the subject's previous tests and controlled by visual feedback. The cyclists used their own pedals and racing shoes and adjusted the seat height and handlebar positions to the individual morphological characteristics and habitual racing position. The test ended when the cadence could no longer be maintained within 10 rpm of the preferred cadence for more than 5 seconds. Standard room temperature (18-22° C) and air humidity (40-50%) were established. Verbal encouragement was given to motivate the subjects to perform their best effort for as long as possible. All tests were performed in the conventional seated posture.

Experimental measurements

After the venous blood samples were withdrawn, plasma was immediately separated by centrifugation (10 min at 3,500 revolution/min; 4° C), deposited in aliquots, and directly stored at -80° C for subsequent analysis. Insulin, testosterone, and ACTH were assessed in venous blood collection using the immuno-chemiluminescence method (Kricka, 2003). The 24-hour urinary-free cortisol was analysed after collecting the entire urine volume for a whole day, not counting the first urine of the day, and ended after collecting the first urine of the following day. The sample was then centrifuged (5 min at 2,500 revolution/min;

15° C), with a prior preparation with Dichloromethane, and then analysed in Cobas (cobas® 6800 System, Roche, Switzerland). Cortisol was then detected using the immuno-chemiluminescence method (Kricka, 2003). After assessing all the concentrations, the participants were classified by presenting hypo and normal-insulinemia (using > 3.0 µIU/mL as a cut-off threshold) based on the ranges from the laboratory that performed the analysis (Daly & Hackney, 2005; Tohidi et al., 2014).

Respiratory and pulmonary gas-exchange variables were continuously measured during the ramp test (Metalyzer 3B, Cortex, Germany). The gas analyser was calibrated before each test with gases of known concentration (15% oxygen and 5% carbon dioxide). According to the manufacturer's instructions, the turbine volume transducer was calibrated with a 3-L syringe. Collected ventilatory data were processed to exclude errant breaths (e.g., caused by swallowing, coughing, and signal interruptions). For the analysis, only data between the mean $\pm$ 3 SD were considered, which were subsequently smoothed with a moving and time average of three breaths and 10 s, respectively (Monteiro et al., 2022; Sousa et al., 2015). The $\dot{V}O_{2\max}$ (measured over the last 30 s) was defined when (despite an increase in power) occurred an occurrence of an oxygen uptake plateau (< 2.1 mL/kg/min), respiratory exchange ratio $\geq$ 1.0, heart rate > 90% of (220 minus age) or volitional exhaustion controlled visually (Monteiro et al., 2022).

Statistical analysis

The normality and homogeneity of distribution were checked using the Shapiro–Wilk test for all the assessed variables, and mean values $\pm$ SD were calculated. T-test for independent groups were performed to determine differences between hormonal concentrations between the elite and non-elite groups with effect size estimation (Cohen, 2013). Subgroup analyses were performed between elite and non-elite with normal and hypo-insulinemia values. Based on the low number of subjects in the subgroup elite with hypo-insulinemia group, Mann-Whitney U test was performed to compare it to non-elite. Pearson correlation coefficients were calculated for cortisol, insulin, total testosterone, and ACTH concentration values.

The threshold used to assess the correlation values qualitatively was based on the following criteria (Hopkins et al., 2009): < 0.1 (trivial), 0.1-0.3 (small), 0.3-0.5 (moderate), 0.5-0.7 (large), 0.7-0.9 (very large) and > 0.9 (nearly perfect). Magnitude-based inference and precision of estimation were calculated to assess practical significance with 95% confidence intervals (CI; Hopkins et al., 2009), and correlations are presented as $r \pm 95\% \text{ CI}$. All statistical procedures were conducted with SPSS 29.0, and the significance level was set at 5%.

Results

Based on the $\dot{V}O_2\text{max}$ of the thirty cyclists, eleven were considered elite and nineteen non-elite, and three and eleven presented hypoinsulinemia (for elite and non-elite, respectively). The physical and fitness characteristics and concentration values of 24-hour urinary cortisol, ACTH, insulin, total testosterone and testosterone-cortisol ratio are presented in Table 1. The higher cardiovascular fitness of the elite group was confirmed and it was observed that they were lighter than their non-elite counterparts. The correlations between cortisol, insulin, total testosterone and ACTH concentration values are presented in Table 2. Cortisol showed a clear and nearly perfect negative correlation with insulin in the elite and a moderate negative correlation in the non-elite group, where higher 24-hour urinary cortisol consistently corresponded to lower insulin values. Also, a large negative correlation between cortisol and testosterone was observed only in the non-elite group, where higher 24-hour urinary cortisol values consistently corresponded to lower testosterone values. Insulin and testosterone only showed large positive correlations in the non-elite group, with higher insulin concentrations corresponding to higher total testosterone levels. Additionally, a clear and nearly perfect negative correlation between ACTH and insulin was observed only in the elite group.

Table 1. Physical characteristics, cardiovascular fitness and hormonal concentration values with comparisons between elite and non-elite groups.

Variables (mean $\pm$ SD)	All (n=30)	Elite (n = 11)	Non-elite (n = 19)	P value	Effect size (95% CI)
Height (cm)	175.2 $\pm$ 5.9	172.8 $\pm$ 4.7	176.6 $\pm$ 6.1	0.085	0.7 (-0.1 to 1.4)
Body mass (kg)	70.9 $\pm$ 9.6	64.4 $\pm$ 5.4	74.7 $\pm$ 9.5*	0.003	1.2 (0.4 to 2.0)
Body fat (%)	10.2 $\pm$ 2.9	10.0 $\pm$ 1.6	10.3 $\pm$ 3.4	0.709	0.1 (-0.6 to 0.9)
$\dot{V}O_2$ max (mL/Kg/min)	65.0 $\pm$ 9.9	76.4 $\pm$ 3.3	58.4 $\pm$ 5.3*	< 0.001	-3.9 (-5.0 to -2.6)
Cortisol (μ g/24h)	131.9 $\pm$ 33.3	134.0 $\pm$ 27.7	130.7 $\pm$ 36.9	0.802	-0.1 (-0.8 to 0.6)
Insulin (μ IU/mL)	4.0 $\pm$ 1.5	4.3 $\pm$ 1.5	3.9 $\pm$ 1.5	0.444	-0.3 (-1.0 to 0.5)
ACTH (pg/mL)	33.6 $\pm$ 16.1	31.7 $\pm$ 15.1	34.7 $\pm$ 17.0	0.641	0.2 (-0.6 to 0.9)
Total testosterone (ng/mL)	5.8 $\pm$ 1.9	5.6 $\pm$ 1.4	5.8 $\pm$ 2.1	0.772	0.1 (-0.6 to 0.9)
Testosterone/cortisol ratio	4.8 $\pm$ 2.2	4.4 $\pm$ 1.4	5.0 $\pm$ 2.5	0.450	0.3 (-0.5 to 1.0)

*Significant differences compared to elite cyclists. Abbreviations: ACTH, Adrenocorticotrophic hormone; SD, standard deviation.

Table 2. Correlations between 24-hour urinary cortisol, fasting morning plasmatic insulin, total testosterone and ACTH concentration values.

Pearson r (95% CI)	All (n=30)	Elite (n = 11)	Non-elite (n = 19)	P value
Cortisol-insulin	-0.61 (-0.79 to -0.32)	-	-	< 0.001
	-	-0.94 (-0.99 to -0.79)	-	< 0.001
	-	-	-0.51 (-0.79 to -0.07)	0.027
Cortisol-testosterone	-0.61 (-0.79 to -0.32)	-	-	< 0.001
	-	-0.25 (-0.74 to 0.41)	-	0.452
	-	-	-0.70 (-0.88 to -0.37)	< 0.001
Insulin-testosterone	0.51 (0.18 to 0.73)	-	-	0.004
	-	0.44 (-0.22 to 0.82)	-	0.174
	-	-	0.58 (0.17 to 0.82)	0.009
ACTH-testosterone	-0.20 (-0.53 to 0.17)	-	-	0.282
	-	-0.50 (-0.85 to 0.14)	-	0.118
	-	-	-0.12 (-0.54 to 0.36)	0.632
ACTH-insulin	-0.29 (-0.59 to 0.01)	-	-	0.124
	-	-0.94 (-0.99 to -0.78)	-	< 0.001
	-	-	0.08 (-0.39 to 0.52)	0.743

Table 3 compares the elite and non-elite groups with normal and hypo insulin values. The cyclists who presented normal insulin values, those in the elite group showed higher 24-hour urinary cortisol values, lower testosterone, similar insulin concentrations and lower testosterone-cortisol ratio compared to non-elite. From the cyclists who presented hypoinsulinemia values, those in the elite group showed lower insulin levels than non-elite ones. In addition, contrarily to elite, the non-elite cyclists from the hypoinsulinism group had lower testosterone values (7.8 ± 0.9 vs 4.4 ± 1.6 ng/mL, $p < 0.001$) than those with normal insulin.

Discussion

In this study, the primary objective was to investigate the potential protective role of cardiovascular fitness in mitigating the cortisol-induced anti-anabolic effect, specifically impairing insulin and testosterone release. The hypothesis posited that higher cardiovascular fitness could counteract this detrimental effect. The results revealed that elite cyclists with normoinsulinism exhibited higher cortisol levels compared to non-elite counterparts, despite similar insulin concentrations, but lower total testosterone concentrations and testosterone/cortisol ratio. Among cyclists with hypoinsulinism, the elite group displayed lower insulin levels, while testosterone levels remained like the non-elite group. Additionally, in the non-elite context, the hypoinsulinism group had significantly lower testosterone values compared to the normoinsulinism group.

The study confirmed our hypothesis since an inverse relationship was observed between 24-hour urinary free cortisol and plasmatic testosterone and insulin and, athletes with greater aerobic capacity, the cortisol effect on insulin concentrations occurred at a higher threshold. This suggests that cardiovascular fitness could mitigate this detrimental effect, promoting greater insulin sensitivity and cortisol tolerance, since considerably higher cortisol values were needed to reflect the decrease in insulin (only verified on three of the eleven elite cyclists). This heightened sensitivity can be attributed to training-induced adaptations in the vasculature, skeletal muscle, and adipose tissue, justifying lower basal insulin

Table 3. Comparisons between the elite and non-elite groups with normal and hypo insulin concentration values.

Variables (mean $\pm$ SD)	Elite normal (n=8)	Non-elite normal (n=8)	P value	Effect size (95% CI)	Elite hypo (n=3)	Non-elite hypo (n=11)	P value	Effect size (95% CI)
Height (cm)	171.5 $\pm$ 4.1	173.8 $\pm$ 3.7	0.268	0.6 (-0.4 to 1.6)	176.3 $\pm$ 5.1	178.7 $\pm$ 6.8	0.584	0.4 (-0.9 to 1.6)
Body mass (kg)	62.5 $\pm$ 5.2	70.1 $\pm$ 7.9*	0.040	1.1 (0.1 to 2.2)	69.3 $\pm$ 1.1	78.0 $\pm$ 9.5*	0.013	1.0 (-0.4 to 2.3)
Body fat (%)	9.2 $\pm$ 1.0	8.6 $\pm$ 2.4	0.527	-0.3 (-1.3 to 0.7)	12.0 $\pm$ 0.4	11.5 $\pm$ 3.6	0.700	-0.1 (-1.4 to 1.1)
$\dot{V}O_2$ max (mL/Kg/min)	77.2 $\pm$ 2.8	56.8 $\pm$ 5.4*	< 0.001	-4.7 (-6.7 to -2.7)	74.4 $\pm$ 4.1	59.6 $\pm$ 5.1*	< 0.001	-3.0 (-4.7 to -1.2)
Cortisol (μ g/24h)	118.7 $\pm$ 8.6	107.4 $\pm$ 11.7*	0.046	-1.1 (-2.1 to 0.0)	174.9 $\pm$ 10.5	147.8 $\pm$ 39.9	0.067	-0.7 (-2.0 to 0.6)
Insulin (μ U/mL)	5.2 $\pm$ 0.5	5.2 $\pm$ 1.6	0.968	0.0 (-1.0 to 1.0)	2.1 $\pm$ 0.7	2.8 $\pm$ 0.2*	0.008	2.0 (0.5 to 3.5)
ACTH (pg/mL)	23.7 $\pm$ 6.2	34.4 $\pm$ 17.0	0.129	0.8 (-0.2 to 1.9)	53.3 $\pm$ 6.7	34.9 $\pm$ 17.9	0.113	-1.1 (-2.4 to 0.3)
Total testosterone (ng/mL)	5.8 $\pm$ 1.3	7.8 $\pm$ 0.9*	0.003	1.8 (0.6 to 3.0)	5.2 $\pm$ 1.9	4.4 $\pm$ 1.6	0.474	-0.5 (-1.8 to 0.8)
Testosterone/cortisol ratio	4.9 $\pm$ 1.1	7.3 $\pm$ 0.9*	< 0.001	2.3 (1.0 to 3.5)	3.0 $\pm$ 1.3	3.4 $\pm$ 1.9	0.774	0.2 (-1.1 to 1.5)

*Significant differences compared to elite cyclists. Abbreviations: ACTH, Adrenocorticotrophic hormone; SD, standard deviation.

levels in well-trained athletes (Goodpaster et al., 2001). Also, the increased pituitary resistance to cortisol is a known adaptation of the HPA axis to chronic endurance training (Duclos et al., 1997; Lehmann et al., 1993), as well as the variation in tissue sensitivity to glucocorticoids associated with training intensity (Duclos et al., 2003).

Moreover, in the context of hypoinsulinism, no differences in testosterone levels were observed between elite and non-elite groups, implying that testosterone may be a less critical biomarker of allostatic load for elite athletes compared to insulin. Testosterone's relevance appears to be inversely proportional to the cardiovascular fitness level since, in the non-elite group, cyclists with hypoinsulinism had significantly lower testosterone values compared to the ones with normoinsulinism. In this sense, additional data collection corroborating our results may eventually suggest a higher cut-off value for hypercortisolism determination in elite endurance athletes since the 100 mcg/24h currently assigned (UCLA-Health, 2023) does not appear significant enough to affect anabolic processes negatively.

The selection of 24-hour urine-free cortisol collection provided an integrated measure of cortisol secretion, correcting for circadian rhythm variations of the HPA axis since resting cortisol is not a reliable measure to accurately assess daily cortisol release (Duclos et al., 1997; Duclos et al., 2007). Additionally, the data collection, conducted during spring, aimed to minimize potential seasonal rhythmicity effects on cortisol release (Gouarne et al., 2005).

Prolonged endurance training, over the macrocycle, increases cortisol circulation as a response to the organic stress associated with increased training loads (Alves et al., 2020; Daly & Hackney, 2005). Moreover, in conditions of energy deficiency, cortisol concentrations typically rise, especially during severe caloric restriction or fasting periods (Elliott-Sale et al., 2018). This hypercortisolism can inhibit local anabolic stimulation in the muscle, decreasing the production of insulin and insulin growth factor (IGF-1) and the signalling mediated by amino

acids involved in regulating muscle protein synthesis. This anabolic compromise can be accompanied by inhibition of the mammalian target of rapamycin (mTOR) (Liu et al., 2004). Others have suggested the inhibition of the Phosphoinositide 3-kinases / Protein kinase B (PI3K/Akt) pathway by glucocorticoids as responsible for the impairment of the anabolic actions of insulin/IGF-1 (Braun & Marks, 2015; Hu et al., 2009; Nakao et al., 2009), thus compromising the regenerative synthesis of proteins (Liu et al., 2004).

Additionally, low insulin levels have been observed during prolonged endurance exercise (catecholamines inhibition effect) (Kindermann, 1982) and in energy-deficient athletes (Elliott-Sale et al., 2018), potentiating the anabolic commitment to the anti-anabolic effect of cortisol mentioned above. A longitudinal study in well-trained endurance runners demonstrated a decrease in basal insulin values throughout the season, more noticeable in periods of higher training volume (Alves et al., 2020). Other studies reported a negative correlation between insulin and training volume in elite rowers (Jürimäe et al., 2006), a significant reduction in exercise-induced insulin level (Elliott-Sale et al., 2018), and an increase in insulin resistance in overtraining periods (Petibois & Déléris, 2004). Nevertheless, low fasting morning insulin levels, as above mentioned, have been reported as an adaptive mechanism among aerobic endurance athletes, enhancing its sensitivity and optimizing lipid oxidation (Horton et al., 2006). Nonetheless, a nuanced analysis is warranted, as excessively low values may eventually jeopardize its efficacy (Borghouts & Keizer, 2000).

