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Special Issue Reprint

Training and Nutrition for Performance

Males, Females, and Gender Differences

Edited by
Valentín E. Fernández-Elías and Olga López Torres

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This is a reprint of the Special Issue, published open access by the journal *Nutrients* (ISSN 2072-6643), freely accessible at: https://www.mdpi.com/journal/nutrients/special_issues/42H1I903VN.

For citation purposes, cite each article independently as indicated on the article page online and as indicated below:

Lastname, A.A.; Lastname, B.B. Article Title. *Journal Name* **Year**, *Volume Number*, Page Range.

ISBN 978-3-7258-2815-9 (Hbk)
ISBN 978-3-7258-2816-6 (PDF)
https://doi.org/10.3390/books978-3-7258-2816-6

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Editorial

Training and Nutrition for Performance: Males, Females, and Gender Differences

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As sports nutrition research evolves, a growing body of evidence highlights the importance of sex-based differences in responses to dietary interventions for athletic performance. In particular, the distinct nutritional needs of female athletes and the impact of chronic energy deficits emerge as central themes. Different studies underscore the complexity of nutritional requirements across genders, emphasizing tailored interventions that can support both performance and long-term health outcomes.

Chronic low energy availability (LEA) is a pervasive issue, particularly among female athletes, which can lead to relative energy deficiency in sport (RED-S) [1]. This condition, marked by insufficient caloric intake to meet energy expenditure, not only impairs athletic performance but also affects bone density, menstrual health, and immune function [2]. The female athlete triad is a serious condition that can lead to health problems [3]. For female athletes in weight-sensitive sports, such as wrestling, maintaining energy balance is crucial yet challenging. In this context, research reveals that female wrestlers often resort to extreme weight-control practices that increase the risk of disordered eating behaviors [4]. The implications of these findings are profound; by focusing on education and structured dietary support, coaches and practitioners can encourage healthy weight management strategies and minimize the risk of nutritional deficiencies and psychological stressors that frequently accompany restrictive eating practices in competitive sports. In addition, adequate protein intake is essential for muscle repair and recovery, particularly following strenuous training [5]. However, sex-specific differences in muscle protein synthesis and amino acid metabolism highlight the need for targeted nutritional interventions [6]. While both male and female athletes benefit from increased protein intake post-exercise, studies indicate that women may require adjusted protein doses to fully support recovery and performance gains, especially in sports with high aerobic and anaerobic demands [7]. Furthermore, the metabolism of branched-chain amino acids (BCAAs) and other essential amino acids is influenced by hormonal factors, which vary significantly across sexes. Research suggests that while amino acid supplementation supports muscle recovery in both male and female athletes, women may require specific attention to amino acid intake to prevent central fatigue and support sustained energy availability, especially in endurance sports [8]. Moreover, the role of amino acids extends beyond immediate muscle repair. Amino acids such as leucine and glutamine play key roles in immune function and energy production, both of which are critical during recovery. Sex differences in the metabolism of these amino acids may affect the efficiency of recovery strategies. While men may metabolize certain amino acids more rapidly, women may benefit from adjusted timing and types of amino acid supplementation to optimize recovery and reduce inflammation [9]. Understanding these nuances in amino acid metabolism offers practical insights into developing personalized nutrition plans that optimize performance while safeguarding health.

However, lipid metabolism also reveals significant insights into recovery, particularly in sports demanding high aerobic capacity [10]. The lipid profile of red blood cells, specifically their glycerophospholipid composition, has been linked to aerobic performance.

Citation: López Torres, O.; Fernández-Elías, V.E. Training and Nutrition for Performance: Males, Females, and Gender Differences. *Nutrients* **2024**, *16*, 3979. https://doi.org/10.3390/nu16233979

Received: 6 November 2024
Accepted: 8 November 2024
Published: 21 November 2024

Lipid-rich cell membranes enhance red blood cell deformability and oxygen transport, directly influencing endurance capacity. Interestingly, female athletes show different lipid utilization patterns than males, which could impact energy availability and recovery. The findings in recent research indicate that optimizing lipid profiles through targeted dietary interventions, potentially with a higher emphasis on omega-3 and omega-6 fatty acids, may help improve endurance and reduce exercise-induced inflammation, thus supporting long-term performance in endurance sports [11].

On the other hand, some supplements can be considered useful for increasing performance [12]. However, not all supplements or ergogenic aids have the same effects in men and women [13]. For instance, creatine supplementation, a well-researched ergogenic aid [14], also appears to impact recovery and muscle strength differently across sexes. While creatine is widely known for its benefits in high-intensity and strength-based activities, studies show that men tend to experience more significant strength gains from creatine supplementation than women. These differences may stem from hormonal variations that influence creatine's efficacy on muscle strength and mass. For example, men may have a more pronounced response to creatine due to higher basal levels of testosterone, which interacts with creatine to promote muscle synthesis. For female athletes, creatine still provides benefits, but dosages and timing might need adjustment to maximize its effectiveness without leading to excessive water retention or muscle fatigue. The gender-specific outcomes of creatine supplementation underscore the need for tailored approaches that consider the physiological differences in muscular adaptations to strength training [15].

Additionally, hormonal factors contribute to the differential effects of various ergogenic aids and nutrients on muscle strength and endurance across genders. Hormones such as estrogen play a protective role in muscle damage, allowing female athletes to recover faster from intense training, yet this same factor may mitigate some of the hypertrophic responses seen in male athletes [16]. Recent findings suggest that hormonal modulation may play a role in the efficacy of nutritional interventions such as protein and creatine supplementation [7,15]. By considering these hormonal influences, sports nutritionists can create more precise and effective nutritional protocols that account for sex-specific metabolic responses, ultimately aiding in the prevention of overtraining and enhancing recovery. In terms of overall muscle strength, meta-analytical data support the effectiveness of protein and creatine supplementation in strength improvements [16]. However, these effects vary by sex, with men generally experiencing more significant gains in upper and lower body strength compared to women. The reasons for these differences are multifaceted, including variations in muscle fiber composition, hormonal response to resistance training, and differences in baseline creatine storage. These findings advocate for sex-specific supplementation protocols in sports nutrition, where the nuances of muscle physiology are respected to ensure that both male and female athletes can reach their strength potential.

The culmination of these findings underscores the importance of individualized dietary strategies that consider both physiological and psychological factors [17]. Female athletes, particularly in sports that emphasize weight control, require comprehensive support that addresses energy needs, protein intake, and safe weight management strategies [1,3,18]. For male athletes, maximizing gains in strength and endurance through targeted supplementation may be more straightforward but still requires a deep understanding of timing, dosage, and nutrient interactions.

In conclusion, the literature reveals the complexity of training and nutrition interactions influenced by sex-based differences. By integrating these insights into practice, sports nutrition can advance toward more personalized approaches, ultimately promoting optimal performance and health outcomes across diverse athletic populations. Such individualized strategies are not only critical for enhancing individual performance but are also essential for supporting the long-term health and well-being of athletes in high-demand sports environments.

Conflicts of Interest: The authors declare no conflicts of interest.

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Sportomics Analyses of the Exercise-Induced Impact on Amino Acid Metabolism and Acute-Phase Protein Kinetics in Female Olympic Athletes

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Citation: Muniz-Santos, R.; Bassini, A.; Falcão, J.; Prado, E.; Martin, L., III; Chandran, V.; Jurisica, I.; Cameron, L.C. Sportomics Analyses of the Exercise-Induced Impact on Amino Acid Metabolism and Acute-Phase Protein Kinetics in Female Olympic Athletes. *Nutrients* **2024**, *16*, 3538. https://doi.org/10.3390/nu16203538

Academic Editor: Valentín E. Fernández-Elías

Received: 25 September 2024
Revised: 12 October 2024
Accepted: 14 October 2024
Published: 18 October 2024

Abstract: Background: Exercise can be used as a model to understand immunometabolism. Biological data on elite athletes are limited, especially for female athletes, including relevant data on acute-phase proteins and amino acid metabolism. Methods: We analyzed acute-phase proteins and amino acids collected at South American, Pan-American, and Olympic Games for 16 Olympic sports. We compared female and male elite athletes (447 vs. 990 samples) across four states (fasting, pre-exercise, post-exercise, and resting) to understand sex-specific immunometabolic responses in elite athletes. Results: Considering all states and sports, we found that elite female athletes exhibited higher concentrations of C-reactive protein, lipopolysaccharide-binding protein, myeloperoxidase, haptoglobin, and IGF1, with ratios ranging from 1.2 to 2.0 ($p < 0.001$). Women exhibited lower concentrations of most amino acids, except for glutamate and alanine. Although almost 30% lower in women, branched-chain amino acids (BCAAs) showed a similar pattern in all states ($\rho \geq 0.9$; $p < 0.001$), while aromatic amino acids (AAAs) showed higher consumption during exercise in women. Conclusion: We established sex dimorphism in elite athletes' metabolic and inflammatory responses during training and competition. Our data suggest that female athletes present a lower amino acid response towards central fatigue development than male athletes. Understanding these differences can lead to insights into sex-related immuno-metabolic responses in sports or other inflammatory conditions.

Keywords: amino acid metabolism; elite female athlete; exercise immunology; acute-phase protein; Olympic Games; dried blood spot; mass spectrometry; sportomics

1. Introduction

Exercise-induced impacts on amino acid metabolism and acute-phase protein kinetics have been a topic of many investigations [1–3].

Amino acids (AAs) play multiple roles related to exercise, such as serving as energy sources, signaling protein synthesis during recovery, or facilitating neurotransmitter synthesis. AA supplementation in athletes has been extensively investigated as a performance-enhancing and recovery strategy, especially for glutamine, alanine, and the branched-chain amino acids (BCAAs; valine, isoleucine, and leucine) [4–7]. The role of amino acids in different central nervous system physiopathological conditions has been widely discussed [7–10]. A pivotal role of amino acid metabolism in developing exercise-induced hyperammonemia and central fatigue during exercise has also been investigated [8,11,12]. This effect seems to be caused by amino acid metabolism and AMP deamination through the myokinase reaction (E.C. 2.7.4.3) [13]. Both processes can result in the release of ammonia into the blood, exceeding its clearance capacity and subsequently impairing physical and mental functions due to ammonia's toxic properties [14–16]. The altered proportion of amino acids in the bloodstream can change their concentrations within the central nervous system, potentially impacting neurotransmitter synthesis and leading to fatigue (as proposed by Fischer and Newsholme) [17,18]. AA profile changes can also reveal underlying regulation of biochemical pathways, such as gluconeogenesis, ketogenesis, and lipolysis [19,20]. Acute-phase proteins (APPs) may be upregulated (positive APPs) or downregulated (negative APPs) in response to inflammation-triggering events [21]. They have been considered markers indicating the systemic effects of cytokine regulation, which, conversely, are difficult to measure clinically due to their lower concentrations (~pg/mL) and short half-lives. Intense exercise can induce a state often called hypermetabolic stress and affect the inflammatory acute-phase response [22]. Assessing APPs' response in exercise can shed light on hemolysis, gut permeability, bacterial translocation, and the innate immune response [23–25]. Select proteins not categorized as acute-phase proteins can be evaluated with APP analysis, providing other information regarding kidney function and volemia changes [23]. Understanding APPs' kinetics in response to exercise can be an excellent model for understanding the immunometabolic responses in human and animal physiology and pathophysiology [26,27].

Dried blood spots (DBSs) can be used to carry out accurate and effective blood AA and APP measurements [23,28,29]. The convenient sample storage and collection from fingertip capillary blood in DBS have enabled multiple collections in real conditions faced by athletes [30]. DBS can be easily used to monitor athletes' responses to different exercise and recovery phases and analyze the impact of different exercise sessions during training and competition, supporting individually tailored precision interventions.

It has been acknowledged that women may exhibit distinct metabolic responses to exercise compared to men [31]. There is evidence of sex-specific differences in the regulation of BCAA catabolism in mouse models [32], and various studies have revealed sex-based differences and suggested different interventions for male and female athletes [33]. Considering that energy metabolism is linked to amino acid metabolism, it is reasonable to hypothesize that amino acid and acute-phase protein metabolism could also show sex-specific differences in elite athletes. Indeed, professional athletes have been shown to exhibit different concentrations of metabolites, such as amino acids, compared to control individuals [34]. However, most studies on AA, APP, and other metabolic parameters have focused on male and non-elite athletes and neglected to investigate their responses to different sports in real competitions or training sessions [35,36]. In fact, data related to elite athletes and teams—not only metabolic data—are often not published due to privacy protection, competitive secrets, and lack of incentives or interest from clubs and federations [37,38].

More information concerning AA and APP metabolism in high-level sports needs to be provided, particularly for elite female athletes since increasing numbers of female athletes participate in Olympic events [39,40]. Data from the International Olympic Committee

(IOC) show that the proportion of women participants in the Olympic Games has seen a notable rise—from 34% of the total in Atlanta in 1996 to a new high of 48% in Tokyo 2020, with a pledge to achieve complete sex equality in the Games of the XXXIII Olympiad in Paris 2024 [41]. It has been shown that female athletes are exposed to a higher risk of injury [39], which is also partially dependent on hormonal and metabolic changes related to the menstrual cycle [40]. In this sense, we previously detected gaps in the existing literature on the interplay between exercise metabolism and the menstrual cycle in elite female athletes [42]. Due to the lack of female-specific data, data retrieved from male athletes are used to manage women's training and recovery, which is, at minimum, inefficient and potentially harmful.

Our main objective was to investigate AA and APPs' responses in Olympic athletes from 16 Olympic sports across different states (fasting, pre-exercise, post-exercise, and resting) during training sessions and competitions. By performing sex-based stratification, we aimed to identify and characterize potential sex-specific differences and present all quantitative data. Due to the similarity of metabolic and inflammatory pathways in exercise and certain pathological conditions, our results may provide a better understanding of these responses in different scenarios.

2. Materials and Methods

2.1. Participants

Using our biobank of dried blood spot (DBS) samples collected during major sports events and training sessions of athletes from the Brazilian Olympic Committee (BOC), we identified all female athletes who had provided samples for amino acid (AA) and acute-phase protein (APP) analysis between January 2014 and January 2016. All procedures involving human subjects were approved by the ethics committee for human research at the Federal University of the State of Rio de Janeiro (117/2007, updated and renewed in 2011, 2013, and 2016) and the Federal University of Mato Grosso (2017–2021) and met the requirements regulating research on human subjects [43]). Participants were obligated not to use any prohibited performance-enhancing substances or methods, as confirmed by the negative results from various doping control analyses. Moreover, according to BOC policy, all athletes were advised not to use supplements, even from compounding pharmacies, due to the risk of contamination. We retrieved 54 female athletes and 272 samples for APP analysis, and 17 female athletes and 175 samples for AA analysis. The female athletes represented 11 different sports disciplines (athletics, boxing, cycling, modern pentathlon, karate, taekwondo, softball, gymnastics, basketball, archery, and swimming).

Using the same biobank, we identified all male athletes who had provided samples for AA and APP analysis during the same period. In total, we retrieved 76 male athletes with 471 samples for APP analysis, and 47 male athletes with 520 samples for AA analysis. The male athletes represented 16 different sports disciplines (athletics, boxing, beach volley, canoeing, cycling, handball, modern pentathlon, karate, taekwondo, softball, gymnastics, basketball, archery, diving, ultra-marathon, and swimming).

The athletes provided the blood mainly in four different states: fasting, pre-exercise or post-exercise (meaning before or after training or competition), and resting (mostly one hour after the end of exercise) for different sports, both women and men. The sample collections were performed under field-of-play conditions that the athletes face in their training sessions or competitions, i.e., an environment that cannot be controlled as in laboratory conditions. The competitions in which our collections were performed encompassed the Pan American and South American Games. Most of the athletes analyzed are also Olympic medalists.

2.2. Sample Collection and Amino Acid and APP Quantifications

Samples were collected using lancet finger-pricks (Microtainer Contact-Activated lancet, BD, Franklin Lakes, NJ, USA) dried on Whatman 903 Protein Saver DBS cards

(Merck Sigma-Aldrich, Darmstadt, Germany). The cards were dried at 4 °C in the presence of a desiccant and processed daily.

We used the AbsoluteIDQ® p180 Kit (Biocrates, Innsbruck, Austria) to quantify the concentrations of 21 amino acids (alanine, arginine, asparagine, aspartate, citrulline, glutamine, glutamate, glycine, histidine, isoleucine, leucine, lysine, methionine, ornithine, phenylalanine, proline, serine, threonine, tryptophan, tyrosine, and valine).

We used SISCAPA-MRM mass spectrometry to analyze selected acute-phase proteins as described [25] (Alpha-1-acid glycoprotein—A1AG11; Cystatin C—CST3; C-reactive protein—CRP; Hemoglobin beta chain—HBAA; Haptoglobin—HPTT; Insulin-like growth factor 1—IGF1; Lipopolysaccharide binding protein—LBP; Mannose-binding lectin—MBL2; Myeloperoxidase—MPO and Serum amyloid A1—SAA11).

2.3. Bioinformatic Analysis

Raw data were analyzed using the SciPy Python library (ver. 1.12.0) in Python 3.11.1. We used non-parametric Spearman's rank-order correlation (rs, scipy.stats.spearmanr method) as in previous studies [23]. The off-diagonal entries represent direct positive or negative correlations of pairwise proteins (the correlation matrix is symmetric; diagonal entries represent self-correlations). Supplementary Table S1 includes all data, but the discussion focuses only on protein pairs with $\rho > 0.5$ and significance of $p < 10^{-3}$. Correlation matrices were visualized using Matlab R2023b (Mathworks, Natick, MA, USA) and the PyPlot Python library (ver. 3.1.2). To emphasize correlations with statistical significance, only correlations with $p < 0.01$ or $p < 10^{-3}$ were used in the resulting image, as indicated. The color of each cell was then determined by linearly interpolating the correlation (blue for positive correlation, red for negative correlation).

Violin plots were generated using Matlab ver. 2023b (Mathworks, Natick, MA, USA) and grpandplot ver. 1.0.0 [44].

Sports-specific changes in AA and APP from pre- to post-exercise across all sports—cycling, karate, and modern pentathlon—were visualized as a network using NAViGaTOR ver. 3.0.19 [45]. The final figure with legends was prepared from an exported SVG file in Adobe Illustrator ver. 28.4.

3. Results

3.1. Acute-Phase Proteins

We measured and analyzed 10 proteins to examine the exercise-induced inflammatory acute-phase response and other markers among elite female athletes, highlighting the differences from their male counterparts. A comprehensive analysis of blood concentrations considering all collection states unveiled significant sex dimorphism for most of the measured APPs (Figure 1a,b). It is essential to highlight that CRP, LBP, HP, SAA1, MBL2, and MPO exhibited a broader range of concentrations in women than in men (Figures 1a and 2a). Overall, IGF1, HP, LBP, MPO, and CRP (in ascending order of dimorphism impact) were higher in women, with a female-to-male median ratio varying from 1.2 to 2.0 ($p < 0.001$) across all states and sports (Figure 1b). In contrast, ORM1 and MBL2 were significantly lower in women (1.2-fold and 2-fold, respectively; $p \leq 0.001$) (Figure 1a,b). Three proteins—SAA1 ($p = 0.1$), HBA ($p = 0.075$), and CST3 ($p = 0.219$)—were not different between women and men (Figure 1a).

Proteins linked to general inflammation (CRP), gut permeability (LBP), neutrophil activity (MPO), and hemolysis (HP) exhibited similar patterns of intersexual variations across states (Figure 2a). While significantly higher in women in most analyzed states, sex-based differences in APP appear to be attenuated by exercise. CRP concentrations were significantly higher in women during fasting and pre-competition (3-fold and 2.1-fold, respectively), whereas post-exercise collection states were more similar between sexes (~1.8-fold higher in women post-exercise, with no significant difference in resting) (Figure 2b). Although not statistically significant, SAA1 displayed a trend toward higher concentrations

in women from fasting to post-exercise (1.4–1.1-fold) and lower concentrations in resting (1.5-fold), mirroring CRP's sex-based kinetic differences (Figure 2b).

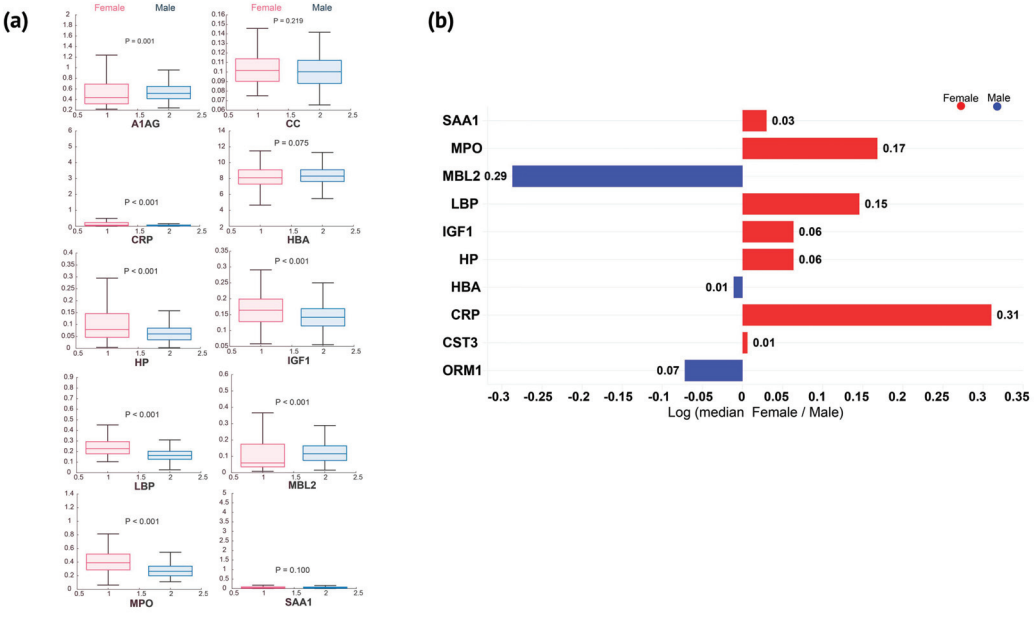

Figure 1. Female athletes presented higher concentrations of inflammatory proteins than male athletes. (**a**) Protein concentration distribution and comparison between sexes across all states and sports unveiled significant sex dimorphism. (**b**) Ratio of the median values for each measured protein between women and men, considering all states together.

Exercise also attenuated sex-based differences in LBP, MPO, HP, and IGF1. LBP and MPO were significantly higher across all states and sports in women (1.5-fold and 1.4-fold, respectively) (Figure 1a and 1b). During post-exercise states, LBP's sex-based difference progressively decreased, remaining statistically significant post-exercise (1.4-fold), but no longer in resting (Figure 2a). MPO was higher in women pre-exercise, but exercise acutely attenuated the sex-based difference, which was no longer significant post-exercise, returning to the pre-exercise women-to-men ratio during resting (Figure 2a). HP's women-to-men median ratio was also elevated from fasting to post-exercise, with no significant sex-based difference in resting (Figure 2a,b). IGF1's response appeared to follow a similar pattern but with a smaller magnitude of intersexual difference (varying from 1.3-fold higher in fasting to 1.1-fold higher post-exercise, with no significant difference in resting) (Figure 2a,b).

In contrast, MBL2 and ORM1 were predominantly lower in female than in male athletes. MBL2 concentrations were not statistically significantly lower in the women during fasting and pre-exercise. Exercise induced a sex-based difference during post-exercise collections, from 1.8-fold post-exercise to 2.6-fold lower in women during resting. ORM1 was significantly different pre-exercise, and exercise increased the dimorphism, albeit to a lesser extent than it did for MBL2 (Figure 2a,b).

To explore potential relationships between specific APPs, we performed Spearman correlation analyses of the proteins, considering all states and sports together. We chose to discuss correlations with $p < 10^{-3}$ and $\rho > 0.5$. Both female and male athletes presented significant positive correlations between CRP and SAA1, CRP and LBP, and SAA1 and ORM1 (ρ = 0.5–0.6) (Figure 3). In addition, women also presented significant positive

correlations between SAA1 and LBP, MBL2 and ORM1, LBP and CST3, and CST3 and HBA (ρ = 0.5–0.6). All correlations can be found in Supplementary Table S1.

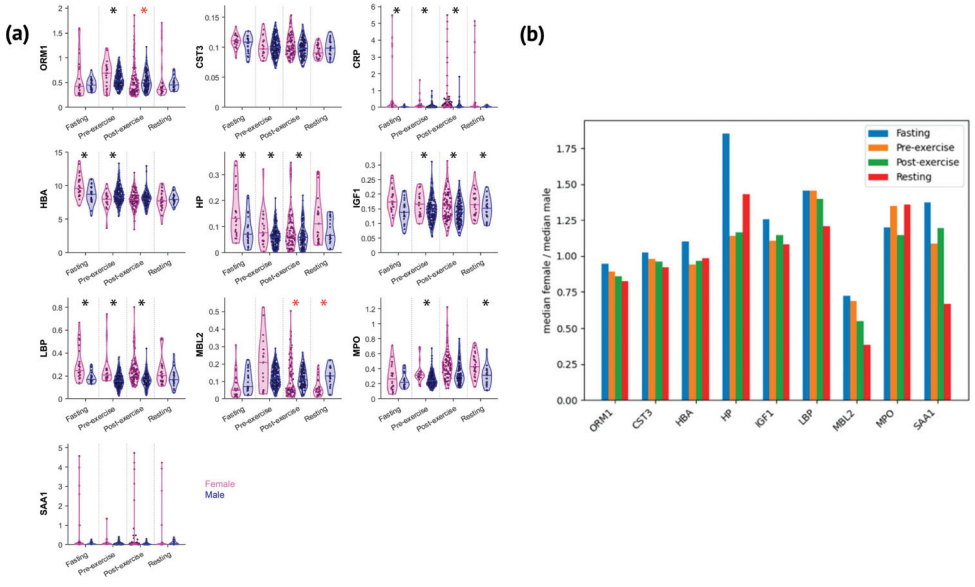

Figure 2. Female athletes presented a broader range of acute-phase protein concentrations than men in different states but seemed to exhibit a lower acute-phase response following exercise. (**a**) Protein changes and distribution across states adjusted for sex. (**b**) Ratio of the median values for each measured protein between women and men across all analyzed states. * = significantly higher in women. * = significantly lower in women.

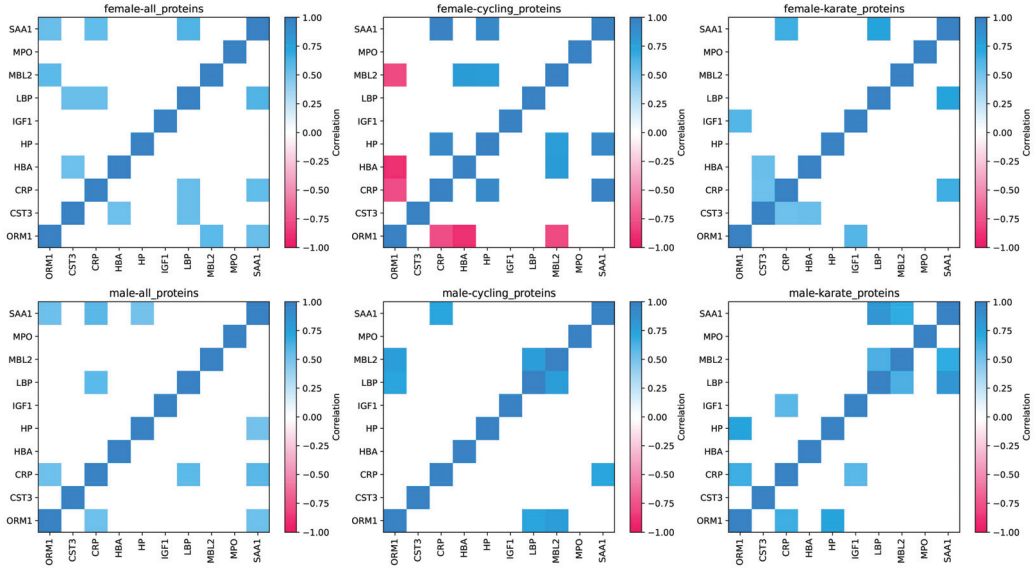

Figure 3. Spearman pairwise correlation matrices for proteins, considering all states together across different sports. Only correlations with $p < 0.001$ and ρ > 0.5 are highlighted.

We also explored correlations within specific sports due to their different physical and skill demands. We chose to investigate potential correlations in modern pentathlon, cycling, and karate, as they represent different types of exercise in terms of intensity, duration, movements, and biomechanical aspects. As hypothesized, the three analyzed sports presented different APP correlations for both women and men (Figures 3 and 4). As expected, correlations within specific sports were stronger than correlations across all sports combined. In cycling, the pair CRP-SAA1 was the only one that showed a positive correlation for women and men ($\rho > 0.9$, $\rho > 0.7$, respectively). Meanwhile, the only discordant pair was MBL2-ORM1, showing a negative correlation for women and a positive one for men ($\rho > -0.7$, $\rho = 0.8$) (Figure 3). Women exhibited two additional pairs with negative correlations, also involving ORM1 (ORM1-CRP $\rho = -0.8$ and ORM1-HBA $\rho = -0.9$). In karate, only LBP-SAA1 was correlated for both female and male athletes ($\rho > 0.7$, $\rho > 0.8$, respectively) (Figure 4). CRP-SAA1 was correlated across all analyzed sports for women but not for male athletes (Figure 3).

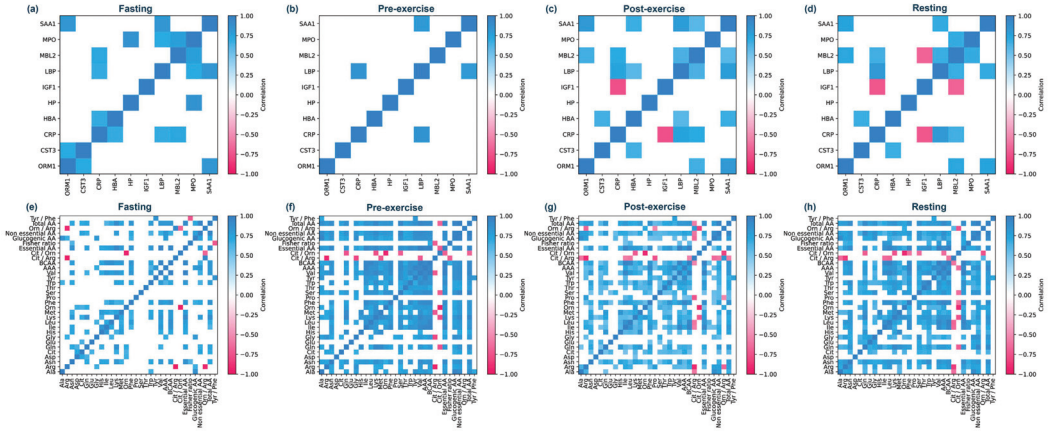

Figure 4. Spearman pairwise correlation matrices for proteins and amino acids, considering samples collected from female modern pentathlon athletes. Panels (**a**–**d**) show the measured protein correlations in different states; panels (**e**–**h**) show the amino acids correlations at different exercise moments. Only correlations with $p < 0.001$ and $\rho > 0.5$ are highlighted.

To emphasize changes in correlation among female athletes across states, we computed Spearman correlations within the four different states for modern pentathlon (Figure 4). Exercise produced an increase in significant correlations among APPs, with the most notable being SAA1-ORM1 ($\rho = 0.8$), SAA1-LBP ($\rho = 0.8$), and LBP-CRP ($\rho = 0.7$). In MP, we found two pairs with significant negative correlation involving IGF1. IGF1-MBL2 were negatively correlated in post-exercise and resting ($\rho = -0.5$, $\rho = -0.7$), while IGF1-CRP were negatively correlated in pre-exercise, post-exercise, and resting ($\rho = -0.6$, $\rho = -0.8$, $\rho = -0.7$). It is noteworthy that IGF1 in modern pentathlon (MP) tended to be negatively correlated with all other proteins, especially after the fasting period, even though it did not reach statistical significance based on our predetermined threshold for discussion ($p \leq 0.01$) (Supplementary Table S1).

3.2. Amino Acids

We measured and analyzed 21 amino acids across the four examined states. Elite female athletes exhibited lower concentrations of most amino acids, except for glutamate and alanine, which had similar concentrations in both sexes across all sports and states (Figure 5a,b). The concentration of selected amino acids followed a similar pattern in both sexes but at different magnitudes.

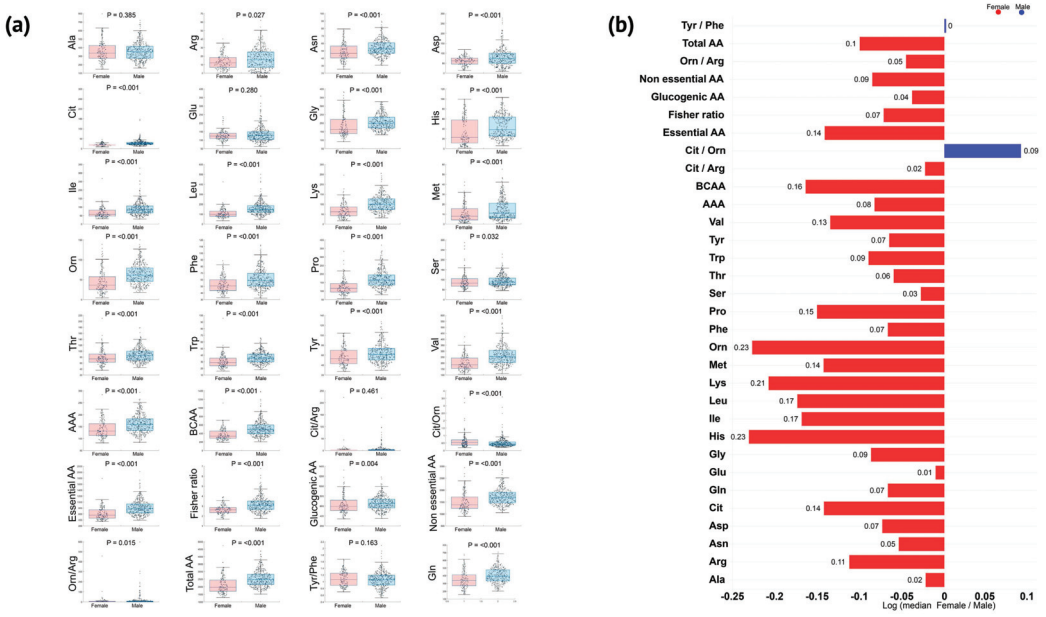

Figure 5. Women present lower amino acid blood concentrations than male athletes. (**a**) Amino acid distribution and comparison between sexes across all states and sports unveiled significant sex dimorphism. (**b**) Ratio of the median values for each measured amino acid between women and men considering all states together.

We did not find significant sex-specific differences in blood alanine in any analyzed state (Figure 6a,b). Alanine increased roughly 1.4-fold after breakfast (from fasting to pre-exercise) for both groups and additionally significantly increased by 6–12% with exercise, remaining 30–40% above fasting concentrations in resting (Figure 6a). Feeding promoted a rise in glutamine concentration of 51% in women and 29% in men, which decreased in response to exercise. Glutamine was significantly lower in female athletes, although they appeared to show greater glutamine conservation during resting than men (0.83 vs. 0.77, compared with pre-exercise), also maintaining a 25% higher concentration for the amino acid compared to fasting (while men reached the fasting concentration) (Figure 6a). Blood glutamate was mostly unchanged by feeding or exercise in both groups of athletes and was not significantly different between the sexes (Figure 6a,b).

Although almost 30% lower in women (Figure 5b), BCAA showed a similar response to exercise in both sexes, while aromatic amino acids (AAAs; the sum of tyrosine, tryptophane, and phenylalanine) were consumed during exercise in men but not in women (Figure 6a,b). A decreased blood ratio between BCAA and AAA (Fischer ratio) may play a role in developing exercise-induced central fatigue. Although the Fischer ratio was significantly lower in women in every analyzed state, the acute exercise-induced decrease occurred only in male athletes and remained lower in resting. Another theory related to central fatigue development is the serotoninergic theory, which is related to the ratio between tryptophan and BCAA blood concentration. Female athletes did not present significant changes in this ratio, while male athletes exhibited an 11% decrease from pre- to post-exercise and 16% from pre-exercise to resting). Selected correlation pairs were maintained across the sex vs. state analysis (Figure 7). Among the BCAAs; valine concentration consistently exceeded that of isoleucine and leucine. Total BCAA exhibited strong correlations with each BCAA in every analysis conducted ($\rho \geq 0.9$), mirroring the correlations observed among the individual BCAAs themselves ($\rho \geq 0.8$). Interestingly, tyrosine, tryptophan, and phenylalanine were correlated with BCAAs for both women and men, and these

correlations were maintained in cycling and karate for women but not for men. Arginine also correlated with BCAAs only for women ($\rho = 0.7$) but not for men. Blood amino acids involved in the urea cycle were highly affected. Arginine was greatly affected by breakfast in both groups. The amino acid concentration in the blood rose 1.5-fold in female and 1.4-fold in male athletes (Figure 6). Women conserved arginine more than men (85 vs. 53%). Ornithine showed a different pattern per group. In women, ornithine doubled in response to breakfast, returning to fasting concentrations after exercise (post-exercise and resting). For male athletes, ornithine rose by 40% after breakfast, with a slower decrease caused by exercise (a further 20% drop post-exercise, returning to fasting concentrations in resting). Like ornithine, exercise caused a drastic reduction in citrulline concentrations in women. Exercise almost doubled the sex-specific difference in ornithine concentration from 1.1- to 1.9-fold lower in women (also mainly driven by a decrease in women from 63.1 to 32.1 µmol/L). Also, it increased the difference in citrulline concentration from 1.3- to 1.4-fold lower in women. Aspartate and asparagine were slightly affected by either breakfast or exercise. Interestingly, histidine was one of the most affected amino acids showing sex dimorphism. Blood histidine concentration in elite female athletes showed a very distinctive pattern compared to their male counterparts. The amino acid rose 5.4-fold after breakfast, dropping to 40% compared to the pre-exercise condition, with an additional 16% drop during resting. Male athletes had a minor increase after breakfast followed by a delayed drop in histidine blood concentrations (22.4, 62.7, 58.2, and 28.3 µmol/L for fasting, pre-exercise, post-exercise, and resting, respectively). The substantial drop in the women-to-men ratio in the post-exercise phase was primarily driven by a substantial decrease in women's histidine concentration, from 61.6 to 25.7 µmol/L (Figure 6). Serine decreased by 21% in elite female athletes, while it did not change in men.

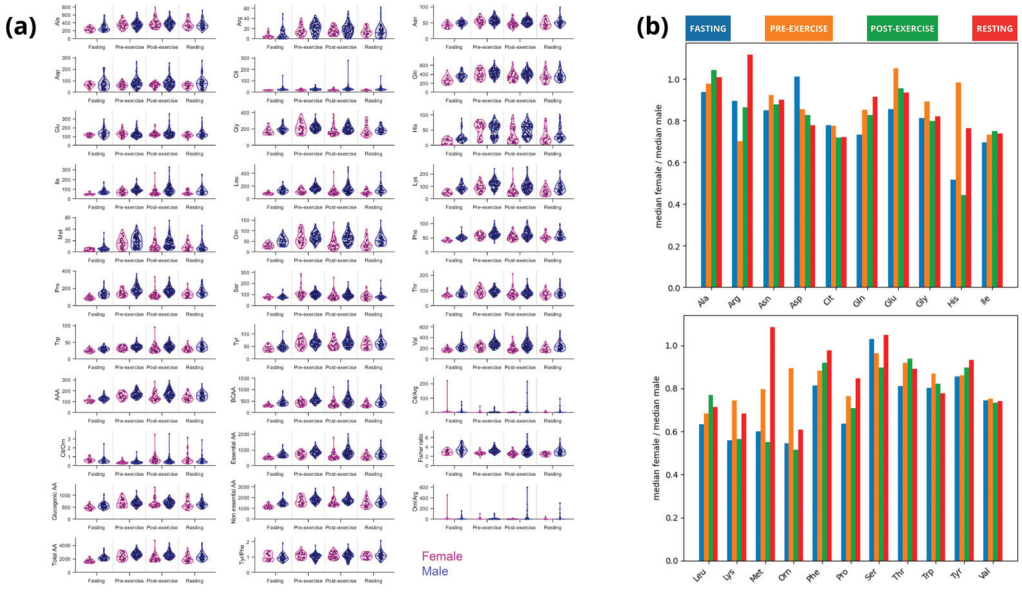

Figure 6. Selected amino acids involved in energy metabolism, protein synthesis, recovery, and central fatigue exhibit sex-based differences in response to exercise. (**a**) Amino acid kinetics and distribution across the states for each analyzed sex. (**b**) Ratio of the median values for each measured amino acid between women and men across all analyzed states.

Amino acid correlations were affected by sports modality. Interestingly, female athletes presented a considerably higher number of correlated pairs than men in HOLO analyses and the specific sports analyzed (cycling and karate) (Figure 7).

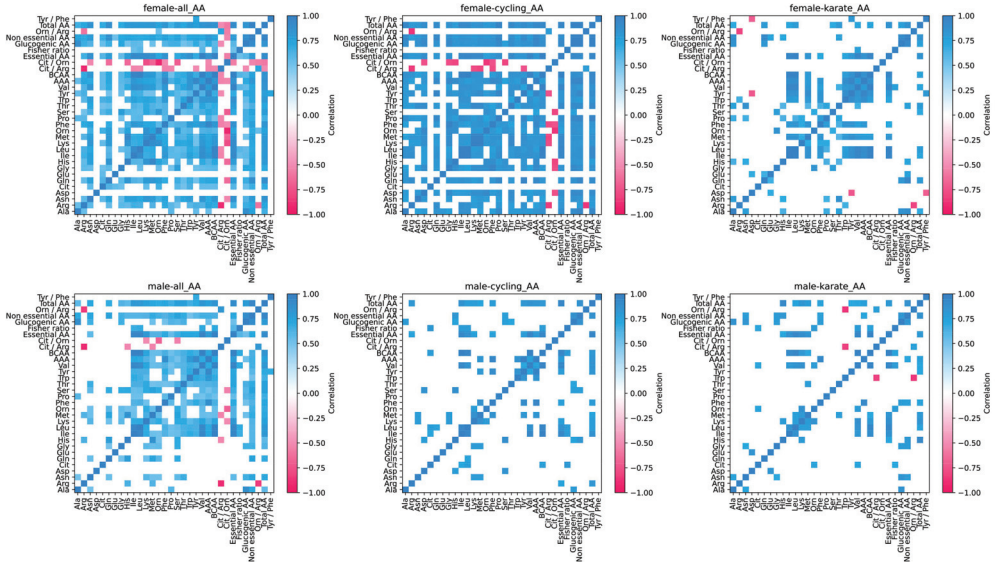

Figure 7. Spearman pairwise correlation matrices for amino acids, considering all states together across different sports. Only correlations with $p < 0.001$ and $\rho > 0.5$ are highlighted.

In MP, as observed in the previous APP analysis, exercise increased the number of correlated pairs. However, in the amino acid evaluation, we observed that the predominant factor driving the increase in correlated pairs was primarily dietary intake, occurring between the "fasting" and "pre-exercise" states (Figure 4).

4. Discussion

Here, we demonstrated that selected acute-phase proteins showed sex-specific differences in response to exercise stress in elite athletes, either in direction or magnitude, highlighting the importance of exercise as a model for understanding immunometabolism.

There are limited data on elite athletes' biological parameters, partially due to the need for anonymity or to avoid revealing data that other competitors can use to improve performance [35]. Such data on women are even more scarce. Sharing data on elite athletes (both women and men) is critical for improving the understanding of metabolic and inflammatory responses, and thus advancing exercise science and pathophysiology. Here, we explored parameters in elite athletes during world-class training and competitions, comparing sex-dependent inflammatory and metabolic responses. Our data are reinforced by mass-spectrometry-based anti-doping control in all analyzed athletes, ensuring the presented results are not affected by drugs, which could alter metabolic responses. Moreover, we used DBS due to its advantages, such as being easy to collect and less invasive, requiring a small amount of blood, the cost of shipping/storage being significantly lower, analyte stability, and reduced risk of infection [46].

4.1. Acute-Phase Protein Response in Elite Female Athletes

CRP concentrations are widely evaluated in clinical settings to screen, diagnose, and monitor inflammatory conditions [47–49]. CRP analysis can provide insights into the impact of a specific exercise session on an individual and their recovery process [43,50]. It has been shown that blood CRP changes are affected by multiple, often conflicting factors, such as ethnicity, medications, age, and sex [51–53]. Women present higher CRP concentrations than men, even when adjusting for age, medication, and cardiovascular risk factors [54]. Other studies have reported higher CRP concentrations in women, with a stronger correlation between CRP concentration and central adiposity, with the difference

being maintained across ethnic groups [55–58]. It is widely accepted that acute exercise can lead to a transient increase in serum CRP depending on factors such as exercise intensity and individual adaptation to the exercise [59,60]. Our results show that this sex-based difference is maintained across elite athletes, with women presenting higher concentrations of CRP (Figure 1a). However, the sex-based difference was attenuated during post-exercise periods. The CRP median women-to-men ratio progressively decreased from 3.1 in fasting to 1.1 in resting, being no longer different between sexes in the last analyzed state. Similarly, the SAA1 median women-to-men ratio decreased from 1.4 in fasting to 0.7 in resting. However, SAA1 was not significantly different between sexes in any state and only increased in male athletes from pre-exercise to resting. In fact, it has been reported that female soccer and netball athletes might present little acute-phase response to exercise under typical training challenges (not competition), with CRP likely being the most sensitive protein [26]. Also, our data suggest that women exhibit a broader range of CRP values (Figure 2a). The higher fasting values for women's CRP and the different patterns in the post-exercise state seem valuable when tracking the impact of training and recovery. Tailoring training sessions and recovery protocols considering these sex-specific inflammatory responses might reduce the risk of overtraining or injury and improve recovery time (so important to elite athletes).