Some studies have reported an inhibitory effect of high cortisol levels on testosterone production (Daly & Hackney, 2005; Lane et al., 2015), possibly compromising the recovery and performance of athletes due to its role in protein synthesis and erythropoiesis (Shahani et al., 2009). Chronic endurance training has been reported to negatively affect basal testosterone concentration, with chronically decreased values due to the accumulation of endurance training over the years (Hackney & Lane, 2018). In a recent study it was reported a significant decrease in basal testosterone levels three months into the season in runners

and in their pituitary stimulation (reduction in lutein hormone concentrations) (Alves et al., 2020), suggesting central inhibition (Krsmanovic et al., 2009; MacConnie et al., 1986). The authors (Alves et al., 2020) justified this response as an effect of the highest volume of km per week of the entire season, forcing them to carry out longer training sessions with less recovery, promoting the maintenance of high cortisol concentrations during that period. Other studies also suggested a negative relationship between testosterone concentrations and high volumes of endurance training in runners when exceeding 100 km/week (De Souza et al., 1994; Hooper et al., 2017), and a decrease in swimmers testosterone when it was implied an increase of training volume of 88% during two weeks (Flynn et al., 1997).

The literature reported corroborates, in part, our results, namely the reduction of insulin and testosterone in the context of hypercortisolism in chronic high-volume endurance training. However, it was suggested recently that volume of training alone is insufficient to predict low testosterone concentrations (Hackney, 2020), and it was proposed that changes in the gonadal hormonal system in endurance runners may be more linked to the development of LEA (Dipla et al., 2021). It has also been reported recently that athletes who perform chronic strenuous exercise (such as high-volume endurance training) have difficulty meeting the energy required by their daily training causing changes in the hypothalamic-pituitary-gonadal axis (De Souza et al., 2019). Cycling is a sport commonly characterised by all these above-mentioned endocrine imbalances, with elevated cortisol and reduced testosterone (Torstveit et al., 2018; Torstveit et al., 2019) and IGF-1 (Heikura et al., 2019; Torstveit et al., 2019), mainly because it is practiced by athletes with a significant risk factor for LEA, even in male practitioners, due to inherent characteristics such as low impact, high energy demand, and a common perception that leanness is mandatory (Schofield et al., 2021), leading to constant energy deficits throughout the season (Barry & Kohrt, 2008; Olmedillas et al., 2012), however, the chronic effect of endurance training may mitigate the risk of the aforementioned imbalances.

Additionally, our results showed an inverse relationship between ACTH and insulin for the elite group, confirming that these catabolic/anabolic dynamics are influenced by various factors such as their peripheral concentration or of other hormones or factors like caloric balance. This feedback relationship emphasizes the need to assess hormonal status in a context rather than through isolated assessments, considering the integrated nature of endocrine balance (Barat et al., 2008; Duclos & Tabarin, 2011).

Conclusion

This study highlighted two key messages. Firstly, elite road cyclists with higher cardiovascular fitness, particularly above 70 mL/kg/min, exhibit a higher threshold for the negative effects of cortisol on anabolic processes compared to their non-elite counterparts (lower cardiovascular fitness). This suggests that superior cardiovascular fitness may provide protection against the elevated catabolic hormonal concentrations, characteristic of this modality. Secondly, insulin emerged as a more sensitive biomarker than testosterone in controlling the catabolic/anabolic balance in elite male cyclists, suggesting its potential utility in training monitoring for high-endurance athletes.

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Hormonal and rate of perceived exertion behaviour during an ultramarathon event. Are cortisol and insulin real markers of extreme exertion?

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Abstract

BACKGROUND: The current work aimed to describe and compare the hormonal behaviour (based on cortisol and insulin concentrations) and rate of perceived exertion (RPE) during a 115 km ultramarathon race. **METHODS:** Twelve ultrarunners (ten males) were evaluated six times (0, 37, 60, 76, 89 and 115 km). At each moment, saliva samples (for cortisol and insulin assessment) and RPE (CR10 scale) were collected. Statistical analysis included correlation, one-way repeated measure ANOVA, and Statistical Parametric Mapping to compare hormonal and RPE profiles. **RESULTS:** Our main findings revealed an early peak in cortisol and RPE, accompanied by a decline in insulin responses (402 ± 49 min of the race, $p < 0.05$). Cortisol and insulin only showed magnitude differences with inverse behaviours until ~6% of the ultramarathon duration. Cortisol and RPE presented similar behaviours, rising initially and remaining elevated throughout the race ($\eta^2 = 0.91$ and $\eta^2 = 1.0$, $p < 0.001$). Insulin levels initially decreased, remaining below 60% of baseline values from the midpoint to the end of the race ($p = 0.04$). **CONCLUSIONS:** The study showed an imbalance in the catabolic/anabolic hormone profile during an ultramarathon race. Assessing the behaviour of these biomarkers and their correlation with RPE could help regulate stopping and intensity strategies during these extreme endurance races.

Keywords: ultramarathon, hormonal balance, health, performance, running

Introduction

Ultra-endurance sports, such as ultramarathons (distances exceeding 42.192 km), have recently gained popularity (Berger et al., 2023; Knechtle & Nikolaidis, 2018). The increasing number of participants and finishers in these long-distance running or hiking, which push the human body to its limits, has made ultramarathons increasingly attractive from a scientific perspective (Knechtle & Nikolaidis, 2018), allowing investigation and greater understanding of the boundaries of human performance (Millet & Millet, 2012; Saugy et al., 2013). As ultramarathons cover a wide range of distances, from 50 km to over 1,600 km, across varied terrains and formats of one and multi-day races, it is challenging to generalize and compare the results.

While the benefits of regular exercise are well-documented (Warburton & Bredin, 2017), the extreme nature of ultra-endurance raises questions regarding their impact on hormonal balance and overall health (Hohl et al., 2019; Ramos-Campo et al., 2016). Hormones regulate body functions, including metabolism, energy production, and immune response. Understanding how ultramarathons influence hormone levels is crucial for assessing potential health implications for athletes. Cortisol, known as the stress hormone, is the primary glucocorticoid in the body and is essential for the integrative adaptive response to stress (Smith & Vale, 2022). It represents the physiological reaction to exercise demands (Duclos & Tabarin, 2016), acting as a potent anti-inflammatory and immunomodulator agent (Hackney & Walz, 2013). Its endogenous secretion shows a variation of diurnal activity and nocturnal rest, representing a prominent circadian rhythm, with a daily peak around the sleep-wake transition and reaching minimal levels in the evening and early night (Oster et al., 2017). Despite this rhythm, cortisol significantly increases during endurance exercises to maintain an appropriate energy supply (Hill et al., 2008; Viru & Viru, 2004).

In contrast, insulin, the primary anabolic hormone, promotes the deposition of energy substrates from food into the form of glycogen, playing a central role in

muscle growth and protein turnover, promoting muscle recovery (O'Neill et al., 2015). Interactions between insulin and exercise are essential for metabolic regulation and homeostasis. In this sense, prolonged exercise has been reported to decrease plasma insulin levels due to increased catecholamines, which inhibit its secretion. This regulation is critical for glucose homeostasis and energy availability to the central nervous system (O'Neill et al., 2015). In addition, insulin concentration rises post-exercise to enhance glycogen replacement, decreasing carbohydrate oxidation and increasing lipid oxidation (Horton et al., 2006).

From a psychological point of view, resilience, and endurance are essential in these extreme sports to overcome pain and succeed (Ferrer et al., 2015). It is common in training and research to assess fatigue through the subjective rate of perceived exertion (RPE), which is recognized as a valid indicator of exercise intensity and physical homeostasis disruption (Eston, 2012). It has also been helpful in elucidating pace variations and strategies, as the rate at which RPE progresses during competition correlates with race duration and endpoint confidence (Eston, 2012; Ferrer et al., 2015).

To ensure that the participants' health is not compromised in events that combine extreme stress, exertion, and high pain tolerance, monitoring and understanding hormonal responses and fatigue perception are essential (Belli et al., 2018; Roebuck et al., 2018). Therefore, the study aimed to determine the behaviour of cortisol and insulin concentrations and the RPE during an ultramarathon and compare them since, as far as we know, no studies have investigated it in real competitive conditions. It was hypothesized that: i) cortisol and RPE would increase during an ultra-endurance race and insulin decrease, and ii) cortisol would have a direct relationship with RPE and an inverse relationship with insulin.

Material and Methods

Twelve ultrarunners athletes (ten males and two females) with the following characteristics (age 39.6 ± 5.0 vs. 46 / 43 years; height 178.2 ± 7.3 vs. 174 / 163

cm, weight 71.5 ± 5.6 vs. $58.0 / 58.0$ kg, % body fat 12.4 ± 2.4 vs. $15.6 / 19.6\%$, maximal oxygen uptake 58.0 ± 4.5 vs. $63.3 / 52.1$ mL·kg⁻¹·min⁻¹, for male and females respectively) entered the Madeira Island Ultra-Trail (MIUT) and voluntarily participated in this study. The MIUT is a continuous ultra-trail running competition, spanning a 114.8 km course over rough terrain at medium altitude (altitude range between 5 and 1740.5 m), with a total positive/negative elevation of + 7060 /- 7070 m. In the MIUT event analysed in the current work, of the 785 starters, 590 (~75%) completed the event.

Participants provided written informed consent after being briefed on all experimental procedures and possible risks associated with the experiments before the commencement of the study. Prior to the study, all subjects underwent a medical questionnaire to ensure the absence of any exogenous anabolic-androgenic steroids, drugs, or other supplements that could affect their physical performance or hormonal balance during the study. Ethical approval was obtained by the local Ethics Committee (CEFADE 20_2023), and all procedures complied with the Declaration of Helsinki Ethics Code and the Standards for Ethics in Sports and Exercise Science Research.

Procedures

Athletes were evaluated six times: pre-race (0 km) and at five designated stops (moments 37, 60, 76, 89, and 115 km, corresponding to 32, 52, 66, 77, and 100% of the race). At each moment, saliva samples were collected before food ingestion for subsequent analysis of cortisol and insulin, and RPE was assessed using the CR10 scale (Ritchie, 2012). Saliva samples were collected without stimulation by putting a cotton tube into the mouth until saturated, stored in the plastic tubes and then frozen at -70° C. Before analysis, saliva samples were clarified by centrifuging at 12 000 g for 5 min at 4° C. Cortisol and insulin levels were measured using commercially available ELISA kits (Salimetrics, Newmarket Suffolk, UK). Figure 1 presents a schematic diagram of the data collection done during the MIUT.

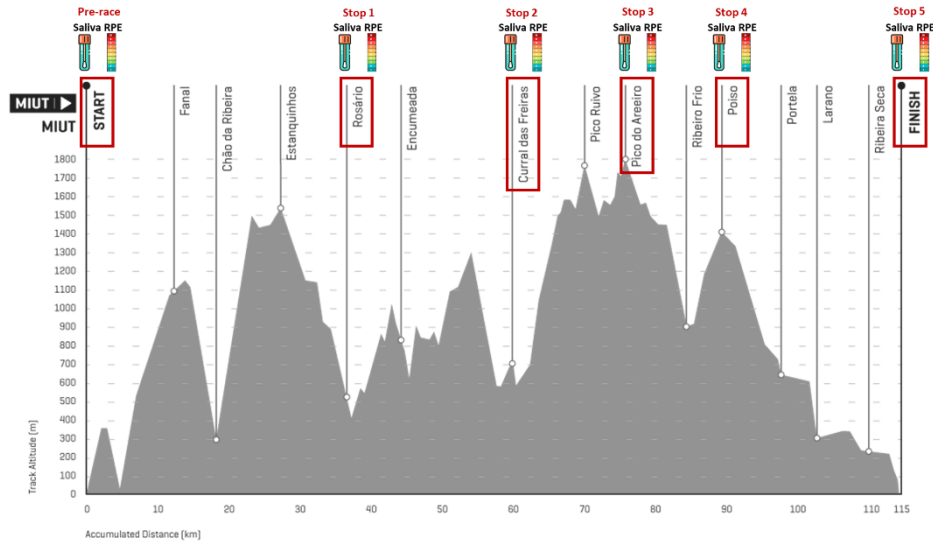


Figure 1. Schematic representation of the testing timeline during MIUT. Abbreviations: RPE, rate of perceived effort.

Race results were obtained from official reports, and running velocity was assessed dividing the distance covered by the time of each section, minus the stop durations. MIUT started at 00:00 am and the beginning running velocity was assessed at the first checkpoint (13.9 km). To allow the comparisons with previous studies in different ultramarathons, we also calculated the equivalent flat distances: $\text{positive elevation (m)} / 100 + \text{distance (km)}$ and velocities: $\text{equivalent flat distances (km)} / \text{race time (h)}$, as proposed by Saugy (Saugy et al., 2013).

Statistical Analysis

Statistical analysis was performed with the software SPSS 29.0 (IBM Statistics, USA) for Windows. The normality and homogeneity of distribution were checked using the Shapiro-Wilk test for all the assessed variables, and mean values $\pm$ SD were calculated. One-way repeated measures ANOVA with pairwise comparisons with Bonferroni correction was used to determine cortisol, insulin, RPE, and running velocity behaviour across the MIUT event, using partial eta-squared (η^2) for effect size calculation: 0.05-0.25 (small), 0.25-0.64 (moderate) and > 0.64 (strong; Ferguson, 2016). Also, a paired samples student's t-test with effect size estimation (Cohen, 1988) was used to assess the differences between the beginning and the end of the MIUT event.

Pearson correlation coefficients were calculated between all the assessed variables at each moment of the collection. The threshold used to determine the correlation values qualitatively was based on the following criteria (Hopkins et al., 2009): < 0.1 (trivial), 0.1-0.3 (small), 0.3-0.5 (moderate), 0.5-0.7 (large), 0.7-0.9 (very large) and > 0.9 (nearly perfect). Magnitude-based inference and precision of estimation were calculated to assess practical significance with 95% confidence intervals (CI; Hopkins et al., 2009), and correlations are presented as $r \pm 95\% \text{ CI}$. The Statistical Parametric Mapping (SPM) one-way repeated measure ANOVA and Bonferroni correction for pairwise comparisons was used to compare cortisol, insulin, RPE, and running velocity profiles in MATLAB (MATLAB R2019b, The MathWorks Inc., Natick, MA, USA) using the open-source code SPM1d (v.M0.1, www.spm1d.org; Pataky, 2010). All variables were interpolated, transforming the six moments into 100 data points using the spline transformation. Afterward, the data series were normalized based on their maximal value (100%), and the duration of MIUT was transformed to 0-100% of the event. For all statistical procedures, the significance level was set at 5%.

Results

Among the twelve ultramarathon runners, nine completed the total 115 km (75%, aligning with overall finishers), where three withdrew at km 70 (two males and one female). Finisher's performance data, RPE, and cortisol and insulin levels at each collection point are presented in Table 1. Running velocity changed along the event ($F_{5,45} = 68.01$, $\eta^2 = 1.0$, $p < 0.001$) but was similar between the initial 14 km and the final 25 km of the race ($p = 1.00$). However, when converted to flat running velocity, it decreased from the start to the end of the race ($F_{5,45} = 211.60$, $\eta^2 = 1.0$, $p < 0.001$). Increases in cortisol ($F_{5,45} = 8.10$, $\eta^2 = 0.91$, $p < 0.001$) and RPE ($F_{5,45} = 210.64$, $\eta^2 = 1.0$, $p < 0.001$), along with a decrease in insulin ($t = 2.44$, $ES (d) = 0.82$, $p = 0.04$), were observed from the race's beginning to the end. Only four correlations were found: two very large positive correlations between total time (minutes) and RPE at 37 km and between cortisol and RPE at the 60 km stop, and two very large negative correlations between running velocity and RPE at 37 km and between insulin and RPE at the 115 km (Table 2).

Table 1. Mean $\pm$ SD of the time of exercise, stop durations, running velocity, rate of perceived exertion, cortisol, and insulin concentration before and during MIUT.

Variables (n = 9)	Pre-race	Moment 14 km	Moment 1 37 km	Moment 2 60 km	Moment 3 76 km	Moment 4 89 km	Moment 5 115 km
Race (% of final distance)	-	12	32	52	66	77	100
Total time (minutes)	-	138 $\pm$ 14	402 $\pm$ 49	666 $\pm$ 89	957 $\pm$ 131	1152 $\pm$ 162	1419 $\pm$ 219
Total time (% of final time)	-	10 $\pm$ 1	28 $\pm$ 1	47 $\pm$ 2	68 $\pm$ 2	81 $\pm$ 1	100
Stop duration (min)	-	-	4.3 $\pm$ 3.1	12.1 $\pm$ 8.5	6.3 $\pm$ 5.7	6.1 $\pm$ 3.3	-
Running velocity (km/h)	-	6.1 $\pm$ 0.6	5.6 $\pm$ 0.6	5.3 $\pm$ 0.7	3.8 $\pm$ 0.7*	4.5 $\pm$ 0.8	5.9 $\pm$ 1.1*
Flat running velocity (km/h)	-	12.8 $\pm$ 1.2	10.4 $\pm$ 1.2*	8.5 $\pm$ 1.2*	7.6 $\pm$ 1.3	6.7 $\pm$ 1.2	6.2 $\pm$ 1.2
Flat running distance (km)	-	29.4	68.7	105	139.2	158.6	185.4
Total elevation gained (m)	-	1546	3127	4540	6331	6956	7060
Rate of Perceived exertion (CR10 Borg scale)	0 $\pm$ 1	-	5 $\pm$ 1 [#]	6 $\pm$ 1	7 $\pm$ 1	7 $\pm$ 1	7 $\pm$ 2
Cortisol (μ g/dL)	0.13 $\pm$ 0.07	-	0.88 $\pm$ 0.71 [#]	1.39 $\pm$ 0.67	1.46 $\pm$ 0.77	1.30 $\pm$ 0.80	1.15 $\pm$ 0.67 [#]
Insulin (μ IU/mL)	2.14 $\pm$ 1.50	-	0.62 $\pm$ 0.58 [#]	1.52 $\pm$ 0.78*	1.11 $\pm$ 0.92	0.94 $\pm$ 0.79	1.13 $\pm$ 0.98 [#]

*,[#] Significant differences between the previous moment and the pre-race (respectively), $p < 0.05$.

Table 2. Significant partial Pearson correlations between the pairs: running velocity - rate of perceived exertion, cortisol - rate of perceived exertion and insulin - rate of perceived exertion at different race moments.

Partial Pearson correlations (sex controlled)	r (95% CI)	p
Running velocity - rate of perceived exertion 37 Km	-0.81 (-1.00 to 0.49)	0.016
Total time (minutes)- rate of perceived exertion 37 Km	0.86 (-0.13 to 1.00)	0.006
Cortisol - rate of perceived exertion 60 Km	0.94 (0.79 to 0.99)	< 0.001
Insulin - rate of perceived exertion 115 Km	-0.74 (-0.96 to -0.32)	0.035

Figure 2 shows the mean behaviours (based on SPM1d) of running velocity, cortisol, insulin, and RPE along the MIUT event. Running velocity slightly decreased until 40% of the race, then reduced at a higher pace, reaching its minimum value at around 60%, followed by an increase to a new peak at the race's conclusion. Cortisol presented a crescent behaviour until 50% of the race, stabilized from 50 to 60%, slightly dropped until 80%, and remained stable again until the end, with maximal values presented between 40 and 60% of the race. Insulin initially decreased to 20% of the race, then increased and peaked at 40% before declining again until 80%, remaining 60% below initial levels until the end. RPE also presented a crescent behaviour until approximately 65% of the race, with peak values between 60-65%, stabilizing thereafter.

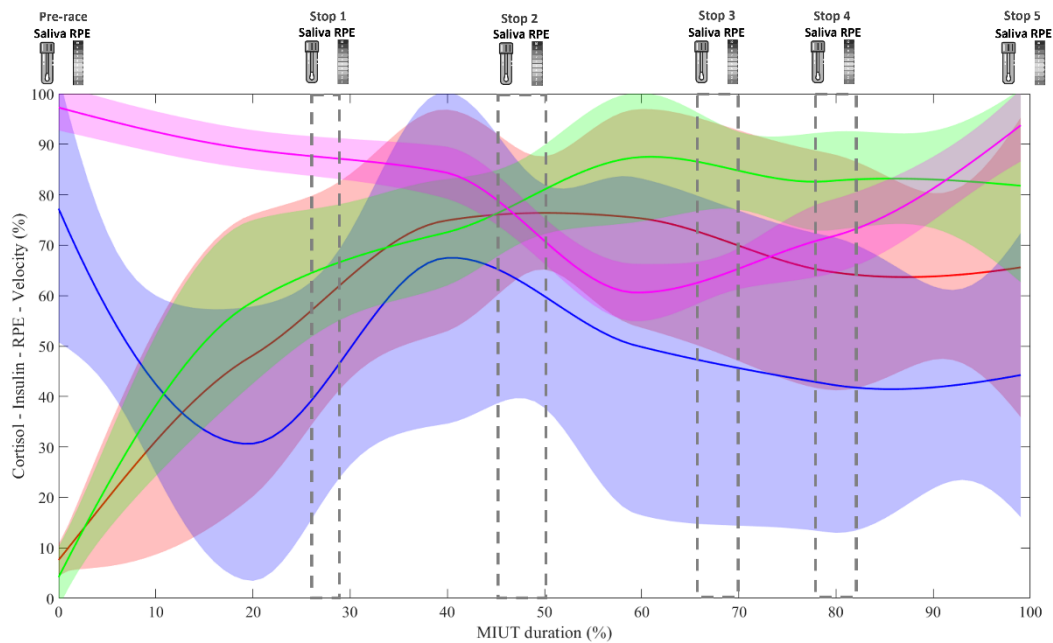


Figure 2. Mean and standard deviation curves of the behaviours of running velocity (magenta line) and cortisol (red line), insulin (blue line) concentrations and RPE (green line) along the MIUT event.

Figure 3 shows the ANOVA test based on SPM1d of running velocity, cortisol, insulin, and RPE magnitudes along the MIUT event. The differences were found in the first 38, at 50, and beyond 70% of the race duration. Figure 4 shows the pairwise comparisons of the magnitudes and behaviours of cortisol and insulin and both with RPE along the race. Initially (0 to 6,24%), cortisol and insulin

presented magnitude differences with inverse behaviour (cortisol rising and insulin decreasing). After that first moment, cortisol and insulin showed similar concentrations magnitudes. Insulin and RPE also presented magnitude differences with an inverse behaviour initially and near the race's end (0 to 6,43% and 96.51 to 100%), while cortisol and RPE presented similar magnitudes and behaviours throughout the event (from 0 to 100%). Figure 5 shows the pairwise comparisons of the magnitudes and behaviours of cortisol, insulin, and RPE with running velocity along the MIUT event. The running velocity presented a higher magnitude with an inverse behaviour compared to cortisol at the beginning of the race (0 to 17.5%), decreasing velocity and increasing cortisol. A higher magnitude was also observed compared to insulin (2.8 to 30.3 and 87.3 to 99%) and to RPE (0 to 31.7 and 53.0 to 73.6%).

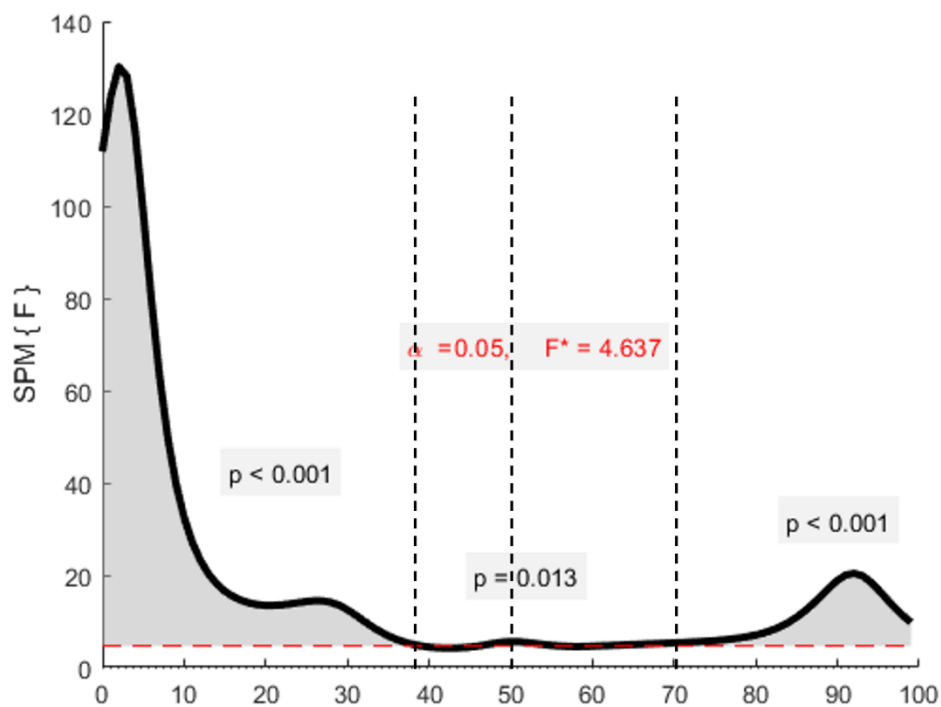


Figure 3. ANOVA test based on SPM1d of running velocity, cortisol, insulin and rating of perceived exertion behaviours along the MIUT event. Dashed lines represent the cut for the significant differences.

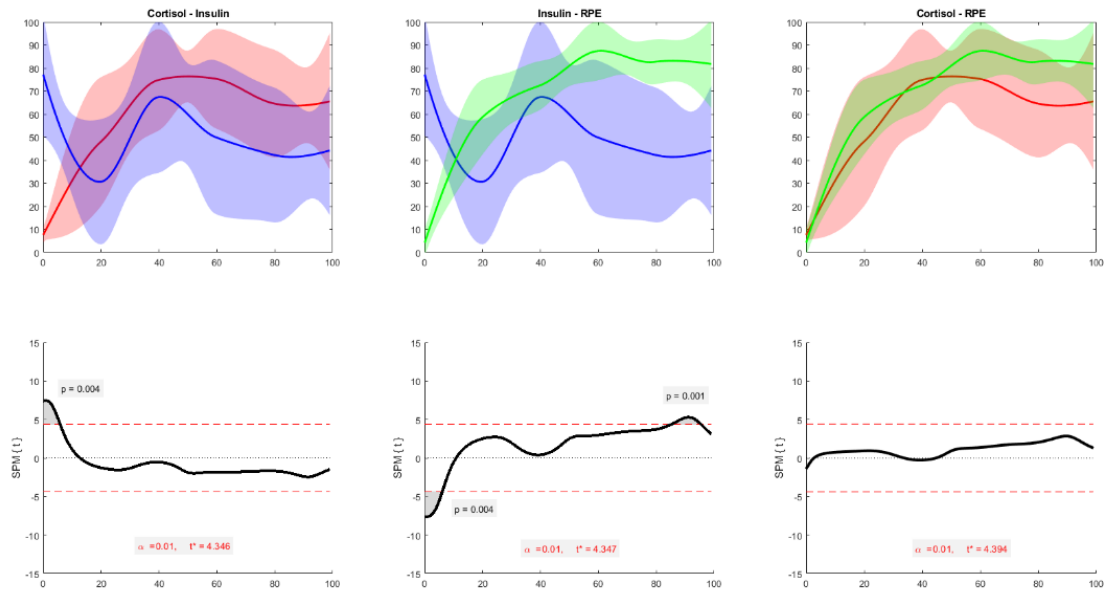


Figure 4. Comparisons of the behaviours of cortisol (red) and insulin (blue) concentrations (top left) and both with RPE (green: top middle and right panels) along the MIUT event.

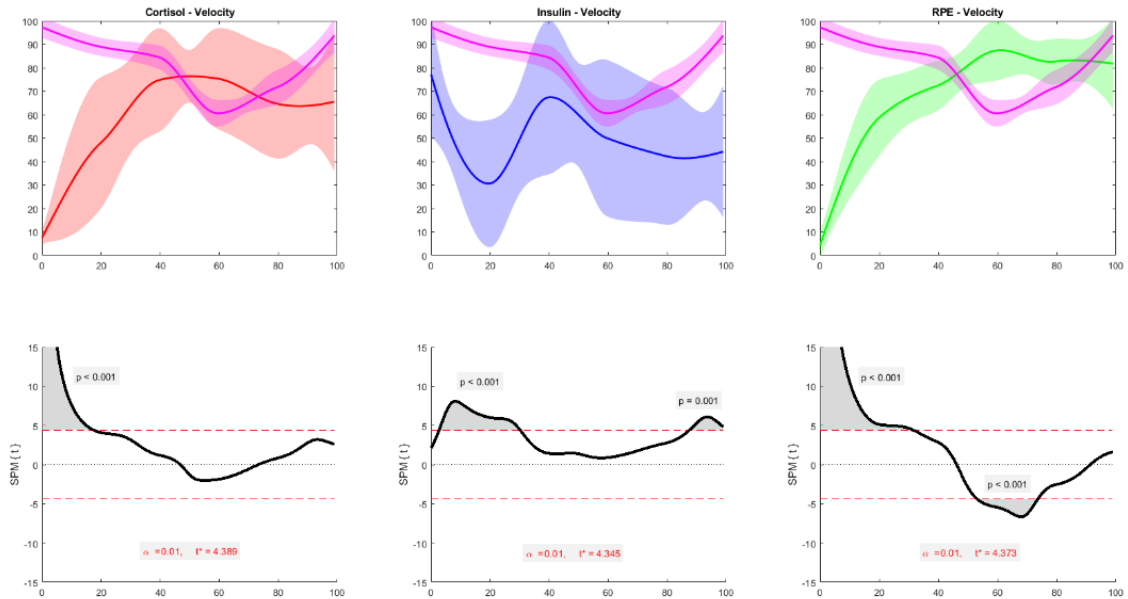


Figure 5. Comparisons of the behaviours of running velocity (magenta) and cortisol (red), insulin (blue) concentrations (top left and middle panels) and RPE (green: top right panel) along the MIUT event.

Discussion

The growing participation in ultramarathons such as MIUT in recent years underscores the importance of describing the hormonal responses during these extreme duration exercises (close to 24 h) as a disease prevention strategy (Ramos-Campo et al., 2016; Tauler et al., 2014). The current work aimed to describe and compare the hormonal behaviours (cortisol and insulin) and the RPE during a 115 km ultramarathon race. Our findings indicated an early peak from the catabolic and perceived exertion responses and a drop in the anabolic response. The primary hypothesis was confirmed since the increase in cortisol and RPE and the decrease in insulin were observed from the beginning to the end of the MIUT event. The second hypothesis was partially confirmed since cortisol and RPE presented similar magnitude and behaviours during the entire MIUT event; however, cortisol and insulin only presented a magnitude difference with inversed behaviour until approximately 6% of the race duration.

The hormonal behaviour analysis was possible due to statistical parametric mapping, interpreting the data as a continuous event rather than looking for each moment of stop isolated. The salivary hormonal assessment was chosen because it has already been deemed valid in comparing salivary and serum levels of hormones such as cortisol both in basal and under exercise conditions, presenting high correlations, being a reliable and non-invasive method to determine the physiological response to exercise (Cadore et al., 2008; Papacosta & Nassis, 2011).