LBP is released in response to LPS entering the bloodstream, following the presence of bacteria or LPS translocation [61,62]. Intense exercise can increase epithelial wall permeability to LPS, supporting LBP as a marker for gut permeability changes during exercise [63,64]. These findings support the idea of exercise as a model for immunometabolism during metabolic stress. We have previously shown that LBP positively correlated with CRP under inflammatory conditions (~1700 DBS samples collected during infections, vaccinations, surgery, intense exercise, and Crohn's disease) [30]. Unlike CRP, there are limited data on sex differences in LBP concentrations among the general population. Even though LBP has been increasingly used in clinical research, we could not find any relevant data on elite athletes. A recent systematic review showed no sex-based difference in indirect markers of gut damage or permeability following different types of exercise [65]. Unfortunately, LBP was not included in the meta-analysis [65]. Marriot et al. reported in an in vivo study a lower increase in LBP following intraperitoneal injections of LPS in female mice [66]. In that study, the authors collected samples 24 h after injection, which can be partially compared with our dataset's "resting" state. Our data show that female athletes have significantly higher LBP concentrations across all states than men (Figure 1a), with an exercise-induced decrease in the sex-based difference, from 1.5 in pre-exercise (significantly different between sexes) to 1.2 in resting (not different). Moreover, our data showed that LBP correlates with CRP in women across all sports, with stronger correlations observed in modern pentathlon (a sport combining a long-duration exercise with different intensities and skills).

It is known that blood haptoglobin decreases in response to hemolytic conditions due to it forming complexes with free hemoglobin [67]. Exercise-induced hemolysis can be intensity-dependent due to mechanical or metabolic mechanisms, thus reducing haptoglobin concentration [68]. In our study, we did not find significant HP changes from pre- to post-exercise for both sexes; however, from pre-exercise to resting, HP significantly decreased in women and increased in male athletes (Figure 2a). Women also presented higher HP concentration across all states, with the median women-to-men ratio varying from 1.9 to 1.2 (fasting and pre-exercise/post-exercise, respectively). This finding suggests that elite female athletes may experience an opposite hemolysis response to exercise than men. It is critical to highlight that interpreting changes in the women-to-men ratio of HP is challenging since modifications can be due to hemolysis, inflammatory response, or their combination. HP concentrations can increase in reactive states, such as infection and trauma [69,70]. In fact, HP was not different between sexes in resting, similar to CRP and LBP response findings (Figure 2a). Myeloperoxidase (MPO) is an enzyme found in granulocytes, particularly in neutrophils and monocytes. Previously, we demonstrated that neutrophil count increase correlates with CRP in male amateur triathletes during a 200 km cycling race [71]. It has also been shown that different types of exercise and sports, as well

as different intensities (e.g., 45% VO_2 max/4 h, 60% VO2 max/3 h, and 75% VO_2 max/2 h), increased MPO and neutrophil counts in male athletes [72,73]. However, it seems that MPO and neutrophil counts do not correlate in the post-exercise collection. However, the low number of participants and the absence of sex-specific analysis limit the study's conclusions [73]. The increase in MPO in response to exercise has also been described in animal models [74]. Our data show that exercise acutely decreased the MPO women-to-men ratio from 1.4 in pre-exercise to 1.1 in post-exercise, returning to the pre-exercise ratio during resting. Also, MPO increased significantly in both sexes from pre- to post-exercise, but the elevation remained significant only among female athletes during resting. Although MPO did not correlate with any APPs measured across all sports, it did show a positive correlation with CRP (pre-exercise), MBL2 (post-exercise), and HP, LBP, and MBL2 (resting) in female modern pentathlon athletes (Figure 4). Conversely, MBL2 was approximately 2-fold lower in elite female athletes and significantly different in post-exercise collections (1.8-fold lower in women post-exercise and 2.6-fold lower in resting). The literature in the field is scarce, but a previous study indicated that healthy non-athlete women presented similar MBL2 values to men and did not identify changes in MBL2 induced by ~25 min of progressive-load exercise on a cycle ergometer [75].

We found that women exhibit a broader APP concentration range than men, which occurred without reported injuries or inflammatory events in the analyzed athletes. This broader APP response in women can influence sports and clinical translational studies. We reported evidence of sex-based differences in APP concentrations, with women appearing to be at a higher risk of suffering inflammation and, subsequently, injuries, as repeatedly described [60,76]. Sex-based differences must be considered in APP responses and N-of-1 trials for athletes are paramount to implementing tailored protocols (training, competition, resting, and rehabilitation). These trials are even more necessary considering that different sports may induce different immunometabolic responses, as shown in this study (Figure 8).

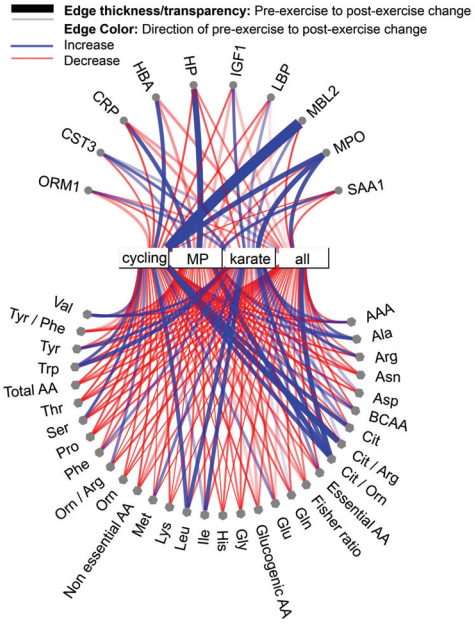

Figure 8. Chord diagram showing exercise-induced trend changes from pre-exercise to post-exercise for each analyzed analyte in female samples across cycling, karate, modern pentathlon, and all sports combined. Node shape signifies AAs and APPs, while edge thickness/transparency represents the change, and color shows the direction of the change pre- to post-exercise, as per legend. Visualized in NAViGaTOR.

4.2. Amino Acid Metabolism in Elite Female Athletes

In addition to their structural role, amino acids can perform various metabolic and signaling tasks. Different models of metabolic stress can impact and be impacted by amino acid metabolism, such as sepsis, cancer, burn injury, hepatitis, diabetes, or obesity [77–84]. Exercise has been used as a suitable model for studying metabolic stress [13,35,85–87]. Amino acid metabolism has been mainly assessed by studies focusing on amino acid concentrations in the muscle or plasma [88]. However, geolocation and seasonal variations throughout the year have been shown to impact the amino acid pool during exercise [89]. Additionally, the microbiome can influence the general metabolite concentrations, including amino acids [64,90]. We analyzed sex-related ex post facto changes in elite athletes' plasma amino acids from various sports during different metabolic states in a highly diverse country. This investigation may provide insight into the crucial role of amino acids during elite-level athletic performance.

Our data align with the current view, indicating that women's amino acid concentrations are lower than men's [91–93]. However, some studies have found controversial results regarding specific plasma amino acids in women of different ages and ethnic populations [94–96]. Alanine, glutamine, and glutamate significantly influence both anaplerosis and gluconeogenesis. Additionally, they play an essential role in ammonia metabolism, a key metabolite in exercise-induced central fatigue [14,16,97]. We did not detect sex-related differences in alanine blood concentrations in elite athletes. It has been shown in untrained individuals that alaninemia is altered by exercise in a duration-dependent manner, increasing in exercises lasting up to 80 min and decreasing in longer ones [98], a phenomenon that seems to mirror alanine concentration changes within the skeletal muscle [88,99]. We found a significantly higher increase in blood alanine in women (12% vs. 6%), as expected for non-prolonged exercise.

Our data did not show an exercise-induced change in glutamate concentrations in the blood, while elite female athletes exhibited a significantly lower decrease in glutamine. Glutamine can be depleted during extended exercise (like alanine), while it may increase after short bouts of exercise, especially with high output [100]. The decrease in glutamine blood concentration can be observed during prolonged exercise and other models of hypermetabolic stress, such as post-surgery and overtraining syndrome [101,102]. Glutamine plays an essential role as an ammonia transporter from muscle to the liver and is critical for exercise maintenance [97]. This finding may suggest a sex-based difference in the role of amino acids in the acute energy metabolism response during exercise. Due to glutamate's pivotal role in energy metabolism within skeletal muscle, exercise can lead to blood glutamate depletion by increasing its uptake into muscle [103]. Here, we showed that blood glutamate concentration did not significantly change in female athletes, while it increased by 9% in men post-exercise. Indeed, we previously reported in a windsurf trial that glutamate was less affected by exercise than alanine and glutamine [104]. We confirmed that elite athletes presented minor changes in blood glutamate, alanine, and glutamine after dietary adjustments and training. More importantly, these amino acids were associated with a less fluctuating state, along with decreasing biomarkers of muscle injury, as previously shown by our group [104]. Elite female athletes presented significantly lower blood concentrations of BCAA in all collection states than their male counterparts. In our study, BCAA responses were highly correlated among the individual amino acids ($\rho \geq 0.9$; $p < 0.001$) without differences between sexes in their response to exercise. Since BCAAs are ketogenic or glycogenic amino acids (or both), their role in metabolism depends on nutritional status and training. We did not observe an increase in BCAA response to exercise, probably because we analyzed changes induced by acute exercise bouts, as previously described [105,106]. Margolis et al. (2021) showed that BCAAs significantly increased before exercise following a glycogen-depleting protocol and a low-carbohydrate diet [107]. In addition, lower BCAA concentrations in women have been observed in the military during basic combat training, while ten weeks of training caused an increase in

both BCAA and total amino acid concentrations in both sexes, with higher levels observed in women among the military population [108].

As previously described in animal models and the general population, we also found that exercise decreased the Fischer ratio for both female and male elite athletes, favoring the development of central fatigue [109,110]. However, our data show that elite female athletes had a smaller decrease in Fischer ratio in response to exercise, although they also presented a significantly lower ratio pre-exercise. Women's Fischer ratios did not change acutely post-exercise. Still, they significantly decreased by 5% from pre-exercise to resting, while men's Fischer ratio decreased by 9% already in post-exercise and then 10% from pre-exercise to resting. In addition, BCAA and AAA had a high correlation in female athletes for cycling and karate but not in men, suggesting sex-based differences in exercise metabolism during specific sports (Figure 7). To the best of our knowledge, this is the first study showing sex-based differences in the Fischer ratio during exercise. This finding can be of importance for understanding exercise metabolism, as well as the role of BCAA/AAA in liver failure and encephalopathies.

In the 1980s, Newsholme proposed a possible link between the increased tryptophan/BCAA ratio and central fatigue development, now commonly referred to as the "serotonin hypothesis", since tryptophan is a serotonin precursor [18,111]. We did not detect exercise-induced changes in tryptophan blood concentration in female or male athletes; however, women presented a significantly higher tryptophan/BCAA ratio in pre-exercise collections. In addition, elite female athletes did not exhibit a significant exercise-induced increase in the tryptophan/BCAA ratio, while men showed a significant increase of 11% from pre- to post-exercise and 16% from pre-exercise to resting. While prolonged exercise can decrease blood BCAA concentrations due to potential utilization as an energy source within muscles or hepatocytes, prolonged exercise may increase blood free-tryptophan concentrations. While the influence of tryptophan on central fatigue remains controversial, clinical studies have been evaluating it. Maciejak et al. observed that intragastric administration of FFAs increased the seizure threshold and induced sedation, an effect abolished when tryptophan passage into the brain was blocked [112].

We highlight that sex comparisons regarding central and peripheral fatigue development have attracted the scientific community's attention [31,113–115]. However, data retrieved from elite athletes have not been published. After evaluating clinical tests, Jo et al. proposed that women presented a trend of being less affected by exercise-induced central fatigue than men following sustained isometric ankle plantar flexion [116]. Together with our analysis regarding the Fischer ratio and tryptophan/BCAA ratio, we add that elite female athletes exhibit weaker exercise-induced amino acid responses, which have been evaluated as indirect central fatigue markers. However, our data showed that female athletes can be at a higher risk for developing central fatigue.

4.3. The Immune-Metabolic Response

The decrease in glutamine concentration and subsequent availability has been linked to the decline in immune function due to the energetic needs of both intestinal and white blood cells for glutamine as a fuel. This effect was seen in the four individually analyzed sports and was concurrent with increased MBL2 and MPO in cycling.

It is interesting to highlight that the metabolism of BCAA, either collectively or by sport, was different and was not associated with a particular immune response. Otherwise, the relationship between the urea cycle intermediates was observed across all sports and MPO [117]. Citrulline has been related to inflammatory response, including during rheumatoid arthritis [118]. The relationship between the NO precursors and MPO has been discussed, and the enzyme's activity can be related to different causes of joint inflammation [119]. These events relate to our previous idea of differentiating HP increases due to hemolysis from those caused by inflammation [120]. The influence of amino acid metabolism and its integration with the immune response can be of great interest for understanding the response to hypermetabolic states, as we described previously [8].

5. Conclusions

Here, we demonstrate that the sex-based disparity in APP concentrations in each state appears more critical than the sex-based difference in the APP response.

Our data reveal significant sex-specific and sex-agnostic correlations between proteins and amino acids across specific sports. This emphasizes the need for investigations focusing on sex-based differences in metabolism across different sports or even grouping sports by similarities (e.g., intensity, duration, power output). Previous evaluations on sex differences during fatigue have relied on controlled exercises rather than field-of-play situations, which can limit data translation to elite athletes. Moreover, we suggest the need for more molecular investigations to understand sex differences in central fatigue development, utilizing other markers.

Taking the results together, we propose the importance of exercise as a model for understanding immunometabolism in physiological and pathological conditions.

6. Limitations

This study has several inherent limitations. First, as an ex post facto sportomics analysis conducted in real-world training and competition settings, we had no control over various conditions, which introduced variability in factors such as diet, sports activity, intensity, and athlete age. Another limitation is the broad scope of the study, which covered multiple sports and a wide range of analytes. While this provides a comprehensive overview, it lacks the specificity that could be achieved in controlled experiments. These confounding factors limit the external validity of the findings and hinder the ability to infer causality. Therefore, the study's results should not be used to discuss mechanisms or draw definitive conclusions, but rather to observe real differences in a non-controlled environment, raise new hypotheses for future investigation, or reinforce previously published data.

Supplementary Materials: The following supporting information can be downloaded at https://www.mdpi.com/article/10.3390/nu16203538/s1, Table S1: Spearman pairwise correlation matrices for acute phase proteins and amino acids, considering different states and sports. In this table, correlations were not filtered for statistical significance or correlation strength, as was done in the main figures.

Author Contributions: R.M.-S.: writing original draft, visualization, and writing—review and editing; A.B.: writing original draft, conceptualization, methodology, and data acquisition; J.F.: writing original draft and visualization; E.P.: writing original draft, review, and editing; L.M.III: methodology, investigation, and writing—review and editing; V.C.: writing—review and editing and formal analysis; I.J.: conceptualization, writing—review and editing, visualization, and formal analysis; L.C.C.: conceptualization, methodology, writing original draft, project administration, writing—review and editing, visualization, formal analysis, and funding acquisition. All authors have read and agreed to the published version of the manuscript.

Funding: L.C.C. and this work were supported by the Brazil Olympic Committee (BOC); Conselho Nacional de Desenvolvimento Científico e Tecnológico (CNPq); BIOCRATES; Coordenação de Aperfeiçoamento de Pessoal de Nível Superior (CAPES); Financiadora de Estudos e Projetos (FINEP); Fundação Carlos Chagas Filho de Amparo à Pesquisa do Estado do Rio de Janeiro (FAPERJ); INCT-TeraNano (CNPq #403193/2022-2, -FAPEMIG CBB #APQ-03613-17); Merck-Sigma-Aldrich; SISCAPA; Universidade Federal do Estado do Rio de Janeiro (UNIRIO); and Waters Corporation. I.J. was supported in part by funding from the Natural Sciences and Engineering Research Council (NSERC RGPIN-2024-04314), the Canada Foundation for Innovation (CFI #225404, #30865), and the Ontario Research Fund (RDI #34876, RE010-020). V.C. is supported by a Clinician Scientist Salary Support Award from the Department of Medicine, University of Toronto, Toronto, Canada. The funders had no role in study design, data collection and analysis, publication decisions, or manuscript preparation.

Institutional Review Board Statement: The study was conducted in accordance with the Declaration of Helsinki. All procedures involving human subjects were approved by the ethics committee for human

research at the Federal University of the State of Rio de Janeiro (117/2007 CAEE: 0053.0.313.000-07, updated and renewed on 25 May 2011; 18 February 2013; and 15 May 2016) and the Federal University of Mato Grosso (2017–2021; approval code: 2,230,073) and met the requirements regulating research on human subjects [43]. Written informed consent was obtained from each subject, who was informed about the nature of the research.

Informed Consent Statement: Informed consent was obtained from all subjects involved in the study.

Data Availability Statement: The data supporting this study's findings are available from the corresponding author upon reasonable request.

Acknowledgments: The authors are incredibly grateful to all the athletes who participated in this study. Unfortunately, their names cannot be disclosed here due to a previous agreement regarding anonymity. The authors are grateful to SISCAPA, especially Leigh Anderson, Terry Pearson, and Morty Razavi, for participating in the sample analyses. We acknowledge Waters Corporation Canada's Chris Stadey and Stephane Montpas, who performed the amino acids analysis.

Conflicts of Interest: The authors declare no conflicts of interest. LeRoy Martin III is an employee of Waters Corporation and received no compensation for his work on this paper. The authors declare that this study received funding from BIOCRATES, SISCAPA, and Waters Corporation. The funder was not involved in the study design, collection, analysis, interpretation of data, the writing of this article or the decision to submit it for publication.

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Protein Supplementation Increases Adaptations to Low-Volume, Intra-Session Concurrent Training in Untrained Healthy Adults: A Double-Blind, Placebo-Controlled, Randomized Trial

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Abstract: Combined endurance and resistance training, also known as "concurrent training", is a common practice in exercise routines. While concurrent training offers the benefit of targeting both cardiovascular and muscular fitness, it imposes greater physiological demands on the body compared to performing each modality in isolation. Increased protein consumption has been suggested to support adaptations to concurrent training. However, the impact of protein supplementation on responses to low-volume concurrent training is still unclear. Forty-four untrained, healthy individuals (27 ± 6 years) performed two sessions/week of low-volume high-intensity interval training on cycle ergometers followed by five machine-based resistance training exercises for 8 weeks. Volunteers randomly received (double-blinded) 40 g of whey-based protein (PRO group) or an isocaloric placebo (maltodextrin, PLA group) after each session. Maximal oxygen consumption (VO_{2max}) and overall fitness scores (computed from volunteers' VO_{2max} and one-repetition maximum scores, 1-RM) significantly increased in both groups. The PRO group showed significantly improved 1-RM in all major muscle groups, while the PLA group only improved 1-RM in chest and upper back muscles. Improvements in 1-RM in leg muscles were significantly greater in the PRO group versus the PLA group. In conclusion, our results indicate that adaptations to low-volume concurrent training, particularly leg muscle strength, can be improved with targeted post-exercise protein supplementation in untrained healthy individuals.

Keywords: low-volume exercise; HIIT; resistance training; interference effect; whey protein

Citation: Reljic, D.; Zieseniss, N.; Herrmann, H.J.; Neurath, M.F.; Zopf, Y. Protein Supplementation Increases Adaptations to Low-Volume, Intra-Session Concurrent Training in Untrained Healthy Adults: A Double-Blind, Placebo-Controlled, Randomized Trial. *Nutrients* **2024**, *16*, 2713. https://doi.org/10.3390/nu16162713

Academic Editor: Gary D. Miller

Received: 24 June 2024
Revised: 6 August 2024
Accepted: 13 August 2024
Published: 15 August 2024

1. Introduction

Adequate levels of cardiorespiratory [1–4] and muscular fitness [1,5,6] are crucial determinants for the maintenance of general health and for preventing numerous chronic diseases. It has been documented, for example, that the degree of maximal oxygen consumption (VO_{2max}) as an indicator of cardiorespiratory fitness is a key predictor of cardiovascular disease and overall mortality, even stronger than traditional risk factors, such as obesity, hypertension, type 2 diabetes mellitus, or nicotine abuse [7,8]. Additionally, research indicates that muscle strength is an independent and significant factor related to morbidity and mortality [6,9]. Thus, guidelines [10,11] advocate that individuals should participate in both regular aerobic and muscle-strengthening activities to maintain/improve cardiorespiratory as well as muscular fitness. Accordingly, prescriptions for structured exercise programs typically involve combined endurance and resistance training—also termed "concurrent training"—to promote holistic fitness and health benefits.

Despite the well-accepted additive benefits of combined exercise programs with regard to overall fitness and health outcomes, research has highlighted that concurrent training—particularly when both modalities are carried out consecutively in the same session (commonly referred to as "intra-session concurrent training")—can lead to greater physiological stress by challenging multiple systems (cardiovascular, muscular) simultaneously [12,13]. It has been suggested that previous endurance training may compromise subsequent resistance exercise quality and vice versa, due to residual fatigue and/or reduced substrate availability (e.g., depleted glycogen levels resulting in increased skeletal muscle protein breakdown) [14–16]. Moreover, there is a body of evidence suggesting that adaptations to endurance and resistance training may interfere with each other under certain circumstances [14,15,17–19]. This so-called interference effect was first observed in a pioneering study by Hickson [20], who found that simultaneous endurance and resistance training resulted in a reduced capacity to develop muscle strength when compared to resistance training alone in recreationally active subjects. Although this finding was not always confirmed in follow-up studies, multiple investigations showed similar results, indicating that particularly muscle strength development and hypertrophy can potentially be diminished by concurrent training [14,15,17–19], most likely due to antagonistic molecular mechanisms underlying adaptations to both types of exercise [14,15,17]. Additionally, it has been demonstrated that untrained individuals can experience lower VO_{2max} improvements with concurrent versus endurance training only [21].

Practical recommendations to balance the increased physiological demands or mitigate potential interference effects of intra-session concurrent training are related to training variables (e.g., type of exercise, volume, and intensity) [19] and nutritional strategies [22]. Regarding nutrition, protein supplementation has been particularly highlighted in recent systematic reviews as a potential approach to optimize synthesis of muscle protein, aid muscle repair and growth, and support strength adaptations during concurrent training [22–25]. There is evidence, for example, that ingesting 20–40 g of protein immediately after an exercise session can provide a beneficial impact on muscle protein synthesis and performance responses to concurrent training [24]. Furthermore, it has been reported that post-exercise protein intake may contribute to improvements in cardiovascular fitness by supporting, for example, the forming of new capillaries, oxygen-transporting proteins, and mitochondrial proteins [26]. However, in this context, it must be pointed out that the majority of previous trials investigating the effects of protein supplementation or increased dietary protein intake on concurrent training adaptations involved athletes or trained/physically active individuals [27–47] and used higher-volume exercise programs, such as prolonged continuous endurance training or longer-duration interval training protocols combined with multiple-set resistance training regimens [27–33,35,37–40,42–52].

Currently, only a small number of investigations [28,48–53] have been conducted with sedentary/untrained samples, of which only one trial examined the influence of protein supplements on changes in cardiorespiratory fitness compared to concurrent training without supplementation [51]. In that study, Lockwood et al. [51] found that absolute VO_{2max} only improved in conjunction with whey protein supplementation over a period of 10 weeks of concurrent endurance and resistance exercise in a group of sedentary, overweight females and males. Given that VO_{2max} improvements following concurrent training, in comparison to isolated endurance exercise, were found to be particularly blunted in untrained individuals [21], there is clearly a need for more research to investigate whether protein supplementation can improve adaptations to concurrent endurance and resistance training in novice exercisers. Moreover, to our knowledge, it has not yet been investigated whether individuals engaged in more time-efficient, "low-volume" training programs may also benefit from targeted protein intake after completion of the exercise session. Low-volume training types, including low-volume high-intensity interval training (LOW-HIIT) [54,55], a specific form of interval endurance exercise (involving, by definition, $\leq$10 min of intensive exercise during a training session of $\leq$30 min duration, including periods of warm-up and cool-down [55]) and low-volume resistance training (LOW-RT, previously defined as

<12 weekly exercise sets per muscle group [56]), have gained increasing popularity among exercisers who have tight time schedules and thus have become a fruitful topic of research in recent years [57,58].

The aim of this study was therefore to address these research gaps by examining the effects of protein supplementation (40 g of whey-based protein) post-exercise on adaptations of VO_{2max}, muscle strength and body composition after an 8-week low-volume concurrent training program comprising LOW-HIIT and LOW-RT (two sessions per week), in previously sedentary, healthy men and women. We hypothesized that both groups would show improve physical fitness indices, but that post-session protein supplementation would increase the concurrent training-induced responses of cardiorespiratory and muscular fitness compared to an isocaloric placebo.

2. Materials and Methods

2.1. Design of the Study

This investigation was a randomized, placebo-controlled, double-blind trial involving a concurrent training intervention (LOW-HIIT followed by LOW-RT) of 8 weeks duration with two arms (experimental group and placebo control group). The experimental condition consisted of 2 weekly sessions of concurrent LOW-HIIT and LOW-RT plus post-exercise whey-based protein supplementation (PRO group). The control condition consisted of the same concurrent training program plus post-exercise supplementation of an isocaloric placebo (PLA group). Primary outcomes of the study were VO_{2max}, maximum strength values, defined as one-repetition maximum (1-RM) for the five main muscle groups (chest, upper back, abdominals, lower back, and legs), and overall fitness (Fit score, computed as the mean of VO_{2max} and the average 1-RM value of the five muscle groups). Secondary outcomes were body composition parameters, described in more detail in Section 2.3.3.

The outcome measurements were conducted 1 week preceding the onset of the exercise program (i.e., week 0, T-1) and in the first week after completion of the exercise program (i.e., week 9, T-2). The timeline of the trial is illustrated in Figure 1. After T-1, volunteers were allocated to the two groups by stratified-randomization based on their baseline VO_{2max} (<35 mL/kg/min, or ≥35 mL/kg/min), age (<30 years, or ≥30 years), and sex (male or female) using the software MinimPy (GNU General Public License version 3.0 [59]). Randomization was conducted by a researcher not engaged in the collection of data. All volunteers in the trial were fully briefed on the study's scope, which complied with the Declaration of Helsinki, and signed an informed consent prior to study inclusion. The study was authorized by the Medical Faculty Ethics Committee of Friedrich–Alexander University Erlangen—Nürnberg (approval 147_19B) and registered at ClinicalTrials.gov (ID NCT04359342).

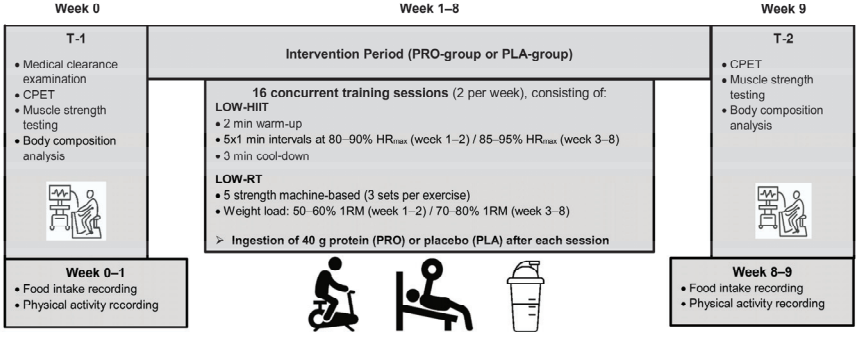

Figure 1. Study timeline. T-1 = pre-intervention; T-2 = post-intervention; LOW-HIIT = low-volume high-intensity interval training; LOW-RT = low-volume resistance training; CPET = cardiopulmonary exercise testing.

2.2. Study Volunteers

Participant recruitment involved advertising in local newspapers and social media platforms. Interested persons contacted study staff via email or by telephone to determine if they were eligible to participate. Eligibility criteria for the study included that volunteers were at least 18 years of age, led a mostly sedentary lifestyle as defined elsewhere [60], and were not participating in any other exercise or nutrition intervention. Exclusion criteria included pregnancy, clinical diagnosis of coronary disease, oncological disease, major orthopedic disorders, or other serious health problems that would rule out safe involvement in physical exercise. All volunteers consented to maintaining their current lifestyle habits during the study to minimize possible bias. We based our sample size calculation on results of a recent meta-analysis [24], which demonstrated a large pooled effect size (d = 0.89) of the impact of protein supplementation on improvements in performance outcomes in response to concurrent training. Accordingly, an a priori estimation of sample size, expecting a large effect size for repeated-measure ANOVA (f = 0.45), indicated that a total of 20 participants (N = 10 for each group) would be adequate to yield a power of 95% with a significance level of 5% (G*Power, version 3.1.9.2). In order to take possible dropouts into account, the aim was to recruit 20 participants per group.

2.3. Outcome Measurements

The baseline testing procedures (T-1) were conducted 1 week prior to the onset of the exercise program. The post-testing (T-2) took place within the first week after the completion of the 8-week exercise program, with at least 3 days between the last exercise session in order to ensure sufficient recovery. T-1 and T-2 were scheduled at a similar time of day to reduce potential circadian influences. Additionally, care was taken that both testing days were performed within the same menstrual cycle in all female volunteers. Volunteers were advised to report to the laboratory in an overnight-fasted state, to abstain from alcohol, and avoid vigorous physical activity for a minimum period of 24 h prior to their visit. Measurements were strictly standardized as specified below and performed in stable ambient conditions (22–24 °C, and 30–50% air humidity). At T-1, study outcome measurements were preceded by a medical clearance examination, including medical history recording, blood pressure measurements, 12-lead resting electrocardiography, and evaluation of standard blood and urine laboratory values to assure the safety of participation in the training program. All measurements and examinations were executed investigator-blinded, meaning that personnel collecting the data had no knowledge of volunteers' group assignment.

2.3.1. Body Composition Measurements

Upon arrival at the laboratory, volunteers were requested to void their bladder, and afterwards, to remain in a seated position for 5 min. Subsequently, multifrequency segmental bioelectrical impedance analysis was performed using a validated analyzer (seca mBCA 515, Seca, Hamburg, Germany) [61] to assess the body weight, body fat mass, skeletal muscle mass, and total body water of the volunteers. Furthermore, volunteers' waist circumference was obtained in the standing position to the closest millimeter. Measurements were performed approximately midway between the lower edge of the last palpable rib and the upper iliac crest along the mid-axillary line using a flexible tape.

2.3.2. Cardiopulmonary Exercise Test (CPET)

CPETs were carried out using a stationary electronically braked cycle ergometer (Corival cpet, Lode, Groningen, The Netherlands) to assess VO_{2max}, maximal power output (W_{max}), and maximal heart rate (HR_{max}). Additionally, volunteers' power output at the ventilatory threshold (W_{VT}) was assessed by means of the V-slope method (i.e., plot of carbon dioxide release versus oxygen consumption) to determine submaximal endurance capacity. Following a 1 min adaptation, CPET commenced at 50 W, with the power output progressively increasing by 12.5 W/min (females) and 15 W/min (males) until reaching voluntary exhaustion. Exhaustion was reached within 8–12 min in most volunteers, as

per suggested guidelines for exercise testing [60]. Heart rate was recorded constantly with a 12-lead ECG device (custo cardio 110, custo med, Ottobrunn, Germany). Oxygen consumption and carbon dioxide release were obtained constantly with a breath-by-breath, open-circuit metabolic cart (Metalyzer 3B-R3, Cortex Biophysik, Leipzig, Germany). Oxygen consumption and carbon dioxide release data were averaged every 10 s. To verify that maximal exertion had been achieved, volunteers had to meet a minimum two of the specified criteria: plateauing of oxygen consumption, a respiratory exchange ratio of $\geq$1.1, an age-related HR_{max} of $\geq$90% (computed according to the formula 220—age), and a rate of perceived exertion of $\geq$19 on the Borg scale [62], as recommended elsewhere [63]. CPET data were used to set the volunteers' personalized LOW-HIIT heart rate zones.

2.3.3. Determination of One-Repetition Maximum Strength and Overall Fitness Z Score

After a brief familiarization with test procedures and local warm-up of the target muscles, volunteers conducted a modified 1-RM test of the following muscles: chest, upper back, abdominals, lower back, and legs. While a "classical" 1-RM test typically aims to determine the maximal weight load that can be lifted for one complete repetition, the modified 1-RM test utilized in the present trial involved performing multiple repetitions to predict 1-RM. This method is considered to have a lower a lower risk of injury and is therefore advocated for untrained collectives [64]. The tests were supervised by certified physiotherapists or sports therapists on five machines in the following standardized order: chest press, lat pulldown machine, lower back machine, abdominal crunch, and leg press (TechnoGym, Neu-Isenburg, Germany). On each machine, volunteers were required to raise the applied weight until reaching muscular failure. As recommended elsewhere [65], the number of repetitions was not to exceed six to ensure accurate 1-RM predictions. If more than six repetitions were completed, the weight was increased and a following attempt was executed after a 3 min recovery. The load that could be lifted for six repetitions was usually determined within three tries. Afterwards, 1-RM values were estimated based on the following formula [66]:

$$\text{1-RM} = 100 \times \text{load rep}/(102.78 \times 2.78 \times \text{rep})$$

The test results were utilized to determine volunteers' weight load for the resistance training exercises as specified below (2.5). At the beginning of training week 4, 1-RM tests were repeated to account for progression and to reestablish the respective weight loads. Furthermore, an overall fitness (Fit score) was computed at T-1 and T-2 as the mean value of each fitness sub-component (cardiorespiratory and muscular fitness) as follows:

$$\text{Fit score} = (VO_{2max} + \text{average 1-RM from the five muscle groups})/2$$

2.4. Daily Nutrition and Physical Activity Monitoring

Volunteers were instructed to record their dietary intake on three days in a row during the week prior to the onset of the exercise program and during the last training week with the help of a standardized 24 h food protocol (Freiburger Ernährungsprotokoll; Nutri-Science, Freiburg, Germany). A registered dietitian analyzed all dietary records using software (PRODI 6 expert, Nutri-Science, Freiburg, Germany). Furthermore, volunteers recorded their habitual physical activities on a daily basis in an activity diary. All recorded physical activities were categorized based on metabolic equivalents (METs), according to Ainsworth et al. [67]: light (<3 METs), moderate (3–6 METs), or vigorous (>6 METs). The average MET score over 24 h was used to assess the daily physical activity level (PAL).

Based on the individual PAL and anthropometric values, volunteers received personalized dietary advice to keep a consistent nutritional intake throughout the intervention period. The dietary advice adhered to guidelines from the German Nutrition Society [68,69].

Volunteers' resting metabolic expenditure (REE) was calculated by the following established equations [70]:

$$\text{Men: REE (kcal/day)} = 66.5 + 13.8 \times \text{weight (kg)} + 5.0 \times \text{size (cm)} - 6.8 \times \text{age (years)}$$

$$\text{Women: REE (kcal/day)} = 655 + 9.6 \times \text{weight (kg)} + 1.8 \times \text{size (cm)} - 4.7 \times \text{age (years)}$$

Caloric requirements per day were computed by multiplication of REE with PAL values. Volunteers were instructed to ingest 10–15% of daily energy from protein, 30–35% from fat, and $\geq$50% from carbohydrates [68]. Handouts containing meal planning advice and detailed instructions were provided to help volunteers implement the dietary recommendations at home.

2.5. Concurrent Training Program

During the 8-week intervention period, volunteers conducted two weekly concurrent training sessions for a total of sixteen sessions. To maximize compliance, volunteers could schedule all exercise sessions on an individual basis throughout the opening hours of the Training Center, with at least 1 day's rest in between to ensure proper recovery. The exercise sessions consisted of sequential LOW-HIIT and LOW-RT, which were all supervised by certified sports therapists or physiotherapists.

All sessions commenced with a LOW-HIIT cycle ergometer protocol, which was adapted from Reljic et al. [71]. Briefly, volunteers warmed up with low-intensity cycling for 2 min. Subsequently, volunteers performed 5 intervals of 1 min duration at 80–90% of HR_{max} in week 1 and week 2. From week 3 on, training intensity was increased to a target heart rate range of 85–95% of HR_{max}, to be achieved during the intervals. Throughout each session, volunteers wore a heart rate chest strap (acentas, Hörgertshausen, Germany) to measure their individual heart rate during exercise. Heart rate data were saved for later analysis using a software program (HR monitoring team system, acentas, Hörgertshausen, Germany). During each interval bout, volunteers were directed to adjust the cadence and/or the load resistance of the ergometer to achieve their pre-defined heart rate zones. Intervals were separated by a 1 min recovery period with low-intensity cycling. The last interval bout was followed by a 3 min cool-down period at a self-selected low-intensity pace. According to previous definitions of "low-volume HIIT" [55], the total duration of LOW-HIIT (warm-up and cool-down included) was 14 min/session.

After completing LOW-HIIT, volunteers performed five resistance exercises targeting the major muscle groups, including chest muscles, upper back muscles, abdominal muscles, lower back muscles, and leg muscles, on the following training devices: chest press, lat pulldown machine, lower back machine, abdominal crunch, and leg press (TechnoGym, Neu-Isenburg, Germany). Each exercise was performed with 3 sets according to the following pattern: 2 s of concentric, 2 s of eccentric muscle work until the volunteer reached muscle failure, and 2 min rest between each set. As recommended for novice exercisers [72], the initial weight load during weeks 1–2 was set at 50–60% of 1-RM to achieve ~15–20 repetitions per set to accustom volunteers to resistance training and thereafter progressed to 70–80% of 1-RM, targeting 8–12 repetitions per set. Previously defined as "low-volume resistance training" [56], the resistance training part of the exercise session involved only 6 sets per muscle group per week. Thus, total time per session, including both LOW-HIIT and LOW-RT, was ~57 min per session (~114 min exercise per week).

2.6. Supplementation

Following the conclusion of every exercise session, volunteers received (double-blinded) 40 g of a whey-based protein supplement (Fresubin Protein, Fresenius Kabi, PRO group) or an isocaloric placebo (maltodextrin) with the same taste (MaltoCal 19, MetaX, PLA group). According to previous research, consumption of 40 g of protein after

termination of exercise appears to be a very effective approach to increase synthesis rates of muscle protein in healthy subjects [73]. Table 1 presents the calorie and macronutrient composition of each supplement. Both supplements were prepared with 150 mL of low-fat milk (46 kcal/100 mL, 3.4 g protein, 4.8 g carbohydrates, 1.5 g fat) and administered in the form of a shake in identical non-transparent drinking cups. Based on the medical history survey obtained at study entry, none of the volunteers reported being lactose-intolerant or experiencing any associated clinical symptoms after milk consumption. Supplement preparation and delivery were carried out by staff members who were not engaged in the collection and analysis of study outcomes. Once data collection was complete, the group allocation of each volunteer was revealed. Volunteers were requested to document their personal responses with the supplements, including any relevant observations pertaining to their taste and any adverse effects they may have experienced as a result of taking them (e.g., nausea, bloating, or stomach pain). Furthermore, to assess blinding success, volunteers were asked to estimate which supplement they thought they had received after completion of the intervention using the following answer options: "protein", "placebo". or "I do not know".

Table 1. Caloric and macronutrient contents of supplements (per serving size).

Variable	Protein Shake [1,3]	Placebo Shake [2,3]
Caloric value (kcal)	213	222
Protein (g)	40	5
Carbohydrates (g)	7.5	46
Fat (g)	2.6	2

[1] Whey protein, [2] maltodextrin, [3] both shakes prepared with 150 mL low-fat milk.

2.7. Statistical Analysis

SPSS version 24.0 software (SPSS Inc., Chicago, IL, USA) was used for analyses. The data were first checked for whether they exhibited a normal distribution using the Shapiro–Wilk test. A 2 × 2 repeated-measure ANOVA was subsequently conducted to examine the data, with the objective of analyzing the main effects of group (PRO vs. PLA), time (T1 vs. T2), and their interaction. To assess whether sex influenced changes in the primary outcomes (VO_{2max}, 1-RM-values, and Fit score), male and female sub-analyses were conducted. Levene's test was utilized to confirm the homogeneity of variance. In instances where ANOVA revealed the existence of a significant main effect or interaction, Holm–Sidak post hoc tests were used for multiple and between-group comparisons and post hoc paired *t*-tests were applied to identify changes within groups [74,75]. In cases where the data exhibited a skewed distribution, logarithmic or square root transformations were applied, and the identical analyses described above were conducted on the transformed data. If normalization was not achieved through transformation (W_{VT}, 1-RM chest press, 1-RM lat pulldown machine, Fit score and PAL values), Friedman two-way analysis of variance was employed. In cases of significant results, Dunn's Bonferroni post hoc tests were used for comparisons between groups, and Wilcoxon and Mann–Whitney post hoc tests were carried out for comparisons within groups, respectively. Moreover, effect sizes were determined using partial eta-squared ($\acute{\eta}p^2$) for ANOVAs and Kendall's coefficient of concordance (*W*) for the Friedman tests. Based on the established literature [76], effect sizes were deemed small ($\leq$0.01), medium ($\geq$0.06) and large ($\geq$0.14) for $\acute{\eta}p^2$, and small ($\leq$0,10), medium ($\geq$0.30), and large ($\geq$0.50) for *W*. In all statistical tests, the threshold of significance was defined at $p < 0.05$. Data are reported as means ± standard deviation (SD). Changes between T-1 and T-2 are presented with 95% confidence intervals (95% CI).

3. Results

3.1. Study Flow

In total, 46 individuals were screened for eligibility, of whom 44 were included and randomly allocated to the PRO group (N = 23) or PLA group (N = 21). Two dropouts of

eligible candidates occurred due to the onset of the COVID-19 pandemic. All participating volunteers were free of medications, except for two women (N = 1; each group), who were taking contraceptives. During the study, eight volunteers dropped out (PRO group, N = 4; 25% females, PLA group, N = 4; 100% females). The specific reasons for dropout are illustrated in Figure 2. Thus, the study concluded with a total of 36 volunteers having been analyzed (PRO group, N = 19, 63% females, 26 ± 4 years; PLA group, N = 17, 53% females, 27 ± 6 years).

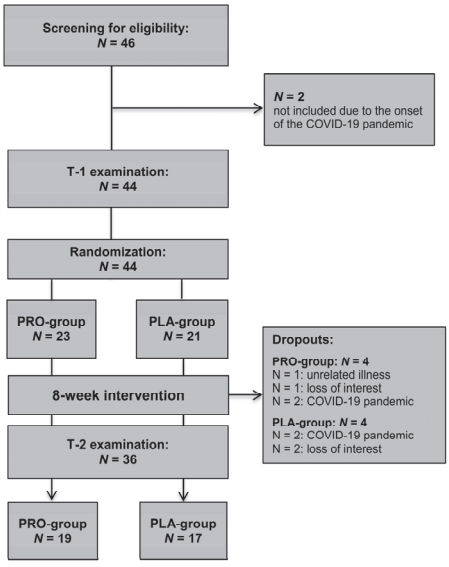

Figure 2. Study flowchart.

3.2. Training Data, Adverse Events, and Volunteers' Evaluations

There were no significant between-group differences in primary outcomes at T-1. Moreover, no notable sex-based differences were identified in the observed alterations in VO_{2max} and 1-RM values. Thus, the results for both sexes were combined in all analytical procedures. Training compliance (indicated by the percentage of scheduled sessions attended) was notably high (PRO group: 93% ± 10%, PLA group: 93% ± 8%). The mean peak heart rate reached during the intervals of the LOW-HIIT protocol corresponded to 95% ± 2% of volunteers' HR_{max}, indicating successful attainment of the targeted intensity of exercise. The mean heart rate throughout the whole LOW-HIIT protocol (warm-up, intervals, recovery between intervals, and cool-down calculated together) equaled 78% ± 3% of volunteers' HR_{max}. All volunteers managed to lift the prescribed weight loads, and completed 17 ± 2 repetitions per set during weeks 1–2 and 10 ± 1 repetitions per set during weeks 3–8.

Throughout the whole intervention period, no adverse events associated with the exercise program were documented. A mean score of 6.0 ± 0.7 was recorded on a 7-point rating scale, ranging from 1 (indicating that the exercise program was "not enjoyable at all") to 7 (representing "extremely enjoyable"). This indicates that the training program was rated as highly enjoyable by the volunteers. Moreover, 90% of volunteers expressed an intention to continue with low-volume concurrent training after the study. In the PRO group, no complaints or intolerance were reported after consuming the protein supplement. In the PLA group, only a small number of minor adverse events were documented following consumption of the maltodextrin supplement, including mild gastric discomfort (N = 1), flatulence (N = 2), and mild nausea (N = 1). A 7-point Likert scale was employed to assess the palatability of the supplements, with an average rating of 5.0 ± 1.6 for the protein

supplement and 5.5 ± 1.0 for the placebo. The majority of volunteers (N = 26, 76%) declared that they were unsure which supplement they received during the intervention. Five (15%) volunteers correctly identified which supplement they received (PRO group: N = 3, PLA group: N = 2). Three (9%, all PLA group) volunteers incorrectly identified the supplement they received.

3.3. Nutritional Intake and Daily Physical Activity

There were no significant differences in regular diet or physical activity habits within the groups or between them. Table 2 presents the dietary intake and physical activity data for each group, recorded both before the intervention period and during the final week of training.

Table 2. Dietary intake and physical activity during week 0 and week 8.

Outcome	PRO Group (N = 19)		PLA Group (N = 17)		Main Effect of Time (*p*-Value)	Group × Time Interaction (*p*-Value)
	Week 0	Week 8	Week 0	Week 8		
Nutrition [1]						
Energy (kcal/day)	1987 ± 482	1965 ± 406	2052 ± 473	2000 ± 528	0.548	0.798
Protein (g/day)	81 ± 17	76 ± 11	79 ± 22	81 ± 28	0.656	0.237
Protein (g/kg/day)	1.2 ± 0.2	1.2 ± 0.3	1.1 ± 0.3	1.1 ± 0.3	0.786	0.511
Fat (g/day)	81 ± 28	78 ± 22	81 ± 23	80 ± 33	0.632	0.723
Fat (g/kg/day)	1.2 ± 0.4	1.2 ± 0.4	1.1 ± 0.4	1.1 ± 0.4	0.644	0.930
Carbohydrates (g/day)	207 ± 52	210 ± 78	216 ± 70	219 ± 63	0.745	0.995
Carbohydrates (g/kg/day)	3.2 ± 0.9	3.2 ± 1.0	3.0 ± 1.2	3.0 ± 1.0	0.929	0.990
Fiber (g/day)	21 ± 8	20 ± 8	22 ± 9	21 ± 9	0.527	0.830
Physical activity [2]						
Light PA (h/week)	2.3 ± 1.4	2.4 ± 0.7	2.9 ± 0.7	2.9 ± 0.7	0.310	0.640
Moderate PA (h/week)	1.1 ± 0.2	1.1 ± 0.2	1.2 ± 0.5	1.4 ± 0.9	0.063	0.781
PAL	1.40 ± 0.02	1.41 ± 0.02	1.47 ± 0.01	1.47 ± 0.01	0.234	[3]

Data shown as means ± SD. Week 0 = 1 week before T-1, Week 8 = final week of intervention, PA = physical activity, PAL = estimated physical activity level. [1] Nutrition excluding the supplements, [2] PA excluding the study exercise program, [3] non-parametric testing.

3.4. Anthropometric Data

There were no significant main or interaction effects for any anthropometric parameter, except for a significant main effect of time in waist circumference ($p = 0.033$, $\eta^2 = 0.13$). Post hoc tests revealed a reduction in waist circumference by 2.0 cm (95% CI: –3.3 to –0.1 cm, $p = 0.038$) in the PRO group. Table 3 displays the data specific to each group at both T-1 and T-2.

Table 3. Anthropometric data at T-1 and T-2.

Outcome	PRO Group (N = 19)		PLA Group (N = 17)		Main Effect of Time (*p*-Value)	Group × Time Interaction (*p*-Value)
	T-1	T-2	T-1	T-2		
Body weight (kg)	65.9 ± 11.16	65.7 ± 11.6	75.9 ± 13.7	76.0 ± 12.9	0.936	0.530
Body mass index (kg/m^2)	21.8 ± 2.2	21.8 ± 2.3	25.0 ± 4.3	25.0 ± 4.3	0.426	0.982
Fat mass (kg)	15.7 ± 4.3	15.4 ± 4.6	21.9 ± 10.2	22.0 ± 10.3	0.726	0.340
Fat mass (%)	24.0 ± 6.6	23.6 ± 6.6	28.3 ± 10.2	28.4 ± 10.4	0.535	0.238
Skeletal muscle mass (kg)	23.9 ± 6.1	24.0 ± 6.2	25.7 ± 5.5	25.9 ± 5.4	0.304	0.344
Total body water (L)	36.9 ± 7.6	36.0 ± 10.6	39.7 ± 7.2	39.8 ± 7.1	0.381	0.321
Waist circumference (cm)	74 ± 8	72 ± 8 [a]	81 ± 7	80 ± 7	0.033	0.291

Data shown as means ± SD. T-1 = pre-intervention, T-2 = post-intervention. [a] $p < 0.05$: significant difference vs. T-1.

3.5. Cardiorespiratory Fitness Data

Significant main effects of time were found for relative VO_{2max} ($p < 0.001$, $\eta^2 = 0.30$) and absolute VO_{2max} ($p < 0.001$, $\eta^2 = 0.28$), as well as for relative W_{max} ($p < 0.001$, $\eta^2 = 0.61$) and absolute W_{max} ($p < 0.001$, $\eta^2 = 0.66$). Post hoc tests identified increases in absolute VO_{2max} (PRO group: 0.3 L/min, 95% CI: 0.1 to 0.4 L/min, $p = 0.005$; PLA group: 0.1 L/min, 95% CI: 0 to 0.2 L/min, $p = 0.011$), relative VO_{2max} (PRO group: 2.7 mL/kg/min, 95% CI: 0.9 to 4.5 mL/min, $p = 0.003$; PLA group: 1.4 mL/min, 95% CI: 0.1 to 2.6 mL/min, $p = 0.032$) (Figure 3), absolute W_{max} (PRO group: 22 W, 95% CI: 15 to 29, $p < 0.001$; PLA group: 17 W, 95% CI: 10 to 24, $p < 0.001$) and relative W_{max} (PRO group: 0.4 W/kg, 95% CI: 0.2 to 0.5 W/kg, $p < 0.001$; PLA group: 0.2 W/kg, 95% CI: 0.1 to 0.3 W/kg, $p < 0.001$) in both groups. Group-specific values of all cardiorespiratory fitness outcomes at T-1 and T-2 are presented in Table 4.

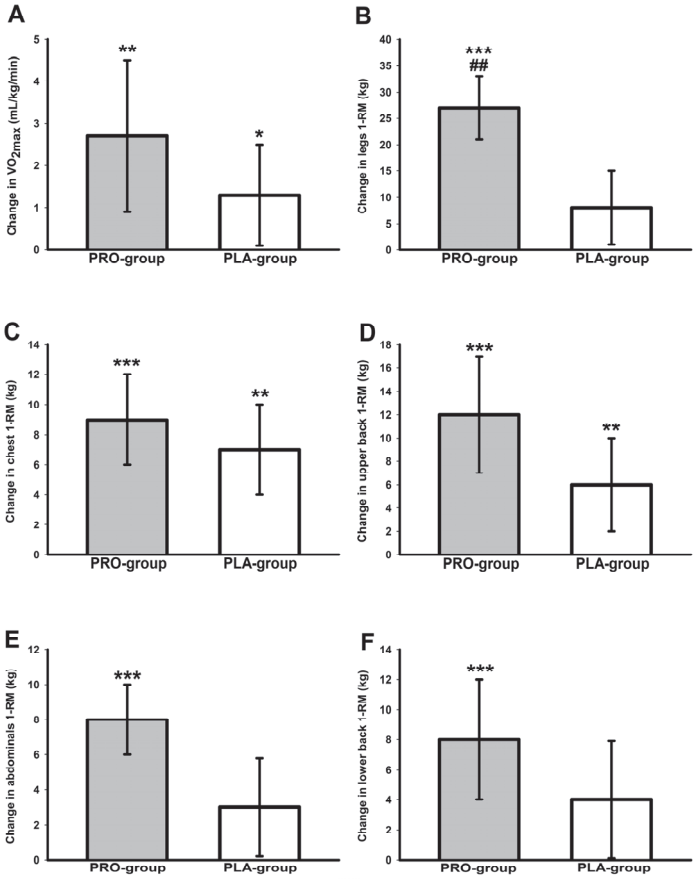

Figure 3. Changes in VO_{2max} (**A**), legs 1-RM (**B**) chest 1-RM (**C**), upper back 1-RM (**D**), abdominals 1-RM (**E**), and lower back 1-RM (**F**). * ($p < 0.05$), ** ($p < 0.01$), *** ($p < 0.001$): significant change between T-1 and T-2. ## ($p < 0.01$): significant difference between PRO group and PLA group.

Table 4. Cardiorespiratory fitness data at T-1 and T-2.

Outcome	PRO Group (N = 19)		PLA Group (N = 17)		Main Effect of Time (p-Value)	Group × Time Interaction (p-Value)
	T-1	T-2	T-1	T-2		
VO_{2max} (mL/kg/min)	40.1 ± 6.3	42.8 ± 7.2 [b]	36.8 ± 8.2	38.2 ± 8.1 [a]	<0.001	0.210
VO_{2max} (L/min)	2.6 ± 0.8	2.9 ± 0.8 [b]	2.8 ± 0.7	2.9 ± 0.7 [a]	<0.001	0.166
W_{max} (W/kg)	3.2 ± 0.5	3.6 ± 0.5 [c]	2.8 ± 0.6	3.0 ± 0.6 [c]	<0.001	0.062
W_{max} (W)	213 ± 60	235 ± 64 [c]	211 ± 43	228 ± 52 [c]	<0.001	0.306
W_{VT} (W)	82 ± 36	90 ± 49	76 ± 12	78 ± 19	0.275	0.532

Data shown as means ± SD. VO_{2max} = maximal oxygen consumption, W_{max} = maximal power output, W_{VT} = power output achieved at ventilatory threshold. [a] $p < 0.05$, [b] $p < 0.01$, [c] $p < 0.001$: significant difference vs. T-1.

3.6. One-Repetition Maximum Strength Data

Main effects of time were significant for 1-RM of chest ($p < 0.001$, $W = 0.86$), upper back ($p < 0.001$, $W = 0.69$), abdominals ($p < 0.001$, $\acute{\eta}^2 = 0.46$), lower back ($p = 0.001$, $\acute{\eta}^2 = 0.26$) and legs ($p < 0.005$, $\acute{\eta}^2 = 0.52$). There was a significant group-by-time interaction for 1-RM in leg muscles ($p = 0.007$, $\acute{\eta}^2 = 0.20$). Additionally, a strong trend for a group-by-time interaction was noted for 1-RM of abdominal muscles ($p = 0.05$, $\acute{\eta}^2 = 0.11$). Post hoc tests revealed that the PRO group showed significantly ($p < 0.001$) improved 1-RM in all tested muscle groups (Figure 3), while the PLA group only improved 1-RM significantly in chest ($p = 0.001$) and upper back muscles ($p = 0.002$). Improvements in 1-RM of leg muscles (15 kg, 95% CI: 4 to 25 kg $p = 0.003$) were larger in the PRO group in comparison to the PLA group (Figure 3). Group-specific 1-RM values are shown in Table 5.

Table 5. One-repetition maximum strength data and Fit scores at T-1 and T-2.

Outcome	PRO Group (N = 19)		PLA Group (N = 17)		Main Effect of Time (p-Value)	Group × Time Interaction (p-Value)
	pre	post	pre	post		
Abdominals (kg)	28 ± 12	35 ± 14 [c]	30 ± 11	34 ± 10	<0.001	0.050
Lower back(kg)	39 ± 13	47 ± 15 [c]	49 ± 15	53 ± 14	0.001	0.276
Chest (kg)	34 ± 16	43 ± 16 [c]	39 ± 19	47 ± 20 [b]	<0.001	[1]
Upper back (kg)	47 ± 18	58 ± 22 [c]	50 ± 20	57 ± 2 [b]	<0.001	[1]
Legs (kg)	125 ± 42	152 ± 43 [c]	132 ± 32	139 ± 35	<0.001	0.007
Fit score	47 ± 11	54 ± 13 [c]	48 ± 9	52 ± 11 [b]	<0.001	[1]

Data shown as means ± SD. [b] $p < 0.01$, [c] $p < 0.001$: significant difference vs. T-1. [1] Non-parametric testing.

3.7. Overall Fitness Z Score

A significant main time effect was detected for the Fit score ($p < 0.001$, $W = 0.69$). The Fit score significantly increased in both groups (PRO group, $p < 0.001$; PLA group, $p = 0.002$) (Figure 4). Group-specific Fit scores are presented in Table 5.

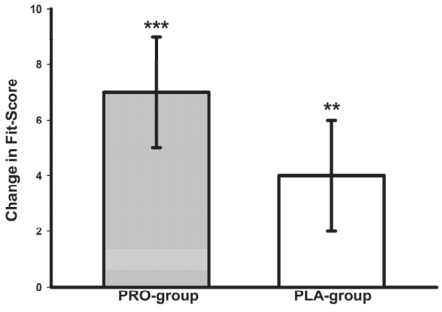

Figure 4. Changes in overall physical fitness Z score. ** $p < 0.01$, *** $p < 0.001$: significant change between T-1 and T-2.

4. Discussion

To our knowledge, this trial is the first to evaluate the impact of a post-exercise protein supplementation following a low-volume concurrent training program consisting of combined LOW-HIIT and LOW-RT on physical fitness outcomes in untrained healthy individuals. Our findings provide important insights into the effectiveness of nutritional strategies in optimizing training adaptations in individuals performing low-volume concurrent cardiovascular and muscular training programs. The main results were as follows: (i) in accordance with our assumption, 8 weeks of low-volume concurrent training improved VO_{2max} and muscular strength in our examined cohort—irrespective of protein or placebo supplementation, (ii) improvements in leg muscle strength were significantly larger in the PRO group in comparison to the PLA group, pointing to a beneficial effect of post-exercise protein supplementation on lower body strength adaptations to combined intra-session LOW-HIIT and LOW-RT in previously untrained individuals.

The increase in VO_{2max} following the 8-week low-volume concurrent training program (~2.1 mL/kg/min, average of both groups) was in the range of the values observed in other investigations examining the impact of LOW-HIIT in healthy untrained or recreationally active individuals (1.2 to 7.2 mL/kg/min), including previous trials from our laboratory applying the identical LOW-HIIT protocol as in the current study [61,77–83]. Thus, in conjunction with previous findings, our data provide further evidence for the effectiveness of LOW-HIIT in improving cardiovascular health with relatively little time invested. Given the paramount importance of VO_{2max} for health and longevity [7,8], this finding has clinical significance and supports the role of LOW-HIIT in cardiometabolic disease prevention.

The observed improvements in 1-RM values (11–33%) in our study cohort are consistent with other research findings, suggesting that untrained individuals can experience notable increases in muscle strength following LOW-RT programs within a few weeks. A systematic review by Grgic et al. [84] found that LOW-RT can result in an average increase in 1-RM ranging from 20% to 35% over 6–12 weeks. A study by Schoenfeld et al. [85] reported that participants engaging in LOW-RT exhibited approximately a 25% increase in their 1-RM for both lower and upper body exercises after 8 weeks. Similarly, another study by Jenkins et al. [86] observed a 30% improvement in 1-RM values following a 12-week LOW-RT regimen in untrained young adults. These findings are important because increasing 1-RM is not only a measure of improved physical fitness but also a significant indicator of overall health. For instance, a study by Volaklis et al. [87] found that higher 1-RM is negatively correlated with the incidence of cardiovascular disease. Specifically, the study demonstrated that each standard deviation (SD) increase in muscle strength (equivalent to an approximate 15% increase in 1-RM) was associated with a 20–30% reduction in overall mortality risk and cardiovascular disease events. A meta-analysis [88] indicated that each 10% increase in muscle strength reduced the risk of type 2 diabetes by 12%. Evidence also suggests that higher muscle strength is associated with lower cancer mortality. Leong et al. [89] found that each 5 kg increase in handgrip strength was linked to a 17% decrease

in cancer mortality. Moreover, the Health ABC study [90] reported that greater leg strength was associated with lower mortality rates in older adults. For every 10% increase in leg strength, there was an 11% reduction in the risk of death from all causes.

Taken together, our findings indicate that two weekly sessions of low-volume concurrent training, requiring less than 2 h of total time effort per week, can yield significant improvements in both cardiorespiratory and muscular fitness within only 8 weeks, which most likely translates into improved health status. However, when comparing the PRO group and the PLA group, it is a major finding of this study that the two groups noticeably differed regarding their improvements in leg muscle strength. This finding is of importance, since robust lower-extremity strength is not only essential for performing daily activities but also linked to several health outcomes, including the prevention and management of chronic diseases and a reduction in mortality risk [9,90–92]. For instance, it has been demonstrated that lower limb muscle strength is significantly associated with a lower risk of cardiometabolic disorders [91], cardiovascular disease [92] and all-cause mortality. Interestingly, it has been suggested that loss of strength in the lower limb muscles, in particular, significantly affects overall body functionality and may have a greater impact on mortality compared to upper limb muscle strength [9], which further underscores the relevance of our findings.

Regarding cardiorespiratory fitness, the improvement in VO_{2max} was ~1.3 mL/kg/min larger in the PRO group compared to the PLA group. Although this difference was not statistically significant, it can be deemed clinically meaningful [93], because it has been suggested that a 1 mL/kg/min improvement in VO_{2max} is related to a reduced risk of cardiovascular disease-related premature death by approximately 9% [94]. Moreover, it is of note that the PRO group showed substantial increases in 1-RM across all major muscle groups, while the PLA group showed improved 1-RM values only in the chest and upper back muscles. Although the changes in muscle strength (aside from leg muscle changes) were not statistically different between the two groups, these differences may be of clinical relevance [93]. Research suggests that small differences in 1-RM can be associated with important health benefits. For instance, in patients with chronic obstructive pulmonary disease (COPD), 1-RM improvements of approximately 5 kg in leg extension and 6 kg in chest press were identified as clinically significant. These improvements were correlated with better performance in functional tests such as the six-minute walk test [95]. In healthy populations, even small 1-RM differences, such as 1 kg, can translate to better performance in daily activities and reduce the risk of injury. It has been reported that minor gains in maximum strength can lead to improved balance, reduced fall risk, and better overall mobility, which are crucial for maintaining independence, especially in older adults [58,96]. Moreover, modest differences in muscle strength can have positive psychological effects, including enhanced self-esteem, reduced symptoms of depression and anxiety, and overall better mental health [96]. These findings collectively suggest that even small-to-modest differences in 1-RM values can be clinically relevant, contributing to better physical function, injury prevention, and mental health. Therefore, the observed differences in muscle strength changes between the PRO group and PLA group may be of clinical interest, even when the differences in most muscle groups did not reach statistical significance.

Consequently, our findings highlight the beneficial effects of post-exercise protein supplementation on leg strength adaptations, a critical variable linked to physical fitness and general health [9,90–92]. The observed increase in leg strength underscores the potential of protein supplementation to support lower-extremity muscle improvements and performance in response to low-volume concurrent training and aligns with previous research [24]. The absence of statistically significant differences in other measured physical fitness outcomes suggests that while protein supplementation may specifically benefit leg muscle strength, its effects on the adaptation of other muscle groups and cardiorespiratory fitness in response to low-volume concurrent training may need further investigation, potentially requiring larger samples and/or longer interventions. In this context, several hypothesized physiological mechanisms have been postulated to account for the beneficial

influence of post-exercise protein supplementation to improve adaptations to concurrent training that primarily relate to muscle mass and strength adaptations [22–25]. First, protein intake after exercise stimulates synthesis of muscle protein by providing essential amino acids, which activate the mechanistic target of rapamycin (mTOR) pathway [97]. The mTOR activation is crucial for initiating the translation process necessary for muscle repair and growth. During concurrent training, the increased muscle protein synthesis response from protein supplementation can help offset the catabolic effects of previous endurance training, thereby promoting muscle hypertrophy and strength gains through subsequent resistance training [29]. Second, both endurance training (especially high-intensity training such as HIIT) and resistance training can cause exercise-induced muscle micro-trauma. Post-exercise protein supplementation provides the necessary substrates for muscle repair and promotes faster recovery [98]. This accelerated recovery allows for more effective subsequent training sessions, enhancing overall training adaptations. Additionally, post-exercise protein supplementation can positively influence the hormonal environment conducive to muscle anabolism. Protein ingestion has been shown to elevate the release of anabolic hormones such as insulin and growth hormone, which facilitate muscle protein synthesis and hypertrophy [99]. These impact on hormones can counteract the potential catabolic effects of endurance training, promoting a net anabolic state that supports resistance training adaptations. It is of note that neither of our two study groups experienced significant increases in skeletal muscle mass following the exercise program, suggesting that 8 weeks of LOW-RT may not be enough to induce substantial hypertrophic muscle changes, particularly when combined with additional LOW-HIIT within the same session. However, muscle strength increase results from both changes in muscle structure (in particular muscle hypertrophy) and neuronal adaptations, such as improved recruitment of motor units [100]. Although protein supplementation is primarily associated with beneficial effects on muscle hypertrophy, it has been reported that it may also play a role in neuronal adaptations by facilitating the repair and growth of neural tissues, ensuring optimal nerve function, and promoting the release of neurotransmitters involved in muscle contraction [101]. Moreover, it has been reported that protein supplementation can enhance muscle quality by promoting myofibrillar protein synthesis, thus increasing the density of contractile proteins within the muscle fibers and leading to more efficient force production per unit of muscle mass [102].

While the role of carbohydrates and fats in supporting endurance training adaptations is well established, emerging evidence suggests that protein availability and supplementation may also play a critical role. Changes in VO_{2max} are largely dependent on adaptations in cardiac output, stroke volume, capillary density, blood volume and mitochondrial capacity, which adapt at different rates in response to regular endurance training [103]. Protein intake supports repair and remodeling processes that are essential for cardiovascular and mitochondrial adaptations. Adequate protein availability, for example, is necessary to sustain the synthesis of new contractile proteins and enzymes that facilitate increased cardiac output and stroke volume [104]. In this regard, previous research has demonstrated that protein ingestion after endurance training can support formation of new capillaries and may enhance mitochondrial adaptations by promoting mitochondrial biogenesis and function [104–106].

There are some potential limitations of this study. First, we acknowledge that we did not rigorously monitor volunteers' dietary intake or habitual activity patterns outside of the prescribed training sessions, except during the 3-day assessments at the beginning and end of the study. Also, volunteers were given general nutritional guidelines and recipes at the study's onset, but their diets were not strictly standardized throughout the 8-week period. Thus, despite the absence of notable discrepancies in dietary intake and daily physical activities between the two observation periods or between the groups, it is not possible to entirely discount the potential influence of variations in habitual nutrition or physical activity on non-monitored days on the adaptations to the training program. Nonetheless, we note that our study aimed to determine if targeted protein supplemen-

tation post-exercise could enhance the adaptations to a low-volume concurrent exercise program without significantly altering volunteers' habitual diets. Second, some conclusions are drawn from self-reported dietary intake and activity records. In this respect, it has been reported that people typically tend to underestimate their dietary intake and overestimate their engagement in physical activities, and that the act of recording itself may unconsciously alter behaviors [107]. However, we believe that the comprehensive guidance provided on the accurate recording of record dietary intake and daily activities likely minimized the potential for errors. Third, volunteers received a standardized dose of 40 g of protein following each training session. While it could be argued that matching the supplement dose to each volunteer's body weight would have been more precise, it is not uncommon for post-exercise nutrient recommendations to be given as absolute values [108]. Further, previous studies using fixed protein supplementation have reported significant effects on myofibrillar muscle protein synthesis [73,109] and VO_{2max} [110,111], supporting the effectiveness of this approach. Fourth, volunteers' body composition was determined with BIA. Although the utilized device has shown high accuracy in assessing skeletal muscle mass when compared to magnetic resonance imaging (MRI) and dual X-ray absorptiometry (DXA) (63), this method can have some limitations, including, for example, its sensitivity to hydration changes [112]. It is therefore possible that some pertinent distinctions in skeletal muscle adaptations between the PRO and PLA groups have been overlooked in this study. Fifth, one notable limitation of our study is the absence of biochemical markers, such as mTOR, which play a crucial role in muscle protein synthesis and hypertrophy. The inclusion of such markers would have allowed for a more comprehensive understanding of the molecular mechanisms driving the observed physiological changes. Future research should integrate biochemical analyses to provide deeper insight into the anabolic signaling pathways and their contribution to muscle strength and adaptation in response to exercise interventions. Finally, the 8-week duration of this study leaves questions about longer-term effects of protein supplementation following exercise on responses to low-volume concurrent training unanswered. Future research with extended intervention as well as with different training protocols (e.g., variations in the order of concurrent low-volume endurance and resistance training) is necessary to evaluate these questions. Despite these limitations, this study is the first double-blind, randomized, placebo-controlled investigation to evaluate the impact of targeted protein supplementation following a concurrent low-volume exercise program on key variables of physical fitness in previously untrained individuals.

5. Conclusions

Our study suggests that supplementation with 40 g of whey-based protein after a session of low-volume concurrent training can improve adaptations to low-volume concurrent training in previously untrained healthy individuals. Individuals combining low-volume endurance and resistance training in the same session may benefit from targeted protein supplementation, particularly to maximize leg muscle strength improvements.

Author Contributions: Conceptualization, D.R.; methodology, D.R. and Y.Z.; validation, D.R., H.J.H. and Y.Z.; data collection, N.Z.; supervision of data collection, D.R.; data curation, N.Z.; formal analysis, D.R.; writing—original draft preparation, D.R.; writing—review and editing, N.Z., H.J.H., M.F.N. and Y.Z.; project administration, D.R. and Y.Z.; funding acquisition, D.R., H.J.H. and Y.Z. All authors have read and agreed to the published version of the manuscript.

Funding: This study has been supported by the Hector Foundation II (funding number: MED1710), the Manfred Roth Foundation (funding number: N.N.) and Research Foundation for Medicine at University Hospital Erlangen (funding number: N.N.).

Institutional Review Board Statement: The study was conducted according to the guidelines of the Declaration of Helsinki and approved by the Medical Ethical Committee of Friedrich–Alexander University Erlangen—Nürnberg (protocol code: 147_19B, 14 May 2019).

Informed Consent Statement: Informed consent was obtained from all subjects involved in the study.

Data Availability Statement: The datasets generated and analyzed during the current study are not publicly available, but are available from the corresponding author on reasonable request.

Acknowledgments: We would like to thank Alisia Gerl, Melanie Klaußner, Maike Tobschall and Kerstin Weidlich for supervising and instructing the exercise sessions. We are especially grateful to all study volunteers for their willingness to participate in this study. Parts of this manuscript are used in the medical dissertation of co-author Nilas Zieseniss.

Conflicts of Interest: The authors declare no conflict of interest.

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Article

Effectiveness of Sports Nutrition Education Based on Self-Determination Theory for Male University Rowing Athletes: A Randomized Controlled Trial

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Abstract: To resolve problems in the dietary life of university athletes, education is essential to enable athletes to change their own dietary behavior. The purpose of this research was to verify the effectiveness of sports nutrition education based on self-determination theory (SDT). The participants were 36 male university rowers. A stratified randomized comparison test was conducted by student year (SDT group and control group). Sports nutrition education was held three times, via an Internet conferencing system. Furthermore, group work over social media was used for the SDT group. Four evaluations were carried out based on anthropometric measurements, a brief self-administered diet history questionnaire (BDHQ), sports nutrition knowledge test (SNK), and treatment self-regulation questionnaire (TSRQ). The results showed no differences between the two groups. However, for the intragroup factor, "Protein", a significant difference was evident in the self-determination theory group (50.0 ± 28.5, 78.6 ± 28.1, 81.0 ± 21.5, $p < 0.000$, units: %) and improved knowledge ($p = 0.002$, $p = 0.002$). And for the BDHQ, the self-determination theory group also showed significant differences and increased their intake of green and yellow vegetables, fruits, and dairy products (159.1 ± 74.2–126.7 ± 70.6, $p = 0.009$, 306.0 ± 196.2–195.2 ± 146.1, $p = 0.020$, 257.0 ± 147.0–183.3 ± 167.9, $p = 0.040$, units: g). In conclusion, sports nutrition education based on SDT improved dietary knowledge and increased food requirements for athletes.

Keywords: sports nutrition knowledge; brief self-administered diet history questionnaire; intervention study; nutrition education; male athletes

Citation: Enatsu, N.; Seino, J.; Tsuji, T.; Ogata, M. Effectiveness of Sports Nutrition Education Based on Self-Determination Theory for Male University Rowing Athletes: A Randomized Controlled Trial. *Nutrients* **2024**, *16*, 799. https://doi.org/10.3390/nu16060799

Academic Editors: Valentín E. Fernández-Elías, Olga López Torres and Miguel Mariscal-Arcas

Received: 1 February 2024
Revised: 25 February 2024
Accepted: 6 March 2024
Published: 11 March 2024

1. Introduction

1.1. Background

Rowing is a competitive sport that requires both anaerobic and aerobic capacity. Although a race lasts only 6–8 min, daily practice sessions are long [1]. In addition, there are lightweight and heavyweight categories, which require different body masses and compositions, requiring individualized nutritional strategies and plans. Additionally, Michael et al. reported that there is an association between patterns of achievement-motivated behavior and performance and future success in rowing [2]. Karen et al. suggested that a well-planned nutrition strategy that includes the careful timing and selection of appropriate foods and fluids helps to maximize training adaptations and, thus, should be an integral part of the athlete's training program. And they also tell us that food choice is influenced by physiological, social, psychological, and economic factors and varies both within and between individuals and populations, and they say that research is needed to investigate the motivations for athletes' food choices [3].

Hamaguchi et al. reported that university students often eat takeaway foods or eat outside, and thus have a low awareness of balancing meals and cooking for themselves. Therefore, they pointed out that to raise awareness among university students, practical hands-on guidance is important in order for them to concretely understand and become aware of problematic areas in various directions by learning about problems related to food on their own and understanding their own dietary situation [4]. In addition, Akamatsu suggested that education on eating behavior is an important aspect of nutrition education that incorporates a perspective on eating behavior [5]. Jagim et al. reported a large discrepancy between the perceived amount of energy and nutritional intake and the actual intake among university athletes [6]. These findings highlight the importance of sports nutrition education for university students.

Concerning nutrition education, Contento suggested that to facilitate changes in eating behavior, it is necessary to properly address various factors influencing or determining food choice and eating behavior and to use programs based on educational strategies, learned experience, and evidence [7]. In addition, a nutrition intervention protocol that incorporates a behavioral change model to boost motivation for the 5As (Ask, Advise, Assess, Assist, and Arrange) and motivational interviewing (MI) has been tested for adolescent athletes and has been reported to potentially promote changes in dietary behavior and inform nutrition strategies [8]. This 5A behavior change model, which has also been tested in smoking cessation [9] and weight loss counseling [10], has been reported as an effective tool for behavioral counseling [11]. MI is a client-centered approach developed by Miller [12] that focuses on assisting participants in planning and achieving autonomy to lead healthier and fulfilling lives. Regarding this motivation, it has been reported that Olympic medalists in judo "became intrinsically motivated by developing an interest in diet through a recognition that diet and athletic performance are linked" [13]. Therefore, research on nutrition education for athletes focusing on motivation has attracted attention in a broad range of sports. The formation of athletes' eating behaviors and assessment of dietary intake is very important because an appropriate dietary intake improves athletes' health and sports performance [14]. However, few studies have investigated deeper associations. Therefore, we focused on nutrition education research for athletes, focusing on motivation.

This study examines this motivation in terms of intrinsic and extrinsic motivation and focuses on Ryan and Deci's self-determination theory (SDT), which explains human behavior from the perspective of autonomy [15]. They said that intrinsically motivated behaviors are experienced as being volitional and emanating from one's self, a point made early on. And in contrast, they also said that extrinsically motivated behaviors occur because of externally imposed reward or punishment [15].

One of its sub-theories, the Basic Psychological Needs Theory, involves three basic psychological needs—autonomy, competence, and relatedness—that lead to intrinsic motivation. This theory is employed in a wide range of areas, including health management and diet therapy, smoking cessation, dentistry, sports, physical activities, physical education, and work and organization.

Contento asserted that nutrition education helps students achieve motivation, self-control, and self-determination when it provides active feedback for the experience of autonomy (Autonomy) and situations where students feel responsible for acting with confidence (Competence) and provides support in the educator–student relationship (Relatedness) [7]. Markland et al. also suggested adopting the SDT perspective to deepen our understanding of the psychological processes involved in MI [16]. Furthermore, Patrick and Williams suggested that SDT and MI, although developed for different purposes and areas, have a great deal of conceptual overlap [17]. An SDT study on athletes reported that autonomy-supportive coaching strengthened the motivational orientation of behavior in high school and college athletes [18]. It has been reported that an SDT-informed intervention for young endurance athletes significantly increased knowledge but did not lead to changes in food intake [19]. In addition, motivation underlies feeding regulation, and the satisfaction and inhibition of basic psychological needs in SDT show how disor-

dered eating occurs and how a person can optimally regulate ongoing eating patterns [20]. Leblanc et al. suggest that nutrition education utilizing self-determination theory appears to be particularly compatible with male individuals [21]. The results of a meta-analysis by Ntoumanis et al. indicate that SDT-based interventions have a positive impact on health parameters [22]. However, the examples of this type of intervention study focusing on male athletes are scarce. And Simona et al. reported that research on the effectiveness of nutrition education and behavior change interventions in athletes is lacking; therefore, they report that additional studies of sufficient rigor (i.e., randomized controlled trials) are needed to demonstrate the benefits of nutrition counseling in athletes [23].

1.2. Purpose

Against this background, we speculated that sports nutrition education, by incorporating SDT and creating opportunities to understand an individual's own diet, might help students improve their food knowledge and improve and sustain their autonomous eating behavior. Therefore, the purpose of this study was to implement an SDT-informed sports nutrition education intervention and identify its effectiveness in inducing autonomous eating behaviors among male university athletes.

2. Materials and Methods

2.1. Experimental Design

The intervention period was six months, from 13 June 2021 to 27 November 2021. This study comprised a two-group randomized controlled trial.

The groups were blocked, randomized, and assigned according to grade level after the pre-survey. Individuals were assigned code names by the author, and assigned to the intervention group (SDT group) or the control group (hereafter referred to as the COT group). It used the RAND function in Excel to generate a random number and sort the members into two groups. The power calculations were based on the results from a prior study on nutrition knowledge [24]. In this study, the mean difference in knowledge between athletes and coaches was $8 \pm 9\%$, with athletes correct $73 \pm 9\%$ and coaches correct $81 \pm 9\%$. Thus, we estimated that the change in knowledge scores that would be achievable and beneficial for the participants was about 8%. ($\alpha = 0.05$, desired power = 0.80). Based on this, in this study, we have just confirmed G-power; we calculated that there should be 17 athletes in each group, with an expected dropout of 10%. Therefore, we estimated that we needed at least 38 athletes.

2.2. Participants

The participants were defined as members of the University of Rowing Club in April 2021 who were training continuously, and excluded were those who had left the club, taken a leave of absence, or switched to being coxswains. Thirty-six male athletes and four staff members, including a coach, a coxswain, a new trainer coach, and a manager, participated in the survey. The male athlete members were assigned to two groups, with 18 members in the SDT group and 18 in the COT group.

An explanatory meeting was held for the participants two weeks before the study program began. An explanation of the study and a consent form were distributed to the participants, who provided free and voluntary informed consent. For the consent of minors, we mailed an explanation of the study, together with a consent form, to their parents or guardians and asked them to give their consent by returning the completed forms to us in a self-addressed envelope. The participants were informed that they would not know which group they would be assigned to at the time of the briefing session. A preliminary survey was conducted on 30 May, two weeks before the program began. The overall study design is shown in Figure 1.

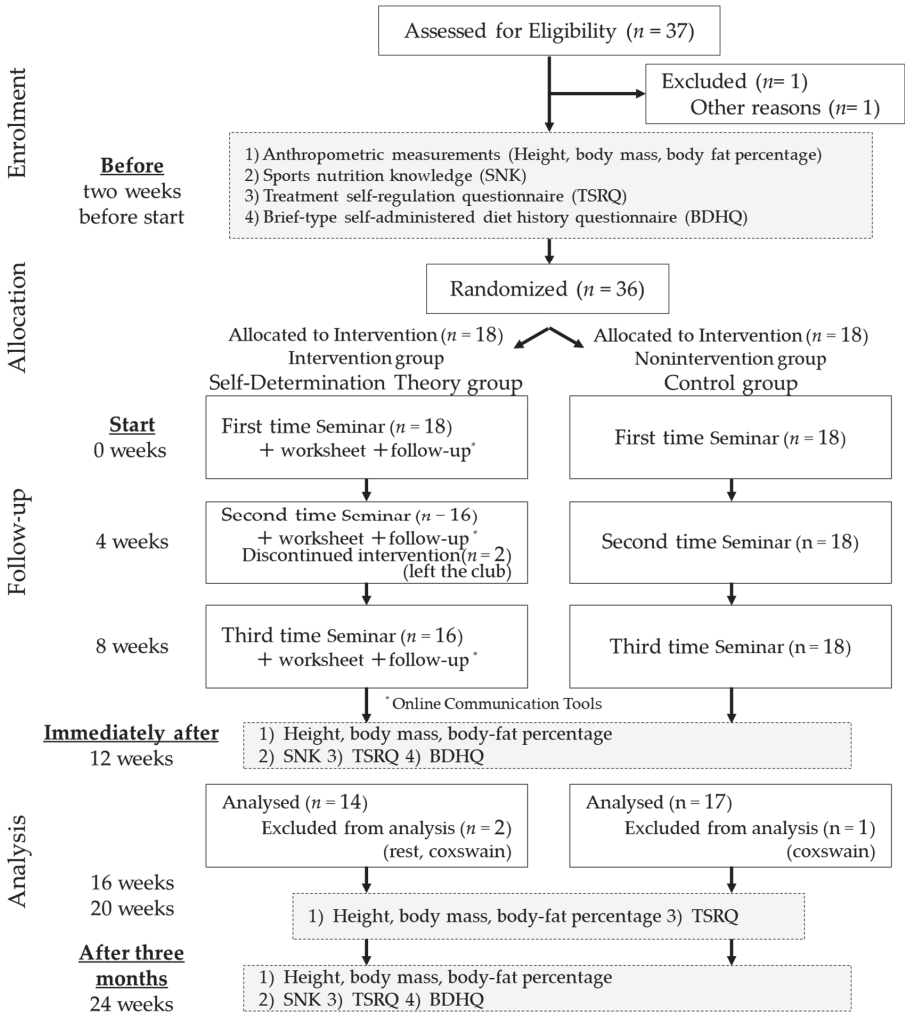

Figure 1. Research design.

2.3. Intervention Program

2.3.1. Program Overview

This study created an intervention program centered on SDT that incorporated the elements of the 5As and MI. The aim of the SDT program was to enhance autonomy, leading to a change in intrinsic behavior, and it included the following elements: (1) Desire for Autonomy, which is a desire for the self-determination of one's own experiences and actions, and a sense of self-determination of one's own actions; (2) Desire for Competence, which is a desire to effectively demonstrate one's ability and competence, and the feeling of being able to demonstrate one's abilities and talents; and (3) Desire for Relationships (relations and interactions), which is the desire to form good relatedness with others, to be cared for by significant others, and to contribute something for the benefit of others [15]. For the intervention program that incorporated elements of the 5As and MI, we also incorporated elements of the nutrition intervention protocol validated by Lee and Lim [8].

In this study, group sports nutrition education seminars were held once every four weeks for a total of three sessions, using a web-conferencing system. Table S1 presents

the overall flow of the program. The program time was 90 min for the SDT group and 60 min for the COT group. After the sports nutrition education seminar, information was shared once a week in small groups (four or five participants), including one manager for both groups, using an online communication tool. The content of the program was decided by two university faculty members specializing in physical education, sports management, coaching, and sports nutrition. One of the authors, a sports nutritionist, provided the guidance.

2.3.2. Sports Nutrition Education Seminars

Educational content and handouts were similar for both the SDT and COT groups, with the SDT group receiving an additional worksheet that included work on Autonomy, Competence, and Relatedness.

The common educational content for both groups was original and based on the content of the nutrition education session adopted by Lee and Lim [8]. In the first month, the Basic Nutrition Concept program included (1) the need for sports nutrition and how to use nutritional information, (2) nutrition and training, (3) regular eating habits, (4) body image, and (5) knowledge of sports nutrition (protein, carbohydrates, and water supplementation). In the second month, the Basic Food Skills program included (1) knowledge of sports nutrition (including supplements), (2) grocery shopping (ingredients and food selection), (3) cooking methods and food hygiene, (4) meal planning, and (5) eating out. In the third month, the Performance Enhancement program consisted of (1) planning light meals (recovery from fatigue and muscle pain), (2) meals before practice and games, (3) meals during practice and games, (4) meals after practice and games, (5) meals on the move, and (6) meals for various purposes (weight gain, weight loss, and weight maintenance). All participants were encouraged to participate in interactive communication through a web conferencing system.

The additional work performed by the SDT group was designed to promote autonomy based on (1) providing rational motivation for the participant activities, (2) understanding the participants' feelings, and (3) giving the participants a choice of activities [25]. The participants then undertook question-and-answer sessions and group discussions, and used a worksheet that allowed them to discover problems and issues with their own food profiles, their usual cooking, and light meals before and after practice and games. To enhance autonomy, support was provided by clearly communicating expectations and clarifying the process of achievement [26]. Concrete suggestions for improvement were provided before the worksheet was filled out. We ensured that this led to their usual eating habits by asking them to write down, in their own words, the areas for improvement and by actually taking action. The worksheets were collected after the sports nutrition education seminar and returned after checking their content. The checked content was used to send advice via e-mails. Sports, nutrition, and education seminars were held in the evenings after practice, and the participants attended seminars at the boatyard and from their homes.

2.3.3. Group Support Using Online Communication Tools

For the online communication tool, both groups shared one day's worth of photographs of their meals once a week and were able to consult with the author regarding any questions they had.

In addition, for the SDT group, progress in the tasks was reported within the group based on worksheets completed at the monthly sports nutrition education seminars, allowing the groups to advise each other. The SDT group managers reported their progress to the authors each week, and the authors sent 12 advice e-mails to each group within 2–3 days of the report. This program was designed to link sports nutrition education with group support using online communication tools.

2.4. Survey and Measurement Instruments

The survey/measurement items included anthropometric measurements (height, weight, and body fat percentage), a brief self-administered diet history questionnaire (BDHQ), a sports nutrition knowledge questionnaire (SNK), and a treatment self-regulation questionnaire on the self-regulation of eating habits (TSRQ). The primary outcome comprised anthropometric measurements and the BDHQ; the secondary outcome comprised the SNK and TSRQ.

Anthropometric measurements were taken using equipment that estimates body composition using bioelectrical impedance analysis (BIA). The following seven points were standardized when measuring body mass and body fat percentage: (1) measurements were taken two hours after eating; (2) urination and defecation were completed before measurement; (3) measurements immediately after exercise were avoided; (4) measurements in the presence of dehydration or swelling were avoided; (5) measurements at low temperatures or during hypothermia were avoided; (6) measurements at high temperatures were avoided; and (7) measurements immediately after bathing were avoided. Anthropometric measurements were taken with a manager who was thoroughly familiar with this method.

Kobayashi et al. [27] developed the BDHQ, which retains the features of the self-administered dietary history questionnaire developed by Sasaki et al. [28] by simplifying its structure, responses, and data processing. We used the BDHQ to survey nutrient and other intakes in the previous month. The manager distributed the BDHQs and collected them within three days. The manager and authors checked for omissions and errors on the day of collection; if recompletion was required, the manager requested correction within a few days and the resulting responses were collected once more.

The SNK was based on a questionnaire (40 questions) developed by Walsh et al. [29] and surveyed food knowledge, categorized into five sections: (1) Training Schedule and Positioning with Training, (2) Eating and Hydration Habits and Awareness, (3) Attitudes Toward Nutrition Intake, (4) Nutrition Knowledge, and (5) Nutrition Information Sources and Nutrition Education that Might be Needed in the Future.

The TSRQ was developed by Ryan and Connell [30] to assess autonomous self-regulation, and was first used for health purposes by Williams et al. [31]. It is now widely used in behavioral change studies in medical settings. Subsequently, Levesque et al. validated the utility of healthy behaviors [32]. The TSRQ consists of a series of questions about why people engage in or attempt to engage in healthy behaviors, undergo treatment for illness, change unhealthy behaviors, follow a treatment plan, or engage in other health-related behaviors (Center for Self-Determination Theory online) [33]. There are four types of TSRQ: smoking cessation, dietary modifications, regular exercise, and responsible drinking. In this study, we used a modified version of the diet. Furthermore, respondents were asked to respond to the questions by viewing their health as "health on the playing field". The TSRQ was used after we reconfirmed its contents, which had been translated by a company specializing in translating research papers.

These questionnaires have been developed and validated [27,29,34].

These surveys and measurements were conducted two weeks before the start of the program (before), immediately after the program's conclusion (immediately after), and three months after the program's conclusion (after three months). During the program's implementation period, the participants' body composition in terms of height, weight, and body fat percentage was measured every four weeks, and anthropometric measurements and diet self-control were conducted every four weeks as a continuing evaluation for three months after the program ended.

2.5. Statistical Analysis Methods

Normality assumption was confirmed using the Shapiro–Wilk test. The program was subjected to parametric tests before it started, immediately afterward, and three months later. Anthropometric data were obtained as mean and standard deviation.

In this study, the nutrient intake of ten items and the nutrient intake by food group of fifteen items were used in the BDHQ. The nutrients selected were "iron, vitamin D, calcium, and the antioxidant vitamin C", which are reported to be particularly important for athletes [35]. In addition, vitamin B1 and dietary fiber, which are easily deficient in the university's rowing club, were selected.

The 14 SNK questions were divided into five items (Total Knowledge Score, Energy and Replenishment, Hydration, Supplements, and Protein), and the percentages of correct answers were analyzed for each item.

The TSRQ of 15 questions was divided into four items. The means of the responses to each question were calculated for three items: autonomous motivation, externally controlled motivation, and non-motivation. The fourth item, the Relative Autonomous Motivation Index, is calculated by subtracting the mean value of externally controlled reasons from that of autonomous reasons.

The results of the BDHQ, SNK, and TSRQ were expressed as means and standard deviations. Unpaired t-tests were used for pre-program group comparisons. The Levene test was checked before the ANOVA to confirm equal variances. Repeated two-way analysis of variance was used for between-group and within-group factors, and the Bonferroni method was used for multiple comparison tests when significant differences were confirmed. The statistical significance level was set at 5%. Statistical analysis software was IBM® SPSS Statistics 28.0 for Windows.

2.6. Ethical Considerations

The University of Tsukuba Research Ethics Committee reviewed the ethical considerations for conducting this study in accordance with the Declaration of Helsinki and approved it on 20 May 2021 (issue no. Tai 020-176) [36].

3. Results

3.1. Participant Flow and Number of Participants Analyzed

Two members left the club, and there was one leave of absence and two coxswain transfers, resulting in a total of 31 participants for analysis: 14 in the SDT group and 17 in the COT group.

3.2. Differences between Groups before the Program

The results of the anthropometric measurements, SNK, and TSRQ are listed in Table S2. No significant differences were observed in any parameters between the SDT and COT groups. The BDHQ results are shown in Table S3. Before, The SDT and COT groups had no significant differences in nutrient intake, but there were significant differences in meat ($p = 0.043$) and fat and oil ($p = 0.004$) intake by food group.

3.3. Immediately after and after Three Months—Anthropometric Measurements, Sports Nutritional Knowledge Questionnaire (SNK), and Treatment Self-Regulation Questionnaire (TSRQ)

The results of the anthropometric measurements, SNK, and TSRQ are listed in Table S2. No significant differences were observed in any parameters between the SDT and COT groups. Table S4 lists the results of the anthropometric measurements, SNK, and TSRQ. Between the SDT and COT groups, there were no significant differences in body mass, body fat percentage, lean body mass, or lean body mass/m. There were no differences between the SDT and COT groups in intragroup factors for immediately after and before, after three months and before, or after three months and immediately after. There was no significant difference in the total SNK knowledge score between the SDT and COT groups in the between-groups factor. On the other hand, there was no significant difference in the main effect of time on the within-group factor ($p = 0.004$). The SDT group had $78.6 \pm 12.5\%$ for before and $87.2 \pm 7.5\%$ for immediately after, whereas the COT group had $75.6 \pm 10.1\%$ for before and $83.6 \pm 8.3\%$ for immediately after. Thereafter, there was a significant difference ($p = 0.003$) between immediately after and before in a subsequent multiple comparison

test, with an increase in immediately after compared with before. In the between-group factor for protein, there was no significant difference between the SDT and COT groups. In contrast, there was a significant difference in the main effect of time on the within-group factor ($p < 0.001$).