Despite the increasing scientific interest in ultramarathons (Knechtle & Nikolaidis, 2018), data on exercise intensity and physiological demands during real competitive contexts is still scarce and almost non-existent (Berger et al., 2023). Nevertheless, in the few related studies, ultra-endurance exercises have been consistently characterized by intensities below the anaerobic threshold/onset of blood lactate accumulation, representing 64 to 82% of maximal heart rate (HR_{max}) in efforts between 7 and 14 h (Fornasiero et al., 2018; Ramos-Campo

et al., 2016), or even at lower HR for events of longer duration (Gimenez et al., 2013). Even though we did not collect the HR data, we can assume that, on average, in our subjects, it would be close to the previously reported since their maximal oxygen uptake was similar to other finishers of mountain ultramarathons (higher than 50 mL·kg⁻¹·min⁻¹) and the duration of the event was in the range mentioned above (Fornasiero et al., 2018; Ramos-Campo et al., 2016).

Ultra-endurance races impose an immense caloric requirement/deficit, necessitating a sufficient provision of metabolic fuels (Bizjak et al., 2022). Since the body's carbohydrate stores are limited, prolonged muscular activity amplifies the demand for carbohydrates. Cortisol, in its broad spectrum of tasks for metabolic processes control (Viru & Viru, 2004), increases significantly during prolonged endurance exercise to elevate blood glucose levels and facilitate fat and protein processing, providing energy to the muscles (Belli et al., 2018; Hackney & Walz, 2013; Kupchak et al., 2014). Elevated cortisol levels are also expected in conditions of energy deficiency with a severe caloric deficit, which is typical in these events (Elliott-Sale et al., 2018). This phenomenon could justify the behaviour found in our results, with an early increase and peak in the midsection and maintaining elevated throughout the race. These findings can be supported by previous studies that reported increases in cortisol levels following 160 km foot races (Kraemer et al., 2008; Kupchak et al., 2014), early surge during a 217 km mountain ultramarathon (highest value observed at 16 h; Belli et al., 2018), and others that showed that cortisol levels did not only increase at the beginning of the race but also remained augmented until the finish line (Belli et al., 2018; Bizjak et al., 2022; Hohl et al., 2019).

Furthermore, as the race began at midnight, with participants' cortisol at the predicted minimal levels, and as a response to the sustained stress imposed by the effort and to the circadian rhythm, it was expected that cortisol values would increase along the event, this way justifying the behaviour found and the high maintenance throughout the race (Oster et al., 2017). Also, in line with our data, it has been reported that in very long exercise, when the intensity is below the

anaerobic threshold (but still at 60% of maximal oxygen uptake), the activation of cortisol is not due to the intensity but to the duration of the exercise (Hill et al., 2008).

Other studies on ultramarathons have reported similar effects on cortisol levels, as well as increases in muscle damage and inflammation biomarkers (higher creatine kinase, lactate dehydrogenase, aspartate aminotransferase, and C-reactive protein levels) (Belli et al., 2018), depletion of the antioxidative capacity and increased inflammation (higher neopterin levels; Bizjak et al., 2022) and suppression of the anabolic testosterone levels (Kraemer et al., 2008; Tauler et al., 2014), thus enhancing the catabolic process. In addition, the hyperproduction of cortisol can also compromise the anabolic stimulation in the muscle by inhibiting insulin and IGF-1 production and decreasing signalling mediated by amino acids involved in the muscle protein synthesis regulation. This anti-anabolic effect is accompanied by the inhibition of the mammalian target of rapamycin (mTOR), compromising the regenerative synthesis of proteins (Liu et al., 2004), thus enhancing the anti-anabolic process.

Although insulin is mainly used in research related to diabetes, recent studies are giving it a new relevant role as a biomarker for training monitoring and assessing catabolic/anabolic balance, with the interaction between insulin and exercise being an example of the need to modify and adjust the effects of two opposing metabolic forces under varying conditions (Richter et al., 2021). Its decrease during exercise is critical for mobilizing glucose and fatty acids from the liver and adipose tissue (Richter et al., 2021; Wasserman et al., 1991). Therefore, in prolonged muscular activity, such as ultramarathons and with the depletion of glycogen reserves, the demand for carbohydrates from processing fats and proteins is amplified, suppressing insulin's effects on fuel storage (Richter et al., 2021).

Conversely, after exercise, there is an inverse need to replenish the fuel reserves mobilized during exercise, particularly the glycogen reserves in the muscle,

reducing carbohydrate oxidation and increasing lipid oxidation (Horton et al., 2006). This process is facilitated by increased insulin sensitivity of muscles previously involved in physical activity that direct glucose for glycogen resynthesis. This sensitivity is optimized with training due to vasculature, skeletal muscle, and adipose tissue adaptations, justifying lower insulin baseline levels in well-trained athletes (Goodpaster et al., 2001; Richter et al., 2021). Decreased insulin levels have also been reported during prolonged exercise (catecholamine inhibition effect) and in energy-deficient athletes (Elliott-Sale et al., 2018).

In our study, insulin initially decreased up to 20% of the race following cortisol rise. However, it then increased and peaked at 40%, which can be explained by the first stop (see Table 1) and can be assumed to be the end of the exercise and a new beginning. Another possible explanation can be the unavoidable food intake during this stage to avoid the energy deficit that characterizes this type of race (Bizjak et al., 2022). Even so, this behaviour was not repeated in the remaining stops, which can be interpreted either by the anti-anabolic cortisol effect or by the energy deficiency with a severe caloric deficit mentioned above. These factors may also justify the insulin behaviour after 40% of the race, decreasing until the end, reaching levels 60% lower than the initial levels. It is essential to consider that all the saliva collections in the programmed stops were performed before any food intake. Therefore, spikes in blood glucose did not influence the analysis.

RPE analysis plays a significant role in understanding athletes' perception of external load during competition. It was reported that the most motivated runners perform more distance in a 24-hour marathon (Ferrer et al., 2015), associated with an abnormally high pain tolerance, which could partially explain ultrarunners' ability to persist with endurance exercise for extremely long periods (Roebuck et al., 2018). Our findings suggest that RPE behaviour was influenced by the race's more challenging altitude sections, as it stabilized after 65% of the event, similar to the elevation gains diminishing.

Evidence suggests a link between the elevation of both cortisol and RPE during exercise (McGuigan et al., 2004). Cortisol, released in response to stress, tends to increase during and immediately after high-intensity exercise (Anderson et al., 2016; McGuigan et al., 2004). At the same time, the RPE, a subjective measure of how hard someone feels during an effort, follows the same behaviour (Eston, 2012). One possible explanation for the relationship between them is that cortisol may contribute to feelings of fatigue and exertion during exercise (Anderson et al., 2016) and also may interact with other stress-response hormones like adrenaline and noradrenaline and amplify perceived exertion (Duclos & Tabarin, 2016). These latter hormones are involved in the body's stress response, increasing heart rate, blood pressure, and respiration. While the relationship between cortisol levels and RPE during exercise is not fully understood, our results showed a link between the two. Further research is needed to fully explore the mechanisms underlying this relationship and its implications for exercise performance.

Complementary, along with the evaluated MIUT event, changes in running velocities (real and flat conversion) were consistent with previous studies on ultramarathons (Belli et al., 2018; Waśkiewicz et al., 2012) and had similar behaviours in all the evaluated participants. The more demanding phases of the race made it impossible to maintain constant velocities (e.g., altitude changes and rough terrains), which can explain the drop from the midsection compared to the beginning and the end. The flat conversion analysis confirmed the results, with the last race section (with a lower altitude gained compared to the rest of the race, +104 m) showing a lower flat running velocity than the midsection. The combined real or flat conversion analysis of distance and velocities showed the effect of the terrain difficulty and the intensity of the race and justified the stabilization of the RPE (one of the runners decreased RPE in the last section of the race).

The present study has the limitation of not considering food intake during the event, which may have influenced the interpretation of insulin levels.

Furthermore, the varying durations for different runners (19 to 30 hours) have the potential to influence circadian hormonal concentrations like cortisol (Oster et al., 2017). However, evaluations made during a real event help better understand the effects of such extreme efforts on hormonal balance. Future research should consider food intake and break times during ultramarathons and try to find the best relationship between them, and the intensity performed. Also, the recovery process of hormonal alterations should be analysed.

Conclusion

The present study found a relationship between hormonal and RPE responses to an ultramarathon lasting an average of 24 hours, with the increase and maintenance of high cortisol and RPE perceived by the athletes and the decrease and maintenance of low insulin levels during the race. The imbalance observed in the catabolic/anabolic hormonal profile during the ultramarathon should be further investigated, and its possible detrimental effect on the athlete's health should be considered. Assessing the behaviour of these biomarkers and the interpretation based on their RPE could help regulate stopping and intensity strategies during these extreme races.

Catabolic-anabolic dynamics of professional cyclists during the Tour of Portugal

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Abstract

Objectives: The study aimed to investigate hormonal responses and psychological states during a twelve-day stage road cycling competition.

Methods: Eight professional male cyclists (average age 26.8 ± 3.2 years and maximal oxygen uptake 72.2 ± 4.5 mL/kg/min) underwent evaluation throughout the twelve-day Tour, involving body mass, fat percentage, basal heart rate, and Profile of Mood State questionnaire (POMS) in the morning. Saliva cortisol samples were collected upon waking (AM) and before bedtime (PM), while serum was collected six times for insulin, insulin-like growth factor 1 (IGF-1), testosterone, adrenocorticotrophic hormone (ACTH), and hemoglobin analysis.

Results: Notably, body mass, body fat, cortisol PM, and POMS displayed no substantial changes during the cycling event ($p > 0.05$). A decrease in testosterone ($F_{3,27} = 4.95$, $\eta^2 = 0.41$, $p = 0.040$) and IGF-1 ($F_{3,27} = 11.6$, $\eta^2 = 0.62$, $p < 0.001$) until the morning of rest day (8) and subsequent increase until the end, was observed. ACTH ($F_{3,27} = 10.93$, $\eta^2 = 0.61$, $p < 0.001$) and hemoglobin ($F_{3,27} = 12.70$, $\eta^2 = 0.65$, $p = 0.003$) showed a decreased behaviour until the morning of day 8 and maintained low until the end. Cortisol AM maintained high concentrations between day 1 and day 12. However, an additional increase was observed between day 8 and day 12 ($F_{3,27} = 3.53$, $\eta^2 = 0.34$, $p = 0.032$). Insulin decreased until day 8, normalized on day 10, and decreased again on day 12 ($F_{3,27} = 6.27$, $\eta^2 = 0.47$, $p = 0.003$). **Conclusion:** The rest day during these long endurance competitions enabled the body to achieve catabolic and anabolic efficiency again. High cortisol values and the corresponding anabolic commitment may result from adaptations, specifically cortisol tolerance and insulin sensitivity, enabling the body to maintain overall performance.

Keywords: Road cycling, professional, hormonal dynamics, Tour of Portugal

Introduction

The Volta a Portugal, spanning twelve days of intense endurance effort, imposes significant organic demands on cyclists, leading to alterations in adaptive biomarkers and hormonal responses. Inadequate recovery from this demanding load poses risks, including potential compromises to neuroendocrine function (Faria et al., 2005; Kreher & Schwartz, 2012; Meeusen et al., 2013a). External stressors unrelated to the exercise, such as inadequate nutrition and low energy availability, further amplify the risk of imbalances (Cadegiani et al., 2020; Meeusen et al., 2013a; Schofield et al., 2021). The catabolic-anabolic hormonal dynamics during such events are crucial for organic balance and sports success. However, topics like hormonal sensitivities or tissue resistances, positive consequences of prolonged endurance training, remain insufficiently studied and analysed particularly in real competition conditions, despite the fundamental role of endocrine adaptations in performance.

The well-documented adrenal catabolic response to prolonged endurance exercise, mediated through the hypothalamic-pituitary-adrenal (HPA) axis, and the subsequent release of ACTH and cortisol is key (Meeusen et al., 2013b). Similarly, this type of exercise is associated with anabolic recovery responses and adaptations through the hypothalamic-pituitary-testicular (HPT) axis and pancreatic function. However, all these hormonal dynamics are released under feedback control, and their status is influenced by several factors such as their peripheral or other hormone concentrations, circadian rhythms, circulating cytokines, metabolites, osmolality, caloric balance, or mood (Kraemer et al., 2020; Lastella et al., 2015; Oster et al., 2017). This emphasizes the need to assess hormonal status in context rather than through isolated assessments, considering the integrated nature of endocrine balance at multiple levels (Barat et al., 2008; Duclos & Tabarin, 2011).

Multi-week professional cycling tours, such as the Tour de France, Giro d'Italia, Vuelta a España, or Volta a Portugal, strategically include rest days (Lucía, Hoyos, et al., 2001). These breaks are essential for the athletes' recovery and

overall well-being due to the intense physical demands of the race (Lucía, Díaz, et al., 2001; Raya, 2015) and are usually scheduled after a series of particularly challenging stages, such as those involving multiple mountain climbs (Lucía, Díaz, et al., 2001; Lucía, Hoyos, et al., 2001). However, the proper effect of each rest day of the Tours on hormonal, physiological, and psychological remains scarce (Lastella et al., 2015).

A professional cycling Tour is a highly stressful event due to the physical burden and emotional aspects, as it is a professional sport, and future contracts depend on the results. Furthermore, cyclists are naturally prone to a catabolic state due to excessive cortisol production from volume training and caloric deficit to maintain necessary leanness (Heikura et al., 2019; Lucía, Díaz, et al., 2001; Schofield et al., 2021). Despite these challenges, there is a limited understanding of the effects of continuous endurance and exhaustive exercises on hormonal responses, especially the catabolic/anabolic status, in real competitive conditions. Therefore, this study aims to analyse and quantify the catabolic-anabolic dynamics in cyclists during a twelve-day competition with one day of rest, as well as the effects on the profile of mood state. We hypothesized that in high-endurance athletes, during the prolonged competitive period, elevated cortisol production could impair the anabolic response influencing insulin, testosterone, and IGF-1 release, and that the rest day could have positive effects on recovering the hormonal balance.

Materials and Methods

Eight professional male cyclists of the same professional cycling team with the following characteristics (age 26.8 ± 3.2 years; height 176.4 ± 6.4 cm, weight 62.1 ± 6.4 kg, body fat $10.7 \pm 2.8\%$, maximal oxygen uptake [$\dot{V}O_{2\max}$] 72.2 ± 4.5 ml/kg/min and power at $\dot{V}O_{2\max}$ 494 ± 21 W), participated in the *Volta a Portugal* (a long-distance race, performed in stages) and voluntarily integrate the current study. The participants were regularly involved in the Grand Tours events in the past years. They gave their written informed consent after being informed of all experimental procedures and possible risks associated with the experiments

before the commencement of the study. All the cyclists were under WADA regulations, meaning no exogenous anabolic-androgenic steroids or drugs that could affect hormonal balance were taken during the study period. The current study was approved by the Institutional Review Board committee of the University of Porto (CEFADE 20_2023) and conducted in accordance with the Declaration of Helsinki.

Study Design

During the 12 days duration of the Volta a Portugal (11 stages plus one day off at day 8) the following parameters were assessed: body mass and fat percentage (in the morning in fasting state), saliva samples upon waking (AM) and before bedtime (PM) for posterior analysis of cortisol, basal heart rate, and subjects completed a reduced version profile of mood state questionnaire (POMS) to evaluate fatigue and potential mood disturbances throughout the competition. Additionally, serum samples were collected six times (cf. Table 1).

Procedures and data Analysis

Saliva samples were collected without stimulation by putting a cotton tube into the mouth until saturated and then put back into the plastic salivate tubes for storage (Kirschbaum & Hellhammer, 1989). All saliva samples were stored at -70°C until further analysis. Before analysis, saliva samples were clarified by centrifuging at 12 000 g for 5 min at 4°C . Cortisol (AM and PM) was measured in these clarified saliva samples using commercially available ELISA kits (Salimetrics, Newmarket Suffolk, UK). Serum samples allowed analyse insulin, insulin-like growth factor 1 (IGF-1), total testosterone, adrenocorticotrophic hormone (ACTH), and hemoglobin. After the venous blood samples were withdrawn, plasma was immediately separated by centrifugation (10 min at 3,500 revolution/min; 4°C), deposited in aliquots, and directly stored at -80°C for subsequent analysis, and hormonal concentrations were assessed in venous blood collection using the immuno-chemiluminescence method (Kricka, 2003).

Table 1.- *Volta a Portugal* stages characterization and schematic representation of the testing timeline.

Stage	Distance (km)	Testing day	Tests performed	Terrain
Prologue	6.8	Day 1	AM: saliva, blood, HR and POMS PM: saliva	 Individual time trial
1 st	183.5	Day 2	AM: saliva, HR and POMS PM: saliva	 Flat
2 nd	171.8	Day 3	AM: saliva, blood, HR and POMS PM: saliva	 Semi-mountainous
3 rd	180	Day 4	AM: saliva, HR and POMS PM: saliva	 Mountain
4 th	192.5	Day 5	AM: saliva, HR and POMS PM: saliva	 Mountain
5 th	161.3	Day 6	AM: saliva, blood, HR and POMS PM: saliva	 Semi-mountainous
6 th	155	Day 7	AM: saliva, HR and POMS PM: saliva	 Semi-mountainous
Rest day	-	Day 8	AM: saliva, blood, HR and POMS PM: saliva	-
7 th	172.5	Day 9	AM: saliva, HR and POMS PM: saliva	 High Mountain
8 th	194	Day 10	AM: saliva, blood, HR and POMS PM: saliva	 Flat
9 th	38.9	Day 11	AM: saliva, HR and POMS PM: saliva	 Individual time trial
10 th	167.1	Day 12	AM: saliva, blood, HR and POMS PM: saliva	 Flat

* AM, morning; PM, evening; HR, heart rate; POMS, profile of mood state.

Statistical Analysis

The normality and homogeneity of distribution were checked using the Shapiro–Wilk test for all the assessed variables, and mean values $\pm$ SD were calculated. Pearson correlation coefficients were calculated for data collected during the competition's first and last days to explore the relationships between the hormonal variables and the POMS score. The threshold used to assess the correlation values qualitatively was based on the following criteria (Hopkins et al., 2009): <0.1 (trivial), 0.1-0.3 (small), 0.3-0.5 (moderate), 0.5-0.7 (large), 0.7-0.9 (very large) and >0.9 (nearly perfect). Standardized effect sizes (Cohen, 2013) and precision of estimation were calculated to assess practical significance with

95% confidence intervals (CI) (Hemming & Taljaard, 2021), and correlations are presented as $r \pm 95\%CI$.

A single-factor repeated measures ANOVA was used to test whether there were differences in the variable's mean values between days 1, rest day (8), 10, and 12. Partial eta-squared (η^2) values were computed for effect size calculation: 0.05-0.25 small, 0.25-0.64 moderate, and > 0.64 strong (Ferguson, 2016). Post-hoc pairwise comparisons with Bonferroni correction were applied, and sphericity was determined in advance using the Mauchly test. Intraclass correlation coefficient (ICC) was used to assess the reliability and stability between all measurements. The thresholds used to assess the ICC and autocorrelation values qualitatively were based on the following criteria: < 0.5 poor, 0.5-0.75 moderate, 0.75-0.9 good, and > 0.9 excellent (Koo & Li, 2016).

The Statistical Parametric Mapping (SPM) was used to compare hormonal, basal heart rate, and POMS global score profiles in MATLAB (MATLAB R2019b, The MathWorks Inc., Natick, MA, USA) using the open-source code SPM1d (v.M0.1, www.spm1d.org) (Pataky, 2010). All variables were interpolated, transforming the six or twelve moments of the collection into 100 data points using the spline transformation. Afterwards, that data series were normalized based on their maximal value (100%), and the twelve-day duration of de Volta de Portugal transformed to 0-100% of the competition. For all statistical procedures, the significance level was set at 5%. The conduct and presentation of statistical analyses in this study are in accordance with the checklist for statistical assessment of medical papers statement (Mansournia et al., 2021).

Results

The body mass, body fat, basal heart rate, POMS scale, cortisol (AM and PM), insulin, total testosterone, IGF-1, hemoglobin, and ACTH values assessed during the twelve days are presented in Table 2. Body mass, body fat, basal heart rate, POMS scale, and cortisol PM did not present substantial changes during the cycling event ($p > 0.05$). Cortisol AM was maintained in high concentrations

between day 1 and the end of the Tour. However, an additional increase was observed between the rest day and the end of the Tour ($F_{3,27} = 3.53$, $\eta^2 = 0.34$, power = 0.7, $p = 0.032$). Insulin concentrations decreased until the morning of rest day, normalized on day 10, and decreased again at the end of the Tour ($F_{3,27} = 6.27$, $\eta^2 = 0.47$, power = 0.9, $p = 0.003$). A decrease in testosterone ($F_{3,27} = 4.95$, $\eta^2 = 0.41$, power = 0.6, $p = 0.040$) and IGF-1 ($F_{3,27} = 11.6$, $\eta^2 = 0.62$, power = 1.0, $p < 0.001$) until the morning of rest day and subsequent increase until the end of the Tour were observed. The ACTH ($F_{3,27} = 10.93$, $\eta^2 = 0.61$, power = 1.0, $p < 0.001$) and hemoglobin ($F_{3,27} = 12.70$, $\eta^2 = 0.65$, power = 1.0, $p = 0.003$) showed a decreasing behavior until the morning of the rest day and maintained low until the end of the Tour. Also, it was only observed a large positive correlation ($r = 0.81$, 0.25 to 0.96 CI 95%, $p = 0.015$) between POMS global score and hemoglobin concentrations, where better scores on the fatigue index were associated with higher hemoglobin concentrations. In general, high stability of results with good to excellent ICC was observed along the twelve days of Tour, except for insulin and ACTH concentrations (see Table 2).

Figures 1, 2, 3 and 4 show the pairwise comparisons (based on spm1d) of the magnitudes and behaviors of the variables assessed during the twelve days of the Tour. POMS values presented a stable magnitude through the event without decreasing substantially to the variations of the cortisol AM magnitude changes, particularly on days 5-6 and 9-10, showing the consistent resilience of the cyclist along the Tour (cf. Figure 1). IGF-1 and cortisol showed magnitude differences in the first three days of competition ($p = 0.002$), and until day 6, they presented inverse behaviors (cf. Figure 2). ACTH only showed magnitude differences with testosterone (cf. Figure 3). Testosterone and insulin showed substantial differences in magnitudes towards the end of the Tour where insulin dropped again and testosterone was close to the initial concentration values of day one (cf. Figure 4). It was observed magnitude differences between hemoglobin and total testosterone between days 3 and 10 due to the decrease in testosterone and stability of hemoglobin concentrations (cf. Figure 4).

Table 2.- Mean $\pm$ SD values for body mass, body fat, basal heart rate, POMS scale, cortisol (AM and PM), insulin, total testosterone, IGF1, hemoglobin, and ACTH during the cycling Tour. Reliability (95%CI) and significance level values are also presented.

Variables (mean $\pm$ SD)	<u>Day 1</u>	Day 2	Day 3	Day 4	Day 5	Day 6	Day 7	<u>Day 8</u> (day off)	Day 9	<u>Day 10</u>	Day 11	<u>Day 12</u>	<u>ICC (95%CI)</u>	<u>ICC p value</u>
Body mass (kg)	62.1 $\pm$ 6.4	61.6 $\pm$ 6.2	61.5 $\pm$ 6.1	61.7 $\pm$ 6.2	61.8 $\pm$ 6.8	62.1 $\pm$ 7.0	62.4 $\pm$ 6.7	62.3 $\pm$ 6.5	63.0 $\pm$ 6.3	62.1 $\pm$ 6.3	61.8 $\pm$ 6.4	62.3 $\pm$ 6.4	0.999 (0.998 - 1.000)	< 0.001
Body fat (%)	10.7 $\pm$ 2.8	10.7 $\pm$ 2.8	10.7 $\pm$ 2.7	10.3 $\pm$ 2.6	9.6 $\pm$ 2.5	9.9 $\pm$ 2.6	9.6 $\pm$ 2.5	9.8 $\pm$ 2.5	10.1 $\pm$ 2.3	9.8 $\pm$ 2.3	9.7 $\pm$ 2.5	10.0 $\pm$ 2.6	0.996 (0.990 - 0.999)	< 0.001
Heart rate (bpm)	40.5 $\pm$ 5.2	41.5 $\pm$ 4.8	44.4 $\pm$ 8.6	44.5 $\pm$ 6.4	45.9 $\pm$ 6.5	44.1 $\pm$ 6.6	43.4 $\pm$ 5.2	41.5 $\pm$ 5.4	42.0 $\pm$ 5.3	43.9 $\pm$ 6.0	42.4 $\pm$ 4.7	43.0 $\pm$ 6.3	0.980 (0.951 - 0.995)	< 0.001
POMS score	104.3 $\pm$ 4.4	103.1 $\pm$ 5.1	106.9 $\pm$ 4.6	105.8 $\pm$ 4.7	104.8 $\pm$ 5.9	99.4 $\pm$ 7.7	100.4 $\pm$ 7.8	99.6 $\pm$ 10.3	99.6 $\pm$ 10.3	92.1 $\pm$ 12.1	97.9 $\pm$ 5.0	98.6 $\pm$ 5.5	0.794 (0.494 - 0.951)	< 0.001
Cortisol AM (μ g/L)	5.3 $\pm$ 2.8	4.6 $\pm$ 2.1	4.7 $\pm$ 2.0	4.8 $\pm$ 2.1	5.5 $\pm$ 1.8	5.2 $\pm$ 2.1	3.8 $\pm$ 1.4	3.7 $\pm$ 0.9	4.8 $\pm$ 1.5	4.8 $\pm$ 1.6	4.4 $\pm$ 1.0	5.6 $\pm$ 2.5 [#]	0.914 (0.791 - 0.980)	< 0.001
Cortisol PM (μ g/L)	0.5 $\pm$ 0.4	0.5 $\pm$ 0.2	0.5 $\pm$ 0.2	0.5 $\pm$ 0.1	0.5 $\pm$ 0.4	0.5 $\pm$ 0.3	0.4 $\pm$ 0.2	0.8 $\pm$ 0.7	0.4 $\pm$ 0.4	0.4 $\pm$ 0.3	0.3 $\pm$ 0.2	0.3 $\pm$ 0.2	0.764 (0.425 - 0.944)	< 0.001
Insulin (μ U/mL)	3.5 $\pm$ 1.3	-	2.6 $\pm$ 1.1	-	-	1.8 $\pm$ 1.2	-	1.7 $\pm$ 1.3*	-	3.2 $\pm$ 1.1 [#]	-	1.9 $\pm$ 1.0*	0.619 (-0.001 - 0.911)	0.025
Total testosterone (ng/mL)	9.1 $\pm$ 3.6	-	7.9 $\pm$ 2.8	-	-	6.5 $\pm$ 2.5	-	6.2 $\pm$ 2.2*	-	8.4 $\pm$ 2.8 [#]	-	8.5 $\pm$ 2.4 [#]	0.903 (0.745 - 0.977)	< 0.001
IGF-1 (ng/mL)	223.4 $\pm$ 71.4	-	218.5 $\pm$ 54.1	-	-	149.9 $\pm$ 34.7	-	136.8 $\pm$ 26.5*	-	176.5 $\pm$ 48.9 ^{*#}	-	182.6 $\pm$ 51.0 [#]	0.838 (0.575 - 0.962)	< 0.001
Hgb (g/dL)	15.4 $\pm$ 0.5	-	15.3 $\pm$ 0.6	-	-	14.4 $\pm$ 0.9	-	14.3 $\pm$ 0.9*	-	14.3 $\pm$ 0.8*	-	14.4 $\pm$ 0.7*	0.790 (0.448 - 0.951)	< 0.001
ACTH (pg/mL)	27.7 $\pm$ 9.1	-	15.1 $\pm$ 3.5	-	-	22.2 $\pm$ 8.7	-	14.6 $\pm$ 4.0*	-	16.1 $\pm$ 5.4*	-	15.7 $\pm$ 6.7*	0.592 (-0.070 - 0.905)	0.034

*.# Differences compared to days 1 and 8, respectively (p < 0.05). Legend: AM, morning; PM, evening; POMS, profile of mood state; IGF-1, insulin like growth factor 1; Hgb, Hemoglobin; ACTH, adrenocorticotrophic hormone

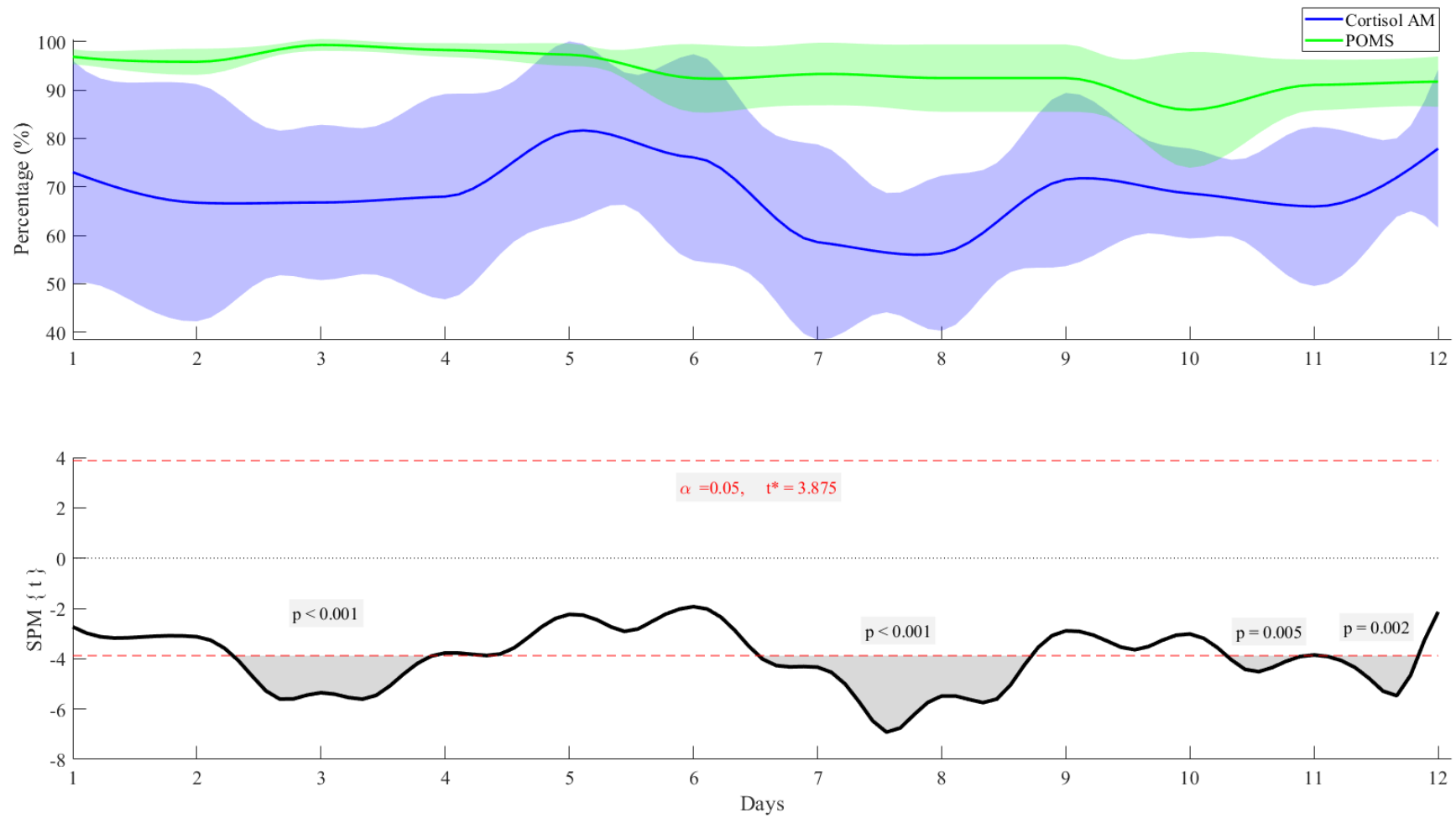


Figure 1.- Comparisons of the behaviors (with twelve measures) of cortisol AM concentrations (blue) and POMS (green) along the cycling event.