In the SDT group, the results were 50.0 ± 28.5% for before, 78.6 ± 28.1% for immediately after, and 81.0 ± 21.5% for after three months. In the COT group, the results were 56.9 ± 22.9% for before, 72.5 ± 27.0% for immediately after, and 68.6 ± 24.9% for after three months. In the subsequent multiple comparison tests, there was a significant difference between immediately after and before ($p = 0.002$), and between after three months and before ($p = 0.002$). There were no significant differences between or within the groups in terms of energy, supplementation, hydration, or supplements. There were no significant differences between the SDT and COT groups on the TSRQ autonomous motivation subscale, externally controlled motivation subscale, non-motivation subscale, or the Relative Autonomous Motivation index in terms of between-group and within-group factors.

3.4. Immediately after and after Three Months—Brief Dietary History Questionnaire (BDHQ)

The BDHQ results are shown in Table S3. The BDHQ results are presented in Table 1. In terms of between-group factors, there was no significant difference in carbohydrate intake between the SDT and COT groups. However, only the main effect of time was significant ($p = 0.021$), and a subsequent multiple comparison test revealed no significant difference. For the between-group factor, there was no significant difference in calcium between the SDT and COT groups. However, only the main effect of time was statistically significant ($p = 0.026$). Thereafter, subsequent multiple comparison tests showed no significant differences. Moreover, there were no significant differences in energy, protein, fat, iron, vitamin D, vitamin B1, vitamin C, or dietary fiber levels between or within groups.

There were no significant differences in the intake of green and yellow vegetables between the SDT and COT groups in terms of the intake of each food group and the between-group factors. However, the main effect of time on the within-group factors showed a significant difference ($p = 0.009$). The intake of green vegetables in the SDT group was 126.7 ± 70.6 g for before and 159.1 ± 74.2 g for immediately after, whereas in the COT group, it was 99.8 ± 69.2 g for before and 159.9 ± 99.3 g for immediately after. A subsequent multiple comparison test revealed a significant difference ($p = 0.009$) immediately after and before. Fruits were not significantly different between the SDT and COT groups in terms of between-group factors. However, there was a significant difference in the main effect of time on within-group factors ($p = 0.013$).

In the SDT group, the intake of fruits was 195.2 ± 146.1 g for before and 306.0 ± 196.2 g for immediately after, whereas in the COT group, it was 133.2 ± 152.4 g for before and 215.3 ± 208.6 g for immediately after. Thereafter, multiple comparison tests showed a significant difference ($p = 0.020$) immediately after and before the intervention.

There were no significant differences in the between-group factors for dairy product production between the SDT and COT groups. However, there was a significant difference in the main effect of time on within-group factors ($p = 0.012$). The intake of dairy products was 183.3 ± 167.9 g for before and 257.0 ± 147.0 g for immediately after in the SDT group, and 177.3 ± 93.3 g for before and 262.6 ± 134.4 g for immediately after in the COT group. A subsequent multiple comparison test revealed a significant difference ($p = 0.040$) immediately after and before.

There were no significant differences between or within the groups for cereal grains, potatoes, sugar and sweeteners, pulses, other vegetables, fish and shellfish, meats, eggs, fats and oils, confectioneries, beverages, or seasonings and spices.

Table 1. Results of between-group and within-group factor analysis for the BDHQ.

	Grouping Factor		Within-Group Factor			
	Reciprocal Action Group × Time p †	Time Main Effects p †	Immediately After–Before p ‡	After Three Months–Before p ‡	After Three Months–Immediately after p ‡	Multiple Comparison Test
BDHQ (Nutrient intake)						
Energy (kcal)	0.413	0.059				
Protein (g)	0.652	0.114				
Fat (g)	0.853	0.810				
Carbohydrate (g)	0.307	0.021 *	0.059	0.090	1.000	n.s
Calcium (mg)	0.939	0.026 *	0.054	0.178	1.000	n.s
Iron (mg)	0.757	0.056				
Vitamin D (μg)	0.314	0.059				
Vitamin B_1 (mg)	0.832	0.080				
Vitamin C (mg)	0.854	0.050				
Dietary fiber (g)	0.967	0.295				
BDHQ (Intake by food group)						
Cereal grains (g)	0.305	0.068				
Potatoes (g)	0.806	0.553				
Sugar and sweeteners (g)	0.864	0.051				
Pulses (g)	0.407	0.121				
Green and yellow vegetables (g)	0.450	0.009 *	0.009 *	0.204	0.735	Before < Immediately After
Other vegetables (g)	0.769	0.435				
Fruits (g)	0.735	0.013 *	0.020 *	0.619	0.267	Before < Immediately After
Fish and shellfish (g)	0.467	0.070				
Meats (g)	0.521	0.527				
Eggs (g)	0.973	0.871				
Dairy products (g)	0.657	0.012 *	0.040 *	0.770	0.076	Before < Immediately After
Fats and oils (g)	0.252	0.322				
Confectioneries(g)	0.688	0.418				
Beverages (g)	0.713	0.052				
Seasonings and spices (g)	0.394	0.148				

* $p < 0.05$: Significant difference. n.s.: Not significant. † A two-way ANOVA was conducted on the between-group and within-group factors. ‡ Multiple comparison tests using the Bonferroni method were performed for before, after, and after three months for within-group factors. < less than. BDHQ: brief self-administered diet history questionnaire.

4. Discussion

This study implemented SDT-informed sports nutrition education among a male university rowing club with the goal of not only improving sports nutrition knowledge but also establishing an autonomous dietary environment. The results showed no differences between the two groups in terms of the anthropometric measurements, BDHQ, SNK, and TSRQ. Thus, the effectiveness of education has not yet been demonstrated. However, anthropometric measurements included the summer months, when appetites are more likely to decrease, but weight and lean body mass did not decrease significantly. In an intervention program combining nutrition education and food environment intervention in a randomized controlled trial similar to this study, the authors reported that the main outcome, the BDHQ survey, confirmed changes in intake by food group [37]. In our study, too, for the BDHQ results for intake by food group, from the time immediately before the intervention to the time immediately after it, the green and yellow vegetable, fruit, and dairy product intake increased for both groups. In terms of food knowledge, the aggregate knowledge score from the SNK and the scores for protein items improved immediately after the intervention compared with those before the intervention and, with respect to protein items alone, remained higher for three months. However, the results indicated no significant differences between the two groups.

For intrinsic motivation, internalization, where a person integrates extrinsically motivated behaviors as their own, plays an important role, which requires the basic psychological needs of Autonomy, Competence, or Relatedness to be fulfilled [15]. Roth et al. [38] argued that autonomous motivation for learning is promoted by expanding support for autonomy. Based on these reports, in this study, support was provided to the SDT group so that the participants used a worksheet in the sports nutrition education seminars to review their everyday meals, write down areas for improvement in their own words, and link

these exercises to actual actions. We attempted to strengthen this mechanism by providing group support using online communication tools. However, our results do not support the effectiveness of this mechanism. According to Murray et al., the factors that contribute to problematic cooking practices among university students include insufficient knowledge and skills in cooking, financial insecurity, inadequate information on healthy eating, and time and lifestyle constraints. Therefore, it is important to design an effective and strategic program to promote motivation [39]. In the case of university rowing clubs, some clubs provide meals in their dormitories, but if the environment is not conducive, they often cook their own meals. Therefore, specific suggestions were needed to create a food environment for university rowers. In this study, food choices and cooking methods were proposed to improve cooking skills at home. This might explain why the effects were observed in both groups immediately after the intervention, leading to changes in eating behavior. Heikkilä et al. [19] reported, in a study on SDT-informed nutrition education intervention, that undergoing the education three times significantly increased nutrition knowledge, but did not lead to changes in food intake. However, in the current study, the participants in both groups established learning goals in a sports nutrition education seminar. This led not only to improved nutritional knowledge, but also to an increase in food intake. The SDT group was asked to look at pictures of their own food, examine "the items that I am missing" and set goals so that they would be intrinsically motivated. Thus, the program was unique, in that it was designed to use learning goals to make students "aware". The approach based on the 5As and MI, which encouraged food selection, was effective, as we believe that it brought about such behaviors and practices in the SDT group.

All seminars were conducted using synchronous distance education and a web conferencing system. As this learning format was entirely new to the participants, we believe that it played a role in making it more difficult for the variances between the groups to manifest. In contrast, Liyun et al. reported that for knowledge acquisition, synchronous distance education was not significantly different from conventional education in terms of learning effectiveness, and the level of satisfaction was also high [40]. The adoption of synchronous distance education in this study led not only to knowledge, but also to action and practice in both groups. We can infer from this that the results demonstrate the effectiveness of synchronous distance education, regardless of the SDT-based approach.

Because intrinsic framing of goals produces effects such as deeper engagement in learning activities, better conceptual learning, and higher persistence in learning activities [41] and learning goals, in which individuals seek to increase their competence, promote challenge-seeking behaviors and a mastery-orientated response to failure [42], the intrinsic framing of goals is likely to contribute to the subsequent continuation of specific habits. Therefore, considering the need for practical hands-on guidance, as discussed by Hamaguchi et al. [4], providing face-to-face nutrition education involving cooking practice and food tasting still leads to the creation of distinctive eating experiences: feeling the taste of food, thinking about how to achieve that taste, and discovering and being impressed by great food. This, in turn, might lead to the continuation of such experiences. In order to implement effective sports nutrition education, we would like to construct an ongoing program that can maintain the intrinsic motivation of athletes.

This study is limited in a number of dimensions. In this study, we focused on feasibility studies [43] and we designed the study to fully consider acceptability, demand, implementation, and practicality, but we believe that we did not fully consider adaptation, integration, extension, and the limited efficacy evaluation. This is because it was a randomized controlled trial administered within a single university rowing club by grade, and we needed to guarantee the quality of the content and make it uniform to a certain extent between the two groups. Nutrition education had to be provided without significantly varying it between different participants, and the manager and all other staff members had to treat all participants similarly. In addition, because they shared the same practice environment and ate in the same place, they inevitably interacted with one another and influenced each other. Within the club, athletes were mutually supportive, and the team's

group cohesiveness was high. The design of a controlled trial, "creating a COT group that does nothing", might, therefore, have limitations in sports practice. In this study, the research design was developed without eliminating existing positive team practices. As Tanaka and Shigematsu [44] state, "rather than creating a control group that is not allowed to do anything, we should focus on participant front of us", and we believe that researchers should pay attention to their research participants and have flexible thinking and broad perspectives. We also used expert-translated questionnaires for the SNK and TSRQ, which have not been validated in our country and need to be validated. As for the BDHQ, it is a questionnaire that has been validated in our country, but it has several issues. Since the amount of food is not specified and the method is to select the frequency, the difference may be large if the amount of food per meal differs [27]. Regarding food weight, rice and cereal grains include water from cooking, and tofu and soy milk, which are legumes, also include water. And in Japan, rice is often eaten as polished rice and fruits are peeled, so the fiber content may be low relative to the food weight. Other limitations are that the university students in this team do not have the habit of drinking cola and other sugar-containing beverages when actually surveyed about their eating habits, probably due to team rules and culture, so the amount of beverage-derived carbohydrates is not so high. However, as reported by Androniki, it is undeniable that the dietary questionnaire may have underestimated the number of subjects, and some sources of error still remain [45]. Therefore, in this study, as in the numerous previous studies that have been identified, the fact of underestimation is undeniable, and we suggest that it is a limitation of the study and an issue for future research. Finally, at the time of the survey, due to the COVID-19 pandemic, meals could not be provided within the team. To address this issue, both groups received assistance with meals immediately after the outbreak through an "emergency special donation" from the team alumni. We believe that this potentially had an uncontrollable influence on the variance between the SDT and COT groups.

5. Conclusions

The results of this study demonstrate that SDT-informed sports nutrition education, through improvement in nutrition knowledge, can potentially lead to the practice and continuation of improved eating behaviors. These effects were clearly demonstrated by the actual food intake. Therefore, body mass, one indicator of athlete health, was maintained, and lean body mass was not significantly reduced. Additionally, the provision of support designed to promote autonomy allowed us to discover that autonomy potentially facilitates intrinsic motivation. In developing a program utilizing SDT in a department that is student-driven, the cooperation of the student staff may lead to continued food awareness and eating behavior, which may affect the overall team's bottom line. The program used in this study could potentially promote sports nutrition education.

Supplementary Materials: The following supporting information can be downloaded at: https://www.mdpi.com/article/10.3390/nu16060799/s1, Table S1: Program overview; Table S2: Between-group differences in anthropometric measurements, the SNK and the TSRQ (prior); Table S3: Between-group differences in the BDHQ (prior); Table S4: Results of between-group and within-group factor analyses of anthropometrics, SNK, and TSRQ.

Author Contributions: Conceptualization, N.E., J.S. and M.O.; methodology, N.E., J.S. and M.O.; formal analysis, N.E., J.S., M.O. and T.T.; investigation, N.E.; resources, N.E.; data curation, N.E., J.S., M.O. and T.T.; writing—original draft preparation, N.E.; writing—review and editing, N.E., J.S. and M.O.; visualization, N.E., J.S. and M.O.; supervision, M.O.; project administration, N.E. and M.O.; funding acquisition, J.S. and M.O. All authors have read and agreed to the published version of the manuscript.

Funding: This study received no external funding.

Institutional Review Board Statement: The University of Tsukuba Research Ethics Committee reviewed and approved the ethical considerations for this study issue no. Body 020-176 on 20 May 2021.

Informed Consent Statement: Informed consent was obtained from all participants involved in the study. We also express our sincere gratitude to the members of the university rowing club and their coaches for their long-term participation and for their great cooperation. We would also like to thank them for their cooperation in collecting valuable data.

Data Availability Statement: Data are contained within the article and Supplementary Materials.

Acknowledgments: N.E. expresses his deepest gratitude to his supervisor, Mitsugu Ogata, for his guidance in this research. He would also like to express his sincere gratitude to Jun Seino for his careful and precise guidance and to Taishi Tsuji for his careful guidance and advice on statistical analysis.

Conflicts of Interest: The authors declare no conflict of interest.

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Article

Glycerophospholipids in Red Blood Cells Are Associated with Aerobic Performance in Young Swimmers

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Abstract: This study aimed to characterize the composition of lipids in the red blood cells (RBCs) of adolescent swimmers and correlate this lipidome with the aerobic performance of the athletes. Five experimental assessments were performed by 37 adolescent swimmers. During the first session, the athletes went to the laboratory facility for venous blood sampling. The critical velocity protocol was conducted over the 4 subsequent days to measure aerobic performance (CV), comprising maximal efforts over distances of 100, 200, 400, and 800 m in a swimming pool. RBCs were obtained and extracted for analysis using the liquid chromatography—high resolution mass spectrometry untargeted approach. A total of 2146 ions were detected in the RBCs, of which 119 were identified. The enrichment pathway analysis indicated intermediary lipids in the glycerophospholipid, glycerolipid, sphingolipid, linoleic acid, and alpha-linolenic metabolisms, as well as pentose and glucuronate interconversions. A significant impact of the intermediary lipids was observed for the glycerophospholipid metabolism, including phosphatidylethanolamine (PE), phosphatidylcholine (PC), 1-acyl-sn-glycero-3-phosphocholine, sn-glycerol 3-phosphate, and phosphatidic acid. Inverse and significant associations were observed for PE 18:2/18:3 ($r = -0.39$; $p = 0.015$), PC 18:3/20:0 ($r = -0.33$; $p = 0.041$), and phosphatidic acid 18:0/0:0 ($r = -0.47$; $p = 0.003$) with aerobic performance. Swimmers who exhibited higher levels of aerobic performance also had the lowest abundance of PE, PC, and phosphatidic acid.

Keywords: lipidomics; swimming; red blood cells; critical velocity; aerobic performance

Citation: Silva, ÁA.R.; Bertolucci, V.; Scariot, P.P.M.; da Cruz, J.P.; Mendes, F.M.M.; de Oliveira, D.C.; Plumari, C.D.; Dos Reis, I.G.M.; Porcari, A.M.; Messias, L.H.D. Glycerophospholipids in Red Blood Cells Are Associated with Aerobic Performance in Young Swimmers. *Nutrients* **2024**, *16*, 765. https://doi.org/10.3390/nu16060765

Academic Editors: Valentín E. Fernández-Elías and Olga López Torres

Received: 27 January 2024
Revised: 1 March 2024
Accepted: 4 March 2024
Published: 7 March 2024

1. Introduction

Although biofluids are the most preferred biospecimen for biochemical analysis, a paucity of interest has been given exclusively to red blood cells (RBCs). The main role of RBCs is to act as carriers of both oxygen and carbon dioxide [1,2], explaining why these cells are so important for those interested in aerobic exercise performance [3,4]. The relevance of hemoglobin is widespread in scientific studies, but this is not the only aspect that needs to be considered in the transport of respiratory gases. Indeed, the membrane properties of RBCs are also able to affect their oxygen-carrying capacity [5]. Evidence supporting this comes from rheological studies showing that more deformable/flexible RBCs may move through the microcirculation more easily [6–10]. Among the different issues that may be explored regarding membrane components, one that deserves attention is the

composition of lipids, which appears important in RBCs given the absence of nuclei and organelles [11–14].

Given the importance of lipid composition in RBCs, lipidomics appears as an important tool, not only for understanding the biophysics of RBCs but also for providing valuable insights into the determinants of aerobic exercise performance. Although the evaluation of lipids in sports is growing, research on the topic is still incipient. Only a few lipidomic studies have investigated RBCs in the context of physical exercise [15–17]. To the best of our knowledge, there is a lack of studies exploring whether lipids in RBCs are related to aerobic exercise performance. Liquid chromatography coupled to mass spectrometry (LC-MS)-based lipidomics has already been performed outside of the sports context [18–20]. Indeed, MS-based lipidomics has emerged as a promising source of information, with considerable sensitivity and the ability to detect thousands of metabolites simultaneously. Therefore, by using an untargeted lipidomic approach, the first aim of the current work was to characterize the composition of lipids in the RBCs of swimmers, which are known to have a high dependence on aerobic metabolism as an energy requirement [21]. The second and main goal of this research was to verify the possible relationships between RBC lipidome and aerobic performance. For this, we used eigenvector centrality to understand which lipids would be correlated with the critical velocity, a valid measure of aerobic capacity [22,23].

2. Materials and Methods

2.1. Participants

Data from the participants included in this study were published before, but concerning distinct analysis [24]. Five experimental assessments were performed by 37 adolescent swimmers (male, n = 19; age = 15 $\pm$ 2 years; body mass = 61 $\pm$ 11 kg; height = 166 $\pm$ 16 cm; female, n = 18; age = 14 $\pm$ 2 years; body mass = 55 $\pm$ 9 kg; height = 160 $\pm$ 7 cm). The athletes went to the laboratory facility during the first session for venous blood sampling and anthropometric measurements. The critical velocity protocol was conducted over the 4 subsequent days (48 h apart at the same time of day) to measure aerobic performance, that is, the aerobic component of the critical velocity protocol. After the identification of molecules in the RBCs by the lipidomic procedure, the complex networks elicited the lipids with higher relevance for aerobic performance (Figure 1).

According to the training periodization created by coaches, the swimmers were at the start of the general preparation period. Coaches were advised by researchers to not conduct physical training during the experimental time interval. Hence, during the critical velocity protocol, athletes only engaged in light, leisurely activities. Throughout the experiment, researchers instructed athletes to keep the same individual hydration/food habits.

2.2. Determination of Aerobic Performance

The critical velocity protocol was conducted on four randomized maximal efforts over distances of 100, 200, 400, and 800 m in a swimming pool (25 m). Athletes were encouraged by researchers and coaches to provide their best efforts during trials. For determination of the aerobic estimate (e.g., CV), the equation $D = CV \times t + AWC$ was applied, where D is equivalent to distance, t is related to time to cover the distance, AWC (e.g., anaerobic work capacity) refers to the y-intercept, and CV relates to the slope of the regression. Given the purposes of this report, only CV was considered for the relationship with the RBCs' lipids.

2.3. Red Blood Cell Profiles

Before having their blood drawn, adolescent swimmers were instructed to abstain from alcohol and unusual foods and drinks for 3 days. A volume of 5 mL of venous blood was drawn by a skilled nurse for hematological evaluations. Samples were brought to a specialized laboratory facility where the Coulter LH 750 hematology analyzer (Beckman Coulter, Miami, FL, USA) [25] evaluated the red blood cell profile, including red blood cell

count, hemoglobin, hematocrit, mean corpuscular volume, mean corpuscular hemoglobin, mean corpuscular hemoglobin concentration, and red cell distribution.

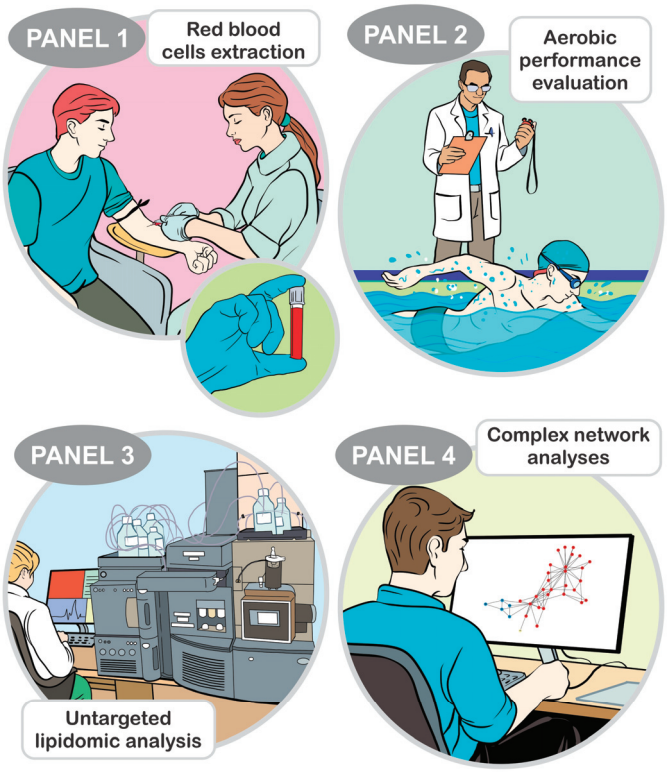


Figure 1. Experimental design of the study. Red blood cells (RBC) were collected by centrifugation of venous blood samples (Panel 1). The RBCs were preserved at −80 °C until further use. Subsequently, the swimmers underwent four predictive trials at 100, 200, 400, and 800 m to determine the critical velocity (Panel 2). Panel 3 shows that untargeted lipidomics were performed using an ultra-high-performance liquid chromatography system coupled to a quadrupole time-of-flight mass spectrometer. In Panel 4, the complex network and eigenvector centrality identified the lipids that were most relevant for the critical velocity, which was the target of this analysis.

2.4. Extraction and Lipidomic Analysis of RBCs

In addition to the blood sample collected for hematological analysis, another 5 mL of venous blood was collected for lipidomic analysis in a separate tube. The sample was centrifuged at 1500 rpm for 10 min, allowing the separation of plasma and RBC. The RBC samples were transferred to other tubes and frozen at −80 °C until extraction. The extraction was based on Gil, et al. [26]. Each sample (200 µL) was then extracted by adding 850 µL of a cold solution composed of methanol (MeOH)/methyl-terc-butyl-ether (MTBE)/chloroform ($CHCl_3$) (1.33:1:1, *v*/*v*/*v*). Afterwards, the samples were homogenized by vortexing for 30 min (2000 RPM at 22 °C), and then vortexed for an additional 30 s. Samples were again centrifuged (13,000 RPM, 10 min, 4 °C), and the supernatant was collected and dried over a nitrogen gas (N2) flow. Samples were resuspended in 200 µL of a solution of isopropanol (ISP)/acetonitrile (ACN)/H_2O (2:1:1 *v*/*v*/*v*).

A total of 25 µL of each resuspended sample was collected to compose a pooled sample used as quality control (QC). To check deviations in extraction and system stability, QC samples were injected after 10 samples. Furthermore, a QC sample was used at the beginning of the experiment to perform instrumental stabilization of the LC-MS system.

Participant samples were extracted and analyzed randomly to observe biological variation and minimize instrumental bias.

The analyses were adapted from Silva et al. [27]. An ACQUITY UPLC was used, coupled to a XEVO-G2XS (QToF) quadruple time-of-flight mass spectrometer (Waters, Manchester, UK) equipped with an electrospray ionization (ESI) source operated in the negative ionization mode. For lipidomic analysis, we employed an ACQUITY UPLC® CSH C18 column (2.1 mm × 100 mm × 1.7 µm, Waters), using the mobile phase A composed of an ACN:H_2O solution (60:40, v/v) with 10 mM ammonium formate + 0.1% formic acid, and the mobile phase B, composed of ISP/ACN (90:10, v/v) with 10 mM ammonium formate + 0.1% formic acid. The flow rate was 0.4 mL min^{-1}. Initially, the column was conditioned with 40% B, increasing to 43% over the next 2 min and subsequently to 50% within 0.1 min. In the next 9.9 min, the gradient was gradually increased to 54% B and then to 70% B in 0.1 min. At the end of the gradient, B was increased to 99% over 5.9 min; after this period, solution B returned to 40% in 0.1 min, balancing the column for the next injection for the next 1.9 min.

The mass spectrometer was operated in MS^E mode with an m/z range of 50–1200 Da, and an acquisition time of 0.5 s per scan. MS^E analysis was operated at 6 V for low-collision energy and a ramp of 20–50 V for high collision energy. Leucine enkephalin (molecular weight = 555.62; 200 pg L^{-1} in 1:1 ACN: H_2O, v/v) was used as the lock mass for mass accuracy, and a 0.5 mM sodium formate solution was used for calibration. Other parameters were as follows: source temperature = 140 °C, desolvation temperature = 550 °C, desolvation gas flow = 900 L h^{-1}, capillary voltage = 2.5 kV, and cone voltage = 40 V.

2.5. Data Processing and Putative Identification of Metabolites

The LC-MS raw files were processed using the Progenesis™ QI software v2.4 (Non-linear Dynamics, Newcastle, UK), which allowed the selection of possible adducts, peak alignment, deconvolution, and annotation of compounds based on MS^E experiments. An alignment score of 95% was adopted. The adducts $[M+H]^+$, $[M+K]^+$, $[M+Na]^+$, $[M+ACN+H]^+$, $[M+H-H_2O]^+$ and $[M+NH_4]^+$ were considered for the positive acquisition mode, and $[M-H]^-$, $[M+Cl]^-$, $[M-H_2O-H]^-$, and $[M+FA-H]^-$ for the negative acquisition mode. Progenesis QI generates an intensity table of the features, which are the ions of each sample, labeled according to their nominal masses and retention times, as a function of their intensity, considered as the areas of the extracted ion chromatogram.

Due to low- and high-energy acquisition enabled by the use of MS^E, we have information on precursor ions (low energy) and fragments (high energy) in the same spectrum. A precursor mass error of $\leq$5 ppm was considered, and a fragment tolerance of $\leq$10 ppm. Fragmentation profile, mass accuracy, mass error, and isotope similarity were evaluated to accept the annotated molecules. To allow the compatibility of Progenesis PQI data and external SDF-based spectra libraries, we used an in-house software named "SDF2PQI" to increase the number of fragment matches [28]. SDF2PQI was recently detailed elsewhere and is available free of charge. External SDF-based spectra libraries were used, such as LipidMaps (http://www.lipidmaps.org/, accessed on 7 December 2023), the Human Metabolome Database (http://www.hmdb.ca/metabolites), and the MoNA—MassBank of North America (https://mona.fiehnlab.ucdavis.edu/).

2.6. Statistical Analyses

The complex network was created based on only significant ($p < 0.05$) correlations [24,29] between CV and erythrocyte lipids, regardless of the correlation coefficient. In the network, each variable that was associated with another was represented as a node, and the associations between the variables' edges connecting these nodes represented connections between nodes. By selecting CV as a target inside the topology, weighted and targeted complex networks were developed. These methods gave positive weights to both positive and inverse correlations equally, regardless of the correlation's direction.

In the eigenvector approach, the centrality of a node is calculated using the target techniques based on the centrality of its neighbors and the weights of its edge connections. The edge weights were determined by multiplying Pearson's correlation coefficient between the nodes connected by the edge (which can vary from 0.01 to 1; higher means closer) by the edge's degree of closeness to the target node CV (which can vary from 0.01 to 1; higher is better). The centrality eigenvector values were acquired using a Python (version 3.9.3) application created specifically for the study and the NetworkX 2.5 package [30]. The Shapiro–Wilk test confirmed the data normality, and the Pearson approach was used for the correlation analysis. Data are expressed as mean ± standard deviation.

To select features based on the eigenvector approach, a threshold of ≥0.0001 was adopted. The principal component analysis (PCA) and enrichment analysis were performed using MetaboAnalyst 5.0 software. The relative standard deviation (RSD) was calculated for the intra-batch QC group, and those features found with an RSD < 30% were not considered for statistical analysis. The dataset was sum-normalized, log-transformed, and scaled by pareto. The false discovery rate (FDR) was applied to control the rate of false positive findings, considering $p < 0.05$. The impact in enrichment analysis refers to a quantitative measure that assesses the biological relevance or importance of enriched pathways.

3. Results

Performances on the 100, 200, 400, and 800 m were obtained at 73 ± 9 s, 170 ± 16 s, 354 ± 43 s, and 747 ± 84 s, respectively. Consequently, the CV was calculated at 1.05 ± 0.11 m/s, with a high R2 (0.999 ± 0.001). Parallel to the mass spectrometry analysis, the red blood cell profile was determined (red blood cell count = 4.83 ± 0.37 10^6/μL; hemoglobin = 13.8 ± 1.01 g/dL; hematocrit = 42.8 ± 2.95%; mean corpuscular volume = 88.6 ± 3.40 fl; mean corpuscular hemoglobin = 28.7 ± 1.04 pg; mean corpuscular hemoglobin concentration = 32.3 ± 0.5 10^6/μL; red blood cell distribution = 13.4 ± 0.4%).

LC-MS raw data were processed, and 2823 signals were detected and filtered by RSD for the QC group. The analytical quality and the LC-MS reproducibility can be observed by the clustering of the QC group in PCA (Supplementary Figure S1). The selection of features was based on eigenvector value (≥0.0001), with 266 indicated as the differential, identified (n = 119), and grouped by chemical subclass.

Table 1 presents the total of erythrocyte lipids detected, identified, and relevant for the CV of adolescent swimmers. Lipids from the glycerophospholipids and sphingolipids classes comprised 81.5% of the identified ones. The complete list of lipids, main classes, sub-classes, fragments, and the respective eigenvector values can be visualized in Supplementary Table S1.

Table 1. Lipids that were detected and identified in RBCs and also relevant (i.e., eigenvector) for the aerobic performance of adolescent swimmers.

	n	Eigenvector Range (A.U)
Total Features with RSD < 30%	2146	0–0.088345
Features selected by eigenvector	266	≥0.0001
Lipids Identified	119	0.000102–0.088345
Glycerophospholipids	65	0.000102–0.085850
Sphingolipids	32	0.000197–0.088324
Fatty Acyls	6	0.000104–0.088345
Neutral glycosphingolipids	3	0.000174–0.068406
Glycerolipids	2	0.001768–0.074106
Others classes *	11	0.000112–0.079785

A.U—arbitrary units; * Others include carboxylic acids and derivatives, organooxygen compounds, lactones, phenols, pyrans, pyridines and derivatives, and dihydrofurans.

Before proceeding with further analysis on the lipids relevant for aerobic performance, a comparison regarding the identified molecules between male and female athletes was performed. The PCA analysis showed that the data variability was not sufficient to discrim-

inate between male and female adolescent swimmers by the lipids (Figure 2). Based on this result, the enrichment and the last analysis considered the total sample regardless of sex.

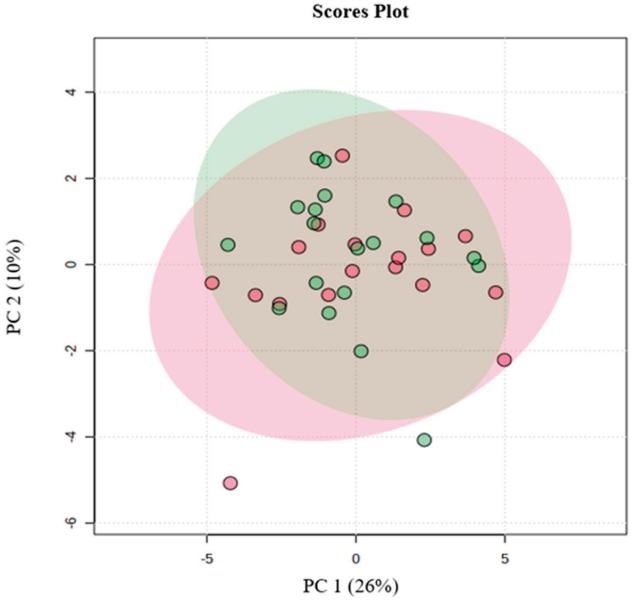
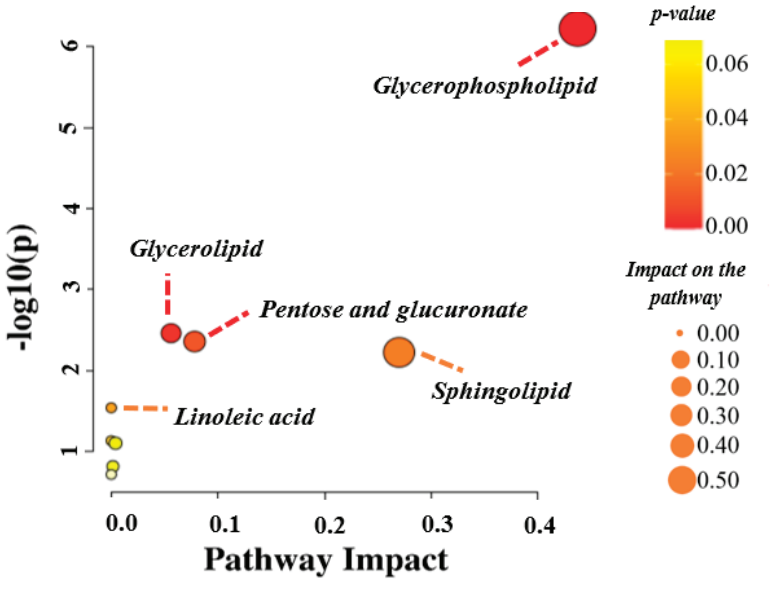


Figure 2. The principal component analysis (PCA) between male and female adolescent swimmers regarding the lipids identified in lipidomic analysis that were relevant for aerobic performance; red—female; green—male.

The enrichment pathway analysis was performed with all metabolites (n = 119), which indicated intermediary lipids in the glycerophospholipid, glycerolipid, sphingolipid, linoleic acid, and alpha-linolenic metabolisms, as well as pentose and glucuronate interconversions (Figure 3). A significant impact of the intermediary lipids was observed for the glycerophospholipid metabolism, including phosphatidylethanolamines (PEs), phosphatidylcholines (PCs), 1-acyl-sn-glycero-3-phosphocholine, sn-glycerol 3-phosphate, and phosphatidic acids. While the latter two classes of lipids also have intermediate glycerolipid metabolisms, sphingomyelin (SM) and ceramides (CERs) were found to be hits of the sphingolipid metabolism. In addition to glycerophospholipid metabolism, PC also has intermediate linoleic acid metabolism, which was highlighted in this pathway. Lastly, D-xylose and D-xylonolactone were the hits in pentose and glucuronate interconversions. The entire glycerophospholipid metabolism is presented in Supplementary Figure S2.

(A)



(B)

Metabolisms	Total	Hits	Raw *p*	FDR	Impact
Glycerophospholipid	36	5	0.00000	0.0005	0.43711
Glycerolipid	16	2	0.00345	0.12241	0.05607
Pentose and glucuronate	18	2	0.00437	0.12241	0.07812
Sphingolipid	21	2	0.00594	0.12487	0.26978
Linoleic acid	5	1	0.02873	0.48273	0
Alpha-linolenic acid	13	1	0.07318	0.94332	0
Glycosylphosphatidylinositol	14	1	0.07861	0.94332	0
Phosphatidylinositol signaling system	28	1	0.15168	1	0
Arachidonic acid metabolism	36	1	0.19108	1	0

Figure 3. The enrichment pathway analysis (**A**) is based on the erythrocyte lipids of adolescent swimmers and the statistics of each highlighted metabolism (**B**); FDR—false discovery rate; $p < 0.05$.

The lipids highlighted in the glycerophospholipid metabolism pathway were correlated with the CV (Figure 4). Inverse and significant associations were observed for PE 18:2/18:3 (Figure 4A), PC 18:3/20:0 (Figure 4B), and phosphatidic acid 18:0/0:0 (Figure 4E) with the CV.

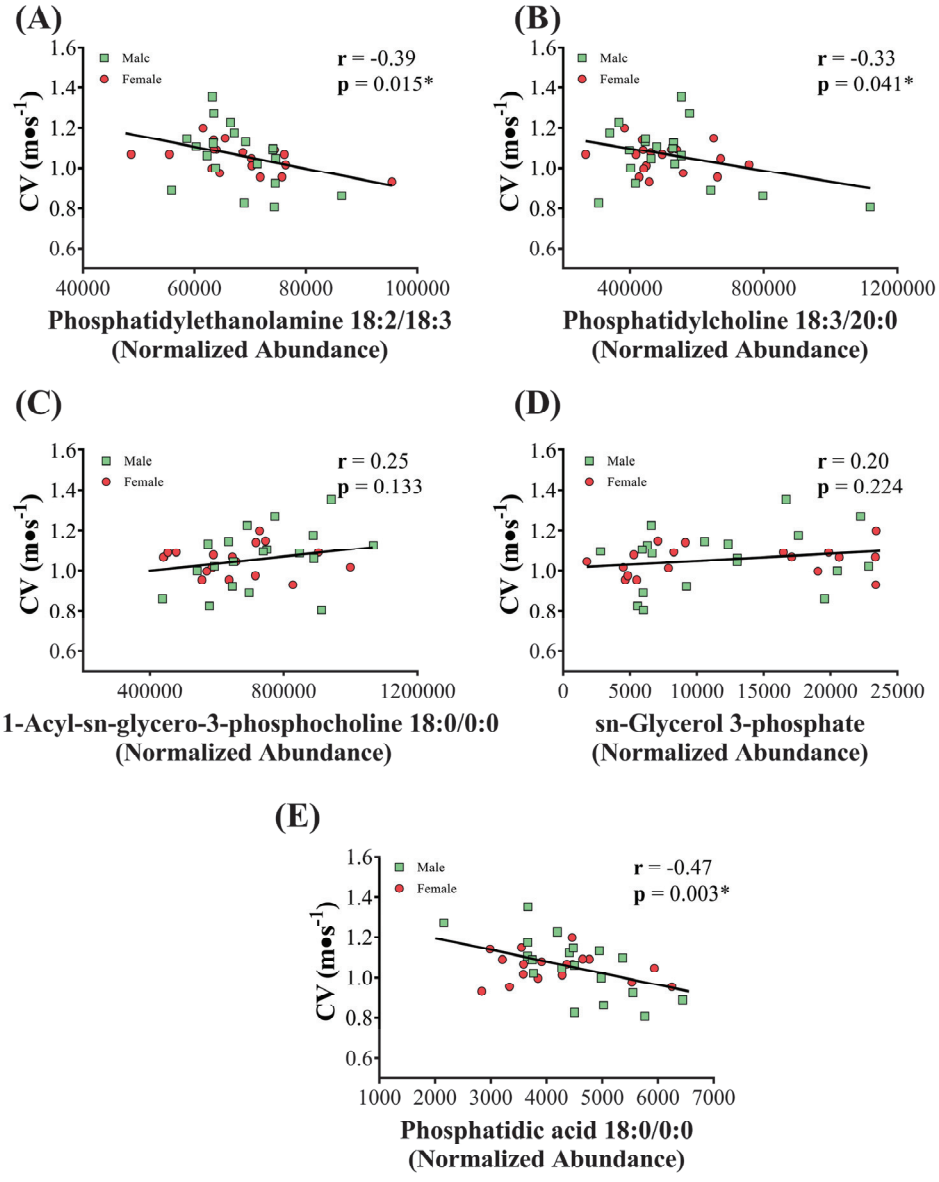


Figure 4. Correlation between the RBC lipids highlighted in the enrichment analysis for the glycerophospholipid metabolism pathway with the critical velocity (CV) of adolescent swimmers. (**A**) correlation between phosphatidylethanolamine 18:2/18:3 with CV; (**B**) correlation between phosphatidylcholine 18:3/20:0 with CV; (**C**) correlation between 1-acyl-sn-glycero-3-phosphocholine 18:0/0:0 with CV; (**D**) correlation between sn-glycerol 3-phosphate with CV; (**E**) correlation between phosphatidic acid 18:0/0:0 with CV. Red—female; green—male; * $p < 0.05$.

4. Discussion

The lipidomics approach, along with the targeted network analysis, revealed 119 RBC metabolites associated with the CV of adolescent swimmers. The differential abundance of lipids resulted in a significant impact on glycerophospholipid metabolism. Among the glyc-

erophospholipids, compounds representing subclasses such as phosphatidylethanolamine, phosphatidylcholine, and phosphatidic acid were inversely correlated with critical velocity. The data presented here can support the view that these glycerophospholipids are downregulated in athletes with high aerobic performance.

In our enrichment pathway analysis, we observed a marked relevance of glycerophospholipid metabolism (Figure 3A). Since glycerophospholipids are a class of lipids that constitute a major component of cell membranes [31], they are expected to have an important role for RBCs. Our finding, together with those of others reported in the literature [14,16,32], reinforces the idea that glycerophospholipids are inexorably linked to the structure of RBCs. The reason why the metabolism of glycerophospholipid was highlighted in the RBCs of swimmers is an interesting finding and deserves further consideration. An enhanced use of fat metabolism can be responsible for the prominence of glycerophospholipid metabolism. Thus, we hypothesize that aerobic activities included in the scope of swimming training may play a role in stimulating fat-oxidative pathways. The results in the present study are not sufficient to demonstrate a causal effect of aerobic training on glycerophospholipids, but it would not be surprising to find an increased flux of lipids in the circulation of endurance-trained swimmers. Given that RBCs are incapable of performing biosynthesis due to the lack of organelles, it is opportune to mention that the membrane lipid composition of RBCs depends on the exchange with the plasma lipids [12–14]. In support of this, hemorheological changes have been demonstrated in rabbits with hypercholesterolemia [33]. Other researchers have proved that RBCs are sensitive to plasma lipids [34–36].

With regard to the correlations, we found a significant inverse relationship between CV and three metabolites of glycerophospholipid metabolism. Swimmers who exhibited higher levels of CV (a measure related to aerobic performance) also had the lowest abundance of phosphatidylethanolamine at 18:2/18:3 ($r = -0.39$; $p = 0.015$), phosphatidylcholine at 18:3/20:0 ($r = -0.33$; $p = 0.041$), and phosphatidic acid at 18:0/0:0 ($r = -0.47$; $p = 0.003$). At the moment, we have no conditions to elucidate these relationships, but it appears appropriate to speculate that changes in RBC glycerophospholipids may be indirectly linked with physical fitness. Phosphatidylethanolamine is a key regulator that we can use as an example. There are reasons to believe that a high amount of phosphatidylethanolamine is indicative of poor health. Supporting this, increased phosphatidylethanolamine levels have been found in non-alcoholic steatohepatitis patients [37]. An increased phosphatidylethanolamine content of platelets has also been found in patients with poorly controlled diabetes [38], and increased phosphatidylethanolamine has been described in cancer [39] and hypertension [40].

There is a growing body of evidence that supports the idea that phosphatidylethanolamines can be modified by glycation, oxidation, and other chemical processes [41–45]. With this in mind, it is reasonable to expect harmful changes in membrane thickness and rigidity, and thus RBC locomotion could be seriously compromised [46,47]. This agrees with previous findings confirming that acute exercise decreases RBC deformability [48–51]. In contrast to acute exercise-related alterations in RBCs, exercise training is known to improve hemorheological parameters [50]. Although we have no measurements of hemorheological parameters (e.g., whole-blood viscosity, RBC aggregation, RBC deformability), we believe that endurance-trained swimmers have enhanced blood flow capacity (likely facilitating oxygen diffusion and tissue repair during rest moments). This, in turn, could be achieved with highly deformable RBCs, which would have conditions to pass (due to reduced membrane stiffness) through the smallest capillaries. All these findings are intriguing and emphasize that the RBC has a complex role, which further depends on the condition in which these cells were harvested, such as exercise or rest (which was our case). Future in-depth studies will need to be conducted to understand mechanisms involving aerobic capacity and RBC glycerophospholipids, comparing both rest and exercise conditions from an integrated perspective. Some of the main findings are sketched in Figure 5.

There are some studies employing metabolomics in the context of swimming [52–54]. Researchers have characterized the serum metabolic profile of swimmers to identify fitness

levels or predict competitive potential. Cai et al. [55] discriminated swimmers with different competitive levels using high-density lipoprotein, glutamine, methanol, and α-glucose. On the other hand, plasma tyrosine was inversely associated with aerobic performance [56]. Although the presented knowledge has contributed to the characterization of different metabolites present in the plasma and serum of swimmers, there is a scarcity of studies exploring RBCs. The novelty of our study was to analyze the RBC lipidome in athletes under rest conditions. The researchers employing lipidomics in RBCs have focused on the metabolic changes that occur after an exercise bout [15,16]. Knowing all the benefits that RBCs can bring to the aerobic physical performance of swimmers and the scientific potential of the lipidomic approach, it becomes of great value to characterize the lipid molecules present in the RBCs of young swimming athletes.

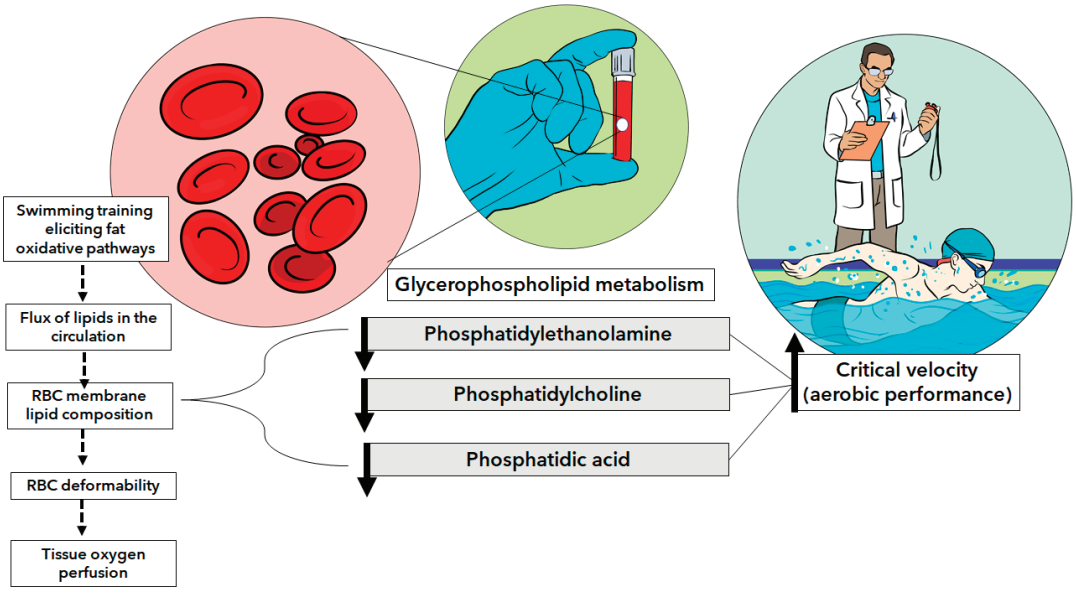


Figure 5. The main findings and possible interpretations (dashed arrows) of the study highlighting the glycerophospholipids subclasses in red blood cells that were inversely correlated with critical velocity (a measure of aerobic performance).

Our study has some limitations. We did not examine whether athletes would have a different RBC lipidome under exercise or diet situations. Although linked to aerobic exercise performance, we did not analyze the shape of RBCs by histological assays. Given that RBC membrane fluidity and lipid composition may be affected by nutrition factors [57–62], it is imperative to account for this factor in a further experimental design. Therefore, more studies are needed to get a better understanding of the impact of athletes' diets on the RBC membrane lipidome. At the moment, we do not have enough information on food ingested by swimmers, despite the fasting state of athletes. Despite limitations, our study is a first step in exploring RBC lipidomics in adolescent swimmers. To our knowledge, our study is the first to demonstrate a connection between some glycerophospholipids in RBCs and aerobic exercise performance (this knowledge could be useful in the fields of medicine and biology). Another approach to be highlighted is the use of critical velocity protocol, which can access aerobic capacity without the necessity of blood collection. Still, we investigated whether the strength and direction of the association were dependent on sex. The PCA analysis found no difference between male and female adolescent swimmers by the lipids, and the dispersion was homogeneous without any agglomeration for females (red circle) and males (green square). This supports the possibility that other factors beyond gender

are responsible for the changes in RBC glycerophospholipids in athletes. We believe that glycerophospholipids in RBCs are an important link between aerobic exercise performance and the regulation of oxygen-carrying capacity. Considering that RBCs regulate several processes that are pivotal in physiology, our findings provide new insights and bring us much closer to understanding RBC adaptations in athletes with different levels of aerobic capacity. The next step will be to explore whether adaptations resulting from aerobic training could be attributed to changes in the RBC lipidome.

5. Conclusions

A significant impact of the intermediary lipids was observed for glycerophospholipid metabolism in the red blood cells of young swimmers. Among these lipids, phosphatidylethanolamine 18:2/18:3, phosphatidylcholine 18:3/20:0, and phosphatidic acid 18:0/0:0 were inversely correlated with CV, suggesting that these are downregulated in athletes with high aerobic performance.

Supplementary Materials: The following supporting information can be downloaded at: https://www.mdpi.com/article/10.3390/nu16060765/s1, Figure S1: Principal Component Analysis (PCA) results. PCA using all metabolites detected. The red point represents the QC Samples, and the green point represents Swimmers Samples; Figure S2: The glycerophospholipid metabolism pathway and the lipids (i.e., red) that were identified in erythrocytes of young swimmers; Table S1: Compounds identified (n = 119) for red blood cell lipidomics.

Author Contributions: Conceptualization, Á.A.R.S., V.B., A.M.P. and L.H.D.M.; data curation, Á.A.R.S., I.G.M.D.R. and L.H.D.M.; formal analysis, Á.A.R.S., I.G.M.D.R. and L.H.D.M.; funding acquisition, Á.A.R.S., A.M.P. and L.H.D.M.; Investigation, Á.A.R.S., P.P.M.S. and L.H.D.M.; Methodology, Á.A.R.S., F.M.M.M., D.C.d.O., C.D.P., A.M.P. and L.H.D.M.; project administration, L.H.D.M.; resources, A.M.P. and L.H.D.M.; software, Á.A.R.S., I.G.M.D.R. and L.H.D.M.; supervision, L.H.D.M.; validation, A.M.P. and L.H.D.M.; visualization, Á.A.R.S., P.P.M.S., J.P.d.C., A.M.P. and L.H.D.M.; writing—original draft, Á.A.R.S., V.B., P.P.M.S., J.P.d.C. and L.H.D.M.; writing—review and editing, Á.A.R.S., V.B., P.P.M.S., A.M.P. and L.H.D.M. All authors have read and agreed to the published version of the manuscript.

Funding: This study was financed, in part, by the Coordenação de Aperfeiçoamento de Pessoal de Nível Superior—Brasil (CAPES)—Finance Code 001 (AMP and AARS—88887.504805/2020-00). We would like to thank the National Council for Scientific and Technological Development—CNPq (LHDM—408680/2021-0) for financial support.

Institutional Review Board Statement: The study was conducted in accordance with the Declaration of Helsinki, and approved by the Ethics Committee of the São Francisco University (protocol code 24892219.3.0000.5514 and 22 November 2019).

Informed Consent Statement: Informed consent was obtained from all subjects and their legal parents involved in the study.

Data Availability Statement: Data are contained within the article and Supplementary Materials.

Acknowledgments: We thank the athletes, their legal parents, and the coaches of the swimming team.

Conflicts of Interest: The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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Article

Eating Behavior Disorders and Disordered Eating Habits in Spanish High-Performance Women's Olympic Wrestling Athletes

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Abstract: Eating disorders (EDs) are a significant health issue in combat sports. This study investigated the differences between the different types of female wrestlers and the frequency at which EDs occur in the elite population, and it also sought to establish which factors are predictors of EDs. This study was comprised of 22 elite, female wrestlers who were selected based on the following inclusion criteria: having previously been the Spanish champion, being part of the Spanish national team, participating in at least one international championship, and having a history of ED. Data collection involved five questionnaires: demographic data, the Eating Attitudes Test-26 (EAT-26), the Bulimic Investigatory Test, the Edinburgh (BITE), the Eating Disorders Inventory (EDI-3), and the Depression, Anxiety, and Stress Scale (DASS-21). The results revealed diverse levels of depression, anxiety, and stress, with BITE scores indicating abnormal eating patterns. Group comparisons exposed significant distinctions in eating behaviors based on competition and training experience. Regression analyses showed competition and training experience as predictors of bulimia severity and symptoms. The study revealed prevalent extreme weight-control practices, including fasting, diuretic and laxative use, and binge eating. This research emphasizes the importance of EDs in Olympic wrestling, urging a comprehensive approach involving education, support, and policy implementation by coaches, health professionals, and sports organizations to prioritize athletes' well-being and discourage unhealthy weight-control practices.

Keywords: female wrestling; eating disorder; nutritional habits; sports nutrition

Citation: Flores, M.R.; Martín-Castellanos, A.; López-Torres, O.; Fernández-Elías, V.E.; García-González, J.; Mon-López, D. Eating Behavior Disorders and Disordered Eating Habits in Spanish High-Performance Women's Olympic Wrestling Athletes. *Nutrients* **2024**, *16*, 709. https://doi.org/10.3390/nu16050709

Academic Editors: Louise Deldicque and Peter WR Lemon

Received: 12 January 2024
Revised: 19 February 2024
Accepted: 23 February 2024
Published: 29 February 2024

1. Introduction

Olympic wrestling, which dates back to 708 BC «International Olympic Committee» [1], requires a unique blend of strength, endurance, agility, and technical skill. Wrestlers are classified by body mass to ensure a fair competition in terms of size, strength, and agility [2]; however, as with many weight-controlled sports, concerns about eating disorders (EDs) loom large, as athletes often undergo drastic measures to gain a competitive edge by competing in lighter weight classes [3,4].

Despite the documented health risks associated with rapid weight loss, aggressive weight-loss methods are prevalent in combat sports, particularly in Olympic wrestling [5]. Wrestlers resort to extreme practices such as severe dietary restrictions and deliberate

dehydration to meet the weight requirements prior to competition [6]. In addition, weight-category sports have been identified as susceptible to EDs [7].

Clinical EDs include anorexia nervosa, bulimia nervosa, binge eating disorder, and other specified and unspecified EDs [8] and require medical and psychological interventions [9]. While both genders are affected, female athletes are at a higher risk [10], although gender parity in ED cases is increasing nowadays [5].

Awareness of the risks of EDs among coaches, health professionals, and wrestlers themselves is critical [11]. Stress, anxiety, depression, and body-image issues are notable concerns for combat athletes, especially for females [12,13]. These factors often predict EDs and unhealthy eating behaviors, which are enhanced by body dissatisfaction and low self-esteem [14,15], resulting from pressure to maintain weight and meet performance expectations [16].

In addition, chronic stress impairs the immune system and increases the risk of physical injury [17], which can result in difficult recoveries and can impair the performance of Olympic wrestlers under intense competitive pressure [18]. Moreover, anxiety manifests as nervousness, excessive worry, and panic attacks, which can also impair performance and recovery [19,20]. These psychological factors, along with the pressure to maintain weight and meet performance expectations, could increase the risk of EDs among wrestlers.

Furthermore, depression decreases motivation, interest in the sport, and adherence to training regimens, which can put athletic performance at risk [21,22]. Age, experience, and weight control significantly influence the development of EDs; thus, younger, less-experienced athletes face weight-category pressures and lack nutritional knowledge [23], while experienced athletes may adopt unhealthy practices that promote dysfunctional eating patterns [24,25].

Thus, addressing these concerns comprehensively through medical, psychological, and emotional support, as well as stress management education, is imperative [11]. Specific research focusing on these issues in female Olympic wrestlers seems to be critical. Hence, the objective of this study was to determine the differences between different categories of female wrestlers, including weight, age, and experience, among others, and the frequencies in which EDs occur in the elite population. In addition, it sought to establish which factors were predictors of EDs.

2. Materials and Methods

2.1. Participants

Thirty elite, female, Spanish wrestlers were contacted through the national coach. Of the total number of athletes, two declined the invitation and six wrestlers were discarded in accordance with the inclusion/exclusion criteria. The two following inclusion criteria were used [26] to identify subjects who could provide relevant information: (1) elite wrestlers who have been the champion of Spain in any age category and have been part of the Spanish national team, having participated in at least one international championship, and (2) wrestlers suffering from or having suffered from EDs. The final sample was 22 female wrestlers with a mean age of 20.82 $\pm$ 2.79 years old. The recruitment process is shown in Figure 1.

All participants received an oral explanation of the study's purpose and signed an informed consent form. The participation was strictly confidential and voluntary. The protocol for this study was approved by the Ethics Committee of the Universidad Politécnica de Madrid (FDRED00000-DML-DATOS-20230609).

2.2. Demographic Data

A questionnaire based on previous similar studies was used to record demographic information, including age, height, competition age category, competition weight category, approximate current weight and age at the start of training, years of experience competing, hours of training per week, placement in Spanish Championships, participation in international competitions, international medals, and educational level.

Regarding the questionnaires, the Eating Attitudes Test (EAT-26), the Bulimic Investigatory Test, the Edinburgh (BITE), the Eating Disorders Inventory-3 (EDI-3), and the Depression, Anxiety, and Stress Scale (DASS-21) were used in this study in their previously validated versions.

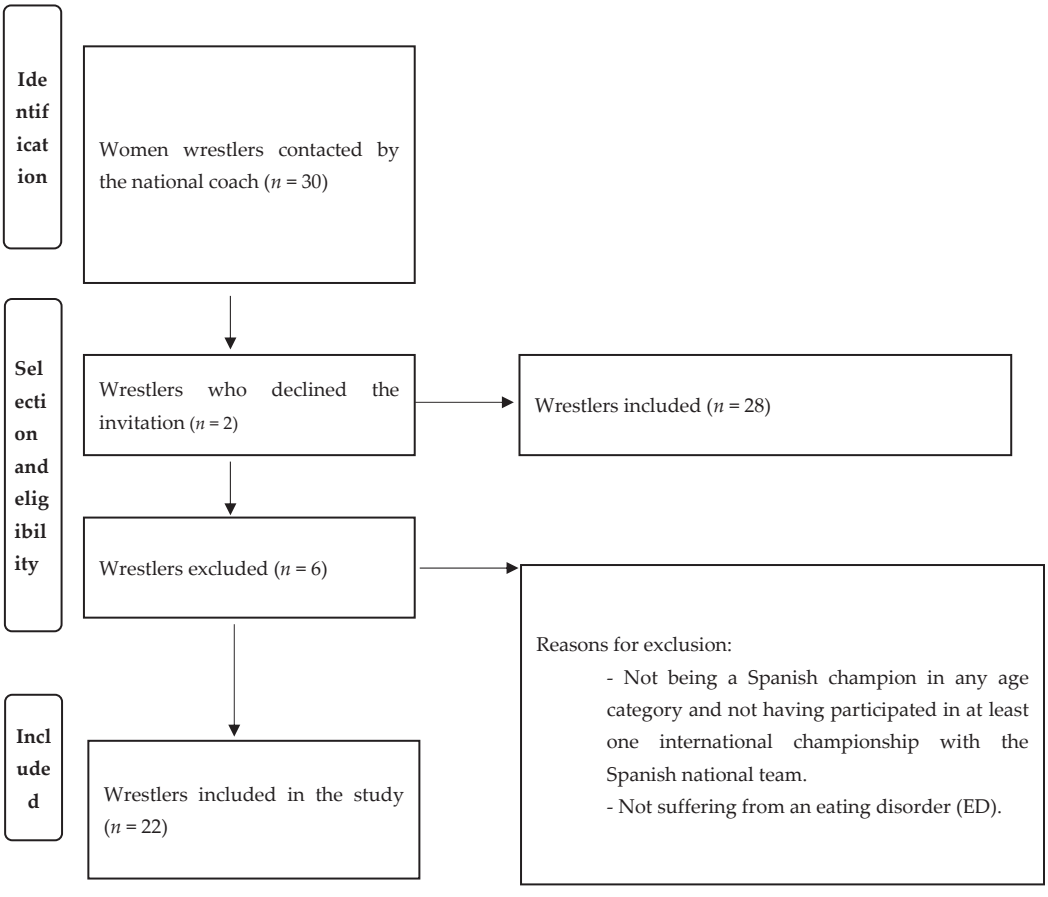

Figure 1. Recruitment process.

2.2.1. Eating Attitudes Test (EAT-26)

The EAT-26 questionnaire [27] was used in this study. The EAT-26 has four response options ranging from 0 to 3 (3 = always, 2 = almost always, 1 = often, and 0 = rarely, almost never, or never). These items were divided into three subscales, each corresponding to a facet of eating behavior: (a) dieting, which refers to a pathological refusal to consume high-calorie foods and a preoccupation with physical appearance; (b) bulimia and attention to food, which refers to episodes of binge eating followed by purging behaviors for the purpose of weight loss or weight control; and (c) oral self-control, which reflects self-control with respect to food and assesses the environmental and social forces that stimulate food intake. The total score of the EAT-26 was the sum of the 26 items. Scores above 20 points indicated an eating disorder risk (EAT+). In addition, the SCOFF questionnaire (answering a single question in the affirmative is sufficient to include the participant in the risk group of EDs) was used.

2.2.2. Bulimic Investigatory Test, Edinburgh (BITE)

The BITE was used to identify subjects with binge eating and compensatory behaviors, providing information on cognitive and behavioral aspects of bulimia nervosa [28]. The scores are classified into two subscales: symptoms and severity. On the symptom scale, a score of 20 or more was indicative of bulimia nervosa; 10 to 19 suggests an unusual eating pattern, and less than 10 was within normal limits. On the severity scale, a score of 5 or more was considered clinically significant, and 10 or more indicates a high degree of severity.

2.2.3. Eating Disorders Inventory-3 (EDI-3)

The EDI-3 [29], which is composed of 91 items distributed into 12 main scales, was used for the assessment of EDs. The following were the specific risk scales for the manifestation of eating pathology: thinness obsession (an obsession with having a thin body, a preoccupation with eating, and an intense fear of gaining weight), bulimia (the presence of binge eating and/or compensatory behaviors), and body dissatisfaction (dissatisfaction with the general shape of the body, as well as the rejection of specific areas). The rest of the scales were general psychological scales linked to EDs: low self-esteem (negative self-perception), personal alienation (feelings of emotional emptiness, loneliness, lack of control, and incomprehension), interpersonal insecurity (apprehension about manifesting one's feelings and thoughts), interpersonal mistrust (feelings of detachment and a propensity to feel trapped in relationships), interoceptive deficits (difficulty interpreting and responding to one's own and others' emotional states), emotional maladjustment (emotional instability, impulsivity, and self-destructive behaviors), asceticism (obsessive behaviors of self-discipline, restraint, and self-sacrifice), perfectionism (self or external demand to achieve excessively high goals and objectives), and fear of maturity (insecurity about maturity and a desire to return to childhood). The questionnaire items were answered on a 6-point Likert-type scale (from 1 = never to 6 = always), and the purpose of the questionnaire was not to provide an ED diagnosis but to provide a measure of psychological traits and symptoms common to EDs.

2.2.4. Depression, Anxiety, and Stress Scale (DASS-21)

The DASS-21 was developed to assess markers of distress, including stress, anxiety, and depression [30]. For this study, the Spanish version of the scale was used [31], in which the total number of items was reduced to 21 (7 items per factor: depression, anxiety, and stress). The response scale for all items ranged from 0 (does not apply to me) to 3 (applies to me most of the time).

2.3. Data Collection

Data collection was carried out during a national training camp at the Centro Gallego de Tecnificación Deportiva in Pontevedra, Spain, in October 2022, using a structured, self-administered instrument with questions on demographic and socioeconomic data. Each day of the week before the first training, only one questionnaire (EAT-26, EDI-3, BITE, or DASS-21) was administered with a stipulated time to complete it. All measurement instruments were presented in booklet form.

2.4. Data Analysis

Participants' data was described using (*M*) and (*SD*). The normality of distribution was tested using the Shapiro–Wilk test, and the homogeneity of variances was tested using Levene's test, both ($p > 0.05$). Participants were divided into two equal halves using the median, and a Student's *t*-test was used to determine the differences in EDs based on age, weight, years of training, and competition experience. The effect size was reported using Cohen's d (small effect [$d = 0.2–0.5$], medium effect [$d = 0.5–0.8$], and large effect [$d > 0.8$]). Cronbach's Alpha coefficient was calculated to evaluate the reliability of each questionnaire scale and subscales. Finally, linear regression was performed to determine which variables predict the occurrence of EDs. Statistical tests were conducted using IBM SPSS Statistics version 29 for Windows, and the level of significance was set at $p < 0.05$.

3. Results

3.1. Consistency

The reliability of all questionnaires and their subscales was tested. Cronbach's Alpha coefficient values are shown in Table 1.

Table 1. Results for each scale and subscale with their level of internal consistency.

Factor	*n*	Items	Min	Max	M	SD	Cronbach's Alpha
BULIMIA							
Symptoms	22	30	3.00	23.00	12.05	6.09	0.883
DASS	22	21	4.00	47.00	17.91	10.56	0.892
Depression	22	7	0.00	14.00	4.82	4.22	0.846
Anxiety	22	7	0.00	15.00	4.86	3.81	0.734
Stress	22	7	2.00	18.00	8.23	4.41	0.776
EAT-26	22	26	11.00	36.00	22.50	8.42	0.912
Dieting	22	13	4.00	23.00	12.88	6.73	0.920
Bulimia	22	6	3.00	9.00	6.13	2.70	0.556
Oral control	22	7	2.00	6.00	3.50	1.51	0.516
EDI-3							
Eating Disorder Risk Scales							
Drive for thinness	22	7	2.00	34.00	18.14	10.77	0.948
Bulimia	22	8	0.00	25.00	12.41	7.04	0.861
Body dissatisfaction	22	10	2.00	47.00	18.73	12.49	0.900
Psychological scales							
Low self-esteem	22	6	0.00	26.00	10.00	7.65	0.939
Personal alienation	22	7	1.00	32.00	10.77	7.23	0.861
Interpersonal insecurity	22	7	5.00	22.00	14.82	4.87	0.480
Interpersonal alienation	22	7	7.00	23.00	12.45	4.34	0.561
Interoceptive deficits	22	9	5.00	34.00	16.23	8.56	0.853
Emotional dysregulation	22	8	2.00	30.00	11.55	6.22	0.773
Perfectionism	22	6	7.00	28.00	17.27	6.16	0.653
Asceticism	22	7	1.00	25.00	12.14	5.88	0.619
Maturity fears	22	8	0.00	32.00	18.18	7.72	0.800
Composites							
EDRC	22	25	10.00	95.00	49.27	25.52	0.940
IC	22	13	1.00	58.00	20.77	14.33	0.944
IPC	22	14	14.00	41.00	27.27	8.10	0.688
APC	22	17	7.00	64.00	27.77	13.70	0.891
OC	22	13	17.00	48.00	30.32	8.81	0.727
GMPC	22	91	76.00	248.00	161.14	51.92	0.947

Note. EDRC: Eating Disorder Risk Composite; IC: Ineffectiveness Composite; IPC: Interpersonal Problems Composite; APC: Affective Problems Composite; OC: Over-control Composite; GPMC: General Psychological Maladjustment Composite.

3.2. Wrestlers' Behaviors

The results showed that, on average, the participant's level of depression ($M = 4.82 \pm 4.22$) did not reach the cut-off point established by the DASS-21 for mild depression ($M > 5$). The participants' level of anxiety ($M = 4.86 \pm 3.81$) met the DASS-21 cut-off point for mild anxiety ($M > 4$). The participants' stress level ($M = 8.23 \pm 4.41$) reached the cut-off point marked by the DASS-21 for mild stress ($M > 8$). The frequency distribution of the wrestlers' behavior is shown in Table 2.

Regarding the BITE questionnaire, the descriptive data of the symptoms scale were ($M = 7.67 \pm 3.39$). Scores of 5–10 were not considered an ED, although they were above what is considered normal. On the other hand, the scores in terms of severity were ($M = 12.36 \pm 6.45$), with values between 10–20 being considered abnormal. In addition, the frequency distribution shows that 50% of the wrestlers have fasted a whole day, 40% of the wrestlers have used diuretics, 33.3% have used laxatives, 10% have made themselves vomit to lose weight, and 50% of the wrestlers binge eat when they are alone, with 10% of them doing this behavior at least two or three times per week.

In addition, the EAT-26 showed values of dieting ($M = 12.88 \pm 6.73$), bulimia ($M = 6.13 \pm 2.70$), and oral control ($M = 3.50 \pm 1.51$), with 40.91% of the total participants having a possible ED risk (EAT+). Lastly, the EDI-3 mean descriptive data were driven for thinness

(*M* = 18.14 ± 10.77), bulimia (*M* = 12.41 ± 7.04), and body dissatisfaction (*M* = 18.73 ± 12.49), with values over 20 points at frequencies of 46%, 22%, and 33.8%, respectively.

Table 2. Frequency distribution of the depression, anxiety, and stress categories.

	Depression		Anxiety		Stress	
	n	**%**	***n***	**%**	***n***	**%**
No symptoms	11	50	9	40.9	10	45.5
Mild	6	27.3	5	22.7	3	13.7
Moderate	2	9.1	3	13.7	6	27.3
Severe	2	9.1	1	4.5	2	9.1
Extremely severe	1	4.5	4	18.2	1	4.5
TOTALS	22	100	22	100	22	100

Notes: *n* = number of participants, % = percentage of affected wrestlers.

3.3. Differences between the Groups—Group Comparison

Differences and effect sizes between the two groups according to the wrestlers' age, competition experience, training experience, and weight were analyzed. No significant differences were found between the groups of younger and older wrestlers ($p > 0.05$) (Table 3). On the other hand, there were significant differences between wrestlers with greater and lesser years of competitive experience (Table 4). Those with more experience scored higher values in dieting ($p = 0.038$; $d = 0.95$), oral control ($p = 0.040$; $d = 0.95$), asceticism ($p = 0.018$; $d = 1.10$), and maturity fears ($p = 0.020$; $d = 1.08$). Additionally, significant differences were observed between wrestlers with different levels of training experience (Table 5). Thus, athletes with more training experience reported higher levels of bulimia ($p = 0.028$; $d = 1.01$) and over-control ($p = 0.047$; $d = 0.90$). Lastly, there were no significant differences among heavier and lighter wrestlers ($p > 0.05$) (Table 6).

Table 3. Results according to the age of the wrestlers (median = 21).

Factor	**Older** [a]		**Younger** [b]				**95% CI**		
	M	**SD**	**M**	**SD**	**t(20)**	***p***	**LL**	**UL**	**d**
BULIMIA									
Symptoms	13.27	6.44	10.82	5.76	0.94	0.357	−2.98	7.89	0.40
DASS	19.82	12.42	16.00	8.46	0.84	0.410	−5.64	13.27	0.36
Depression	6.09	4.41	3.55	3.78	1.45	0.162	−1.11	6.20	0.62
Anxiety	4.18	4.40	5.55	3.17	−0.83	0.414	−4.78	2.05	−0.36
Stress	9.55	5.43	6.91	2.74	1.44	0.171	−1.28	6.55	0.61
EAT-26	23.27	14.70	18.09	11.63	0.92	0.370	−6.61	16.97	0.39
Dieting	13.73	10.15	10.27	7.84	0.89	0.382	−4.61	11.52	0.38
Bulimia	5.82	3.46	4.82	2.52	0.77	0.448	−1.69	3.69	0.33
Oral control	3.73	3.04	3.00	3.69	0.50	0.619	−2.28	3.73	0.22
EDI-3									
Eating Disorder Risk Scales									
Drive for thinness	19.27	9.94	17.00	11.91	0.49	0.632	−7.48	12.03	0.21
Bulimia	12.91	5.79	11.91	8.37	0.33	0.748	−5.40	7.40	0.14
Body dissatisfaction	16.64	12.70	20.82	12.53	−0.78	0.446	−15.40	7.04	−0.33
Psychological scales									
Low self-esteem	9.91	8.07	10.09	7.61	−0.05	0.957	−7.16	6.79	−0.02
Personal alienation	11.36	8.23	10.18	6.43	0.38	0.711	−5.39	7.75	0.16
Interpersonal insecurity	13.73	3.07	15.91	6.14	−1.05	0.309	−6.60	2.24	−0.45
Interpersonal alienation	11.91	5.05	13.00	3.66	−0.58	0.568	−5.01	2.83	−0.25
Interoceptive deficits	15.00	8.53	17.45	8.82	−0.66	0.515	−10.17	5.27	−0.28
Emotional dysregulation	11.64	7.15	11.45	5.48	0.07	0.947	−5.48	5.85	0.03
Perfectionism	18.00	7.32	16.55	4.99	0.54	0.592	−4.12	7.03	0.23
Asceticism	13.64	6.14	10.64	5.48	1.21	0.241	−2.18	8.18	0.52
Maturity fears	15.73	7.96	20.64	6.96	−1.54	0.139	−11.56	1.74	−0.66

Table 3. *Cont.*

Factor	Older [a]		Younger [b]				95% CI		
	M	SD	M	SD	t(20)	*p*	LL	UL	d
Composites									
EDRC	48.82	24.12	49.73	28.03	−0.08	0.936	−24.17	22.35	−0.04
IC	21.27	15.68	20.27	13.59	0.16	0.875	−12.05	14.05	0.07
IPC	25.64	7.27	28.91	8.89	−0.94	0.356	−10.50	3.95	−0.40
APC	26.64	14.78	28.91	13.15	−0.38	0.707	−14.71	10.17	−0.16
OC	29.36	6.56	31.27	10.85	−0.50	0.624	−10.00	6.18	−0.21
GMPC	158.09	44.58	164.18	60.45	−0.27	0.791	−53.33	41.15	−0.12

[a] $n = 11$. [b] $n = 11$. CI = confidence interval; LL = lower limit; UL = upper limit. EDRC: Eating Disorder Risk Composite; IC: Ineffectiveness Composite; IPC: Interpersonal Problems Composite; APC: Affective Problems Composite; OC: Over-control Composite; GPMC: General Psychological Maladjustment Composite.

Table 4. Results according to the competition experience of the wrestlers (median = 7.00).

Factor	More Experience [a]		Less Experience [b]				95% CI		
	M	SD	M	SD	t(20)	*p*	LL	UL	d
BULIMIA									
Symptoms	14.17	5.97	9.50	5.45	1.90	0.072	−0.46	9.80	0.81
DASS	20.08	12.81	15.30	6.75	1.06	0.301	−4.62	14.18	0.46
Depression	6.08	4.93	3.30	2.67	1.60	0.126	−0.85	6.42	0.68
Anxiety	4.92	4.56	4.80	2.90	0.07	0.945	−3.37	3.60	0.30
Stress	9.08	5.30	7.20	2.97	1.05	0.308	−1.89	5.66	0.43
EAT-26	25.67	14.19	14.70	9.38	2.09	0.050 *	0.02	21.92	0.89
Dieting	15.58	9.56	7.70	6.38	2.22	0.038 *	0.49	15.28	0.95
Bulimia	5.42	3.03	5.20	3.12	0.16	0.871	−2.53	2.96	0.07
Oral control	4.67	3.77	1.80	1.81	2.19	0.040 *	0.14	5.59	0.94
EDI-3									
Eating Disorder Risk Scales									
Drive for thinness	22.08	10.37	13.40	9.66	2.02	0.057	−0.30	17.67	0.86
Bulimia	14.00	5.88	10.50	8.13	1.17	0.255	−2.73	9.73	0.50
Body dissatisfaction	22.67	14.75	14.00	7.26	1.79	0.091	−1.56	18.89	0.72
Psychological scales									
Low self-esteem	12.00	8.51	7.60	6.04	1.37	0.186	−2.30	11.10	0.59
Personal alienation	12.58	8.16	8.60	5.56	1.31	0.206	−2.37	10.33	0.56
Interpersonal insecurity	15.75	4.11	13.70	5.66	0.98	0.337	−2.30	6.40	0.42
Interpersonal alienation	13.25	4.97	11.50	3.44	0.94	0.359	−2.14	5.64	0.40
Interoceptive deficits	17.17	8.61	15.10	8.82	0.55	0.586	−5.71	9.84	0.24
Emotional dysregulation	12.42	6.89	10.50	5.46	0.71	0.485	−3.70	7.54	0.30
Perfectionism	16.92	6.57	17.70	5.95	−0.29	0.774	−6.41	4.84	-0.12
Asceticism	14.75	6.05	9.00	4.00	2.57	0.018 *	1.08	10.42	1.10
Maturity fears	17.58	8.83	18.90	6.54	−0.39	0.700	−8.35	5.72	-0.17
Composites									
EDRC	58.75	26.39	37.90	20.12	2.05	0.054	−0.38	42.08	0.88
IC	24.58	15.96	16.20	11.19	1.40	0.178	−4.14	20.90	0.60
IPC	29.00	8.10	25.20	8.01	1.10	0.284	−3.40	11.00	0.47
APC	29.58	14.24	25.60	13.43	0.67	0.510	−8.42	16.38	0.29
OC	32.33	8.62	27.90	8.85	1.19	0.249	−3.36	12.22	0.51
GMPC	178.75	49.97	140.00	48.25	1.84	0.081	−5.20	82.70	0.79

[a] $n = 11$. [b] $n = 11$. CI = confidence interval; LL = lower limit; UL = upper limit. EDRC: Eating Disorder Risk Composite; IC: Ineffectiveness Composite; IPC: Interpersonal Problems Composite; APC: Affective Problems Composite; OC: Over-control Composite; GPMC: General Psychological Maladjustment Composite. * $p < 0.05$.

Table 5. Results according to the training experience of the wrestlers (median = 9.50).

Factor	More Experience [a]		Less Experience [b]				95% CI		
	M	SD	M	SD	t(20)	*p*	LL	UL	d
BULIMIA									
Symptoms	13.91	6.20	10.18	5.64	1.47	0.156	−1.54	9.00	0.63
DASS	19.09	12.00	16.73	9.32	0.52	0.612	−7.19	11.92	0.22
Depression	5.27	3.80	4.36	4.74	0.50	0.625	−2.91	4.73	0.21
Anxiety	4.55	4.37	5.18	3.34	−0.38	0.705	−4.09	2.82	-0.16
Stress	9.27	5.33	7.18	3.16	1.12	0.276	−1.81	5.99	0.48

Table 5. *Cont.*

Factor	More Experience [a]		Less Experience [b]				95% CI		
	M	SD	M	SD	t(20)	*p*	LL	UL	d
EAT-26	24.18	13.11	17.18	12.94	1.26	0.222	−4.59	18.59	0.54
Dieting	14.36	9.52	9.64	8.25	1.24	0.228	−3.20	12.65	0.53
Bulimia	6.18	2.89	4.45	2.98	1.38	0.183	−0.88	4.34	0.59
Oral control	3.64	3.04	3.09	3.70	0.38	0.710	−2.47	3.56	0.16
EDI-3									
Eating Disorder Risk Scales									
Drive for thinness	20.55	10.19	15.73	11.26	1.05	0.305	−4.73	14.37	0.45
Bulimia	15.64	6.31	9.18	6.43	2.38	0.028 *	0.79	12.12	1.01
Body dissatisfaction	19.73	13.56	17.73	11.91	0.37	0.717	−9.35	13.35	0.16
Psychological scales									
Low self-esteem	12.27	8.34	7.73	6.48	1.43	0.169	−2.10	11.19	0.61
Personal slienation	13.45	8.47	8.09	4.72	1.84	0.081	−0.73	11.46	0.78
Interpersonal insecurity	15.55	4.11	14.09	5.63	0.69	0.497	−2.93	5.84	0.30
Interpersonal alienation	12.82	5.06	12.09	3.70	0.38	0.704	−3.21	4.67	0.16
Interoceptive deficits	18.82	9.23	13.64	7.35	1.46	0.161	−2.24	12.60	0.62
Emotional dysregulation	13.00	7.18	10.09	4.99	1.10	0.283	−2.59	8.41	0.47
Perfectionism	17.82	7.03	16.73	5.44	0.41	0.688	−4.50	6.68	0.17
Asceticism	14.27	6.36	10.00	4.71	1.79	0.088	−0.70	9.25	0.76
Maturity fears	19.73	8.33	16.64	7.10	0.94	0.360	−3.80	9.98	0.40
Composites									
EDRC	55.91	25.34	42.64	25.08	1.24	0.231	−9.15	35.69	0.53
IC	25.73	16.35	15.82	10.50	1.69	0.106	−2.31	22.13	0.72
IPC	28.36	8.39	26.18	8.05	0.62	0.541	−5.13	9.50	0.27
APC	31.82	15.05	23.73	11.48	1.42	0.172	−3.81	19.99	0.60
OC	34.00	7.33	26.64	8.90	2.12	0.047 *	0.11	14.62	0.90
GMPC	180.64	49.88	141.64	48.34	1.86	0.077	−4.69	82.69	0.79

[a] $n = 11$. [b] $n = 11$. CI = confidence interval; LL = lower limit; UL = upper limit. EDRC: Eating Disorder Risk Composite; IC: Ineffectiveness Composite; IPC: Interpersonal Problems Composite; APC: Affective Problems Composite; OC: Over-control Composite; GPMC: General Psychological Maladjustment Composite. * $p < 0.05$.

Table 6. Results according to the weight of the wrestlers (median = 63.75).

Factor	Heaviest [a]		Lightest [b]				95% CI		
	M	SD	M	SD	t(20)	*p*	LL	UL	d
BULIMIA									
Symptoms	11.36	6.64	12.73	5.73	−0.52	0.612	−6.88	4.15	−0.22
DASS	13.73	8.20	22.09	11.33	−1.98	0.061	−17.16	0.43	−0.85
Depression	3.27	3.04	6.36	4.78	−1.81	0.085	−6.65	0.47	−0.77
Anxiety	3.64	3.20	6.09	4.11	−1.56	0.134	−5.73	0.82	−0.67
Stress	6.82	3.97	9.64	4.54	−1.55	0.137	−6.61	0.98	−0.66
EAT-26	21.55	13.93	19.82	10.64	0.30	0.767	−10.28	13.74	0.13
Dieting	13.36	10.19	10.64	5.82	0.70	0.492	−5.40	10.85	0.30
Bulimia	4.82	2.23	5.82	3.36	−0.77	0.448	−3.69	1.69	−0.33
Oral control	3.36	3.04	3.36	5.16	0.00	0.999	−3.02	3.02	0.00
EDI-3									
Eating Disorder Risk Scales									
Drive for thinness	19.09	12.30	17.18	9.50	0.41	0.688	−7.86	11.68	0.17
Bulimia	12.45	6.73	12.36	7.67	0.03	0.977	−6.33	6.51	0.01
Body dissatisfaction	23.09	13.38	14.36	10.35	1.71	0.102	−1.91	19.36	0.73
Psychological scales									
Low self-esteem	8.09	6.56	11.91	8.48	−1.18	0.251	−10.56	2.93	−0.50
Personal slienation	9.27	5.41	12.27	8.70	−0.97	0.343	−9.44	3.44	−0.41
Interpersonal insecurity	14.00	4.63	15.64	5.18	−0.78	0.444	−6.01	2.73	−0.33
Interpersonal alienation	11.91	3.18	13.00	5.37	−0.58	0.568	−5.01	2.83	−0.25
Interoceptive deficits	14.73	7.86	17.73	9.34	−0.82	0.425	−10.68	4.68	−0.35
Emotional dysregulation	10.27	4.84	12.82	7.36	−0.96	0.349	−8.09	2.99	−0.41
Perfectionism	17.00	5.62	17.55	6.92	−0.20	0.841	−6.15	5.06	−0.09
Asceticism	11.64	5.80	12.64	6.20	−0.39	0.700	−6.34	4.34	−0.17
Maturity fears	21.18	7.03	15.18	7.48	1.94	0.067	−0.45	12.45	0.83

Table 6. *Cont.*

Factor	Heaviest [a]		Lightest [b]		95% CI				
	M	SD	M	SD	t(20)	*p*	LL	UL	d
Composites									
EDRC	54.64	29.70	43.91	20.55	0.99	0.336	−11.99	33.44	0.42
IC	17.36	11.40	24.18	16.61	−1.12	0.275	−19.49	5.85	−0.48
IPC	25.91	7.02	28.64	9.19	−0.78	0.443	−10.00	4.55	−0.33
APC	25.00	11.76	30.55	15.46	−0.95	0.355	−17.76	6.67	−0.40
OC	32.82	8.85	27.82	8.41	1.36	0.190	−2.68	12.68	0.58
GPMC	162.45	53.91	159.82	52.45	0.12	0.909	−44.67	49.94	0.05

Note. [a] $n = 11$. [b] $n = 11$. CI = confidence interval; LL = lower limit; UL = upper limit. EDRC: Eating Disorder Risk Composite; IC: Ineffectiveness Composite; IPC: Interpersonal Problems Composite; APC: Affective Problems Composite; OC: Over-control Composite; GPMC: General Psychological Maladjustment Composite.

3.4. Predictors of Eating Disorders—Regression Analysis

A multiple linear regression was performed to estimate the wrestlers' factors predicting EDs. The competition experience significantly predicted bulimia severity ($F[1, 7] = 8.197$, $p = 0.024$, and adjusted $R^2 = 0.474$). Moreover, training experience showed a significant relationship with bulimia symptoms ($F[1, 20] = 5.449$, $p = 0.030$, and adjusted $R^2 = 0.175$) and bulimia ($F[1, 20] = 7.432$, $p = 0.013$, and adjusted $R^2 = 0.234$). Similarly, training experience also predicted psychological factors related to EDs, accounting for between 14 and 24% of their variance: personal alienation ($F[1, 20] = 4.760$, $p = 0.041$, and adjusted $R^2 = 0.152$), emotional dysregulation ($F[1, 20] = 6.502$, $p = 0.019$, and adjusted $R^2 = 0.208$), asceticism ($F[1, 20] = 7.517$, $p = 0.013$, and adjusted $R^2 = 0.237$), affective problems ($F[1, 20] = 5.779$, $p = 0.026$, and adjusted $R^2 = 0.185$), and the DASS total score ($F[1, 20] = 4.750$, $p = 0.041$, and adjusted $R^2 = 0.152$). The rest of the variables analyzed were not significant predictors ($p > 0.05$).

4. Discussion

The main objective of this study was to determine the relationship between different variables of female, elite wrestlers (weight, age, and experience, among others) and the frequencies in which EDs occur in this population. In addition, it sought to establish which factors could be predictors of EDs. In this line, EDs are a serious concern in Olympic wrestling and other weight-dependent sports, with wrestlers resorting to extreme and unhealthy weight-control practices. Pressure to maintain low weights, often influenced by coaches, peers, and the desire for athletic success, results in harmful practices such as severe dietary restrictions and dehydration. These practices lead to negative physical and mental health consequences, affecting athletic performance and overall well-being. In addition, competition and training experience seem to be the most critical factors in the development of EDs in female wrestlers; therefore, it seems essential that athletes of all ages and experience levels receive adequate education on nutrition, healthy weight management, and the promotion of positive body image in order to prevent and address EDs in combat sports [11].

While the mean depression score among our female wrestlers did not surpass the threshold for the mild category according to the DASS-21 questionnaire, 50% exhibit depressive symptoms, and 23% experience moderate to extremely severe depression. This data could be interpreted as a comorbidity factor for certain female wrestlers [32]. In contrast, the anxiety and stress reported by the wrestlers reached the mild threshold according to the DASS-21, with most of the sample exceeding this threshold and a high percentage experiencing severe to extreme anxiety levels (22.7%). In consequence, these three factors could be relevant, as they may influence the onset of EDs and clarify why athletes are at a heightened risk for such conditions [33]. In this line, the prevalence of EDs is higher among athletes than in the general population, especially in female athletes compared to male athletes. Moreover, it is also more common in combat sports that emphasize leanness and weight dependency, as opposed to other sports [34].

Regarding the EDI-3 questionnaire, scores above 20 indicate a risk of developing EDs [27]. Our results showed that, on average, the studied female wrestlers did not reach this threshold of clinical EDs but were close (drive for thinness, M = 18.14 and body dissatisfaction, M = 18.73). It is important to note that our values were clearly higher than those of a similar age and gender in the general population [34]. Additionally, a significant proportion of the participants in our study (drive for thinness, 46%; bulimia, 22%; and body dissatisfaction, 33.8%) are at risk of developing EDs. Thus, our findings were more in line with other combat sports, such as judo, which share the characteristics of being combat-oriented and weight-class-regulated [12].

On the other hand, in the BITE questionnaire, a similar pattern emerged. Elevated symptoms were observed on the assessment scale and were consistent with the study of Oliveira [35], who also found abnormally high scores related to unhealthy eating patterns and body-weight concerns in a sample of young, female athletes participating in various sports, some of them being combat sports. This suggests that female wrestlers exhibit a preoccupation and disturbance in their relationship with food, even when the entire sample did not reach the threshold for a clinical diagnosis of EDs. This phenomenon may be linked to the demands of making weight for competitions [36]. The BITE questionnaire could shed light on why EDs are not detected in female wrestlers despite the presence of symptoms and their severity. In addition, this phenomenon can be attributed to training and competition experience, as the more they compete, the more chances they have to reach a specific weight [11].

Furthermore, our results could be linked to how frequently wrestlers resort to unhealthy methods to quickly achieve their weight goals. Specifically, 40% of the participants admitted to using natural diuretics for weight reduction. These results align with the findings of Gullón-López et al. [37], who highlighted that combat sports athletes often employ dehydration as a strategy to meet weight requirements. Moreover, approximately half of the wrestlers in our study revealed practicing fasting for a full day as a weight-loss strategy. Furthermore, 33.3% acknowledged the use of laxatives for the same purpose, and 10% admitted to inducing vomiting to reduce their body weight. Previous studies, such as the one conducted by Rueda et al. [11], indicated that some wrestlers had employed risky methods prior to weigh-ins. Additionally, it was found that half of the wrestlers admitted to experiencing binge-eating episodes when alone, with 10% of them reporting these episodes occurring at least two or three times per week. Studies like those by van Niekerk et al. [38] and Kiefer [39] suggested that these behaviors appeared to be more common in women than in men. Consequently, all of these behavioral patterns could be predictors of future EDs [5].

Regarding group comparisons, it was observed that more experienced wrestlers presented a higher number of predictive factors, possibly due to their increased participation in competitions, which necessitates more frequent weight adjustments [40]. Furthermore, this could be related to the gradual acquisition of unhealthy behaviors to achieve precise weight goals. In contrast to the study by Rouveix et al. [12] with male judo athletes, where beginners resorted to risky methods for weight loss, our results showed that more experienced female wrestlers exhibit a higher propensity to employ hazardous techniques to reduce their body weight [11]. This disparity may be attributed to differences in weigh-in procedures between judo and wrestling. Thus, in judo, official weigh-ins occur the evening before the competition, while in wrestling, they take place a mere two hours prior to the bout, limiting the wrestlers' capacity for recovery. Hence, the timing of weigh-ins could be a determining factor. Additionally, contrary to the study by Nishimaki et al. [4], which found that Japanese female wrestlers in lower weight categories tend to be more overweight compared to those in higher weight categories, which could explain the presence of unhealthy eating behaviors, we did not find any effects of weight category on EDs. This discrepancy could be due to limitations in our sample or the timing of the season in which the data were collected.