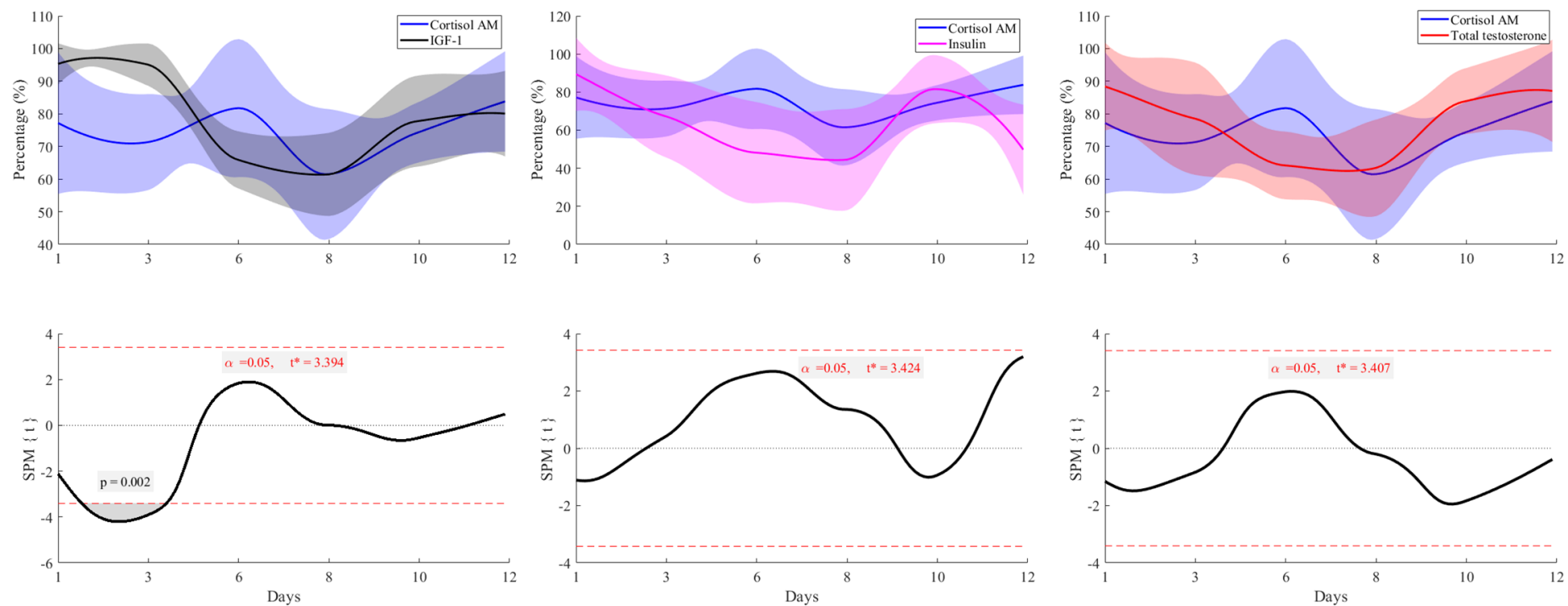


Figure 2.- Comparisons of the behaviors (with six measures) of cortisol concentrations (blue), insulin (magenta), testosterone (red) and IGF-1 (black) along the cycling event.

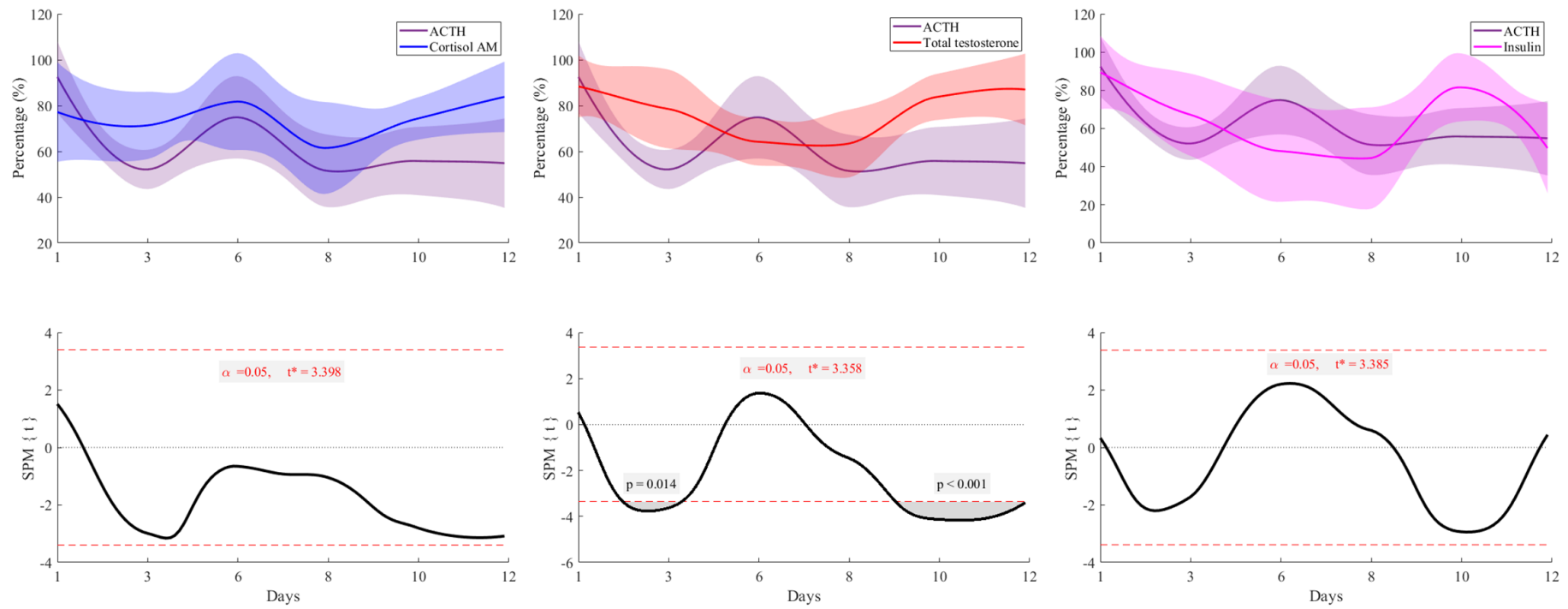


Figure 3.- Comparisons of the behaviors (with six measures) of ACTH (purple), cortisol concentrations (blue), insulin (magenta) and testosterone (red) along the cycling event.

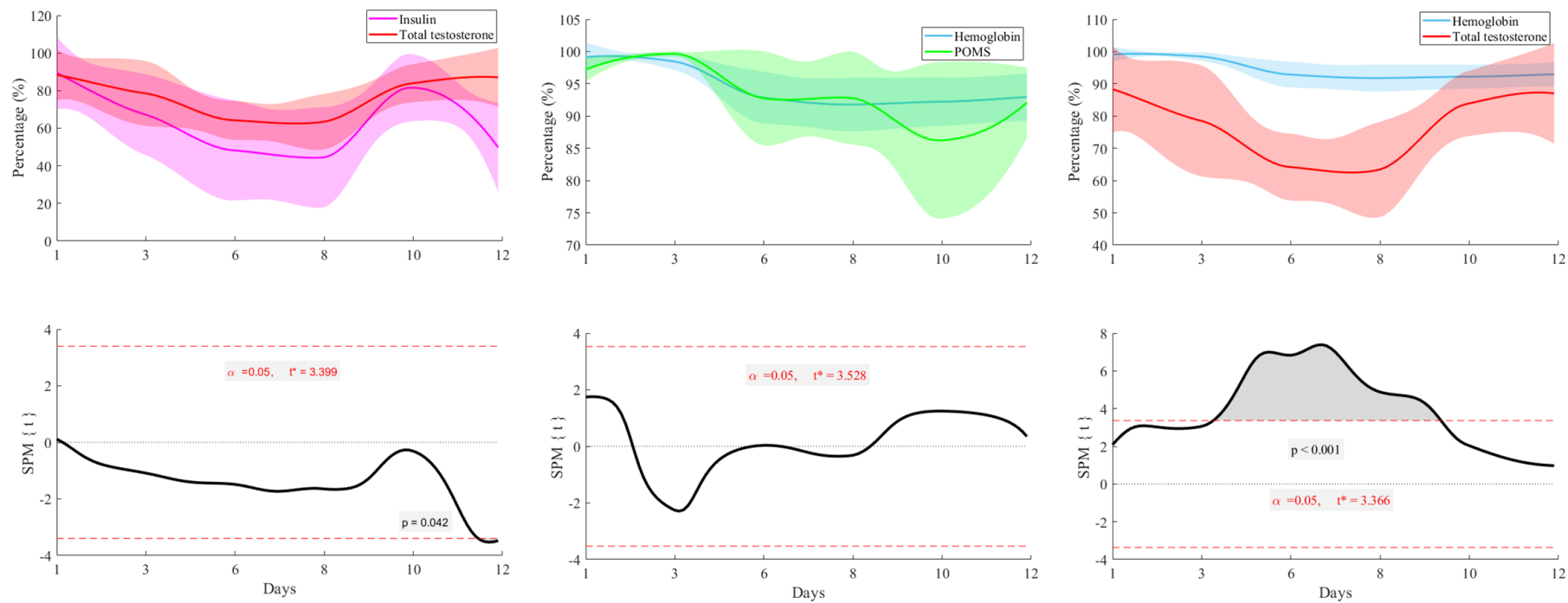


Figure 4.- Comparisons of the behaviors (with six measures) of hemoglobin (cyan), POMS (green), insulin (magenta) and testosterone (red) along the cycling event.

Discussion

The objective of the current study was to analyze and quantify the catabolic and anabolic responses in cyclists subjected to a twelve-day road cycle competition with a rest day. The principal results demonstrated a clear impact on hormonal concentrations, influencing the catabolic/anabolic dynamics. Catabolic cortisol AM release maintained high concentrations from day 1 to the end of the Tour, with an additional increase between the rest day and the end. Conversely, cortisol PM did not show substantial differences during the event. In contrast, a decrease in the anabolic signalling was observed, with a decrease in insulin until the morning of the rest day (day 8), normalizing on day 10, and decreasing again till the end of the Tour. A decrease in testosterone and IGF-1 until the morning of the rest day, with a subsequent increase until the end of the Tour after a day of rest, was also observed. This anabolic commitment suggests a significant impact of prolonged physical exertion on anabolic processes influenced by cortisol. Salivary cortisol assessment was chosen for its validity in comparing salivary and serum cortisol levels in basal and exercise conditions, presenting high correlations and being a reliable and non-invasive method (Cadore et al., 2008; Papacosta & Nassis, 2011). The statistical parametric analysis helped overcome sample and evaluation number issues when comparing the behaviours of the pairs of hormones and the POMS scale.

Cortisol, the principal glucocorticoid in the body, profoundly influences human skeletal muscle in coordination with anabolic hormones. In a physiologically balanced state, cortisol exhibits a circadian rhythm, peaking in the morning, decreasing throughout the day, and reaching its lowest values at night (Oster et al., 2017). Systemically regulation of cortisol levels involves the hypothalamic-pituitary-adrenal (HPA) axis, with local modulation facilitated by 11 β -hydroxysteroid dehydrogenase (11 β -HSD) enzymes (Chapman et al., 2013; Lamberts et al., 1996). It assumes in skeletal muscle during exercise a pivotal role in energy homeostasis and metabolism, enhancing metabolic substrates' availability and increasing circulation in response to heightened endurance training (Alves et al., 2020; Daly & Hackney, 2005).

Additionally, insulin, the primary anabolic hormone, plays a central role in muscle growth and protein turnover for muscle recovery by promoting the deposition of energy substrates from food into the form of glycogen (O'Neill et al., 2015; Urhausen et al., 1995), with its interactions with exercise being fundamental for metabolic regulation and homeostasis (Horton et al., 2006). IGF-I is also a significant player in anabolic signaling, targeting many tissues, including skeletal muscle. While liver-derived IGF-I production is under direct regulation of Growth hormone (GH), local mechanical-stretch mechanisms can activate IGF-I synthesis in tissues. It may be that IGF-I acts as a signal that amplifies or regulates the repair and remodeling of skeletal muscle tissue (Kraemer et al., 2017).

Typically, cortisol exposure antagonizes testosterone, inhibiting protein synthesis and interfering with anabolic signaling (Kraemer et al., 2020; Lee et al., 2017). Furthermore, under conditions of energy deficiency, cortisol concentrations escalate (Elliott-Sale et al., 2018), inhibiting local anabolic stimulation in a muscle, reducing insulin, IGF-1, mammalian rapamycin (mTOR), and amplifying myostatin signaling, a catabolic muscle growth factor. This intricate interplay compromises regenerative protein synthesis, contributing to muscle atrophy (Liu et al., 2004; Patel et al., 2014; Schakman et al., 2013).

On the other hand, one of the positive effects of cardiovascular fitness is to promote greater insulin sensitivity and cortisol tolerance. This heightened sensitivity can be attributed to training-induced adaptations in the vasculature, skeletal muscle, and adipose tissue, justifying lower basal insulin levels in well-trained athletes (Goodpaster et al., 2001). Furthermore, increased pituitary cortisol resistance is another adaptation to chronic endurance training (Duclos et al., 1997; Lehmann et al., 1993), as is variation in tissue sensitivity to glucocorticoids associated with training intensity (Duclos et al., 2003). Increased sensitivity after acute exercise combats muscle inflammation, cytokine synthesis, and muscle damage (Duclos et al., 2003). Subsequently, the decrease in

sensitivity 24 hours after exercise may act to protect the body from prolonged exercise-induced cortisol secretion (Duclos et al., 2003). Additionally, another protective adaptation of endurance training is the cortisol-to-cortisone (inactive form) conversion (Chapman et al., 2013) and the glucocorticoid receptor expression in muscle fibers, being more abundant in the fast-twitch fibers and more sensitive to glucocorticoid-induced catabolism compared to the resistant slow-twitch fibers (Gouarné et al., 2005).

Our study revealed significant magnitude differences in Cortisol AM and IGF-1 in the first three days of competition, with inverse behaviors until day 6. Also, an inverse behaviour between Cortisol AM and insulin and testosterone was found from the first day to the rest day, aligning with similar behaviors of insulin and testosterone, decreasing throughout the competition until the rest day. However, after the rest day, substantial magnitude differences were observed between insulin and testosterone, with insulin dropping and testosterone approaching initial concentrations. This suggests a more significant impact of competition loads on insulin than on testosterone.

ACTH levels showed an early decrease, remaining stable throughout the competition, including post-rest day, which could indicate sustained inflammation or over-physiological stress impacting adrenal function. However, the initial high levels were likely due to pre-competitive anxiety or pituitary resistance, a positive adaptation in endurance athletes. With the start of the competition, ACTH levels decreased (eventually normalized) since cortisol showed the expected increasing behavior in response to the organic needs of the race. Furthermore, the additional increase in cortisol after the rest day until the end of the race may reflect cortisol's complex role in responding to the athlete's global demand, whether organic, energetic, or psychological, and not to a single exercise stimulus. Insulin's behavior, decreasing until day 8, normalizing after the rest day, and decreasing again at the end, may be associated with the anti-anabolic effect of cortisol or changes in caloric availability and metabolic demands during the competition.

Additionally, prolonged endurance training negatively affects hemoglobin concentration and red blood cell (RBC) count, possibly due to exercise-induced plasma volume expansion. Likewise, this type of training negatively affects testosterone levels and its ergogenic and anabolic functions, namely the production of red blood cells. In our study, testosterone and hemoglobin showed similar behaviors only until the rest day (both decreasing). However, after the rest, testosterone normalized, remaining stable until the end of the competition; in contrast, hemoglobin remained at the same decreased level until the end. As mentioned above, this may suggest that the anabolic commitment throughout the competition may be more attributable to other anabolic hormones, implying that testosterone may be a less critical biomarker of allostatic load for well-trained endurance athletes than insulin.

POMS remained stable throughout the event, indicating consistent resilience among cyclists despite variations in Cortisol AM levels. Evaluating mood and fatigue with the POMS questionnaire is crucial, as psychological factors significantly influence physiological responses and vice versa (Lastella et al., 2015), typically altered in an overload or unrecovered state. The sustain of good levels of motivation and mental proactivity is justified by the greater resistance to the negative effects of mental fatigue of professional cyclists (Martin et al., 2016), contributing to the success of extreme events like Tours; nevertheless, in our results, it was not efficient to predict the reported anabolic decrease.

During exercise, cortisol is expected to increase (Oster et al., 2017), particularly in very long exercises below the anaerobic threshold (but still at 60% of $\dot{V}O_2\text{max}$) (Hill et al., 2008). Despite this expectation, research presents mixed outcomes concerning cortisol and testosterone fluctuations in extended endurance exercises. A notable decline in both testosterone and cortisol among endurance athletes was observed over a three-week competition, hinting at potential adrenal gland and testis fatigue (Lucía, Díaz, et al., 2001). Similarly, decreases in testosterone levels and the free testosterone/cortisol ratio were documented across different high-intensity training regimes for cyclists over 12 weeks,

although there was an initial increase in IGF-1 (Sylta et al., 2017). Contractedly, reduced serum cortisol and free testosterone were reported by the end of a 10-day cycling stage race, with an elevated testosterone/cortisol ratio (Simonetto et al., 2016). The latter study also found that lower pre-race testosterone levels might benefit stage race performance, though no direct link between hormonal shifts and race speed was established (Simonetto et al., 2016).

Our study brings a newer outcome where a twelve-day cycling competition revealed sustained high cortisol values, with an additional increase in the latter stages, indicating a necessary organic response to cumulative physical and mental stress stimuli during such efforts. Additionally, it should be noted that the Volta a Portugal is a pivot event in the sporting season, with athletes theoretically entering the competition well-rested and at peak hormonal performance. A decrease in cortisol throughout the race prompts considerations of athletes' pre-race recovery and the competition load's impact on previous adaptation. In line with our results, low insulin levels observed during prolonged endurance exercise (inhibition effect by catecholamines) (Kindermann, 1982) and in energy-deficient athletes (Elliott-Sale et al., 2018), intensify the anti-anabolic commitment to cortisol mentioned above. However, as mentioned earlier, low fasting morning insulin levels are considered an adaptive mechanism in aerobic endurance athletes, enhancing its sensitivity and optimizing lipid oxidation (Horton et al., 2006). Nonetheless, a nuanced analysis is needed, as excessively low values may eventually compromise its efficacy (Borghouts & Keizer, 2000).

Changes in body composition during competitions, such as weight loss or body fat, can indicate caloric deficits and poor hydration status. Furthermore, athletes performing chronic strenuous endurance exercises may struggle to meet energy requirements, risking chronic low energy availability. This energy-deficient state could compromise testosterone (De Souza et al., 2019) and insulin release (Elliott-Sale et al., 2018). This is particularly pronounced in cycling due to its inherent characteristics like low impact, high-energy demand, and mandatory leanness (Heikura et al., 2019; Lucía, Díaz, et al., 2001; Schofield et al., 2021).

In our study, the cyclists maintained their body mass and body fat during the twelve-day event, suggesting a proper nutrition intake (Lucía, Díaz, et al., 2001; Schofield et al., 2021). Nevertheless, it was insufficient to avoid the reported decrease in testosterone and insulin.

To the best of our knowledge, information on hormonal dynamics during Tour events is scarce, and the study from Lucia et al. (2001) was one of the few to demonstrate hormonal behaviors during this competitive context. However, they have only measured at the end of each week, and with that information of what happens within a one-week window, cumulative stages are unknown. Our results bring insights into what is expected to be observed during and within twelve days of competition.

The specific schedule and number of rest days can vary between different Tours and editions, and even for races such as Volta a Portugal that last over one week, at least one or two rest days are included. These stops allow cyclists to recuperate from the cumulative fatigue of consecutive stages and prepare mentally and physically for the remaining stages of the race. However, the proper effect of each rest day of the Tours on hormonal, physiological, and psychological remains scarce (Lastella et al., 2015). Our study was missing the data collection after day 12. So, the effects of the last stage were not accountable, which can be considered a limitation. However, the end of the Tour and the unavailability of the cycling team afterward did not allow assessing those collections. Also, the effects on 24, 48, and 72 hours after the end of the Tour were not measured to understand the post-competition effects on the normal functioning of the endocrine system. The above-raised questions should be addressed in future studies.

Conclusion

This study highlighted three key messages: First is the importance of a rest day during these long extreme endurance competitions, enabling the body to achieve catabolic and anabolic efficiency once again; Secondly, it raises a question of

whether high cortisol values and the corresponding anabolic commitment throughout a twelve-day endurance competition have the expected deleterious effect on performance at that moment, or if well-trained athletes with high cardiovascular fitness develop fundamental adaptations - specifically, cortisol tolerance and insulin sensitivity - that enable the body to maintain overall performance; Thirdly, it underscores the complex relationship between hormones from different axis, confirming that anabolic/catabolic dynamism is influenced by factors such as other hormone concentrations, circadian rhythms, circulating cytokines, metabolites, osmolality, caloric balance, or mood. This feedback relationship emphasizes the need to assess hormonal status in context, considering the integrated nature of endocrine balance - "Instead of thinking about how hormones work, we should think about how they react in these situations."

Chapter 7

General discussion

Resulting from the surge in sports participation at progressively younger ages, coupled with the allure of potential stardom in today's generation—often characterized as the most narcissistic in history—improper sports practices may compromise their anabolic and constructive attributes, which may imply physical and mental repercussions with premature withdrawal from the competition (Emmonds et al., 2023). Given the subtle demarcation between constructive fatigue and overtraining, reliance solely on subjective athlete sensations for assessment poses a considerable risk of misjudgement (Saw et al., 2016).

The fundamental aim of multiple acute bouts of exercise is to induce prompting adaptation to a higher level, creating instability to attain a higher level of stability, aligning with the allostasis concept. However, consistently achieving higher levels of performance is not always guaranteed. One crucial aspect is the hormonal component, precisely the delicate balance between catabolism and anabolism. In contemporary sports, it has become imperative to conduct comprehensive physical, mental, and biochemical monitoring to delineate the trajectory of training, especially for high-performance athletes engaged in individual and endurance sports, although not exclusively (McGuigan et al., 2020, 2021; Saw et al., 2016).

Early identification of prolonged maladaptation to training is pivotal, precluding the athlete's physical, mental, and immune deterioration. This condition, marked by numerous symptoms, is heralded by hormonal shifts, encompassing the inability to release hormones essential for exertion (cortisol-blunted response) and regenerative hormones post-exertion (decreased insulin and its growth factor). Without requisite organic regeneration, athletes experience heightened depressive conditions, severe fatigue, diminished sporting and cognitive functional capacity, an increased likelihood of various organic disorders, and abandonment of competition and sports practice. This condition's timely and

objective diagnosis is paramount for advancing sports performance, necessitating vigilant monitoring by the athlete's multidisciplinary support team.

With the perspective presented in **Chapter 2**, we seek to sensitize coaches and physiologists working with high-performance athletes to the critical importance of hormonal assessment, notably cortisol and insulin. Recognizing these hormones as fundamental biomarkers for analysing adaptation to training, the article advocates for their integration into the category of allostatic biomarkers, urging a more systemic perspective. This perspective acknowledges their profound impact not only on performance but also on overall health. Additionally, athletes are extending their career span while performing at their best and producing extraordinary results (Allen & Hopkins, 2015), specifically those engaged in endurance modalities like road cycling and ultramarathon events, as well as a tendency to older Olympic finalists and medallists (Mallett et al., 2020). Therefore, in **Appendix I**, we extended the theme to the aging-athlete to provide some perspective around the necessity for hormonal evaluation/control for coping with age and maintaining the athlete's optimal health and performance (Martín et al., 2018; Menard & Stanish, 1989).

In 1989, the American Journal of Sports Medicine published an article addressing the theme of "The Aging Athlete." This publication underscored that athletes undergoing the aging process exhibit distinctions from their younger counterparts in various aspects. These disparities encompass physiological, structural, and psychosocial differences, marking them as a distinct entity within the athletic realm. The article emphasized that the primary threat to the health of athletes entering this phase is not aging itself but rather the consequences associated with inactivity. It suggested that regular exercise could potentially defer the physiological decline linked to old age by up to 50%, portraying it as a veritable fountain of youth that merits universal attention. Notably, no mention was made of the hormonal component at that time. Subsequently, the recognition of the pivotal role played by the catabolic and anabolic hormones gained traction, emphasizing the imperative of achieving a delicate hormonal equilibrium.

Later, in the article "Exercise and the HPA Axis," Duclos (2016) emphasized that the physiological processes related to endocrine balance are influenced by more than one single hormone, in an integrated way at various levels, being released under feedback processes, some controlled by their own peripheral concentration and others by other circulating hormones or cytokines, metabolites, osmolality, etc. In other words, hormonal balance should be assessed in a context and not by isolated evaluations, which may question the interpretation and therapy of a single hormonal axis.

Concluding with a prudent perspective from Harvard Men's Health Watch, it was raised pertinent questions: Have alternative factors contributing to fatigue, decreased libido, or other symptoms been considered? Is there adherence to a nutritious and balanced diet? Is regular exercise part of the routine? Is there adequate sleep? The recommendation is to primarily address these lifestyle factors before considering resorting to hormone therapy.

So, the utilization of biomarkers in sports performance has progressively captured the interest of the medical and scientific community and has been driven by the desire to gain a deeper understanding of training dynamics, aiming to mitigate the risk of overtraining. Tracking biomarkers associated with overtraining syndrome is not only crucial for optimizing performance but also serves as a preventive strategy (Herring et al., 2019; Meeusen et al., 2013).

The hormonal catabolic-anabolic balance plays a pivotal role in maintaining the equilibrium between training load and recovery (Cadegiani et al., 2020; Meeusen et al., 2013; Urhausen et al., 1995). Cortisol, a key player in this balance, uniquely influences the catabolic response to exercise, while insulin concentration rises post-exercise to enhance glycogen replacement, reducing carbohydrate oxidation and increasing lipid oxidation (Horton et al., 2006). Despite assumptions that lower insulin baseline levels and higher sensitivity are adaptations in aerobic endurance athletes to optimize lipid oxidation (Goodpaster et al., 2001; Richter et al., 2021), it's crucial to acknowledge cortisol's broad

spectrum of metabolic control, including its anti-anabolic action (Viru & Viru, 2004). Therefore, the regulation of insulin levels becomes crucial for athletes engaged in prolonged endurance training.

Cycling incorporates a lot of this since is considered a significant risk factor for LEA, even in male practitioners, due to the inherent characteristics of this sport: low impact, high energy demands and mandatory leanness (Schofield et al., 2021), with constant energy deficits throughout the season as well as low bone mineral density due to the unloaded bearing nature of the sport correlated with LEA (Barry & Kohrt, 2008; Olmedillas et al., 2012). It is also commonly characterized by an endocrine system dysfunction with elevated cortisol and reduced testosterone (Torstveit et al., 2018, 2019) and IGF1 (Heikura et al., 2019; Torstveit et al., 2019).

In **Chapter 3**, our results with cyclists revealed an inverse relationship between 24-hour urinary free cortisol and plasmatic fasting morning insulin, with maximal cortisol values corresponding to insulin values below the standard lower reference threshold of 3 μ IU/mL (Tohidi et al., 2014). Additionally, an inverse relationship between ACTH and insulin was observed, emphasizing the importance of assessing hormone status in context rather than through isolated assessments. This underscores that multiple hormones influence the physiological processes related to endocrine balance in an integrated manner and at various levels.

Low insulin levels have also been observed during prolonged endurance exercise responding to the increase in catecholamines, inhibiting insulin secretion in order to maintain glucose available for the central nervous system (Kindermann 1982; Richter et al. 2021) and in energy-deficient athletes by locally inhibiting anabolic stimulation in the muscle, decreasing the production of insulin and IGF-1 (Elliott-Sale et al., 2018), thus potentiating the anabolic commitment to the anti-anabolic effect of cortisol mentioned above.

In accordance with this, a longitudinal study during the sports season in high-level endurance runners demonstrated a decrease in basal insulin values compared to initial values throughout the season, more noticeable in periods of higher training volume (Alves et al., 2020). Other studies reported a negative correlation between insulin and training volume in elite rowers (Jürimäe et al., 2006), a significant reduction in exercise-induced insulin level (Elliott-Sale et al., 2018), and an increase in insulin resistance in overtraining periods (Petibois & Déléris, 2004).

We conclude that the low fasting morning insulin levels found in our study, which can be considered as an adaptation mechanism through greater insulin sensitivity, typical of aerobic endurance athletes to optimize lipid oxidation (Goodpaster et al., 2001), should be further analysed as excessively low values may eventually jeopardize its efficacy (Borghouts & Keizer, 2000), knowing its impact in overall health. These catabolic/anabolic imbalances, namely excessive catabolism or insufficient anabolism, align in the maladaptation process described by Selye's General Adaptation Syndrome, representing the chronological development of the response to stressors when their activation is prolonged, consisting of three phases: "the alarm reaction, the state of resistance, and the state of exhaustion" (Selye, 1976). We know that adaptation is not always guaranteed and that analytical changes can precede symptoms (Meeusen et al., 2013), thus gaining vital importance for our study topic. Prevention is essential to avoid the sequelae of prolonged maladaptation (Meeusen et al., 2013), with recovery being key to prevention.

Next, despite the known deleterious effects on the endocrine system due to prolonged periods of endurance training, conversely, it has also been shown to have positive results, namely helping to decrease the risk of cardiovascular diseases and provide direct endogenous cardiovascular protection (Kaminsky et al., 2019; Li et al., 2020). So, the impact of cardiovascular fitness on cortisol tolerance and hormonal ranges for assessing catabolic/anabolic balance was a topic that required further investigation. Therefore, it was evaluated the anti-

anabolic effects of cortisol in elite male cyclists and how cardiovascular fitness could cope with that response. The **Chapter 4** results revealed that elite cyclists (with VO₂max above 70 ml/kg/min) with normoinsulinism exhibited higher cortisol levels compared to non-elite counterparts despite similar insulin concentrations but lower total testosterone concentrations and testosterone/cortisol ratio. Among cyclists with hypoinsulinism, the elite group displayed lower insulin levels, while testosterone levels remained like the non-elite group. Additionally, in the non-elite context, the hypoinsulinism group had significantly lower testosterone values compared to the normoinsulinism group.

The results from this second experimental study corroborate the previous results found in **Chapter 3** and added new information about the effect of competitive level (based on VO₂max) on hormonal dynamics. It was suggested that cardiovascular fitness could mitigate the cortisol anti-anabolic detrimental effect, promoting greater insulin sensitivity and cortisol tolerance since considerably higher cortisol values were needed to reflect the decrease in insulin (only verified on three of the eleven elite cyclists evaluated). Again, this heightened sensitivity could be attributed to training-induced adaptations in the vasculature, skeletal muscle, and adipose tissue, justifying the lower basal insulin levels typical of well-trained athletes (Goodpaster et al., 2001), but also due to increased pituitary resistance to cortisol, a known adaptation of the HPA axis to chronic endurance training (Duclos et al., 1997; Lehmann et al., 1993), as well as the variation in tissue sensitivity to glucocorticoids associated with training intensity (Duclos et al., 2003).

This study highlights two key messages. Firstly, elite road cyclists with higher cardiovascular fitness, particularly above 70 ml/kg/min, exhibited a higher threshold for the negative effects of cortisol on anabolic processes compared to their non-elite counterparts with lower cardiovascular fitness. This suggests that superior cardiovascular fitness may provide protection against the elevated catabolic hormonal concentrations characteristic of cycling. Secondly, insulin emerged as a more sensitive biomarker than testosterone in controlling elite male

cyclists' catabolic/anabolic balance. This suggests its potential utility in training monitoring for high-endurance athletes.