Experience in training and competition positively explained 18–47% of the variability in the severity scale of bulimia symptoms on the BITE questionnaire. These results are in line with the findings of Thompson and Sherman [23], suggesting that many less experienced athletes share similar risk factors to their older counterparts, although they may be less aware of them. These athletes face an "evolutionary" risk factor, as they are in a life stage characterized by a high risk of developing an ED, either when starting their involvement in a sport or taking their sports career seriously. It is important to note that the majority of athletes with EDs did not initiate their problematic eating patterns in adulthood but rather during adolescence [41]. Similarly, Rueda et al. [11] suggest that more experienced female wrestlers face greater pressure to achieve results, as they have to compete more frequently and, consequently, make weight for competition on several occasions, potentially leading to additional pressure. Thus, wrestlers with EDs may feel less fearful about maintaining the disorder rather than trying to overcome it. This fact may be due to different motivating factors, depending on the disorder cycle time [23]. In addition, the desire to achieve optimal health and performance in sports appears to be one of the most important factors for athletes to recover themselves after EDs [42].

Although our study provides valuable and relatively unexplored insights, there are certain limitations that need to be mentioned. First, the sample participants exclusively consisted of members from the national women's wrestling team, raising the question of whether these issues are gender-specific or also apply to male athletes. Another limitation is the temporal nature of the data collection, as it represents a specific point in the season—the preseason—when the weight requirements could be lesser than in competition season periods. Additionally, only some age categories were analyzed. This fact could hide information about how younger wrestlers could be affected by EDs. Consequently, future research should investigate whether the findings of our study are consistent across gender, other weight-category sports, and weight categories. Lastly, the implementation of a longitudinal study would provide a more in-depth understanding of how EDs are developed over time.

5. Conclusions

This study suggests that differences in EDs were found between groups with higher and lower training and competitive experience but not in age and weight categories. Therefore, competitions and training experiences appear to be the most critical variables related to EDs and psychological problems. This implies that competition intensity and training time play a crucial role in the mental health of elite female athletes. Additionally, although the group of wrestlers does not meet the threshold for EDs according to the questionnaire's standard values, there is a high frequency of behaviors associated with EDs; therefore, many athletes are at a high risk of developing EDs during their athletic careers. Lastly, a high percentage of wrestlers experience high levels of anxiety, depression, and stress, which could lead to the development of EDs.

6. Practical Application

To address this issue effectively, a collaborative approach involving coaches, health professionals, and athletes is paramount. Coaches and support staff must undergo comprehensive training to adeptly identify signs that are indicative of EDs, offer necessary support, and seamlessly facilitate referrals to specialized healthcare professionals when warranted. Simultaneously, athletes require thorough education on the risks associated with EDs, coupled with relentless encouragement to prioritize their overall health and well-being over the pursuit of extreme weight-control measures. Consequently, it is imperative for sports organizations and Olympic committees to establish robust policies that champion healthy weight-management practices. These policies should encompass provisions for ample resources and dedicated support systems, thus fortifying athletes' ability to maintain peak health while engaging in elite-level competition. Through the diligent implementation of these recommendations, stakeholders can collaboratively cultivate an environment that

steadfastly upholds the health and well-being of athletes engaged in Olympic wrestling and other combat sports.

Author Contributions: Conceptualization, M.R.F. and D.M.-L.; methodology, M.R.F., J.G.-G. and D.M.-L.; formal analysis, M.R.F., J.G.-G. and D.M.-L.; data curation, M.R.F., J.G.-G. and D.M.-L.; writing—original draft preparation, M.R.F., J.G.-G. and D.M.-L.; writing—review and editing, M.R.F., A.M.-C., O.LT., V.E.F.-E., J.G.-G. and D.M.-L.; visualization, J.G.-G.; supervision, O.L.-T., J.G.-G. and D.M.-L. All authors have read and agreed to the published version of the manuscript.

Funding: This research received no external funding.

Institutional Review Board Statement: The study was approved by the Universidad Politécnica de Madrid Research Ethics Board (FDRED00000-DML-DATOS-20230609).

Informed Consent Statement: Informed consent was obtained from all subjects involved in the study.

Data Availability Statement: The data presented in this study are available on request from the corresponding author. The data are not publicly available due to privacy.

Acknowledgments: We would like to express our deep appreciation to all the collaborators and volunteers whose collaboration and dedication have been fundamental to the successful completion of this study.

Conflicts of Interest: The authors declare no conflicts of interest.

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The Effects of 24-h Fasting on Exercise Performance and Metabolic Parameters in a Pilot Study of Female CrossFit Athletes

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Abstract: Many studies have tested intermittent fasting (IF) in athletes, but its effects on female CrossFit athletes remain relatively unexplored in the existing literature. The aim of this study was to evaluate and compare the effects of 24-h IF on the physical performance of female CrossFit practitioners. Eleven female CrossFit athletes (age: 30.91 ± 3.42, weight: 65.26 ± 7.55 kg, height: 1.66 ± 0.05 m) participated in the study. The study used a crossover design with fasting and eating conditions. Participants completed an exercise test, standing long jump, and handgrip strength assessment. Hydration status, heart rate, blood lactate, blood glucose, rates of perceived exertion, and hunger were measured. Results showed significant differences in blood lactate concentration ($F = 5.435$, $p = 0.042$, $\eta 2p = 0.352$). Resting blood lactate concentration was significantly lower in the fasting trial than in the eating trial ($p < 0.001$), but post-exercise blood lactate concentrations were higher in the fasting trial than in the eating trial ($p < 0.001$). No differences were found in performance times ($p > 0.05$). In conclusion, this pilot study of females suggests that 24-h fasting does not impair exercise performance or negatively affect physiological parameters in CrossFit athletes.

Keywords: intermittent fasting; crossFit; exercise performance; fatigue; blood lactate

Citation: Eroglu, M.N.; Rodríguez-Longobardo, C.; Ramírez-Adrados, A.; Colina-Coca, C.; Burgos-Postigo, S.; López-Torres, O.; Fernández-Elías, V.E. The Effects of 24-h Fasting on Exercise Performance and Metabolic Parameters in a Pilot Study of Female CrossFit Athletes. *Nutrients* **2023**, *15*, 4841. https://doi.org/10.3390/nu15224841

Academic Editors: Benjamin D. Horne and Peter WR Lemon

Received: 11 October 2023
Revised: 10 November 2023
Accepted: 18 November 2023
Published: 20 November 2023

1. Introduction

Intermittent fasting (IF) is a popular dietary practice, which consists of regular alternating periods of unrestricted dietary consumption and abstinence from caloric intake. In recent years, various forms of IF have been extensively investigated with the aim of improving exercise performance and enhancing metabolic health.

There have been several proposed protocols/methods of IF that are in practice. Some of the common ones include alternate-day fasting, which involves fasting for 24-h every other day, and the 5:2 method, which involves a fast of 24-h twice a week and a very low-calorie diet consumed two other days of the week. In these protocols, fasting can take place on consecutive or non-consecutive days [1]. Fasting is also an important part of many religions, a notable example being Muslims fasting during Ramadan, a month-long period during which no food or liquid is consumed during daylight hours [2].

These fasting protocols have been associated with various health benefits such as increasing metabolic flexibility, improving insulin sensitivity, promoting weight loss, and

even slowing down the aging process. However, our knowledge about the effects of IF modalities as a factor influencing athletic performance is limited.

Specifically, short-term (acute) fasting protocols such as the 24-h fasting period can impact exercise performance. During this period, the body utilizes glycogen stores and increases fat metabolism to provide energy. This can lead to changes in energy production mechanisms and potentially affect exercise performance positively or negatively [3].

The effects of short-term fasting on exercise performance have been extensively investigated, and suggest that decreased physical performance occurs during the fasting state [4]. This could be explained (at least in part) by the fasting periods used (>24 to 55 h), dehydration, prolonged exhaustive exercise tests [5–7], and/or very high-intensity levels of exercise [7,8]. However, others [9–12] failed to record significant decreases in performance after shorter periods of fasting (11–24 h).

CrossFit is a high-intensity fitness training program that combines elements of weightlifting, cardiovascular exercise, and bodyweight movements. It aims to improve overall fitness by focusing on functional movements performed at a high intensity. CrossFit workouts, known as "WOD" (Workout of the Day), are constantly varied and incorporate exercises from various disciplines, such as weightlifting, gymnastics, and metabolic conditioning [13].

Different nutritional strategies and approaches are used to improve the performance of CrossFit practitioners, and intermittent fasting can be compatible with CrossFit as individuals can schedule their eating windows around their workouts. Aerobic and anaerobic energy production processes form the basis of CrossFit training, and how these energy systems are affected under IF diets is not yet fully understood since most of them are empirical and lack scientific evidence [14,15]. Studies have focused more on selected nutritional interventions, such as the ketogenic diet [16,17], pre- and post-workout protein, and carbohydrate intake [18–21].

In a study of 2576 CrossFit practitioners, it was reported that 7.7% of the participants were on an IF diet [22]. Considering sex when prescribing dietary strategies is important because men and women have distinct physiological differences (hormonal variations, body composition, and metabolic rates) that can affect their nutritional needs and health outcomes [23]. A recent study [24] surveyed 449 CrossFit practitioners about their workouts and dietary intake and concluded that it is necessary to consider sex when prescribing dietary strategies. Specifically, it has been reported that women more often choose weight loss and fat loss as their nutritional goals, while men are more likely to choose to gain muscle mass and weight. These findings are consistent with previous research on exercise behavior where women exercise more frequently than men to lose weight and men tend to gain muscle mass/weight [25,26].

Based on the information presented, the effects of IF diets on female CrossFit athletes remain relatively unexplored in the existing literature. Therefore, the aim of this study was to assess and compare the effects of 24 h fasting on the physical performance of female CrossFit practitioners. Our hypothesis was that a 24 h fasting period would not negatively affect physical performance compared with a normal pre-exercise diet.

2. Material and Methods

2.1. Participant Selection and Study Design

Eleven female crossfitters (characteristics: age: 30.91 $\pm$ 3.42, weight: 65.26 $\pm$ 7.55 kg, height: 1.66 $\pm$ 0.05 m) participated in the study. The number of participants was determined according to the power analysis results. All were recruited from the same local CrossFit gym. Recruitment and study took place in July 2023. Inclusion criteria were: (1) being female; (2) being 18 or older; and (3) having CrossFit training experience for at least 2 years. The exclusion criteria were: (1) following pharmacological treatment or supplement (including the use of stimulants such as caffeine), (2) muscular, ligamentous, bone, nerve, or joint pathology incompatible with the training program; (3) present cardiovascular or cardiorespiratory problems; and (4) performance of other sports activities during their participation in the study that could influence the study results. All participants were

assessed in the same menstrual cycle phase (late follicular, after the subject's reported menstruation) to avoid the effects of the menstrual cycle phase on exercise capacity [27].

Before commencing the study, all subjects were informed about the experimental procedures and the possible risks and benefits of the study. Each subject signed a written informed consent form prior to participation. The study protocol was approved by the Local Clinical Research Ethics Committee of Madrid (47/764390/17) and was in accordance with the Declaration of Helsinki.

The present study used a within-subject counterbalanced crossover design, as this design has many advantages: each subject acted as their own control, thus reducing possible error variance, while at the same time reducing the required sample size. Each participant was tested under fasting and non-fasting conditions. While the fasting + exercise trial was conducted in the first week, a normal diet + exercise trial was conducted on the same day and time one week later. Participants completed an exercise test, a standing long jump, and a handgrip strength test. Hydration status, heart rate, blood lactate, blood glucose, perceived exertion, and hunger were measured. All participants performed the same set of tests in the same order on each trial. To eliminate the learning effect, the protocols were explained to the participants in advance and practiced. They were asked to refrain from any strenuous exercise 24 h before the test.

2.2. Nutrition Protocol

The participants were provided with the fasting/eating protocol prior to each test period. It was therefore not possible to blind study participants to the testing conditions. In the fasting trial, participants consumed only water for 24 h before exercise. In the eating trial, the instruction given to the participants was to have their last meal previous to the assessment within 2–3 h prior to exercise. On the other hand, participants were provided with nutritional guidelines for the meal before the physical test, which consisted of consuming a meal composed of 50% carbohydrates, 25% fats, and 25% proteins according to the validated visual nutritional tool Athlete's Plate® [28].

With the aim of promoting adherence, all subjects were briefed on the hard training day Athlete's Plate® protocol, as it adjusts the composition of main food groups on the plate to align with high-intensity training days and meet international sports nutrition guidelines [29,30]. Furthermore, a categorized list of foods based on their primary macronutrient content was provided, along with meal ideas, to empower participants to make well-informed dietary decisions.

The athletes were encouraged to contact the dieticians with any questions or concerns regarding the nutritional protocol. All instructions about nutritional guidelines were provided by registered dieticians.

In both test conditions, participants were asked to avoid strenuous physical activity to minimize the potential carry-over effects such as fatigue, muscle damage, or physiological stress. Prior to each testing period, a pre-trial checklist was completed to collect information about the participants' activities over the previous 24-h (e.g., time of last meal, exercise performed, and any injuries since the last contact).

2.3. Physical Testing

2.3.1. Exercise Test

The exercise test consisted of 5 sets of 10 × burpee over bar, 10 × pendlay rowing, 10 × deep squats, and 4 × 20 m sprints. Participants were asked to be as fast as possible, and times were recorded the assess physical performance.

2.3.2. Standing Long Jump

The standing long jump test was used to measure strength and sprint speed [31]. Participants were instructed to perform a standing long jump from a position behind a designated starting line, with the aim of achieving the maximum distance forward using both legs. The distance covered during the jump was quantified in centimeters, determined

by measuring from the front edge of the starting line to the point of initial heel contact with the ground.

2.3.3. Handgrip Strength

Handgrip strength test was performed as an indicator of overall strength [32]. This test was performed using a hydraulic hand-held dynamometer (Takei 5001, Japan) with an accuracy of 0.1 kg. Before and after the test, the subjects held a standardized position (standing, with the elbow in full extension) for 2–3 s at maximum pressure. All participants repeated the test twice, alternating between each hand. The researchers recorded the best score from the two trials.

2.4. Measurements

2.4.1. Hydration Level

Immediately before exercise, the participants were asked to provide a urine sample. A standard well-established urine color scale [33], with demonstrated test-retest reliability and validity [34,35], was used to assess hydration status. The urine samples were held against a white background and the urine color scores were determined using numbers to describe the hydration status, i.e., numbers 1–3 indicate hyperhydrated hydration status, 4 indicate euhyrated hydration status, and 5–8 indicate hypohydrated hydration status.

2.4.2. Heart Rate

The heart rate was monitored during exercise and at rest using a Polar H10 heart rate monitor (Kempele, Finland). The Elite HRV (Heart Rate Variability, NC, ABD) application was used as the data receiver in a Bluetooth connection to record HR (HR average) and measure heart rate variability (HRV). The Elite HRV application has been previously validated for HRV recording [36]. For HRV measurement, the square root of the mean of the sum of the squared differences between normal adjacent RR intervals (RMSSD), the low-frequency band (LF), and the high-frequency band (HF) were analyzed [37].

2.4.3. Blood Lactate

Using a disposable lancet device, the skin was punctured just at the center of the finger pad. The first drop of blood was wiped away, and then approximately 5 μL (2 mm) of blood was applied to the lactate strip and immediately analyzed using the Lactate Pro Analyser (ARKRAY Inc., Kyoto, Japan) [38]. Blood lactate concentrations were measured at rest (pre-exercise) and immediately after the completion of the exercise protocol (post-exercise).

2.4.4. Blood Glucose

Blood glucose measurements were taken from the fingertip using a glucose analyzer (Freestyle Optium Neo, Abbot, Madrid, Spain). The blood glucose was measured both before and immediately after the completion of the 5 sets performed by the participants. Blood sampling was carried out immediately after lactate analysis.

2.4.5. Rate of Perceived Exertion (RPE)

The Borg CR-10 scale was used to assess the perceived exertion. The assessments were made pre- and post-exercise. Participants were given detailed instructions on how to rate the experience in terms of perceived exertion. Each participant rated the perception of physical effort on a scale from "absolutely nothing" (score 0) to "extremely strong" (score 10) [39].

2.4.6. Hunger Feelings

Hunger feeling was assessed pre- and post-training using a Visual Analog Scale (VAS), where participants had to rate their sensation from 0 to 10. A score of 0 represents the "absence of hunger sensation" and 10 represents "very hungry". The VAS method has been validated in several populations [40].

2.5. Statistical Analysis

Statistical analysis was performed with the Statistical Package for the Social Sciences (SPSS, version 24, SPSS Inc., Chicago, IL, USA) in the Windows environment. Data are presented as mean ± SD. The Kolmogorov–Smirnov test was used to confirm a normal distribution of data. To assess variables measured pre- and post-exercise, a two-way analysis of variance (ANOVA) with repeated measures was conducted to determine the main effects of the experimental trials (fasting vs. pre-competition diet) and time (pre- vs. post-exercise) as well as interaction (experimental trial × time). Bonferroni post hoc analysis was performed when a significant F value (Greenhouse–Geisser adjustment for sphericity) was observed. For single measure variables, a Student *t*-test was performed. The effect size was estimated by calculating partial eta squared (η2p) and Cohen's d, for pre- and post-exercise and single measure variables, respectively.

3. Results

The ANOVA analysis revealed significant differences in blood lactate concentration ($F = 5.435$, $p = 0.042$, $\eta 2p = 0.352$). The resting blood lactate concentration in the fasting trial was significantly lower than that in the eating trial ($p < 0.001$). Interestingly, post-exercise blood lactate concentrations were higher in the fasting trial compared to the eating trial ($p < 0.001$, Figure 1). There was no significant difference between trials in RPE ($p > 0.05$, Table 1).

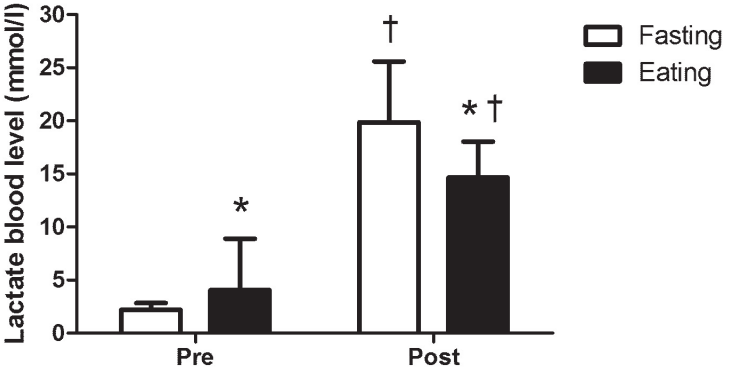

Figure 1. Pre- and post-exercise lactate blood levels. * Significantly different than fasting trial ($p < 0.05$). † Significantly different than pre-exercise ($p < 0.05$).

Table 1. Pre- and post-exercise measurements.

		Pre-Exercise	Post-Exercise	F	*p*	η2p	Post Hoc
RPE (1;10)	Fasting	4.09 ± 2.02	8.18 ± 1.32	0.009	0.928	0.001	Pre-fasting vs. eating $p < 0.001$: Post-fasting vs. eating $p < 0.001$
	Eating	3.09 ± 1.51	7.27 ± 0.78				
Ratings of Hunger (0;10)	Fasting	5.86 ± 3.03	1.90 ± 1.97	8.213	0.017	0.451	
	Eating	1.54 ± 1.91	0.63 ± 1.56				
HGS Right (kg)	Fasting	32.95 ± 3.61	33.68 ± 2.76	0.084	0.777	0.008	
	Eating	33.04 ± 3.32	33.40 ± 3.11				
HGS Left (kg)	Fasting	32.40 ± 4.32	31.81 ± 4.40	0.163	0.695	0.016	
	Eating	31.86 ± 3.49	31.81 ± 4.93				
Jumping (cm)	Fasting	175.11 ± 20.97	168.66 ± 25.63	1.692	0.230	0.175	
	Eating	167.66 ± 18.94	170.11 ± 18.98				

RPE, Rate of perceived exertion; HGS, Handgrip strength. Note: data expressed as the mean ± standard deviation (SD).

In terms of blood glucose concentration (F = 16.80, $p = 0.002$, $\eta 2p = 0.627$), resting levels were significantly lower in the fasting trial than in the eating trial ($p < 0.001$). However, the increase in blood glucose concentration from pre- to post-exercise was not significantly different between the two conditions (Figure 2).

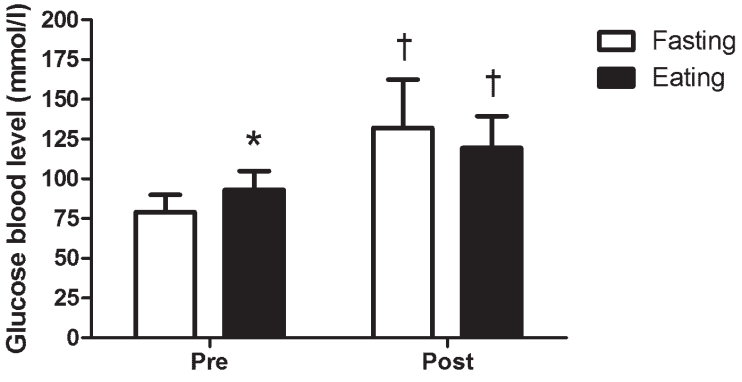

Figure 2. Pre- and post-exercise glucose blood levels. * Significantly different than fasting trial ($p < 0.05$). † Significantly different than pre-exercise ($p < 0.05$).

Participants reported higher levels of hunger both pre- and post-exercise in the fasting trial compared to the eating trial ($p < 0.001$, Table 1).

Handgrip strength values measured with both the right and left hand were not affected by the fasting or eating trials. No significant differences were observed pre- and post-exercise ($p > 0.05$). In addition, there were no significant differences in standing jumping distances between pre- and post-exercise measurements in either the fasting or eating trials ($p > 0.05$, Table 1).

No significant differences in performance times were found between the fasting and eating trials ($p > 0.05$). Resting heart rates before exercise were not significantly different between the fasting and eating trials (71.3 ± 8.3 vs. 67.42 ± 7.44 bpm, fasting vs. eating, respectively, $p = 0.131$, t = 1.647, $\eta 2 = 0.49$). Additionally, heart rate values (RMSSD, LF, HF, Mean HR) during exercise were similar between the fasting and eating trials (Table 2).

Table 2. During exercise HRV and performance time.

	Fasting	Eating	t	*p*	d
Performance Time (min)	12.02 ± 2.4	11.24 ± 2.22	1.911	0.085	0.32
Mean Heart Rate (bpm)	161.00 ± 13.26	160.81 ± 8.43	0.045	0.965	0.01
LF Power (ms^2)	32,493.79 ± 56,646.62	22,355.43 ± 42,151.57	0.442	0.668	0.20
HF Power (ms^2)	88,773.22 ± 200,141.76	52,884.66 ± 139,620.94	0.455	0.659	0.20
RMSSD (ms)	82.70 ± 49.52	76.83 ± 56.82	0.277	0.787	0.11

Min, minute; bpm, beats·min−1; LF, low-frequency power; ms^2, milliseconds squared; HF, high-frequency power; RMSSD, root mean square of the successive differences. Statistical significance set at $p < 0.05$. Note: data expressed as the mean ± standard deviation (SD).

Finally, no significant difference in pre-exercise hydration levels was observed between the fasting and eating trials (2.33 ± 1.33 vs. 2.1 ± 0.87, fasting vs. eating, respectively, $p = 0.662$, t = 0.452, $\eta 2 = 0.17$).

4. Discussion

Our study aimed to investigate the effects of fasting on exercise performance and related physiological parameters in a group of female CrossFit athletes. The results showed that 24-h fasting can lead to lower resting blood lactate concentrations, increased post-exercise lactate levels, and higher subjective hunger levels. However, fasting did not

significantly affect ratings of perceived exertion, post-exercise blood glucose concentration, handgrip strength, jumping performance, performance times, heart rate parameters, or hydration levels.

Blood lactate concentration is an important indicator of the metabolic response to exercise and fatigue levels. Our results showed that resting blood lactate levels were significantly lower in the fasting trial compared to the eating trial. The lower resting lactate levels observed in the fasting trial could be attributed to a decrease in carbohydrate availability, leading to reduced resting glycolytic activity and thus lower lactate production.

Post-exercise blood lactate concentrations were higher in the fasting trial compared to the eating trial. This indicates that fasting may lead to increased lactate production during exercise. Previous research on athletes has shown that fasting can improve lactate production and clearance during exercise [12,41]. The higher post-exercise lactate levels observed in the fasting trial could be attributed to an increased reliance on glycogenolysis and subsequent lactate production as an alternative energy source in the absence of readily available carbohydrates. This increased lactate production may have resulted from the recruitment of a greater mass of higher threshold glycolytic fast-twitch fibers, as the population of slow-twitch fibers became increasingly glycogen-depleted and fatigued. Loy et al. [7] found a significant increase in lactate concentration accompanied by a decrease in skeletal muscle glycogen after a 24-h fasting and exercise to exhaustion. Furthermore, elevated norepinephrine levels in the fasting trial may have impeded lactate clearance by diverting blood flow away from the liver [7,42].

Despite the differences in blood lactate concentrations, we did not observe any significant differences in RPE between the fasting and eating trials. This suggests that subjective perceptions of effort during exercise were similar regardless of the fasting state. Similar findings have been reported in previous studies comparing RPE between fasting and eating states [43]. It is possible that other factors, such as motivational factors or the participants' adaptation to training in a fasted state, may have influenced their perceived exertion levels [44].

Our study also investigated the effects of fasting on blood glucose concentration. Resting blood glucose levels were significantly lower in the fasting trial compared to the eating trial, which is consistent with previous research showing reduced blood glucose levels during fasting [12,45,46]. However, the increase in blood glucose concentration from pre- to post-exercise was not significantly different between the two conditions. This suggests that the body's compensatory mechanisms, such as increased gluconeogenesis and glycogen breakdown, adequately maintain blood glucose levels during exercise regardless of the fasting state [4].

The fasting trial had higher hunger levels both pre- and post-exercise compared to the eating trial. The subjective experience of hunger was influenced by hormonal changes and lack of food intake, which can lead to higher perceived hunger levels in fasting individuals. The feeling of hunger post-exercise decreased in both trials. This effect is due to the ability of acute exercise to temporarily suppress appetite-regulating hormones [47].

A recent meta-analysis of twenty-eight studies suggests that IF does not affect muscle strength and anaerobic capacity [48]. In terms of physical performance, fasting did not significantly affect performance time, handgrip strength values, or standing jumping distances in our study. The effect of short-term fasting on exercise capacity has been extensively investigated, with many studies concluding that fasting prior to exercise results in impaired performance [8,12]. This outcome has been attributed, in part, to the adoption of prolonged fasting periods (>24 to 55 h), dehydration [10], prolonged exhaustive exercise testing [5,6], or engaging in very high-intensity levels of exercise [7,8]. However, some researchers have failed to observe a significant decline in performance after shorter fasting periods (11–24 h) [10,12]. This discrepancy in findings could be potentially explained by the glycogen-sparing effect of fasting before exercise, which is linked to increased availability of free fatty acids, thus accounting for the absence of performance decline during short-term fasting [9,11].

Our study showed that performance time exhibits a nonsignificant trend, being 7% longer after fasting compared to the eating state. As we acclimatized the participants to the exercise protocol, we do not believe that the difference could be due to a learning effect. However, this could be due to the small sample size, which is a limitation of the present study. In this line, the β level for performance analysis was 0.996, which indicates that the statistical power was low. Thus, the null hypothesis may not be rejected.

Resting heart rate and heart rate variability during exercise were not significantly different between the fasting and eating trials. There is conflicting information about the effect of fasting on heart rate during exercise. Sabah Hammoud et al. [49] found that fasting during Ramadan significantly increased HRV in the afternoon. Mzurak et al. [50] confirm that an acute (48 h) total fasting induces parasympathetic withdrawal with simultaneous sympathetic activation. These changes appear to reflect stress, which may be related to feelings of hunger. The most likely explanation of the effect of attenuation of HR in the fasting state compared to the eating state may be related to a higher parasympathetic activity during fasting [51,52]. The variations in these findings may be attributed to differences in fasting duration, exercise intensity, and participant characteristics. Future studies with larger sample sizes and more comprehensive monitoring of heart rate parameters are warranted to provide a clearer understanding of the relationship between fasting and cardiovascular responses to exercise.

Finally, our study found no significant difference in hydration levels between the fasting and eating trials. This suggests that participants maintained adequate hydration status in both conditions. Hydration is crucial for optimal exercise performance. However, as far as we know, no study has investigated the effects of 24-h fasting on hydration status. However, previous research [48] has reported that prolonged fasting has a detrimental effect on hydration status and impairs performance.

The present study is not without limitations. As mentioned earlier, the sample size was small, and a larger number of participants could have yielded different statistical outcomes. Additionally, another limitation is the absence of additional parameters that could be relevant in a fasting-exercise context, such as blood fatty acids and levels of stress-related hormones like cortisol.

5. Conclusions

In conclusion, these female-specific findings in a small pilot study are generally consistent with previous research on athletes and support the concept that short-term fasting does not impair exercise performance or negatively impact physiological parameters in female CrossFit athletes. Future research with larger sample sizes and diverse athlete populations is needed to further validate and extend these findings.

Author Contributions: Conceptualization, O.L.-T. and V.E.F.-E.; methodology, M.N.E., C.R.-L., O.L.-T. and V.E.F.-E.; formal analysis, M.N.E., A.R.-A. and V.E.F.-E.; investigation, M.N.E., C.R.-L., A.R.-A., S.B.-P., O.L.-T. and V.E.F.-E.; resources, O.L.-T. and V.E.F.-E.; data curation, M.N.E., A.R.-A. and V.E.F.-E.; writing—original draft preparation, M.N.E., C.R.-L., A.R.-A., C.C.-C., S.B.-P., O.L.-T. and V.E.F.-E.; writing—review and editing, M.N.E., O.L.-T. and V.E.F.-E.; visualization, M.N.E., C.R.-L., O.L.-T. and V.E.F.-E.; supervision, S.B.-P., O.L.-T. and V.E.F.-E.; project administration, O.L.-T. and V.E.F.-E.; funding acquisition, O.L.-T. and V.E.F.-E. All authors have read and agreed to the published version of the manuscript.

Funding: This research received no external funding.

Institutional Review Board Statement: The study was conducted according to the guidelines of the Declaration of Helsinki and approved by the Local Clinical Research Ethics Committee of Madrid (47/764390/17).

Informed Consent Statement: Informed consent was obtained from all subjects involved in the study.

Data Availability Statement: All the data are presented in the study.

Acknowledgments: We thank the participants for their invaluable contribution to the study.

Conflicts of Interest: The authors declare no conflict of interest.

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Are Supplements Consumed by Middle-Distance Runners Evidence-Based? A Comparative Study between Level of Competition and Sex

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Abstract: Background: Middle-distance running events have special physiological requirements from a training and competition point of view. Therefore, many athletes choose to take sport supplements (SS) for different reasons. To date, few studies have been carried out that review supplementation patterns in middle-distance running. The aim of the present study is to analyze the consumption of SS in these runners with respect to their level of competition, sex and level of scientific evidence. Methods: In this descriptive cross-sectional study, data was collected from 106 middle-distance runners using a validated questionnaire. Results: Of the total sample, 85.85% responded that they consumed SS; no statistical difference was found regarding the level of competition or sex of the athletes. With respect to the level of competition, differences were observed in the total consumption of SS ($p = 0.012$), as well as in that of medical supplements ($p = 0.005$). Differences were observed between sexes in the consumption of medical supplements ($p = 0.002$) and group C supplements ($p = 0.029$). Conclusions: Higher-level athletes consume SS that have greater scientific evidence. On the other hand, although the most commonly consumed SS have evidence for the performance or health of middle-distance runners, runners should improve both their sources of information and their places of purchase.

Keywords: middle-distance; supplementation; nutrition; performance; health

Citation: Del Arco, A.; Martinez Aguirre-Betolaza, A.; Malchrowicz-Mośko, E.; Gogojewicz, A.; Castañeda-Babarro, A. Are Supplements Consumed by Middle-Distance Runners Evidence-Based? A Comparative Study between Level of Competition and Sex. *Nutrients* **2023**, *15*, 4839. https://doi.org/10.3390/nu15224839

Academic Editors: Valentín E. Fernández-Elías and Olga López Torres

Received: 16 October 2023
Revised: 16 November 2023
Accepted: 17 November 2023
Published: 20 November 2023

1. Introduction

Middle-distance running events are highly complex from a bioenergetic, training and tactical point of view [1]. The level of energy intensity is in a middle ground between aerobic and anaerobic metabolism [2], with the aerobic contribution in the 800 m being between 60 and 75% and slightly higher (77–85%) in the 1500 m [3]. In addition, due to the type of muscle fibers these athletes have (Mainly IIX and IIA [4]), most middle-distance runners can reach lactate peaks of >20 mmol/L, leading to muscle pH levels as low as 6.6 [5]. However, the high speed requirements make both aerobic and anaerobic metabolism contribute significantly during these events [6]. This can be reflected in the distribution of training intensities throughout the season. Middle-distance runners work a very wide spectrum of training zones, ranging from low-intensity running sessions to very-high-intensity glycolytic workouts [7]. In this way, elite middle-distance runners develop aerobic capacities similar to those of long-distance runners, mechanical skills close to those of sprinters, as well as a highly enhanced anaerobic capacity [1]. Some of these

characteristics make them adopt different race strategies [8,9]. However, sometimes the difference between being a medalist or not is minimal [10], and the improvements seen with some SS are very worthwhile in terms of performance [11].

Supplements are defined as "A food, food component, nutrient, or nonfood compound that is purposefully ingested in addition to the habitually-consumed diet with the aim of achieving a specific health and/or performance benefit" [11]. Although many athletes use SS to improve their performance, there are other underlying reasons for their use [12]. According to the Australian Institute of Sport (AIS), supplements are classified into four groups using the "ABCD" system [13]. This is based on the latest scientific evidence for determining whether a product is safe, permitted and effective in improving performance or health: (A) supplements with solid scientific evidence in specific situations under established protocols; (B) components with emerging evidence that should be used in research or clinical settings; (C) supplements with limited evidence and effects on performance; (D) prohibited products or those with a high risk of contamination by doping substances. Regarding middle-distance races, some of the supplements that have shown the most evidence in improving performance are caffeine [14,15], β-Alanine [16–18] and sodium bicarbonate [19–21]. However, these SS are not among the most consumed by middle-distance runners, with the consumption of vitamins, minerals and amino acids being higher than the previously mentioned ones [22].

Although SS can provide both health and performance benefits, athletes' knowledge of them is sometimes limited [23,24]. In the same way, it has been shown that the use of some SS with less scientific evidence is greater than those with higher levels of supporting research [25]. Finally, some of the main motivators for their consumption are unqualified individuals, such as friends, teammates or the runners themselves [26–29].

To our knowledge, few studies have been conducted to analyze supplementation patterns in athletes, and no one exclusively in middle-distance runners. Thus, the objective of this research is to know the supplementation trends in those athletes with respect to their level and gender. On the other hand, it aims to assess whether the SS taken by middle-distance runners are those with the most scientific evidence, thus reducing the existing gap in the literature [30].

2. Materials and Methods

2.1. Type of Study

The research was a descriptive and cross-sectional study. The sample was selected using non-probabilistic, non-injurious and convenience sampling among training groups and individual middle-distance athletes at the national level.

2.2. Participants and Study Sample

A total of 106 middle-distance runners (800–1500 m) participated, of which 74 were men and 32 were women (gender assigned at birth). Only two requirements were established to participate in the study, which were as follows: (1) be over 18 years of age (legal age in Spain); (2) be currently performing middle-distance disciplines. The level of the athletes was differentiated by their area of competition, which could be regional (competing at regional or provincial level), national (competitions in Spain) or international (competitions at European and World level). Table 1 describes the age, basic anthropometric data and best performances in middle-distance events of the participants involved in the research.

2.3. Instruments

The questionnaire chosen for this research has been previously used in studies with the same objectives carried out in other sports [26,31,32]. This one was chosen for two main reasons; on the one hand, for its contents, structure, applicability and ease of completion for the athletes. The second reason was the quality of the questionnaire, which was created by 25 experts from different areas and achieved a 54% methodological validity, being one of the 57 questionnaires (out of 167) validated to obtain accurate data on supplement

consumption [33]. The questionnaire has 4 main parts and a total of 32 questions. The first one asks for personal (e.g., sex), anthropometric (e.g., height, weight) and sociodemographic (e.g., region of residence) data, with a total of 8 questions. The second, with a total of 5 questions, covers topics about the sport practice (e.g., years of practice, level of competition). The third part, with a similar objective, collects information about your best times in the different middle-distance disciplines or about your training days and number of competitions and has a total of 8 questions. Finally, the fourth part (11 questions) covers the area of supplementation, with questions such as: what supplements do they consume, reason for consumption, and place of purchase. This questionnaire collects data about all types of supplements, among which we find sports foods (e.g., energy bars, sports gels), medical supplements (e.g., iron, vitamin D, multivitamins) or performance supplements (e.g., caffeine, creatine, ß-Alanine). These different types of supplements are defined as sport supplements in the current study. From this last section, different questions related to diet were eliminated from the original questionnaire because they did not contribute to the objective of the study and in order to limit the response time. This questionnaire can be obtained in: Suplementación nutricional en la actividad físico-deportiva: análisis de la calidad del suplemento proteico consumido [34].

Table 1. Characteristics and personal times of the different subjects.

Sex (n)	Category (n)	Age	Height *	Weight *	BMI *	PB 800 m	PB 1500 m
Male (74)	Regional (29)	22.9 ± 5.7	176.3 ± 8.2	64.8 ± 9.1	18.3 ± 2.0	2:01.74 ± 6.80	4:17.22 ± 16.31
	National (43)	24.5 ± 7.6	177.6 ± 6.4	65.1 ± 6.1	18.3 ± 1.4	1:56.42 ± 5.25	4:02.92 ± 16.50
	International (2)	20.0 ± 2.8	189.0 ± 5.7	68.5 ± 4.9	18.1 ± 0.8	1:48.38 ± 1.15	3:47.50
Female (32)	Regional (11)	23.6 ± 8.2	165.0 ± 4.8	52.4 ± 7.0	15.8 ± 1.7	2:26.04 ± 7.04	5:13.66 ± 21.95
	National (17)	21.8 ± 3.1	164.8 ± 4.3	52.5 ± 4.1	15.9 ± 1.1	2:15.56 ± 6.55	4:59.61 ± 43.06
	International (4)	21.0 ± 2.9	167.5 ± 4.8	55.0 ± 3.2	16.4 ± 0.8	2:05.27 ± 3.84	4:15.23 ± 9.97

Results are expressed as mean ± SD. BMI: body mass index. * Self-reported height and weight. BMI calculated from self-reported height and weight. PB: personal best. Gender assigned at birth.

2.4. Procedures

For the data collection, the questionnaire was distributed via training groups, known athletes and social networks. The questionnaire was distributed online so that runners could complete it remotely, voluntarily and anonymously. The protocol complied with the provisions of the Declaration of Helsinki for human research and was approved by the ethical committee of the University of Deusto (ETK-14/23-24) dated 26 October 2023.

2.5. Statistical Analysis

To verify whether the variables had a normal distribution, a Kolmogorov–Smirnov test was applied, and Levene's test was used to verify homoscedasticity. The quantitative data were presented as mean + SD, while the qualitative variables were expressed as percentages and frequencies. A two-way ANOVA was performed for the sex factor (male–female) and level of competition (regional, national and international) to analyze the differences in the total consumption of SS, as well as the SS consumed from the different categories. To assess sex differences, a *t*-test for independent variables was performed, while to assess differences among competition levels, a one-way ANOVA was performed. For those variables in which significant differences were found, the Bonferroni post hoc analysis was used. Regarding the analysis of the athletes who consumed SS, the reason for consumption, the place where they obtained them and who advised them to consume them, a chi-square ($\chi 2$) test was used to verify the existence or not of differences between athletes of different sex and level of competition. As for the SS that were consumed by at least 10% of the sample, a $\chi 2$ test was performed to verify possible differences according to sex or level of competition. The level of statistical significance was established as $p < 0.05$. The statistical analysis was carried out using the Statistical Package for Social Sciences (SPSS) software v.28.0.0 (IBM, Armonk, NY, USA) for Windows.

3. Results

3.1. General Consumption of Sport Supplements

Of the total sample, 85.85% reported consuming supplements, while 15 of the 106 subjects responded that they did not consume any type of sport supplement. Regarding sex, supplement consumption was higher in men (89.2%) than in women (78.1%), with no statistical differences between them ($p = 0.143$). In the analysis of the results by level of competition, the percentage of autonomous athletes who consumed supplements was 77.5%, in athletes at the national level it was 90.0%, while in athletes who competed at the international level the consumption was 100%, with no differences between levels ($p = 0.126$).

Table 2 shows the supplements consumed according to the different categories established by the AIS. With respect to total supplement consumption, differences were observed at the competitive level between international and regional athletes ($p = 0.011$). However, no differences were appreciated based on sex (F = 2.248; $p = 0.466$), with a total consumption of 4.8 ± 3.7 and 5.4 ± 5.7 for men and women, respectively. No interactions were observed between level and sex (F = 0.306; $p = 0.737$).

Table 2. Descriptive data of the SS consumed according to the different categories defined by the AIS as a function of sex and level of competition.

	Variable	Sex		Level of Competition							
		M	F	R	N	I			Total		
		Mean ± SD	Mean ± SD	Mean ± SD	Mean ± SD	Mean ± SD	Med	IQ	Mean ± SD	Med	IQ
	Total SS	4.8 ± 3.7	5.4 ± 5.7	4.0 ± 3.8	5.1 ± 3.7	9.5 ± 9.6	6.0	26	5.0 ± 4.4	5.0	27
Group A	Sports food	1.1 ± 1.0	1.0 ± 1.2	1.0 ± 1.1	1.1 ± 1.0	1.2 ± 1.5	0.5	3	1.1 ± 1.1	1.0	4
	Medical supplement	0.4 ± 0.6	0.8 ± 0.9	0.3 ± 0.5	0.6 ± 0.8	1.3 ± 1.0	1.0	3	0.52 ± 0.7	0.0	3
	Performance supplement	1.0 ± 1.1	0.9 ± 1.1	0.8 ± 1.1	1.0 ± 1.9	1.7 ± 1.6	1.5	4	1.0 ± 1.1	1.0	4
	Total Group A	2.5 ± 1.9	2.7 ± 2.4	2.1 ± 2.0	2.7 ± 1.9	4.2 ± 3.7	3.0	10	2.5 ± 2.1	2.0	10
	Group B	0.5 ± 0.7	0.5 ± 0.7	0.5 ± 0.6	0.5 ± 0.7	1.2 ± 1.2	1.0	3	0.5 ± 0.7	0.0	3
	Group C	0.5 ± 0.6	0.3 ± 0.4	0.3 ± 0.6	0.5 ± 0.6	0.4 ± 0.6	0.5	1	0.4 ± 0.6	0.0	2

AIS: Australian Institute of Sport; SS: sport supplements; SD: standard deviation; M: male; F: female; R: regional; N: national; I: international; Group A: supplements with solid scientific evidence in specific situations under established protocols; Group B: components with emerging evidence that should be used in research or clinical settings; Group C: supplements with limited evidence and effects on performance; gender assigned at birth.

For Group A, no differences were observed between sexes or levels or for the sex–level interaction for the sports food, performance supplement or total intake. However, differences were observed for the group of medical supplements between competition levels (international athletes, $p = 0.004$ vs. regional and $p = 0.037$ vs. national athletes), with consumption being higher as the level of the athletes increased. Likewise, differences between sexes were noted in this group (F = 3.797; $p = 0.002$), with higher consumption in women than in men (0.4 ± 0.6 vs. 0.8 ± 0.9). Table 3 describes the differences between supplement consumption according to level of competition, sex and the interaction between both. Regarding Group B, no differences were observed between sexes (F = 1.591; F = 0.860), levels of competition (F = 2.656; $p = 0.075$) or the interaction between sex and level of competition (F = 0.279; $p = 0.860$). Finally, for group C supplement consumption, differences were observed with respect to sex (F = 13.297; $p = 0.029$), with 0.5 ± 0.6 vs. 0.3 ± 0.4 for males and females, respectively. However, no differences were seen between levels or for the sex–level-of-competition interaction.

3.2. Most-Consumed Supplements by Competitive Level and Sex

Table 4 shows those supplements that were consumed by more than 10% of the sample. The most-consumed supplements were caffeine (37%), followed by energy bars and sport drinks (34% for both) and creatine (31.1%). With respect to sex, differences were only observed for iron consumption ($p < 0.001$), with higher consumption in women than in men (17.6% vs. 56.3%). Differences between levels were observed for recovery shakes

(83.3% vs. 20% vs. 7.5%, $p < 0.001$; for international, national and regional athletes) and vitamin D (50.0% vs. 18.3% vs. 10.0%, $p = 0.047$; for international, national and regional athletes). The most-consumed supplements in the sport food subgroup for women were sport drinks (34%), contrary to men where the use of sports bars was a little bit higher (36.5%). Regarding medical supplements, iron was the main supplement for both sexes (17.6 vs. 56.3 for male and female).

Table 3. ANOVA of the SS consumed according to the different categories defined by the AIS as a function of sex, level of competition and their interaction.

	Variable	Sex		Level of Competition		Sex–Level-of-Competition (Mean ± SD)							
						R		N		I		F	*p*
		F	*p*	F	*p*	M	F	M	F	M	F		
	Total SS	2.248	0.466	4.582	0.012 #	4.0 ± 3.6	3.9 ± 4.5	5.1 ± 3.4	5.2 ± 4.5	7.5 ± 9.1	10.5 ± 11.1	0.306	0.737
Group A	Sports food	1.330	0.726	0.102	0.903	1.1 ± 1.1	0.8 ± 1.1	1.1 ± 0.9	1.1 ± 1.4	1.5 ± 2.1	1.0 ± 1.4	0.270	0.764
	Medical supplement	3.797	0.002 *	5.693	0.005 #$	0.24 ± 0.4	0.6 ± 0.7	0.5 ± 0.7	0.8 ± 0.9	0.5 ± 0.7	1.8 ± 1.0	1.138	0.325
	Performance supplement	0.014	0.592	2.167	0.120	0.8 ± 1.1	0.6 ± 1.3	1.1 ± 1.1	0.8 ± 0.7	1.5 ± 2.1	1.8 ± 1.7	0.162	0.850
	Total group A	0.671	0.560	3.066	0.051	2.1 ± 2.0	2.0 ± 2.3	2.7 ± 1.8	2.8 ± 2.1	3.5 ± 4.9	4.5 ± 3.7	0.163	0.849
	Group B	1.591	0.860	2.656	0.075	0.5 ± 0.7	0.5 ± 0.5	0.5 ± 0.8	0.4 ± 0.5	1.0 ± 1.4	1.3 ± 1.3	0.279	0.757
	Group C	13.297	0.029 *	1.884	0.157	0.3 ± 0.7	0.1 ± 0.3	0.6 ± 0.6	0.3 ± 0.5	0.5 ± 0.7	0.5 ± 0.6	0.151	0.860

AIS: Australian Institute of Sport; SS: sport supplements; SD: standard deviation; M: male; F: female; R: regional; N: national; I: international; Group A: supplements with solid scientific evidence in specific situations under established protocols; Group B: components with emerging evidence that should be used in research or clinical settings; Group C: supplements with limited evidence and effects on performance; gender assigned at birth. * Statistical difference at $p < 0.05$ between male and female. # Statistical difference at $p < 0.05$ between regional and international athletes. $ Statistical difference at $p < 0.05$ between national and international athletes.

Table 4. Distribution (%) of the most-consumed supplements (>10%) as a function of sex and level of competition according to the categories defined by the AIS.

	Category	Supplement Name	Total (%)	Sex (%)			Level of Competition (%)			
				M	F	*p*	R	N	I	*p*
Group A	Sports foods	Sport bars	34.0	36.5	28.1	0.273	30.0	38.3	16.7	0.451
		Sport drinks	34.0	33.8	34.4	0.561	27.5	38.3	33.3	0.533
		Sports gel	21.7	21.6	21.9	0.582	22.5	21.7	16.7	0.949
		Whey protein	30.2	29.7	31.3	0.525	25.0	31.7	50.0	0.429
		Recovery shakes	18.9	20.3	15.6	0.394	7.5	20.0	83.3	<0.001 *
	Medical supplements	Iron	29.2	17.6	56.3	<0.001 *	22.5	30.0	66.7	0.084
		Vitamin D	17.0	14.9	21.9	0.269	10.0	18.3	50.0	0.047 *
	Performance supplements	β-Alanine	20.8	20.3	21.9	0.521	12.5	23.3	50.0	0.081
		Caffeine	37.7	36.5	40.6	0.424	35.0	36.7	66.7	0.318
		Creatine	31.1	36.5	18.8	0.054	20.0	38.3	33.3	0.151
	Group B	Vit C	19.8	20.3	18.8	0.542	17.5	20.0	33.3	0.662
	Group C	BCAA	10.4	12.2	6.3	0.295	10.0	10.0	16.7	0.873
		Glutamine	11.3	12.2	9.4	0.482	5.0	15.0	16.7	0.276

AIS: Australian Institute of Sport; M: male; F: female; R: regional; N: national; I: international; Group A: supplements with solid scientific evidence in specific situations under established protocols; Group B: components with emerging evidence that should be used in research or clinical settings; Group C: supplements with limited evidence and effects on performance; gender assigned at birth. * Statistical difference at $p < 0.05$.

For performance supplements, differences were observed with caffeine and creatine being the most consumed for men (36.5%) and only caffeine for women (40.6%). Finally, for group C, both BCAA and glutamine were the most-consumed ones for males (12.2%), but not for females (glutamine = 9.4%). As for the level of the athlete, the most-consumed supplements for international athletes were recovery shakes (83.3%), followed by iron and caffeine (66.7%). The national-level athletes' most-consumed supplements were creatine, sports bars and sport drinks (38.3%), while caffeine was the most-consumed one by regional athletes (35%).

3.3. Information about the Place of Purchase, Recommendations and Consumption Patterns

Most athletes took supplements on training and competition days (39.62%). The daily consumption of supplements was 26.42%, followed by training (14.15%) and competition (11.32%). No differences were observed between genders ($p = 0.106$) as opposed to between categories for daily consumption ($p = <0.00$). Thus, 33.3% of the international athletes consumed it daily, while only 18.3% or 7.5% did so in the case of national and regional ones. In analyzing the moment of consumption, most of the sample used them after (56.60%) or before (50.94%) practicing exercise, followed by during training (20.75%). Only a small percentage responded that it was taken during the holiday period (1.89%) or indifferently (7.55%). No differences were observed for levels but between levels for pre- and post-training consumption ($p = 0.007$), which varied according to the level of competition (33% vs. 20% vs. 25% for international, national and regional athletes, respectively).

The principal objective of consumption was to improve performance (70.75%), followed by taking care of their health (35.85%) and palliating dietary deficits (16.98%). Finally, of the 106 middle-distance runners, only 6.60% consumed them for health problems or necessity (3.77%). In this area, no differences were observed between sexes ($p = 0.564$) or levels of competition ($p = 0.086$). The primary place of purchase was the internet (51.89%), followed by specialized stores (26.42%) or a pharmacy (24.54%). Other minority sources of purchase were herbalists (12.26%), sports monitors (3.77%), friends (1.89%) or parapharmacies (0.94%), with no statistically significant differences ($p = 0.082$ and $p = 0.545$ for gender and level). Finally, those who encouraged the use of SS were mainly coaches (37.74%), followed by dieticians–nutritionists (26.42%), teammates (21.70%) or physicians (16.04%). There were other people and sources that recommended its use such as friends and the internet (8.49%) or social network profiles (4.72%). Likewise, there were no differences between levels ($p = 0.919$) or genders ($p = 0.410$).

4. Discussion

The main objective of this study was to analyze the supplementation patterns in middle-distance runners, as well as the differences between genders and level of competition. The results indicate that the main differences between levels are observed both in total consumption and in the intake of medical supplements, with these being greater as the level of the athlete increases. Similarly, differences between levels were also observed in the consumption of medical supplements, as well as in pre- and post-training intake. This indicates that, although most athletes place emphasis on performance enhancement via supplementation, higher-level athletes also use these aids to maintain a better state of health and recover between sessions.

Of the total sample, 85.85% responded that they consumed SS, which was higher than the consumption in other disciplines such as fencing or sailing [26,35], but lower than in sports such as rowing, trail running or tennis (100%, 93.8% and 88.6%, respectively) [25,29,31]. No differences were noted for sexes or competition levels, in line with recent research [25,35]. Comparing the data obtained with a sample of athletes from different disciplines, the consumption of SS in middle-distance runners is higher (85% vs. 77%) [36]. Although there have been previous attempts to investigate supplementation patterns in athletes [22], one contained a limited sample while the other had only a few supplements [33,37] and no one has conducted it exclusively in middle-distance athletes. Therefore, this is the first to do so using a representative sample of middle-distance event participants and a broad list of SS.

With respect to total SS consumption, there were differences between international and regional athletes, which had been previously noted in all types of sportsmen and women [33]. With respect to the different groups established by the AIS according to the level of evidence [13], in group A, no differences were observed between levels and genders, contrary to other recent studies [32,35,38]. However, differences between levels were close to being statistically significant ($p = 0.051$). Within the subgroups that exist in group A, only differences in medical supplements are observed for gender, which may be

primarily due to the higher consumption of iron among women compared to men (56.3% vs. 17.6%). For the level of competition, differences were also observed in this subgroup, being higher as the level increased. These two findings are contrary to the results from other sports, where no differences have been observed for this subgroup between athlete levels or genders [26,29,35,39].

Regarding group B, which includes SS with emerging evidence but in need of future research, no differences are observed for level and gender, in line with the results in other sports [25,26,29,35,39]. Finally, in group C (supplements with insufficient scientific evidence to support its use), differences were noted between sexes, in line with some [25], but not all, recent evidence [26,35,39]. This could be due to the athlete's knowledge, which is worse as the level of competition decreases [40].

Taking into account the days of sport practice when they usually take the SS, 39.62% responded that they take them during training and competition, followed by daily consumption and solely on training days, at 26.42% and 14.15%, respectively. Although the main sporting day is similar to that of other sports such as mountain running or rowing [25,29,39], the second and third causes differ between sports. This could be due to differences in the physiological demands of each event, as well as the average duration and energetic requirements of training sessions. Differences were noted in daily consumption between levels of competition, indicating that the main difference between higher- and lower-level athletes was the use of medical supplements on a daily basis. On the other hand, the majority of middle-distance athletes take SS after (56.60%) or before sports practice (50.94%), while a lower percentage take them during sports practice. The duration of middle-distance sessions rarely surpasses 90–120 min [7], while other sports training sessions usually exceed this time, in which they will need to provide higher nutrition and hydration [25]. Here too, differences between levels are observed for pre- and post-consumption, demonstrating how top-level runners place greater importance on preparing for training or recovering for an upcoming workout. In analyzing the reasons for its consumption, the main one is to improve their performance (70.75%), followed by health care (35.85%) and to palliate a dietary deficit (16.98%), similar to other sport disciplines [26,29,31,32,35,36].

Concerning the person who motivated the consumption of SS, the main motivator was the coach (37.74%), followed by dietitians–nutritionists (26.42%), which showed a worse advisor in the case of middle-distance runners compared to other sports [25,39]. The next advisors were teammates, followed by physicians, indicating the existence of other sports modalities with a worse source of support [26,35]. In this sense, dietitians–nutritionists are the most appropriate when choosing one supplement or another regardless of the level of scientific evidence [26,41,42]. Finally, most athletes purchased SS on the internet (51.89%), followed by specialized stores (26.42%) and pharmacies (24.53%). In this sense, both pharmacy and internet products may contain quantities different from those advertised or contaminated substances, which may also put the athletes at risk of unintended doping [43], so athletes tend to go to specialized stores in order to avoid these problems [12,42].

Finally, with regard to the most-consumed SS, we can appreciate caffeine in the first place. Caffeine is a natural stimulant for the central nervous system, possesses various suggested benefits for enhancing performance and is one of the supplements with the highest scientific evidence supporting its use [15]. These advantages encompass enhanced neuromuscular functionality and a decrease in fatigue and perceived effort levels during physical exertion, among others [44]. The following most-consumed SS were sport drinks (formulated to provide a balanced combination of carbohydrates and liquids, facilitating athletes in rehydrating and replenishing energy simultaneously during and after their workout) and sport bars (created as a portable source of carbohydrates, helping meeting carbohydrate intake goals) [13], which also belong to Group A, such as caffeine. These two supplements help mainly in carbohydrate replenishment post or during training or to reach the recommended daily intake of carbohydrates, which can be up to 70% of the total diet or around 6–12 $CHO \cdot kg^{-1} \cdot BW \cdot day^{-1}$. In this sense, carbohydrate intake both

during [45] and immediately after [46] exercise limits fatigue and improves performance in the following training sessions.

The next most-consumed supplement was isolated protein, with considerable scientific evidence supporting its use [13], which appears necessary both for the recovery and repair of damaged myofibrillar proteins and to optimize mitochondrial and possibly sarcoplasmic protein synthesis [47]. However, this seems unnecessary in most cases, since athletes tend to consume more protein than any high recommendation [47]. Continuing with the SS that can provide more benefits among those consumed by more than 10% of the sample, we find iron or β-Alanine. Iron plays a fundamental role in the transport of oxygen and a high prevalence of anemia has been observed among middle-distance runners [22]. A small decrease in hemoglobin content (subclinical anemia) leads to a significant decrease in oxygen transport capacity and, therefore, a decrease in performance [48]. Thus, it is important to monitor these variables on a recurring basis in order to supplement if necessary. On the other hand, β-Alanine acts as an intracellular buffer by increasing the concentration of muscular carnosine [49]. Since high-intensity exercise (usually performed by middle-distance runners both in training and competition [7]) increases the amount of hydrogen ions and lowers the intracellular pH from 7.0 to 6.6, supplementation with β-Alanine may improve the ability to withstand this drop, limiting muscular fatigue. However, the determination of whether supplementation enhances performance in elite middle-distance athletes is challenging due to insufficient data and non-performance-related tests [47]. Despite this, considering the absence of side effects and potential performance benefits, individual athletes and their support teams may want to try β-Alanine supplementation to assess its effectiveness for them [1]. Finally, it is important to note the very low percentage of athletes using inorganic nitrates or beetroot juice as SS (6.60%). This supplementation seems to improve performance via the bioavailability of nitric oxide, improving exercise efficiency (decreased O^2 cost at the same absolute workload) [50]. However, this low use may be due to variability in the response to its supplementation [1] or decreased effects as the physiological capabilities of the athletes increase [50].

It is important to mention that, although a large part of the SS consumed by middle-distance runners in this study belong to group A, it is also observed that there is still a fairly large consumption of supplements with little or no scientific evidence (groups B and C). This has also been observed in other sports, so it is important that athletes use reliable sources of information when deciding which supplements to consume [25,51]. In addition, the present research has several limitations. First of all, the sample is larger than that of other studies with the same population, but a greater participation of international athletes is necessary. In addition, it was the athletes themselves who responded retrospectively to the consumption of SS, which could lead to errors in the number or type of supplements. Therefore, it is necessary to compare and have the support of different federations or institutions worldwide to check if the consumption is similar depending on the competitive level or gender.

5. Conclusions

Supplement consumption in middle-distance running is similar to that in other sports. The main differences between levels are seen in the total supplement consumption and in the consumption of medical supplements, as well as in daily or pre- and post-exercise consumption, with these being higher as the level of competition increases. On the other hand, the differences between sexes are found in the consumption of both medical supplements and supplements with limited evidence. Middle-distance runners should improve both their sources of information and places of purchase in order to avoid supplements with low scientific evidence or contaminated/fraudulent products.

Author Contributions: Conceptualization, A.D.A. and A.C.-B.; Methodology, A.D.A., A.M.A.-B. and E.M.-M.; Software, A.M.A.-B.; Formal Analysis, A.D.A., A.M.A.-B. and A.G.; Investigation, A.D.A. and A.M.A.-B., Data Curation, A.D.A. Writing—Original Draft Preparation, A.D.A.; Writing—Review

& Editing, A.M.A.-B., E.M.-M., A.G. and A.C.-B.; Supervision; A.C.-B. Project Administration; A.C.-B. All authors have read and agreed to the published version of the manuscript.

Funding: This research received no external funding.

Informed Consent Statement: Informed consent was obtained from all subjects involved in the study. The protocol complied with the provisions of the Declaration of Helsinki for human research and was approved by the ethical committee of the University of Deusto (ETK-14/23-24) dated 26 October 2023.

Data Availability Statement: Data of the article are available in the tables of this paper or on request from the corresponding author.

Acknowledgments: Asier del Arco gives thanks for the distribution of the questionnaire and dedicates the article to the memory of Carlos and Angel Basas, for their contributions to the world of athletics at the national and world levels.

Conflicts of Interest: The authors of the present article declare no conflict of interest.

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Article

Dietary Habits of Elite Soccer Players: Variations According to Competitive Level, Playing Position and Sex

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Abstract: Soccer is a sport practiced worldwide by both men and women, where nutrition plays a fundamental role in the performance of soccer players, providing them with the nutrients necessary for energy, muscle recovery and injury prevention. The aim of this study is to describe the dietary habits in elite soccer players and their association with their competitive level, playing position and sex. A descriptive and non-experimental comparative study was conducted during the 2021–2022 competitive season. A total of 105 players belonging to a Spanish elite soccer team completed a food frequency questionnaire (FCFQ). It was observed that male players presented a higher consumption of carbohydrate-rich foods ($p < 0.05$), fermented foods ($p = 0.014$), frozen foods ($p = 0.049$) and red meat ($p = 0.012$) compared to female players, with the exception of lean meats, which were higher in females ($p = 0.012$). Furthermore, the U16-15 categories stand out for consuming carbohydrate-rich foods such as pasta ($p = 0.000$), bread ($p = 0.004$) and sweets ($p = 0.046$), as well as frozen foods ($p = 0.002$). Finally, alcohol consumption is higher in the senior categories (42.9%), where men are more likely to drink mixed drinks (6.2%), and beer and wine by women (10.7%). Practically no differences were found between the playing positions. In conclusion, differences were found in FCFQ according to competitive level and sex.

Keywords: proteins; football; food; soccer; sport nutrition; carbohydrates; dietary fats

Citation: Sebastiá-Rico, J.; Soriano, J.M.; Sanchis-Chordà, J.; Alonso-Calvar, M.; López-Mateu, P.; Romero-García, D.; Martínez-Sanz, J.M. Dietary Habits of Elite Soccer Players: Variations According to Competitive Level, Playing Position and Sex. *Nutrients* **2023**, *15*, 4323. https://doi.org/10.3390/nu15204323

Academic Editors: Joerg Koenigstorfer, Valentín E. Fernández-Elías and Olga López Torres

Received: 31 August 2023
Revised: 28 September 2023
Accepted: 9 October 2023
Published: 10 October 2023

1. Introduction

Soccer is a team sport played by men and women in constant evolution, as it has experienced an increase in the physical and technical demands, as well as increased economic implications of winning or losing [1]. It consists of 22 male or female players on the field, grouped into two teams of 11 members, that confront each other with the aim of scoring a goal [2]. During training sessions and matches, players engage in a wide range of activities, which encompass both low- and high-intensity efforts. These activities include intermittent exercises of extended duration, such as walking, jogging, running at various speeds (both high and low), sprinting, moving in reverse, kicking, jumping, and tackling [1]. Moreover, studies have determined that, on average, players exhibit an oxygen uptake of approximately 70% of their maximum capacity during a match, with heart rates typically maintained at around 85% of their maximum levels [1,3]. There are two main categories: the base and the professional one, with the base category (U19-10) being the one played

by young people before reaching the professional leagues (1st and 2nd in Spain) [4]. It is well known that body composition [5–7], as well as hydration, healthy eating habits and supplementation [3,8,9], are associated with physical performance among elite athletes. Understanding how these factors, along with playing position, may influence the nutritional intake of soccer players is essential for the design of nutrition education programs.

A study that analyzed the distance covered by soccer players found that wide defenders, central midfielders and wide midfielders covered a greater distance than center forwards, meanwhile the distance covered by center forwards was greater than central defenders' distance [10]. Moreover, the intensity used to cover this distance also changes from one position to another, with the wide defenders, center forwards and wide midfielders being the ones who performed the greatest number of runs at very high intensity [11]. In addition, it has recently been observed that, depending on the playing position, the soccer player shows different body composition characteristics [7]. These results imply that individual differences in playing style need to be taken into account when planning nutritional and training strategies [12].

As mentioned above, nutrition plays a huge role in providing athletes the energy they require to meet their physical demands [3,8]. In addition, young athletes are in a phase of growth, development and dealing with body changes [13,14], which can be a great opportunity to promote healthy behaviors towards physical activity and diet, since these behaviors will continue into adulthood and impact long-term health and weight [15,16]. A few studies [17–19] that assessed the eating habits of professional male and female soccer players found that their nutrition intake was inadequate to sustain optimized performance throughout training and match play. This meta-analysis [20] gave an insight into the development of a macronutrient diet in senior and junior soccer players, finding a higher protein intake than the recommended and a carbohydrates (CHO) intake below the recommendations.

This other study that assessed the eating habits of junior soccer players showed a deficit in nutrient-rich foods, especially vegetables and fruits, and an excessive consumption of low-nutrient foods, highlighting above all the consumption of alcoholic beverages and soft drinks [21]. Those are valid reasons to think that nutrition education interventions are needed in team sports [22], since it has been shown that increasing an athlete's nutrition knowledge can optimize physical performance [19,23,24] and lead to better dietary behaviors [25]. In this matter, there are several intervention tools that can be used to promote healthy eating behaviors in soccer players (i.e., posters, web apps, through activities), but the combination of a poster and a web app seems to be the most practical one in terms of providing the right information and helping them maintain those habits [26].

Lastly, the COVID-19 pandemic affected both daily training and absence from competitions, highlighting the importance of adjusting players' dietary habits and eating patterns in order to preserve both their optimal health and performance in a context characterized by constant change [27,28].

Despite the fact this sport is widely practiced and accepted in our society, there is limited evidence about the eating habits of minors who play soccer and further differentiating between males and females. Therefore, the aim of this study is to describe the eating habits in elite soccer players and their association with their competitive level, playing position and sex. Accordingly, the following was initially hypothesized:

Hypothesis (H1). *Dietary habits will be similar among the different playing positions.*

Hypothesis (H2). *Dietary quality will be worse in the lower categories compared to the senior categories.*

Hypothesis (H3). *Men will consume more CHO, while women will consume more foods rich in healthy fats and lean proteins.*

2. Materials and Methods

2.1. Type of Study

This is a descriptive, cross-sectional and non-experimental study of dietary habits in elite soccer players of both sexes belonging to the Valencia C.F. Academy. The assessment was made in the month of May during the competitive season 2021–2022. The sample size calculation was performed with Rstudio software (version 3.15.0, Rstudio Inc., Boston, MA, USA). The significance level was set a priori at $p = 0.05$. The standard deviation (SD) was set according to the total SS data from previous studies on elite Spanish athletes (DE = 2.1) [29]. With an estimated error (d) of 0.49, the sample size needed was 70 athletes. The study population was selected by non-probabilistic, non-injury, convenience sampling among elite soccer players of both sexes belonging to the Valencia C.F. Academy.

2.2. Participants

A total of 100% of the Valencia Mestalla (n = 21), Juvenil A (n = 22), Juvenil B (n = 23), Cadete A (n = 32), Cadete Fundacions (n = 33), Valencia CF Femenino (n = 15) and Valencia CF Femenino B (n = 13) templates were included for the food consumption frequency questionnaire (FCFQ). However, as it was a voluntary questionnaire, it was finally completed by 110 of 159 soccer players (69.2%) (13 Valencia Mestalla players, 17 Juvenil A players, 16 Juvenil B players, 17 Cadete A players, 18 Cadete Fundacions players and all Valencia CF Femenino players (15) and Valencia CF Femenino B players (13)). All players had at least 4 years of soccer training experience and performed from 4 up to 7 regular training sessions per week (approximately 90 to more than 120 min per day), playing a theoretical official soccer match per week. The criteria for inclusion in this study were as follows: (a) be a healthy subject with medical authorization for the practice of federated sport; (b) belong to a team Valencia C.F. Academy; (c) being federated in soccer; (d) training a minimum of 4 days per week. The exclusion criteria for this study were as follows: (a) having been injured or having become ill during this study. Food portion size was estimated using photographs of a standardized portion, as well as an exact amount in grams or mL.

2.3. Procedure

In order to select the sample, the Valencia C.F. Academy sent a statement informing players of the execution of this study, instructions and inviting resident and non-resident players to collaborate. Before the players filled out/completed the questionnaire, subjects were informed about the purpose of this study. Informed consent was obtained and signed by those responsible for this study, as well as by the medical and coaching staff of the Valencia C.F. Academy. Each participant and their respective parents or legal guardians also signed it. The questionnaire was delivered electronically through a Google Form. The protocol complies with the Declaration of Helsinki for human research and is approved by the Ethics Committee of the University of Valencia (1534145).

2.4. Instruments

This study utilized a questionnaire that had been previously used in similar studies [21,30,31]. The selected questionnaire was validated for content, applicability, structure and presentation by University College Dublin and Crème Software Ltd., (Food4Me FFQ). It is a self-administered, online and semiquantitative food frequency questionnaire [32], with its Spanish version prepared by Bejar LM and collaborators [33]. It contains a total of 24 questions divided into two main sections. The first section collects the age, the team in which the subject plays (which identifies the subject's sex) and the playing position. This section consists of 3 questions. The second section includes 21 questions that collated the consumption of several food and beverage groups over the previous month (average consumption of 157 food items): fruits, vegetables, pulses, white fish, blue fish, white meat, red meat, soft drinks and juices, sweets and 'snacks', 'fast food', and alcohol (beer, wine, mixed drinks, etc.). Frequency of consumption was measured by selecting one of the following options: never or less than once a month, 1–3 times a month, once a week,

2–4 times a week, 5–6 times per week, once a day, 2–3 times per day, 5–6 times per day, and >6 times per day.

The questionnaire can be found in the Supplementary Material section.

2.5. Statistical Analysis

The Kolmogorov–Smirnov normality test was performed to evaluate that all variables had a normal distribution. Kurtosis was also evaluated and Mauchly's test of sphericity was performed to test the hypothesis of sphericity. Given the normal distribution of the data and with the aim of analyzing the frequency of food group and alcohol consumption, the chi-square test (χ^2) was performed, segmenting the sample according to sex, category and playing positions. The minimum level of statistical significance was set at $p < 0.05$. All data were analyzed using the Statistical Package for the Social Sciences (SPSS) version 25.0 (IBM, Armonk, NY, USA).

3. Results

Table 1 shows the frequency of food consumption according to category. Significant differences were found for the consumption of pasta ($p = 0.000$), bread ($p = 0.004$), chicken and turkey ($p = 0.042$), eggs and egg products ($p = 0.040$), red meat ($p = 0.035$), sweets ($p = 0.046$) and prepared or frozen foods ($p = 0.002$). It was observed that cadets had a higher consumption of pasta, bread, sweets and prepared or frozen foods, but a lower consumption of chicken and turkey, while juveniles were the category with the highest consumption of eggs and their derivatives and red meat. In relation to the different playing positions, significant differences were only found for red meat consumption ($p = 0.012$), with a higher consumption by forwards.

Table 2 shows the frequency of food consumption according to sex. Significant differences were found for the consumption of fruits ($p = 0.022$), legumes ($p = 0.001$), pasta ($p = 0.000$), rice ($p = 0.014$), chicken and turkey ($p = 0.012$), red meat ($p = 0.012$), fermented foods ($p = 0.014$) and prepared or frozen foods ($p = 0.049$). In all these food groups, and with the exception of chicken and turkey consumption, it was observed that men had a higher consumption than women, while for chicken and turkey consumption, it was women who had a higher consumption.

Table 3 shows the frequency of alcohol consumption according to category. Significant differences were found for the question of whether or not they consumed alcohol ($p = 0.000$), showing that the frequency increased as the category increased, with the senior category consuming the most. Significant differences were also found for the type of alcohol consumed ($p = 0.000$), with juniors drinking more mixed drinks and seniors drinking more beer and wine. In relation to the different playing positions, significant differences were found for the question of whether or not they consumed alcohol ($p = 0.028$), with goalkeepers (30%) and midfielders (27.6%) consuming the most. Significant differences were also found for the type of alcohol consumed ($p = 0.034$), with beer being the most consumed by goalkeepers, and combined drinks and beer the most consumed by midfielders.

Table 4 shows the frequency of alcohol consumption according to sex. Significant differences were only found for the type of alcohol consumed ($p = 0.024$), with women consuming more beer and wine, and men consuming more mixed drinks.

Table 1. Frequency (%) of food group consumption by soccer players of different competitive levels.

Items	Category (%)																		
	U16-15 (n = 35)						U19-17 (n = 46)						Senior (n = 28)						
	<1 Week	1–2 Week	3–4 Week	5–6 Week	1–2 Day	≥3 Day	<1 Week	1–2 Week	3–4 Week	5–6 Week	1–2 Day	≥3 Day	<1 Week	1–2 Week	3–4 Week	5–6 Week	1–2 Day	≥3 Day	*p*
Fruits	0.0	2.9	8.6	25.7	57.1	5.7	2.2	0.0	15.2	28.3	39.1	15.2	0.0	10.7	21.4	25.0	35.7	7.1	0.228
Vegetables	2.9	17.1	28.6	22.9	28.6	0.0	0.0	15.2	26.1	21.7	28.3	8.7	0.0	3.6	17.9	25.0	46.4	7.1	0.397
Vegetables rich in nitrates	28.6	22.9	31.4	17.1	0.0	0.0	19.6	28.3	28.3	13.0	10.9	0.0	21.4	35.7	32.1	3.6	7.1	0.0	0.441
Legumes	2.9	65.7	28.6	2.9	0.0	0.0	4.3	56.5	34.8	4.3	0.0	0.0	17.9	53.6	21.4	7.1	0.0	0.0	0.246
Tubers	2.9	34.3	48.6	14.3	0.0	0.0	4.3	23.9	45.7	21.7	4.3	0.0	3.6	21.4	50.0	17.9	7.1	0.0	0.834
Pasta	0.0	11.4	42.9	37.1	8.6	0.0	0.0	13.0	56.5	21.7	8.7	0.0	14.3	46.4	21.4	14.3	3.6	0.0	0.000
Rice	0.0	45.7	31.4	17.1	5.7	0.0	0.0	45.7	41.3	8.7	4.3	0.0	14.3	50.0	21.4	10.7	3.6	0.0	0.056
Bread	11.4	17.1	31.4	20.0	20.0	0.0	23.9	19.6	15.2	17.4	19.6	4.3	60.7	10.7	3.6	14.3	10.7	0.0	0.004
Whole grains	8.6	20.0	28.6	17.1	25.7	0.0	17.4	28.3	21.7	4.3	26.1	2.2	25.0	28.6	17.9	7.1	21.4	0.0	0.503
Chicken/turkey	0.0	22.9	45.7	17.1	14.3	0.0	0.0	15.2	39.1	41.3	4.3	0.0	7.1	7.1	53.6	21.4	10.7	0.0	0.042
Eggs and egg products	0.0	34.3	45.7	8.6	8.6	2.9	4.3	17.4	30.4	34.8	13.0	0.0	0.0	42.9	17.9	28.6	10.7	0.0	0.040
Fish	8.6	40.0	37.1	8.6	5.7	0.0	8.7	39.1	39.1	13.0	0.0	0.0	7.1	35.7	50.0	3.6	3.6	0.0	0.755
Red meat	8.6	45.7	34.3	2.9	8.6	0.0	6.5	39.1	41.3	10.9	2.2	0.0	25.9	37.0	25.9	0.0	3.7	7.4	0.035
Nuts	22.9	25.7	20.0	8.6	22.9	0.0	26.1	30.4	17.4	15.2	10.9	0.0	21.4	25.0	10.7	35.7	7.1	0.0	0.189
Fermented foods	2.9	37.1	20.0	28.6	11.4	0.0	8.7	34.8	17.4	26.1	13.0	0.0	25.0	21.4	35.7	10.7	7.1	0.0	0.070
Soft drinks	45.7	37.1	14.3	2.9	0.0	0.0	76.1	19.6	2.2	2.2	0.0	0.0	78.6	17.9	3.6	0.0	0.0	0.0	0.052
Pastries	37.1	54.3	8.6	0.0	0.0	0.0	67.4	30.4	2.2	0.0	0.0	0.0	57.1	42.9	0.0	0.0	0.0	0.0	0.046
Frozen foods	40.0	51.4	8.6	0.0	0.0	0.0	69.6	30.4	0.0	0.0	0.0	0.0	82.1	17.9	0.0	0.0	0.0	0.0	0.002

Table 2. Frequency (%) of food group consumption by male and female soccer players.

Items	Gender (%)												
	Women (n = 28)						Men (n = 81)						p
	<1 Week	1–2 Week	3–4 Week	5–6 Week	1–2 Day	≥3 Day	<1 Week	1–2 Week	3–4 Week	5–6 Week	1–2 Day	≥3 Day	
Fruits	3.6	10.7	25.0	21.4	28.6	10.7	0.0	1.2	11.1	28.4	49.4	9.9	0.022
Vegetables	0.0	7.1	32.1	17.9	32.1	10.7	1.2	14.8	22.2	24.7	33.3	3.7	0.491
Vegetables rich in nitrates	25.0	32.1	21.4	10.7	10.7	0.0	22.2	27.2	33.3	12.3	4.9	0.0	0.671
Legumes	21.4	53.6	14.3	10.7	0.0	0.0	2.5	60.5	34.6	2.5	0.0	0.0	0.001
Tubers	7.1	32.1	42.9	17.9	0.0	0.0	2.5	24.7	49.4	18.5	4.9	0.0	0.518
Pasta	14.3	42.9	25.0	14.3	3.6	0.0	0.0	13.6	49.4	28.4	8.6	0.0	0.000
Rice	14.3	42.9	32.1	7.1	3.6	0.0	0.0	48.1	33.3	13.6	4.9	0.0	0.014
Bread	50.0	17.9	7.1	14.3	10.7	0.0	22.2	16.0	21.0	18.5	19.8	2.5	0.085
Whole grains	25.0	28.6	25.0	3.6	17.9	0.0	13.6	24.7	22.2	11.1	27.2	1.2	0.521
Chicken/turkey	7.1	3.6	39.3	32.1	17.9	0.0	0.0	19.8	46.9	27.2	6.2	0.0	0.012
Eggs and egg products	3.6	28.6	28.6	25.0	14.3	0.0	1.2	29.6	33.3	24.7	9.9	1.2	0.914
Fish	7.1	46.4	39.3	3.6	3.6	0.0	8.6	35.8	42.0	11.1	2.5	0.0	0.720
Red meat	25.9	29.6	25.9	3.7	7.4	7.4	7.4	44.4	38.3	6.2	3.7	0.0	0.012
Nuts	32.1	32.1	10.7	14.3	10.7	0.0	21.0	25.9	18.5	19.8	14.8	0.0	0.603
Fermented foods	28.6	21.4	21.4	21.4	7.1	0.0	4.9	35.8	23.5	23.5	12.3	0.0	0.014
Soft drinks	82.1	17.9	0.0	0.0	0.0	0.0	61.7	27.2	8.6	2.5	0.0	0.0	0.162
Pastries	67.9	32.1	0.0	0.0	0.0	0.0	50.6	44.4	4.9	0.0	0.0	0.0	0.195
Frozen foods	82.1	17.9	0.0	0.0	0.0	0.0	56.8	39.5	3.7	0.0	0.0	0.0	0.049

Table 3. Frequency (%) of alcohol consumption by soccer players of different competitive levels.

Variables	Category (%)						Statistical Value
	U15-16 (n = 35)						
Alcoholic beverages	No 100.0				Yes 0.0		*p* 0.000
Type	Nothing 100.0	Mixed drinks 0.0		Beer 0.0	Wine 0.0	Champagne 0.0	*p* 0.000
Frequency	<1 week 100.0	1–2 week 0.0	3–4 week 0.0	5–6 week 0.0	1–2 day 0.0	≥3 day 0.0	*p* 0.248
Variables	U19-17 (n = 46)						Statistical value
Alcoholic beverages	No 89.1				Yes 10.9		*p* 0.000
Type	Nothing 89.1	Mixed drinks 8.7		Beer 2.2	Wine 0.0	Champagne 0.0	*p* 0.000
Frequency	<1 week 95.7	1–2 week 0.0	3–4 week 4.3	5–6 week 0.0	1–2 day 0.0	≥3 day 0.0	*p* 0.248
Variables	Senior (n = 28)						Statistical value
Alcoholic beverages	No 57.1				Yes 42.9		*p* 0.000
Type	Nothing 57.1	Mixed drinks 3.6		Beer 21.4	Wine 14.3	Champagne 3.6	*p* 0.000
Frequency	<1 week 100.0	1–2 week 0.0	3–4 week 0.0	5–6 week 0.0	1–2 day 0.0	≥3 day 0.0	*p* 0.248

Table 4. Frequency (%) of alcohol consumption by male and female soccer players.

Variables	Gender (%)						Statistical Value
	Women (n = 28)						
Alcoholic beverages	No 75.0				Yes 25.0		*p* 0.161
Type	Nothing 75.0	Mixed drinks 0.0		Beer 10.7	Wine 10.7	Champagne 3.6	*p* 0.024
Frequency	<1 week 100.0	1–2 week 0.0	3–4 week 0.0	5–6 week 0.0	1–2 day 0.0	≥3 day 0.0	*p* 0.401
Variables	Men (n = 81)						Statistical value
Alcoholic beverages	No 87.7				Yes 12.3		*p* 0.161
Type	Nothing 87.7	Mixed drinks 6.2		Beer 4.9	Wine 1.2	Champagne 0.0	*p* 0.024
Frequency	<1 week 97.5	1–2 week 0.0	3–4 week 2.5	5–6 week 0.0	1–2 day 0.0	≥3 day 0.0	*p* 0.401

4. Discussion

The aim of this study was to analyze the differences in the eating habits in elite soccer players by different competitive level, playing position and sex. To our knowledge, this is the first study analyzing these factors in elite soccer players from different level competitions, playing positions, and differentiating males and females. The main results showed a different food and alcohol intake according to the level of competition and sex.

4.1. Influence of Competitive Level on Nutrition

Dietary and physical exercise habits developed during the early years of life can have a lasting impact on long-term health and weight [34]. The nutritional approach aimed at young players faces the particular challenge of targeting individuals whose bodies undergo changes as they mature biologically, a process that does not always coincide with chronological age [3]. It was found that adolescents who were part of youth sports activities presented a greater tendency to consume fruits, vegetables and dairy products, although they also showed a greater inclination to ingest fast food and sugar-sweetened beverages compared to those who did not participate [35]. In addition, parents report poor availability of healthy food and beverage options at sporting events for youth athletes, contributing to the increased consumption of these products [36]. This was similar to our results, as the U16-15 were the soccer players who consumed the most sweets and frozen foods, with 8.6% of the subjects studied consuming both food groups between 3–4 times a week, and 54.3% and 51.4%, respectively, between 1–2 times a week. It is important to comply with the minimum recommendations for the frequency of consumption of some food groups such as ultra-processed foods because, although it has not been established for athletes as such, but for the general population, these are necessary in order to take care of health, physical recovery and body composition [3,37].

However, although there are previous studies highlighting a very low intake of healthy foods such as fruits and vegetables in youth stages, this has not been observed in our results, since in the case of the U16-15 category their daily consumption of one serving or more of fruits and vegetables was 62.8% and 28.6%, respectively, while consumption in the U19-17 categories was 54.3% and 37% [21]. Even so, it is true that there is still margin for improvement and it is necessary to continue applying food education so that all soccer players consume these foods on a daily basis, since fruits and vegetables are essential to obtain a correct supply of water, vitamins, minerals, antioxidants and fiber [3].

Moreover, the consumption of legumes was more frequent compared to other previous studies, where a frequency of 3–4 times a week or more was observed in the U16-15 categories of 31.5%, in the U19-17 categories of 39.1% and in the senior categories of 28.5% [21]. It is important to educate soccer players to consume not only animal protein, but also vegetable protein, especially from legumes, since they are very complete foods with a high contribution of CHO with a low glycemic index, fiber and micronutrients such as iron and vitamins, highlighting that in young ages, the contributions of these are superior in some cases compared to adults [3,30,38]. Regarding animal protein, it was abundant in lean meats, eggs and by-products and fish, with results similar to other previous studies [21,39,40].

In relation to foods rich in CHO such as cereals, whole grains, pasta, rice or tubers, it was high in all categories. There is increasing scientific evidence defending the benefits of high CHO intake compared to low CHO intake in preparation for competitions [3,20,41]. The reason is mainly based on the increase in muscle glycogen which provides (i) a greater endurance capacity in high-intensity exercises [42–44], (ii) an increase in the total distances covered [43,45], (iii) an improvement in specific soccer skills [42] or (iv) an increase in the time required until fatigue is reached [46]. However, it is important to apply CHO periodization during the week according to physical demands in order to properly maintain body composition and promote physiological adaptations to optimize sports performance [3,47].

4.2. Influence of Sex on Nutrition

Although women's soccer has gained popularity and has experienced significant scientific advances, the amount of research on female players in this sport remains markedly lower compared to men's soccer [3]. However, in the last decade, there has been a rapid increase in interest in women's soccer, which has spurred the creation of better professional conditions for training and competition for female players [48,49].

In general, there could be a tendency for women to control their eating habits more than boys and to follow patterns that are considered healthier, both for health reasons

and because of body dissatisfaction, at least in adolescence [50]. In a study that evaluated differences in dietary patterns between male and female soccer players, women showed significantly lower consumption of pork, bread, olive oil and soft drinks compared to men, and significantly higher consumption of seafood, natural fruit juice and fruit [30]. However, the lower consumption of red meat is the only similarity with our results, as women consumed less fruit among other differences. If we focus on micronutrients, it is worth highlighting the dietary needs of iron, where women require higher amounts (18 $mg \cdot d^{-1}$) than men (8 $mg \cdot d^{-1}$), being abundant in foods such as mollusks, red meat or legumes such as soybeans or lentils [3,51]. Our results highlighted that women consumed less red meat and legumes than men, so this could lead, if the athlete's diet is not well structured through food or supplements, to suffering from pathologies such as anemia [3].

If we analyze the most relevant data on the frequency of consumption of the food groups, we observe a lower consumption of CHO (fruit, legumes, rice and pasta) by the female teams, which is in accordance with the literature reviewed in a study conducted with professional athletes who participated in several sports [52] and in another focused on male and female soccer players [30]. Although the reason for our female sample to consume less CHO is unknown, it could be due to misunderstandings about the impact of CHO intake on body composition, fear of weight gain and associated impacts on body image [53,54] and, for this reason, they tend to restrict foods high in this macronutrient.

In general, during prolonged submaximal aerobic exercise, women tend to rely more on fatty acids compared to men, especially when exercising at equivalent relative workload [55–57]. This observation could imply that women may require less CHO than male soccer players [58]. However, future research involving both female and male elite soccer players, matched under match and training conditions, is needed to determine whether energy substrate choice differs between genders. In fact, it is suggested that most female players may even minimize daily protein intake to prioritize higher carbohydrate intake for glycogen restoration, due to the low prevalence of their consumption [58].

Finally, in case the optimal intake of CHO through food is not reached, the use of CHO-rich sports foods such as gels, sports bars, gummies or isotonic drinks is recommended, as they can promote glycogen replenishment and improve performance, as observed in previous studies [3,59,60].

4.3. Influence of Playing Position on Nutrition

It has been seen that widefielder, fullback, center midfielder and center forward seem to be the most physically active playing positions during a match [61–63], which would mean that they are the most demanding in terms of energy expenditure and could explain the higher CHO requirements. Although there is a study where in some of these playing positions a higher consumption of CHO was observed, in our study, significant differences were only found for red meat consumption, with a higher consumption by forwards [12]. However, the questionnaire only includes the weekly frequency of the different food groups and not specific amounts of nutrients, so the results should not be misinterpreted. In addition, it should be remembered that the club has a dietician-nutritionist, so that nutrition, especially around training and matches, will be well advised.

Nutrient intake can have a profound impact on a player's body composition and performance favoring a similarity with elite soccer patterns, where scientific literature advocates that each playing position shows specific characteristics due to different physical demands and roles during training and, especially, in competitions [3,6,7,64]. Therefore, it is recommended to take care of the nutrition of all players and, if possible, to individualize the nutrition based on the playing position.

4.4. Alcohol Consumption

There are multiple factors that are capable of interfering in the recovery of the soccer player, such as high muscle damage caused by eccentric movements (related to an inhibitory effect of muscle glycogen resynthesis in type II fibers [65]), sports injuries, poor sleep quality

or excessive alcohol intake [3,66,67]. After training and/or matches, celebratory events and/or discouragement linked to the ingestion of alcohol in moderate or high amounts may occur. This is a practice that is recommended to be avoided as it seems to cause a reduction in the rate of myofibrillar protein synthesis (even if alcohol is ingested together with dietary protein), which may impair exercise adaptation and recovery by decreasing the anabolic responses of skeletal muscle [3]. Furthermore, let us not forget that it can produce dehydration in the soccer player in situations where rehydration is important for future physical demands (especially when there is more than one match per week) [3,67,68].

In our study, we observed that alcohol intake was higher as soccer players increased in age, which would be consistent due to the legality of consuming this type of beverages. Regarding the type of beverages, male players consumed mixed drinks more often than female players, which can also be seen in this study [30]. However, these authors observed a higher intake in older boys, while it comes from the youngest ones in our results. This could be due to the fact that younger boys have poorer nutritional knowledge, hence why it is important to intervene from an early age so that good decisions are maintained throughout the years [26]. Regardless of the type of beverage, it is advisable to consume the least amount of alcohol in order to ensure proper player health, as well as to optimize athletic performance, recovery from energy demands and body composition [3].

For the first time to our knowledge, it was possible to assess the dietary habits of soccer players differentiated by competitive level, playing position and gender (Figure 1).

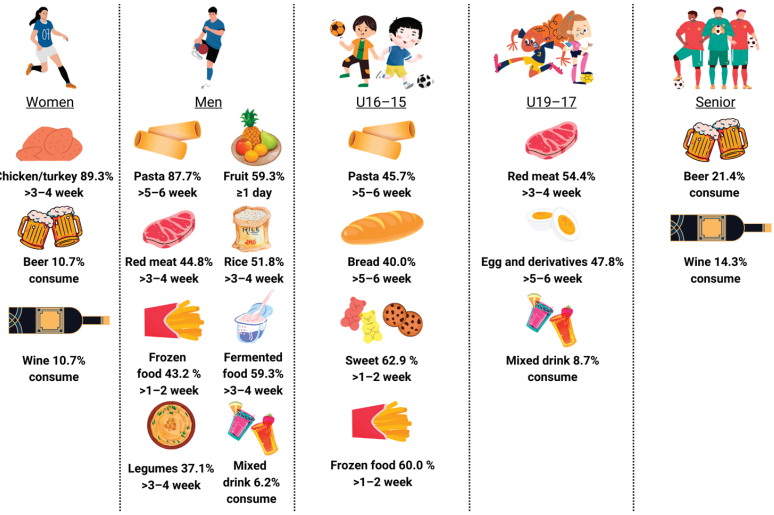

Figure 1. Most-consumed food and beverages classified by competitive level and gender.

4.5. Limitations

This research had some limitations that should be discussed to improve its applicability to soccer sport contexts. Despite having a small study population, 100% of the Spanish soccer players of the selected sample were recruited, which is a significant sample size according to the statistical principles applied.

Using a food frequency questionnaire allows one to estimate the usual intake and is quick and easy to administer since it does not alter the habitual intake of the individual, it does not require trained interviewers, and it has a low administration cost, but the consumption information was collected in a self-reported and retrospective manner based on the memory of the soccer players. This could lead to errors in the number and type of foods declared. Although the questionnaire was useful to describe the eating habits of the sample, it is not accurate to quantify the portions eaten, sports supplements intake or the intake of vitamins and minerals. In addition, the actual intake of CHO, proteins

and fats could not be quantified either. Despite this, this study has achieved its goal of describing the dietary habits and practices of elite soccer players, little studied previously. Another limitation was the sample; it was small in each category and there is heterogeneity between sex groups. Soccer players may change their eating habits over time, whether due to changes in their nutritional needs, professional recommendations or body composition goals among other reasons. The questionnaire may not capture these changes or require periodic updates to reflect changing consumption. Finally, the food frequency questionnaire used was a validated tool, although one of its limitations was that the information on consumption was collected in a self-reported and retrospective manner, based on the memory of the soccer players. In addition, they may have a tendency to respond in a socially desirable manner, which may influence the veracity of the information provided. However, in general, athletes tend to care about their diet and training, as their performance depends on it, and they tend to be knowledgeable about their eating habits. This might make them remember their food intake better compared to the general population.