These initial studies underscore the importance of insulin, which emerged as a more sensitive biomarker than testosterone, particularly in elite male road cyclists. This suggests its potential utility in training monitoring for high-endurance athletes. Following the search for correlations between cortisol and insulin fluctuations based on the athlete's cardiovascular fitness, we delineate our investigation into the dynamics of this hormonal equilibrium during real competitive exercise scenarios.

Ultramarathons cover a wide range of distances, from 50 km to over 1,600 km, across varied terrains and formats of one and multi-day races; it is challenging to generalize and compare the results. While the benefits of regular exercise are well-documented (Warburton & Bredin, 2017), the extreme nature of ultra-endurance raises questions regarding their impact on hormonal balance and overall health (Hohl et al., 2019; Ramos-Campo et al., 2016). Monitoring and understanding hormonal responses and RPE are essential to ensure that the participants' health is not compromised in events that combine extreme stress, exertion, and high pain tolerance (Belli et al., 2018; Roebuck et al., 2018).

Our findings in **Chapter 5** indicated an early peak from the catabolic and perceived exertion responses and a drop in the anabolic response. Utilizing real context analysis with emerging statistics (parametric mapping), it was revealed that insulin initially decreased up to 20% of the race following a rise in cortisol. Cortisol levels and RPE remained elevated throughout the race, with insulin peaking around 40%, likely in response to the longest stop and food intake. However, insulin subsequently decreased again, maintaining low values below 60% of baseline from the midpoint to the race's end, which can be interpreted either by the anti-anabolic cortisol effect or by the energy deficiency with a severe caloric deficit typical of these events.

Insulin once again revealed a new role as a biomarker for training monitoring and assessing catabolic/anabolic balance, with the interaction between insulin and exercise being an example of the need to modify and adjust the effects of two opposing metabolic forces under varying conditions (Richter et al., 2021). Its decrease during exercise is critical for mobilizing glucose and fatty acids from the liver and adipose tissue (Richter et al., 2021; Wasserman et al., 1991). Therefore, in prolonged muscular activity, such as ultramarathons and with the depletion of glycogen reserves, the demand for carbohydrates from processing fats and proteins is amplified, suppressing insulin's effects on fuel storage (Richter et al., 2021).

RPE analysis is crucial role in understanding athletes' perception of external load during competition. Its behaviour closely mirrored cortisol levels, and previous evidence suggests a link between the elevation of cortisol and RPE during exercise (McGuigan et al., 2004). Cortisol, released in response to stress, tends to increase during and immediately after high-intensity exercise (Anderson et al., 2016; McGuigan et al., 2004). Simultaneously, RPE, a subjective measure of perceived effort during an exertion, follows a similar pattern (Eston, 2012). One possible explanation for their relationship is that cortisol may contribute to feelings of fatigue and exertion during exercise (Anderson et al., 2016) and may interact with other stress-response hormones like adrenaline and noradrenaline, amplifying perceived exertion (Duclos & Tabarin, 2016). While our results affirm this link, further research is needed to fully explore the mechanisms underlying this relationship and its implications for exercise performance.

In conclusion, this study established a relationship between hormonal and RPE responses during an ultramarathon lasting an average of 24 hours, with the athletes experiencing increased and sustained high cortisol levels and RPE. Concurrently, insulin levels decreased and remained low throughout the race. The observed imbalance in the catabolic/anabolic hormonal profile during the ultramarathon warrants further investigation, considering its potential detrimental

effects on athlete health. Assessing the behaviour of these biomarkers and interpreting them based on RPE could aid in regulating stopping and intensity strategies during these extreme races.

The final phase of this thesis aimed to comprehend the endocrine and anabolic/catabolic dynamics during extended events such as the Volta a Portugal, which amalgamates high volumes, intense bouts, and diverse terrains (Lucía, Hoyos, et al., 2001). **Chapter 6** results underscored a significant impact on hormonal concentrations, influencing catabolic/anabolic dynamics, with the rest day exhibiting positive effects on restoring hormonal balance. Catabolic cortisol AM release maintained elevated concentrations from day 1 to the Tour's conclusion, with a notable surge post the rest day. Conversely, cortisol PM exhibited minimal variations during the event. Conversely, a decline in anabolic signalling was noted, marked by reduced insulin levels until the rest day, normalization on day 10, and a subsequent decrease until the Tour's end, accompanied by a decrease in testosterone and IGF-1 until the rest day and a subsequent increase post a day of rest. This anabolic commitment implies a substantial impact of prolonged physical exertion on anabolic processes influenced by cortisol.

Our study also uncovered significant magnitude differences in Cortisol AM and IGF-1 in the initial three days of competition, with inverse trends until day 6. Additionally, an inverse correlation between Cortisol AM and insulin and testosterone persisted from the first day to the rest day, aligning with similar patterns in insulin and testosterone, decreasing throughout the competition until the rest day. Post the rest day, substantial magnitude differences emerged between insulin and testosterone, with insulin declining and testosterone approaching initial concentrations, suggesting a more pronounced impact of competition on insulin than on testosterone. It is always crucial to highlight the observed low fasting morning insulin levels throughout the race, considered an adaptive mechanism developed in aerobic endurance athletes, enhancing

sensitivity and optimizing lipid oxidation (Horton et al., 2006), although excessively low values may compromise efficacy (Borghouts & Keizer, 2000).

Furthermore, noteworthy is the stable POMS observed in our study throughout the event, indicating consistent resilience among cyclists despite variations in Cortisol AM levels (Lastella et al., 2015), typically altered in an overload or unrecovered state and the maintenance of their body mass and body fat during the twelve-day event, suggesting proper nutrition intake (Lucía, Díaz, et al., 2001; Schofield et al., 2021). However, this was insufficient to prevent the reported decrease in testosterone and insulin.

This study conveys three key messages: First is the importance of a rest day during these long extreme endurance competitions, enabling the body to achieve catabolic and anabolic efficiency once again; Second, it raises questions about whether high cortisol values and the associated anabolic commitment during a twelve-day endurance competition negatively impact performance, or if well-trained athletes with high cardiovascular fitness develop fundamental adaptations, specifically cortisol tolerance and insulin sensitivity, enabling overall performance maintenance; Thirdly, it underscores the complex relationship between hormones from different axis, confirming that anabolic/catabolic dynamism is influenced by factors such as other hormone concentrations, circadian rhythms, circulating cytokines, metabolites, osmolality, caloric balance, or mood. This feedback relationship emphasizes assessing hormonal status in context, considering the integrated nature of endocrine balance - "Instead of thinking about how hormones work, we should consider how they react in these situations."

Our research presented allowing this academic work underscores the necessity of a nuanced approach to training regimen design, emphasizing the importance of personalized hormone monitoring. By doing so, we can tailor interventions that optimally support the intricate balance between achieving peak performance and maintaining long-term health. The studies presented herein offer a crucial

stepping stone towards more objective and precise control over the training and recovery mechanisms, advocating for an evolution in the research dedicated to understanding hormonal fluctuations and their implications. Preparing an athlete for competitions as demanding as ultramarathons and road cycling transcends mere physical training; it is a meticulously orchestrated process that considers a multitude of interrelated factors, from physiological to hormonal. A significant revelation from our work is the critical need for effective communication and data presentation strategies catering to elite athletes and their coaching teams. Through the rigorous analysis and reporting of hormonal data, we have sought to empower athletes and coaches with actionable insights, enabling them to make informed decisions about training adjustments.

In conclusion, this thesis does not merely contribute to academic knowledge; it strives to serve as a practical guide for athletes, coaches, and sports scientists alike, navigating the complex landscape of sports performance through the lens of hormonal dynamics. By providing detailed analyses and recommendations based on our findings, we aim to facilitate a more informed, health-conscious, and effective approach to training and recovery in ultramarathon running and road cycling. Building on the foundation laid by our current findings, future research should venture into several key areas to further elucidate the complex interactions between hormonal dynamics and endurance performance.

Chapter 8

Conclusions

From our experimental studies it was possible to define several conclusions such as:

- I. Lower values of insulin were associated with higher levels of cortisol and ACTH in high-endurance male athletes, suggesting insulin's new and relevant role as a sports performance training monitoring biomarker;
- II. Elite road cyclists with higher cardiovascular fitness, particularly above 70 mL/kg/min, exhibit a higher threshold for the negative effects of cortisol on anabolic processes compared to their non-elite counterparts (lower cardiovascular fitness);
- III. Superior cardiovascular fitness may provide protection against the elevated catabolic hormonal concentrations found in road cyclists;
- IV. Insulin is a more sensitive biomarker than testosterone in controlling the catabolic/anabolic balance in elite male cyclists, suggesting its potential utility in training monitoring for high-endurance athletes;
- V. Exists a relationship between hormonal and RPE responses to an ultramarathon lasting an average of 24 hours, with the increase and maintenance of high cortisol and RPE perceived by the athletes and the decrease and maintenance of low insulin levels during the race;
- VI. Assessing the behavior of cortisol and insulin and the interpretation based on their RPE could help regulate stopping and intensity strategies during extreme duration races, like the presented ultramarathon;
- VII. The rest day during stage races such as Volta a Portugal enables the body to achieve catabolic and anabolic efficiency once again;
- VIII. It raised the question of whether high cortisol values and the corresponding anabolic commitment throughout a twelve-day stage endurance competition have the expected deleterious effect on performance at that moment, or if well-trained athletes with high cardiovascular fitness develop fundamental adaptations - specifically,

cortisol tolerance and insulin sensitivity - that enable the body to maintain good global performance;

- IX. The complex relationship between hormones from different axis was underlined, confirming that anabolic/catabolic dynamism is influenced by factors such as other hormone concentrations, circadian rhythms, circulating cytokines, metabolites, osmolality, caloric balance, or mood.

Chapter 9

Recommendations for future research

Considering our findings, we believe it is crucial to continue researching this topic, with a specific focus on:

- I. Explore if a significant decrease in the insulin anabolic component when presenting high levels of cortisol and ACTH is confirmed along the seasons and define the training effects on their behaviours;
- II. Explore if hormonal imbalances such as hypoinsulinism, when confirmed as chronic, compromise the athlete's physical and mental health, leading to diseases;
- III. Explore in a longitudinal approach how the gain in cardiovascular fitness changes the sensibility to the cortisol catabolic and anti-anabolic effects;
- IV. The imbalance observed in the catabolic/anabolic hormonal profile during the ultramarathon should be further investigated in different events and repeated times during a season, and its possible detrimental effect on the athlete's health should be considered;
- V. Future research should also consider food intake and break times during ultramarathons and try to find the best relationship between them and the intensity performed, combining it with the recovery process of hormonal alterations;
- VI. Explore the effects 24, 48, and 72 hours after the end of the stage race with more than one-week length to understand the post-competition effects on the normal functioning of the endocrine system;
- VII. Explore the use of glycemic sensors that allow continuous monitoring, particularly in endurance athletes, to better understand the relationship between exercise and nutrition and what type of macronutrient is being utilized, with notable benefits for performance and metabolic health.

Chapter 10

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Chapter 1

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Chapter 4

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Chapter 5

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Appendix I

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The aging athlete

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Em 1989 o *American Journal of Sports Medicine* publicou um artigo com o tema *The Aging athlete* com o objetivo de demonstrar que o atleta que entra no processo de envelhecimento difere do competidor mais jovem em muitas facetas, apresentando diferenças fisiológicas, estruturais e psicossociais, que os distinguem como uma entidade única no mundo atlético. Refere que, apesar das alterações inevitáveis que os anos impõe aos nossos corpos, esses atletas ainda são capazes de performances incríveis de força, habilidade e resistência, apresentando um risco lesional associado ao programa atual e ao seu histórico. Realçam que a literatura sugere fortemente que a maior ameaça à saúde do atleta que entra neste processo não é o envelhecimento em si, mas sim a inatividade, sugerindo que o exercício regular pode ser capaz de retardar o declínio fisiológico associado à velhice em até 50%, chamando-lhe uma verdadeira fonte de juventude, da qual todos nós podemos recorrer. Não faz qualquer referência à componente hormonal.

A testosterona tem um papel importante na fisiologia de vários órgãos e tecidos, mas a concentração sérica diminui gradualmente com o envelhecimento. Assim, o conceito de hipogonadismo de início tardio tem ganho cada vez mais atenção nos últimos anos, promovido pelo aumento da longevidade do cidadão comum e do atleta em particular. Nos tempos atuais, muita da sintomatologia associada ao hipogonadismo natural do envelhecimento é desejavelmente combatida ou atenuada pelo cidadão comum, quer pelo seu desejo de manter a funcionalidade física, mental e sexual, quer pelo objetivo de prevenir a doença, nomeadamente, a doença cardiovascular e/ou metabólica.

Muitos artigos publicados ao longo dos últimos 20 anos apresentam resultados controversos sobre a terapia de reposição com testosterona, havendo, contudo, um certo consenso sobre o cuidado a ter nesta prática a fim de evitar efeitos laterais indesejáveis. A sua utilização é nitidamente crescente. A revista TIME, na edição de agosto de 2014, fez capa com a figura de um atleta no processo de envelhecimento, sob o tema *Manopause*, com a frase: *Feeling deflated? The low testosterone industry wants to pump you up*. Referia que, na altura, tratava-se de

uma indústria que envolvia 2,4 biliões de dólares por ano e que nos EUA, entre 2007 e 2012, o número de prescrições anuais passou de 2,9 para 7,5 milhões. Refere dados de estudo publicado em 2013 pelo pesquisador da Universidade de Sidney David Handelsman no *Medical Journal of Australia*, em que o número de prescrições escritas nos Estados Unidos cresceu quase dez vezes de 2000 a 2011, fazendo parte de um boom mundial. Uma frase curiosa podia ser lida: *não importa o que você pense sobre a terapia de reposição de testosterona, haverá sempre algum cientista, em algum lugar, com dados para apoiá-lo*, realçando as várias posições antagónicas sobre a sua utilização.

Ana Isabel Martin numa publicação de 2018, denominada *Hormones and muscle atrophy*, descreve bem o papel do sistema endócrino no metabolismo muscular, na saúde e na doença, realçando o papel anabólico da testosterona, da hormona de crescimento e do IGF-1, mas igualmente referindo o papel de hormonas catabólicas na proteólise e sarcopenia, nomeadamente o cortisol. No resumo final realça que a atrofia musculoesquelética está associada a grande variedade de condições, onde se inclui o desuso ou a imobilização, bem como a estádios catabólicos crónicos que cursam com caquexia, referindo que a sarcopenia é uma condição complexa e multifatorial e pode ser atribuída às complexas interações entre vários fatores, incluindo alterações do sistema endócrino. Refere, com cuidado, que a suplementação hormonal pode ser uma estratégia terapêutica possível, mas que a sua eficácia e segurança precisam de ser definitivamente estabelecidas por meio de estudos de maior escala.

Martine Duclos na publicação *Exercise and the HPA Axis*, de 2016, realça o facto de os processos fisiológicos relacionados ao equilíbrio endócrino serem influenciados por mais do que uma única hormona, de forma integrada em vários níveis, sendo libertadas sob processo de *feedback*, algumas controladas pela sua própria concentração periférica e outras por outras hormonas ou citocinas circulantes, metabolitos, osmolalidade, etc. Ou seja, o equilíbrio hormonal deve ser avaliado num contexto e não por avaliações hormonais isoladas, o que pode questionar a leitura e terapêutica isolada de um simples eixo hormonal.

Talvez a postura mais sensata de abordagem inicial poderá ser a proposta por *Harvard Men's Health Watch*, que coloca as seguintes questões: Você já considerou outras razões pelas quais pode sentir fadiga, diminuição da libido ou outra sintomatologia? Será que você tem uma dieta nutritiva e equilibrada? Faz exercício regularmente? Dorme bem? Aborde primariamente esses fatores antes de recorrer à terapia hormonal.

Este artigo não deve ser interpretado como um conselho médico. Converse com seu médico ou prestador de cuidados primários antes de fazer qualquer alteração em sua rotina de bem-estar.