It should be noted that the pandemic caused by COVID-19 may have influenced the eating habits of soccer players, but during the pandemic season (2020–2021 season), all soccer players had online counselling by the dietitian-nutritionist in order to avoid changing dietary habits from a healthy eating approach. In the 2021–2022 season, the operation of the club, picnics for matches, etc. functioned normally.

5. Conclusions

In conclusion, differences in the eating habits according to competitive level and sex were found in our sample. Men tend to consume more CHO-rich foods, fermented foods, frozen foods and red meat compared to women, while women frequent lean meats more than men. In terms of competitive levels, a higher intake of certain CHO-rich foods, in addition to unhealthy foods such as sweets and frozen foods, was observed in the lower categories. Practically no differences were found between the playing positions. In summary, it is recommended that daily consumption of alcohol, sweets and frozen food be reduced as much as possible, regardless of competitive level, playing position and gender.

Supplementary Materials: The following supporting information can be downloaded at the following: https://www.mdpi.com/article/10.3390/nu15204323/s1, File S1: Food consumption frequency questionnaire in elite soccer players.

Author Contributions: Conceptualization, J.M.M.-S., J.M.S. and J.S.-R.; methodology, J.M.M.-S., J.M.S., J.S.-R., D.R.-G., J.S.-C., M.A.-C. and P.L.-M.; data curation, J.S.-R., D.R.-G. and J.S.-C.; formal analysis, J.S.-R., D.R.-G., J.S.-C., M.A.-C. and P.L.-M.; investigation, J.M.M.-S., J.M.S., J.S.-R., J.S.-C., M.A.-C. and P.L.-M.; visualization, J.M.M.-S., J.M.S. and J.M.S.; writing—original draft preparation, J.S.-R., D.R.-G., J.S.-C., M.A.-C. and P.L.-M.; writing—review and editing, J.M.M.-S., J.M.S., J.S.-R., D.R.-G., J.S.-C., M.A.-C. and P.L.-M. All authors have read and agreed to the published version of the manuscript.

Funding: This research received no external funding.

Institutional Review Board Statement: This study was conducted in accordance with the Declaration of Helsinki and approved by the Ethics Committee of University of Valencia (file no. (1534145)).

Informed Consent Statement: Not applicable.

Data Availability Statement: The data presented in this study are available in the tables of this article. The data presented in this study are available on request from the corresponding author.

Acknowledgments: Authors are grateful to the Valencia C.F. Academy (soccer players and technical-medical staff) for their support and collaboration in the development of this study. This study is part of the doctoral thesis of Jaime Sebastiá-Rico.

Conflicts of Interest: All authors of the work declare they do not have any conflict of interest.

Abbreviations

CHO: Carbohydrates.

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nutrients

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Review

Female Athlete Triad and Relative Energy Deficiency in Sport (REDs): Nutritional Management

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Abstract: The female athlete triad (TRIAD) is a spectrum of disorders involving low energy availability (LEA), low bone mineral density, and menstrual disorders. It is increasingly common to use the term 'relative energy deficiency in sport' (RED), emphasising the extensive impact of LEA on the body. The aim of this narrative review was to gather original research encompassing female athletes across various sports as well as to collect findings on the potential of a nutrition-focused approach to prevent or treat the aforementioned disorders. A comprehensive search was conducted in PubMed and Scopus. Several challenges were identified regarding the adequacy of the energy availability, protein, and carbohydrate requirements in the diets of female athletes. Moreover, insufficient intake of vitamin D has been observed across all athlete groups studied. This insufficiency also extends to the average requirement for Ca, Mg, the Ca/P ratio, Zn, and Fe. To address those concerns, a nutritional approach is proposed in the latter part of this review. The factors that can improve the absorption of micronutrients have also been discussed. The TRIAD/REDs affect an ever-growing number of women and require appropriate therapeutic management, particularly through nutritional care. Therefore, cooperation within an interdisciplinary team comprising a physician, nutritionist, physiotherapist, and psychologist is crucial.

Keywords: female athlete triad; relative energy deficiency; energy availability; bone mineral density; menstrual disorders; nutrition; nutrient intake; bioavailability

Citation: Grabia, M.; Perkowski, J.; Socha, K.; Markiewicz-Żukowska, R. Female Athlete Triad and Relative Energy Deficiency in Sport (REDs): Nutritional Management. *Nutrients* **2024**, *16*, 359. https://doi.org/10.3390/nu16030359

Academic Editors: Valentín E. Fernández-Elías, Miguel Mariscal-Arcas and Olga López Torres

Received: 23 November 2023
Revised: 18 January 2024
Accepted: 23 January 2024
Published: 25 January 2024

1. Introduction

The female athlete triad (TRIAD) was formerly defined as a cluster of symptoms related to low energy availability (LEA), either with or without coexisting nutritional disorders (including anorexia nervosa), menstrual dysfunction (particularly secondary amenorrhea), and low bone mineral density (BMD) [1]. Currently, the International Olympic Committee, through a consensus, has introduced the term 'relative energy deficiency in sport' (REDs) as the new term to emphasise the fact that a variety of health issues (impairments in the physiological and/or psychological state of the body) resulting from LEA may affect not only female but also male athletes [2]. The presence of significant anatomical and physiological differences between the genders, notably hormonal intricacies, means that the risks of developing health disorders vary between men and women [3,4]. Therefore, for the purpose of this review, the focus will be on women and the most common health concerns caused by LEA. The disciplines that have been studied to identify at-risk groups primarily involve women who practise gymnastics, running, skating, and ballet. However, it is also increasingly common to observe those issues among women participating in leisure sports [1].

Appropriate nutrition for athletes not only enhances sports performance but also safeguards them against injuries and health deterioration [5,6]. Sports nutrition presents a major challenge for sports mentors and athletes themselves. Hence, it is imperative to emphasise the critical role of proper nutritional programming, not only for an athlete's

preparation before events, but also for preventive measures (to mitigate the incidence of adverse health outcomes) and therapeutic action (addressing the nutritional management of LEA's effects). Supporting athletes to retain or regain their health and athletic potential is crucial for the future years of their sports careers [5,7]. The scientific literature contains papers that describe the problem of the TRIAD/REDs and therapeutic approaches based on medical treatment. However, there is a noticeable absence of publications that comprehensively compile and present the pivotal importance of a proper nutritional approach in the prevention or treatment of those disorders.

Consequently, the aim of the study has been to gather and summarise relevant literature in a narrative review focusing on nutritional management concerning the emergence of the TRIAD/REDs. This review discusses the research conducted on women engaged in various sports, evaluating parameters, such as exercise energy expenditure (EEE) and energy availability (EA) as well as the intake of macronutrients and micronutrients crucial for maintaining proper bone density (vitamin D, calcium, phosphorus, magnesium, zinc, and iron). Furthermore, a proposed nutritional approach has been outlined to address each component of the TRIAD. The review also provides a detailed description of those essential nutrients, emphasising their relevant roles in sports and the disorders in question. Furthermore, it discusses their dietary sources and provides insights into factors that may either impair or enhance their absorption.

2. Materials and Methods

The search for publications was conducted across PubMed and Scopus. The primary keywords used included: 'female athlete triad', 'relative energy deficiency in sport', 'female athlete + chosen micronutrient', 'physical activity or female athletes + chosen micronutrient', 'chosen micronutrient + supplementation + sport', 'chosen micronutrient + bioavailability', 'chosen micronutrient + absorption', 'female athlete microelements', 'female athlete serum levels', and 'sports nutrition'. Initially, over 110,000 records were identified, out of which 340 publications were reviewed and pre-selected and retrieved, and 142 were ultimately included in the review. The focus was on original or review papers, mainly regarding women practicing various sports. The exclusion criteria were language other than English, animal/cell studies, and case reports. The primary objectives of the narrative review were to discuss EA and EEE levels, examine macronutrient and micronutrient intake, present the results of various interventions aimed at mitigating health effects associated with REDs, and propose nutritional management strategies for REDs. For this purpose, data such as the type of sport discipline, EA, EEE, and macronutrient and micronutrient intake were extracted from relevant studies. If studies reported those parameters in different units, recalculations were performed wherever it was possible. Interventions were assessed considering sporting discipline, specific issues related to REDs, details of the administered product in terms of quantity and composition, and the outcomes of the experiments. In addition, background information for the review, such as the roles of nutrients, dietary sources, definitions, and general recommendations, was acquired and incorporated using other methods as well, including referencing websites of relevant organisations or citations from related publications. The detailed selection process is illustrated in Figure 1.

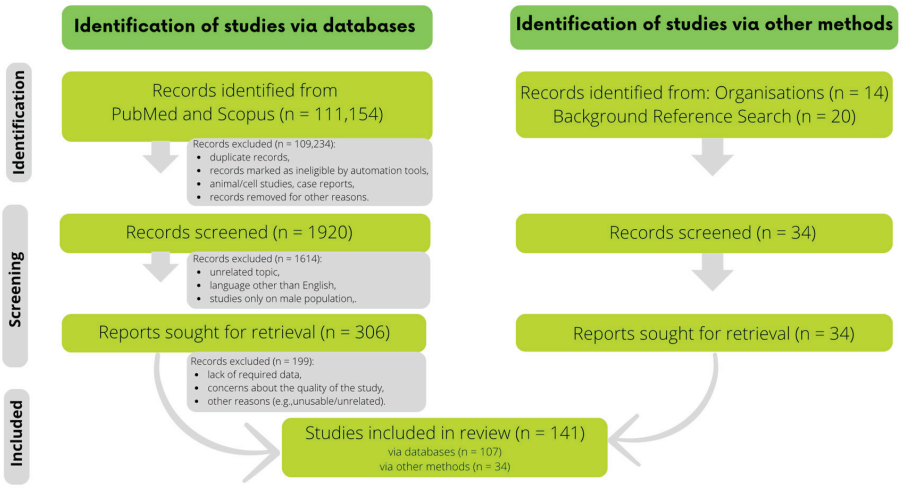

Figure 1. The selection process on the flowchart.

3. Results

The results were divided into several sections. The first three of them present an introduction describing essential information on LEA, BMD, and menstrual disorders. The next one is a summary of nutritional deficiencies prevalent in various sports among female athletes. The subsequent sections provide an overview on dietary management strategies addressing LEA, BMD, and hormonal disorders.

3.1. Low Energy Availability

LEA is one of the three major components of the TRIAD and the main cause of adverse health effects of REDs. Energy availability refers to the amount of energy necessary to sustain essential bodily functions. It is calculated by subtracting exercise energy expenditure (EEE) from the total energy intake throughout the day and subsequently dividing it by fat-free mass (FFM) in terms of kilograms [8,9].

$$Energy\ Availability = \frac{energy\ intake[\text{kcal}] \ - \ exercise\ energy\ expenditure\ [\text{kcal}]}{fat - free\ mass[\text{kg}]}$$

Athletes' inadequate knowledge of nutrition is among the various reasons for the incidence of LEA. However, it may also result from the increasing prevalence of eating disorders (EDs), complex medical conditions characterised by incorrect eating habits adversely impacting health and functional ability, e.g., anorexia and bulimia nervosa. Those disorders often affect athletes in weight-class sports or those that emphasise leanness [8,10]. Muia et al. demonstrated that 75% of elite Kenyan runners exhibited ED-related behaviour [11]. Professional dancers with strict dietary restrictions show a lower EA as compared to those following standard diets [12]. A study by Reed et al. reported high levels of body dissatisfaction among female soccer players experiencing LEA [13]. Similar issues were observed in female endurance athletes with amenorrhea as compared to those with normalised menstruation [14].

A universally accepted gold standard for assessing EA has not yet been established. The prevalent methods chosen in research to measure dietary nutrient intake include 24 h interviews (24HR) and food frequency questionnaires. The most commonly applied method, albeit prone to significant underestimation bias, involves the use of food self-reports. Each method comes with its own set of advantages and limitations, and the choice should be tailored to the specific assessment required. The 24HR method is considered one of the more accurate approaches currently available as it has been designed to estimate present nutrient

intake while addressing the problem of underestimating energy intake. However, a single dietary test may lack reliability due to intra-personal variations and fluctuations throughout the day. Hence, it is crucial to collect data from at least 3 days of intake, including at least 1 weekday, to provide a more comprehensive dietary assessment [15,16]. Minimising measurement errors is paramount as these errors may stem from various factors, such as differing motivation, memory, and the honesty of study participants. Therefore, it is essential that the individuals tasked with data collection possess the requisite qualifications and skills. They must be adept at meticulously instructing participants on accurately completing dietary diaries. Additionally, they should have the expertise to discern which questions to ask and how to frame them, ensuring thoroughness and reducing the likelihood of omission [17–19].

An accurate estimate of the EEE is another component required for calculating the EA. This estimation may vary based on an athlete's training regimen, performance level, and preferred daily lifestyle choices [20]. The EEE is also susceptible to selection bias and survival bias, which is why it is necessary to conduct studies across various groups of sports [20,21]. Researchers have employed diverse methods to calculate the EEE in their studies. It is important to note that there is not only variability in reporting the EA in different units, but also in the use of what is known as lean body mass (FFM plus essential fat) instead of the FFM (defined as the total body mass minus fat) [20,22]. Some studies have utilised the adjusted EEE (i.e., subtracting the energy cost of sedentary exercise behaviour during the exercise period from the EEE) [8]. Attempts have also been made to use the resting metabolic rate (RMR) and the measured/predicted RMR ratio. Nevertheless, predictive equations have proven excessively variable in both genders, rendering them an unreliable marker at this time [20,22].

Recently, validated questionnaires such as the Low Energy Availability in Females Questionnaire (LEAF-Q) have emerged in research, capable of identifying athletes exhibiting symptoms of LEA [23,24]. Given its association with EDs, combining those tools with athlete-specific ED assessments, such as the Female Athlete Screening Tool (FAST) [25] and the Brief EDs in Athletes Questionnaire (BEDA-Q) [26], could prove valuable. Dervish et al. found that 47% of female endurance runners were at risk of LEA, 40% were at risk of disordered eating (a broad term encompassing a disturbed relationship with food, exercise, and the body, including emotional eating [10]), and 9% met criteria for EDs [23]. Similar results were observed in female athletes by Sharps et al., with 53% at risk of LEA, 44% exhibiting disordered eating, and 6% meeting criteria for EDs [27].

Based on the findings of those studies, it is crucial to detect LEA in athletes because its long-term presence represents only the tip of the iceberg concerning further health consequences. Therefore, ongoing efforts are focused on identifying markers that could more directly reveal the occurrence of LEA. Leptin, total and free T3, insulin-like growth factor 1, urinary LH surge, and markers of bone formation and resorption (carboxy-terminal propeptide of type 1 procollagen) are among the most commonly considered markers [28].

3.2. Bone Mineral Density

Age is not the only factor contributing to the loss of bone mass; it can also be experienced among young women. Stress fractures (SFs) typically result from external forces. Under normal circumstances, bones undergo constant remodelling and adapt to various loads. However, abnormal and repetitive loading may lead to the formation of SFs. In such instances, the body is unable to adequately and promptly compensate. Consequently, micro-injuries and fractures occur [29,30]. SFs most commonly affect the lower limb (8–95%) whereas they occur far less frequently in the upper limb (fewer than 10% of SFs) [31]. However, athletes with low BMD face a substantially higher risk of SFs as compared to those with normal BMD. Similar risks emerge when athletes experience nutritional issues related to LEA. Barrack et al. indicated that training for more than 12 h per week was the primary factor contributing to bone stress injury (BSI). The presence of low BMD amplifies this risk 3-fold. When combined with a body mass index <21 kg/m^2 and amenorrhea, the risk

increases 4-fold [32]. This correlation likely stems from low oestradiol levels and insufficient energy supply, leading to an imbalance in bone metabolism. Lappe et al. [33] revealed that calcium and vitamin D supplementation reduced the incidence of SF in female military recruits.

Nutrition plays a pivotal role in ensuring healthy skeletal growth and development, maintaining normal density and thus protecting against conditions such as osteoporosis, bone fragility, and fractures as individuals age. Adequate intake of energy as well as macronutrients and micronutrients—particularly vitamin D, calcium, and phosphorus—is tremendously important [34,35].

3.3. Menstrual Disorders

Nutritional and behavioural changes are essential components of the initial treatment for a hypoestrogenic state and LEA to further influence the return of menstruation and support BMD [1]. The prevalence of menstrual disorders in active women ranges from 19% to 54% [36]. Female athletes are commonly diagnosed with functional hypothalamic amenorrhoea (FHA), where there is inhibition of the hypothalamic–pituitary–ovarian axis function and a reduction in gonadoliberin dipeptide (GnRH) secretion. High training overload and stress, which may result in decreased body weight (BW) and insufficient energy intake, are among the most common factors of FHA [37]. Research reveals that the resumption of menstrual cycles is dependent on nutritional status, including achieving and maintaining adequate BW and FFM [38,39]. A balanced diet and improved energy intake are beneficial not only for weight restoration but also for enhancing bone mass and the GnRH secretion [38,40]. De Souza et al. demonstrated that increasing daily energy intake (330 ± 65 kcal/day; 18 ± 4%) could facilitate the return of menstruation in female athletes with oligomenorrhoea and amenorrhoea [41].

3.4. Consumption of Energy, Macronutrients, and Specific Micronutrients among Female Athletes

3.4.1. Exercise Energy Expenditure (EEE) and Energy Availability (EA)

In the data retrieved (Table 1), EEE ranged from 272 ± 78 to 1300 ± 293 kcal/day. A lowest EEE of 272 ± 78 kcal/day was reported by De Souza et al. [42] for a group of exercising amenorrheic women. In contrast, a highest result of 1300 ± 293 kcal/day was obtained by Schaal et al. [14] for a group of amenorrheic athletes from the United States. The analysis of the EA data (Table 1) showed that EA ranged from 18 ± 6.6 to 42.5 ± 12.1 kcal/kg FFM per day. Schaal et al. [14] demonstrated a value of 18 ± 6.6 kcal/kg FFM per day. In contrast, the highest value (42.5 ± 12.1 kcal/kg FFM per day) was obtained by Melin et al. [43] in a group of female endurance athletes from Denmark and Sweden. To summarise, in various studies, LEA defined by a suggested cut-off point of 30 kcal/kg FFM/day [2,44] was noted among runners [42,45], endurance athletes [14], artistic gymnasts [46], and dancers [47].

Table 1. Exercise energy expenditure (EEE) and energy availability (EA) values among female athletes.

Parameter	Results	Sports Discipline
EEE (kcal/day)	$272 \pm 78^{\text{ExAnov}}$ $480 \pm 53^{\text{ExOvul}}$ $494 \pm 64^{\text{ExLPD}}$	running [42]
	591 ± 95	running [11]
	600 ± 237	running [45]
	$800 \pm 132^{\text{EU}}$ $1300 \pm 293^{\text{AM}}$	endurance sports [14]
	921 ± 256	running [48]
	940 ± 450	endurance sports [43]

Table 1. *Cont.*

Parameter	Results	Sports Discipline
EA (kcal/kg FFM per day)	18.8 ± 3.2 ExAnov 23.3 ± 1.6 ExOvul 26.5 ± 1.8 ExLPD	running [42]
	18 ± 6.6 AM 29 ± 4.8 EU	endurance sports [14]
	23 ± 3 ArtGym 33 ± 10 Swim	artistic gymnastics, swimming [46]
	26 ± 13	dancing [47]
	29.6 ± 17.4	running [45]
	30.7	running [49]
	31.6 (21.2–37.6)	climbing [50]
	33 ± 7	middle- and long-distance running, race walking [51]
	36.5 ± 4.5	running [11]
	37 ± 21	running [48]
	39.6 (35.3–43.9)	endurance sports [52]
	42.5 ± 12.1	endurance sports [43]

Values are expressed as mean (if given: ± standard deviation) or median (interquartile range). Abbreviations: AM—amenorrheic, ArtGym—artistic gymnasts, EA—energy availability, EEE—exercise energy expenditure, EU—eumenorrheic, ExOvul—exercise/ovulatory, ExLPD—exercise/luteal phase deficiency, ExAnov—exercise/anovulatory, FFM—fat-free mass, Swim—swimmers.

3.4.2. Macronutrients

Besides providing adequate energy, the diet should also cover nutrient requirements [7]. Inadequate **protein** intake has been observed in female athletes participating in sports where a lean body composition is important. This deficiency has also been shown in nearly one in three female soccer players (less than 1.2 g/kg BW) [53–55]. Regarding total daily protein consumption, its intake ranged from 13% to 21% (Table 2). The lowest values were documented in three studies [14,46,47], whereas the highest intake was reported by Condo et al. [56] among soccer players. Difficulty in meeting the recommended protein intake of 1.2–2 g/kg BW/day [57] was observed only in a group of artistic gymnasts [46].

Carbohydrate consumption (CHO) is a concern among female athletes. The required amount of this macronutrient varies depending on the duration, intensity, and frequency of training sessions [58]. Nevertheless, reported daily CHO intake values ranged from 41% among female soccer players [56] to 62% among runners [42] (Table 2). Considering the values of the standards used by the authors (in their absence, a CHO target from 5 to 7 g/kg/day was selected [57]), insufficient consumption was evident among various groups of athletes, including soccer players [56,59], artistic gymnasts, swimmers [46], and runners [45].

The last but not least important macronutrient in an athlete's diet is **fat.** Fat intake should follow general guidelines for healthy people (20–35% of total energy intake) and should be considered individually based on training levels (at no less than 20% of total energy intake) [57,60]. Reported daily dietary intake ranged from 22% to 40% (Table 2). The lowest intake (22%) was noted by De Souza et al. [42], while the highest (40%) was noted by Jakše et al. [46] in a group of young Slovenian artistic gymnasts. However, no significant issues in fulfilling fat intake requirements were identified in either study.

3.4.3. Selected Micronutrients

A properly balanced diet, encompassing appropriate proportions of macronutrients and a careful selection of food products rich in micronutrients, is vital in preventing nutritional deficiencies and associated health consequences [3,5].

In the reviewed studies (Table 3), daily **vitamin D** intake ranged from 1.69 µg (among Polish female soccer players [59]) to 8.3 µg (in runners from the USA [48]). Therefore, none of the groups of athletes in the analysed studies demonstrated coverage of the standard for this vitamin. **Calcium** (**Ca**)**,** closely related in its functions to the vitamin D, exhibited a range of intake from 608 mg/day (in athletes with disabilities from Korea [61]) to 1532 mg/day (among long-distance Lithuanian runners [62]) (Table 3). **Phosphorus** (**P**) intake plays a significant role in the adequate absorption of Ca. What is also important is the ratio of both minerals [63,64]. P intake varied between 702 mg/day [61] and 2103 mg/day [62] (Table 3). The **Ca/P ratio** calculated on the basis of the provided data ranged from 0.555 [59] to 0.867 [61] (Table 3). Most of the groups of examined athletes experienced a problem with Ca coverage from the diet. The exceptions were athletes of certain disciplines in the study by Baranauskas et al. [62], McCormack et al. [48], and Soric et al. [65]. The situation was similar for maintenance of a proper Ca/P ratio; only long-distance runners were close to balancing both components [62].

Magnesium (**Mg**) and **zinc** (**Zn**) are crucial in sports nutrition. Studies have indicated that Mg intake ranged between 245 mg/day [59] and 595 mg/day [62] (Table 3). In the case of Zn, consumption varied between 6.0 mg/day (in Brazilian swimmers with eating disorders [66] as well as disabled athletes [61]) and 19 mg/day [62] (Table 3). **Iron** (**Fe**) is also vital for sustaining high-level aerobic capacity [67,68]. Its intake in the reviewed research ranged from 8 mg/day [61] to 27 mg/day [62] (Table 3). In the case of Mg, non-coverage of the average requirement was noted for soccer players [59], artistic gymnasts, swimmers [46], ballerinas, rhythmic gymnasts [65], and runners [45]. There was a similar issue about Zn in the group of swimmers [66] and disabled female athletes [61], and the same was true of Fe among disabled athletes [61] as well as swimmers and artistic gymnasts [46].

Table 2. Macronutrient intake among female athletes.

Macro-Nutrients	Norms		Results			Sports Discipline
	% of Energy or g/Day	g/kg BW	% of Energy	g/Day	g/kg BW	
Proteins	50 g/day	1.2–2.0	17 *	56	n/d	disabled athletes [61]
	n/d	1.2	14 * $^{\text{ArtGym}}$ 13 * $^{\text{Swim}}$	55 * $^{\text{ArtGym}}$ 73 * $^{\text{Swim}}$	1.0 ± 0.2 $^{\text{ArtGym}}$ 1.2 ± 0.2 $^{\text{Swim}}$	artistic gymnastics, swimming [46]
	n/d	n/d	16 * $^{\text{ExAnov}}$ 14 * $^{\text{ExOvul}}$ 14 * $^{\text{ExLPD}}$	58 * $^{\text{ExAnov}}$ 68 * $^{\text{ExOvul}}$ 70 * $^{\text{ExLPD}}$	n/d	running [42]
	n/d	n/d	17	n/d	n/d	ballet [69]
	n/d	1.2–2.0	15 *	71	1.4 ± 0.6	running [45]
	83 g/day	1.4–1.7	19 *	72	1.2 ± 0.44	soccer [59]
	n/d	1.2–1.7	13 *	81	1.3 ± 0.3	dancing [47]
	n/d	n/d	13 $^{\text{AM}}$ 15 $^{\text{EU}}$	68 * $^{\text{AM}}$ 83 * $^{\text{EU}}$	n/d	endurance sports [14]
	n/d	1.2–2	21	98	1.5 ± 0.5	soccer [56]
	71 g/day	1.2–1.7	17	116 ± 26	2.0 ± 0.5	endurance sports [43]
	n/d	1.3–1.7	n/d	80 $^{\text{Interm}}$ 77 $^{\text{Advanced}}$ 82 $^{\text{Elite}}$	1.3 (1.2–1.5) $^{\text{Interm}}$ 1.4 (1.2–1.6) $^{\text{Advanced}}$ 1.4 (1.2–1.9) $^{\text{Elite}}$	climbing [50]

Table 2. *Cont.*

Macro-Nutrients	Norms		Results			Sports Discipline
	% of Energy or g/Day	g/kg BW	% of Energy	g/Day	g/kg BW	
Carbohydrates	n/d	8–12	47 * ArtGym 54 * Swim	181 * ArtGym 305 * Swim	3.3 ± 0.8 ArtGym 5.1 ± 1.6 Swim	artistic gymnastics, swimming [46]
	n/d	5–7 Up to 1 h exercise/day 6–10 1–3 h exercise/day	41 *	192	3.0 ± 0.8	soccer [56]
	296 g/day	5–7	53 *	199	3.3 ± 1.2	soccer [59]
	130 g/day	n/d	49 *	164	n/d	disabled athletes [61]
	n/d	n/d	56	218*	n/d	ballet [69]
	n/d	n/d	62 * ExAnov 60 * ExOvul 57 * ExLPD	229 * ExAnov 285 * ExOvul 288 * ExLPD	n/d	running [42]
	n/d	6–10	53 *	255	4.9 ± 2.1	running [45]
	n/d	n/d	52 EU 57 AM	286 * EU 299 * AM	n/d	endurance sports [14]
	n/d	5–7	52 *	313	5.0 ± 1.0	dancing [47]
	353 g/day	6–10	53	369	6.4 ± 1.6	endurance sports [43]
	n/d	3–7	n/d	282 Interm 228 Advanced 253 Elite	4.6 (4.0–5.4) Interm 4.2 (3.4–5.3) Advanced 4.2 (4.2–5.8) Elite	climbing [50]
Fats	15–30%	n/d	34 *	52 *	n/d	disabled athletes [61]
	n/d	n/d	26	45 *	n/d	ballet [69]
	49.2 g/day	n/d	28	47	0.78 ± 0.39	soccer [59]
	n/d	n/d	22 * ExAnov 26 * ExOvul 29 * ExLPD	36 * ExAnov 54 * ExOvul 64 * ExLPD	n/d	running [42]
	30–40%	n/d	40 ArtGym 38 Swim	n/d	n/d	artistic gymnastics, swimming [46]
	n/d	n/d	28 EU,AM	68 EU 65 AM	n/d	endurance sports [14]
	n/d	n/d	32 *	69	n/d	running [45]
	20–35%	n/d	35	72	n/d	soccer [56]
	<30%	n/d	34	92	1.5 ± 0.4	dancing [47]
	20–35%	n/d	30	93	1.6 ± 0.5	endurance sports [43]
	n/d	n/d	n/d	70 Interm 60 Advanced 68 Elite	1.2 (1.0–1.6) Interm 1.1 (0.9–1.3) Advanced 1.2 (0.8–1.2) Elite	climbing [50]

Values are expressed as mean or median. For the data presented in terms of g/kg, the resulting figures are expressed in terms of the mean ± standard deviation or median (interquartile range). * In cases where the author presented a parameter in a different unit than that established in this review, general recalculations were performed where feasible. Abbreviations: AM—amenorrheic, ArtGym—artistic gymnasts, BW—body weight, EU—eumenorrheic, ExOvul—exercise/ovulatory, ExLPD—exercise/luteal phase deficiency, ExAnov—exercise/anovulatory, Interm—Intermediate, n/d—no data, Swim—swimmers.

Table 3. Micronutrient intake among female athletes.

Micronutrient	Norm	Results			Sports Discipline
Vit. D (µg)	10 [61] 15 [45,60] 20 [46] $\overline{x}$ = 15	1.69			soccer [59]
		2.5 (1.3–5.9) Interm	2.66 (0.9–4.1) Elite	3.9 (1.3–7.2) Advanced	climbing [50]
		2.6			disabled athletes [61]
		2.8 ± 2.2 HCycl	3.2 ± 3.1 Swim	3.4 ± 3.1 Rowers	endurance sports [62]
		3.4 ± 2.7 LDRun	3.7 ± 2.5 Skiers	3.7 ± 3.0 BAthl	
		4.5 ± 0.4			running [45]
		5.5 ± 9.6 Gym	3.5 ± 3.5 Swim		artistic gymnastics, swimming [46]
		8.3 ± 7.2			running [48]

Table 3. *Cont.*

Micronutrient	Norm	Results			Sports Discipline
Ca (mg)	700 [61] 800 $^{19–50\ y}$/1000 $^{>50\ y}$/1100 $^{10–18\ y}$ [59,60] 1200 [46] 1300 [45] $\overline{x}$ = 1000	608			disabled athletes [61]
		629 ± 274 ^{Gym}	806 ± 228 ^{Swim}		artistic gymnastics, swimming [46]
		646 ± 290			soccer [59]
		703 (605–817) ^{Advanced}	706 (537–1097) ^{Interm}	857 (753–1107) ^{Elite}	climbing [50]
		706 (332–1542) $^{DE+\ (15–19\ y)}$	819 (93–1738) $^{DE+\ (11–14\ y)}$		swimming [66]
		843 (269–2305) $^{DE−\ (11–14\ y)}$	909 (329–2563) $^{DE−\ (15–19\ y)}$		
		925 ± 545			soccer [56]
		1000 ± 504 ^{Swim}	1066 ± 407 ^{BAthl}	1117 ± 543 ^{Skiers}	endurance sports [62]
		1163 ± 484 ^{HCycl}	1398 ± 399 ^{Rowers}	1532 ± 1342 ^{LDRun}	
		1013 ± 448 ^{ArtGym}	1052 ± 577 ^{RhytGym}	1680 ± 304 ^{Ballet}	gymnastics, ballet [65]
		1046 ± 58.9			running [45]
		1395 ± 684			running [48]
P (mg)	580 $^{10–18\ y}$/1050 $^{>18\ y}$ [59,60] 700 [61] 1250 [45,46] $\overline{x}$ = 900	702			disabled athletes [61]
		924 ± 192 ^{ArtGym}	1236 ± 188 ^{Swim}		artistic gymnastics, swimming [46]
		1165 ± 357			soccer [59]
		1203 (1044–1451) ^{Advanced}	1370 (1192–1546) ^{Interm}	1535 (1400–1739) ^{Elite}	climbing [50]
		1256 ± 563 ^{RhytGym}	1290 ± 567 ^{ArtGym}	1353 ± 312 ^{Ballet}	gymnastics, ballet [65]
		1341 ± 72			running [45]
		1569 ± 549			soccer [56]
		1646 ± 321 ^{LDRun}	1740 ± 732 ^{Skiers}	1816 ± 552 ^{HCycl}	endurance sports [62]
		1841 ± 520 ^{BAthl}	1865 ± 650 ^{Swim}	2103 ± 546 ^{Rowers}	
Ca/P	0.96 [46] 1 [61] 1.04 [45] 1.05/1.37 [59] $\overline{x}$ = 1	0.555			soccer [59]
		0.589			soccer [56]
		0.536 ^{Swim}	0.579 ^{BAthl}	0.641 ^{HCycl}	endurance sports [62]
		0.642 ^{Skiers}	0.665 ^{Rowers}	0.931 ^{LDRun}	
		0.680 ^{ArtGym}	0.652 ^{Swim}		artistic gymnastics, swimming [46]
		0.780			running [45]
		0.785 ^{ArtGym}	0.838 ^{RhytGym}	0.863 ^{Ballet}	gymnastics, ballet [65]
		0.867			disabled athletes [61]
Mg (mg)	280 [61] 255/300 [59] 350 [46] 360 [45] $\overline{x}$ = 310	245			soccer [59]
		292 ± 80 ^{ArtGym}	333 ± 79 ^{Swim}		artistic gymnastics, swimming [46]
		301 ± 115 ^{RhytGym}	309 ± 64 ^{Ballet}	347 ± 183 ^{ArtGym}	gymnastics, ballet [65]
		317 (245–357) ^{Advanced}	383 (322–505) ^{Interm}	473 (411–510) ^{Elite}	climbing [50]
		351 ± 18			running [45]
		368 ± 138			soccer [56]
		448 ± 191 ^{HCycl}	464 ± 150 ^{BAthl}	480 ± 226 ^{Skiers}	endurance sports [62]
		493 ± 163 ^{Rowers}	503 ± 221 ^{Swim}	595 ± 335 ^{LDRun}	
Zn (mg)	7 [46,59] 8 [61] 9 [45] $\overline{x}$ = 8	6 (1–14) $^{DE+\ (15–19\ y)}$	7 (1–16) $^{DE+\ (11–14\ y)}$		swimming [66]
		7 (2–17) $^{DE−\ (11–14\ y)}$	10 (2–120) $^{DE−\ (15–19\ y)}$		
		6			disabled athletes [61]
		7 ± 2 ^{ArtGym}	9 ± 6 ^{Swim}		artistic gymnastics, swimming [46]
		8 ± 3			soccer [59]
		10 (7–12) ^{Advanced}	11 (10–12) ^{Interm}	12 (12–13) ^{Elite}	climbing [50]
		10 ± 2 ^{Ballet}	11 ± 9 ^{ArtGym}	12 ± 7 ^{RhytGym}	gymnastics, ballet [65]
		12 ± 1			running [45]
		12 ± 4			soccer [56]
		13 ± 2 ^{LDRun}	14 ± 5 ^{Skiers}	15 ± 4 ^{BAthl}	endurance sports [62]
		15 ± 5 ^{HCycl}	16 ± 6 ^{Swim}	19 ± 6 ^{Rowers}	

Table 3. *Cont.*

Micronutrient	Norm	Results			Sports Discipline
Fe (mg)	8 [59] 8/14 [61] 15 [45,46] $\overline{x}$ = 11	8			disabled athletes [61]
		9			soccer [59]
		9 ± 4 ArtGym	14 ± 7 Swim		artistic gymnastics, swimming [46]
		11 ± 3 Ballet	15 ± 7 ArtGym	15 ± 9 RhytGym	gymnastics, ballet [65]
		12 ± 3			soccer [56]
		12 (11–16) Advanced	17 (14–17) Interm	20 (18–21) Elite	climbing [50]
		13 (4–23) DE+, DE− (11–14 y)	13 (6–27) DE+ (15–19 y)	16 (7–35) DE− (15–19 y)	swimming [66]
		16 ± 1			running [45]
		17 ± 5			middle- and long-distance running, race walking [51]
		20 ± 4 LDRun	24 ± 7 BAthl	26 ± 11 Skiers	endurance sports [62]
		27 ± 10 Swim	27 ± 12 HCycl	27 ± 7 Rowers	

Values are expressed as mean (if given: ± standard deviation) or median (interquartile range). To compare the authors' reported results with established norms, the values presented in their respective studies were taken into account. In instances where the standard employed by the authors was unspecified, the results were compared with averaged values. If the author did not specify the Ca/P ratio, it was calculated on the basis of the extracted data. Abbreviations: ArtGym—artistic gymnasts, BAthL—biathletes, DE+—disordered eating, DE−—without disordered eating, HCycl—highway cyclists, Interm—Intermediate, LDRun—long-distance runners, RhytGym—rhythmic gymnasts, Swim—swimmers, y—years.

3.5. Nutritional Management of TRIAD/REDs

3.5.1. Overall Approach

The key approach to TRIAD/REDs nutritional treatment should be holistic; however, it is important to be certain to follow the right steps. Primarily, it is necessary to take care of the appropriate energy density. After providing caloric requirements, the next integral element should be the proper balancing of macronutrients and composing the diet to match the athlete's need for the necessary micronutrients (vitamins and minerals). Next, the timing of intake throughout the day (before, during and after exercise) and type, length, and intensity of exercise should be optimised. For optimal nutritional care of a female athlete, it is necessary to consider not only her training schedule but also the hormonal fluctuations experienced during respective phases of the menstrual cycle [3].

3.5.2. Energy Requirement

LEA is defined to occur when the result is below 30 kcal/kg FFM per day [44]. In the long term, such an insufficient amount may lead to adverse health effects (including interference with reproductive function and bone metabolism) as well as impairing athletic performance [44,70]. The optimal and physiological cut-off point is 45 kcal/kg FFM per day. Intermediate values (30–45 kcal/kg FFM/day) are amounts that can be tolerated for a limited period of time in female competitive athletes who would like to reduce BW with a properly designed and balanced diet and training [2,9,71]. Nevertheless, it is important to remember that those are not specific diagnostic values and there has been no established definitive clinical threshold for EA. The values may vary based on individual variations [2]. The key is to design meal plans that not only enhance the nutrient and energy density of meals without substantially increasing their volume but also take into consideration the athlete's dietary preferences, lifestyle, training regimen, and competition schedules. Cooperation with a psychologist may significantly boost motivation to implement nutritional changes and achieve a balanced diet. Most importantly, the diet should include high-energy-density food, such as dried fruits; dairy drinks fortified with proteins, calcium, and vitamin D; and products rich in essential fatty acids, such as avocado, fish, vegetable oils, nuts, tahini, and chia seeds. Moreover, since athletes commonly experience gastrointestinal issues, it is advisable to recommend an increase in the frequency of small-volume meals [1,3,57,72,73].

3.5.3. Macronutrient Requirements

The recommended daily **protein** intake for female athletes, irrespective of the menstrual phase, should fall within the range outlined in current sports guidelines (1.2–2.0 g/kg BW/day [57] or even 1.8–2.2 g/kg BW/day [74]). In order to maintain or build up FFM in the presence of LEA, a higher intake of protein is recommended (approximately 2 g/kg BW/day), due to its crucial role in muscle protein synthesis and tissue repair [75,76]. However, researchers also suggest that protein requirements in women exercising 1.5 h/day should be at least 1.6 g/day during their follicular phase [77]. During the luteal phase, there is notably higher catabolism of this macronutrient as compared to the follicular phase. To sustain optimal muscle protein synthesis and strength, it is advisable to consume 10 g of essential amino acids (equivalent to 15–25 g of high-biological-value protein) within 2 h after training [74]. If the aim is to prioritise muscle mass growth and repair over using protein oxidation for fuel, it is crucial to ensure an appropriate balance between CHO and energy intake in relation to energy expenditure to accurately address the athlete's requirements [78].

An adequate amount of **CHO** is essential to fuel the brain, support both aerobic and anaerobic metabolism, and maintain hormonal balance [79]. Moreover, it significantly influences performance and aids in recovery [58]. CHO requirements depend on the duration, intensity and frequency of training sessions as well as weather conditions. If an athlete conducts low-intensity training, 3–5 g/kg BW/day is enough. However, for moderate physical activity (more than 1 h/day), the requirement increases to at least 5 to 7 g/kg BW/day. For endurance training (1–3 h/day), 6–10 g/kg BW/day is recommended, while for even more intense exercise (>4–5 h/day), up to 8 to 12 g/kg BW/day [57]. Nevertheless, those are general guidelines for both female and male athletes. However, women in the follicular phase of the menstrual cycle benefit from increased glycogen stores (via a CHO load of 8.4–9.0 g/kg BW) as compared to the luteal phase, where glycogen storage is higher and CHO oxidation is lower [58,74,80]. Initially CHO at a rate of 30–60 g/h during training may help counterbalance the menstrual cycle's effect on glucose kinetics and exercise metabolism. This approach can also aid in minimising the likelihood of gastrointestinal disorders. Furthermore, rapid intake of CHO (at a rate of 1.2 g/kg BW) is important after prolonged physical workouts [74].

Fats play a crucial role in metabolic and hormonal sustainability, in addition to replenishing intra-muscular triglyceride reserves and maintaining energy balance, thereby holding tremendous importance for female athletes [79,81]. In a study by Hausswirth et al. [53] women expended more fat during exercise as compared to men, due to a lower respiratory exchange ratio (RER). In addition, women exhibited enhanced lipolytic activity during prolonged moderate-intensity physical activity. Oestrogen enhances lipid peroxidation during athletic endeavours, resulting in elevated levels of free fatty acids. Manipulating the quantity and source of dietary fat may impact the levels of several anabolic hormones in blood, consequently affecting both body composition and efficiency [81]. Athletes should avoid fat intake below 20% of energy. This practice may reduce dietary diversity and cause deficiencies in fat-soluble vitamins and essential fatty acids, particularly *n*-3 [57].

3.5.4. Micronutrient Requirements

All nutrients are essential and have a key impact on an athlete's health. However, it is crucial to cover the ones (vitamin D, calcium, phosphorus, magnesium and zinc) that influence BMD as well. Proper selection of food groups and inclusion of fortified products may prevent nutritional deficiencies [34,35]. Meals should be composed to include products that support absorption and minimise interactions with products that reduce bioavailability (Table 4). On the other hand, when it is not possible to ensure the intake of adequate amounts of micronutrients from the diet, e.g., due to excessive needs, after consultation with a specialist, appropriate supplementation with preparations containing the most bioavailable forms should be implemented (Table 4).

The benefits of **vitamin D** supplementation are widely recognised, particularly for bone health and immune system support. For athletes, its significance extends to aiding recovery from injuries, optimising performance, and maintaining normal neuromuscular function [57,79]. In cases of deficiency, the absorption of calcium and phosphorus may decrease by up to 15% and 60%, respectively. Notably, a study involving Navy recruits observed a reduction in SF with the supplementation of 800 IU of vitamin D and 2000 mg of calcium in [33]. Additionally, more than three-quarters of injuries among swimmers and divers were associated with decreased levels of 25-hydroxy vitamin D, implying that a preventive dose of 4000 IU might be beneficial [82]. Foods particularly abundant in vitamin D include eggs, dairy products, such as milk and cheese, and fatty fish, such as salmon, herring, and mackerel (Table 4) [60,83–85]. To increase the absorption of this vitamin from the gastrointestinal tract, meals should contain fats and vitamin E, while polyunsaturated and long-chain fatty acids and phytosterols may have a diminishing effect [86–89]. Cholecalciferol represents the most readily absorbed form of vitamin D. However, it is crucial to note that endogenous synthesis, which occurs through exposure to sunlight, remains extremely important [89–92].

Maintaining optimal bone density also requires adequate **Ca** intake. A study found that 85% of female runners with elevated bone turnover did not meet the recommended intake for Ca [93]. Currently, the recommendation stands at 1500 mg/day for athletes with amenorrhea, nutritional disorders, or early risk of osteoporosis [94]. Adequate calcium levels can lower the likelihood of skeletal system injuries. A dose of 2000 mg reduced the number of fractures [33]. Delayed menarche may increase the risk of low BMD due to the effect of oestrogen on Ca transfer to bone [95]. It is essential to incorporate calcium into the diet as research suggests that the beneficial effects on BMD become insignificant once supplementation is discontinued [96]. The main dietary sources of this element include milk and dairy products, as well as plant products such as parsley, kale, spinach, and beans (Table 4) [60,83,97,98]. Nutrients that notably enhance the absorption of Ca are lactose and vitamin D_3. Conversely, it is important to be mindful of foods high in fibre and phosphorus, oxalic acid, and phytic acid, as they can hinder calcium absorption [83,99–101]. When considering supplementation, suitable forms are calcium carbonate, citrate, and gluconate [97,101,102]. For athletes at risk for low calcium levels, a daily intake of 1500 mg is recommended to maintain optimal bone health, particularly relevant for women with LEA and menstrual disorders [103]. It is worth remembering that the intestine cannot absorb more than 500 mg of calcium at one time, necessitating the distribution of the element throughout the day [104].

P holds particular significance in the synthesis of adenosine 5′-triphosphate (ATP) and other high-energy compounds, such as adenosine diphosphate, guanosine triphosphate, and phosphocreatine. Thus, it plays a key role as far as the functions of skeletal muscles are concerned, ensuring their normal contractility. Furthermore, phosphorus is important for neuromuscular conduction. Additionally, it contributes to maintaining a normal acid–base balance [105]. It has been proven that a high dietary supply of P may contribute to endocrine disruption related to parathormone (PTH), leading to lower blood Ca levels. And conversely, a high dietary Ca supply is known to hinder phosphate absorption, consequently lowering blood PTH levels [63,64]. Elevated blood P concentrations were frequently observed in the athletes studied; these can potentially be attributed to high dietary phosphorus intake and rhabdomyolysis associated with intense exercise [106]. Phosphorus is most abundant in legumes, eggs, fish, offal, and wholegrain bread (Table 4). Optimal absorption of this element occurs with organic phosphate esters and ionised inorganic forms [107–111]. Factors that may restrict absorption include vitamin D deficiency and a high dietary Ca intake [107,109].

Mg is equally relevant for athletes to ensure proper oxygen uptake and electrolyte balance, as well as having an impact on the endocrine system [94,112]. Research indicates that athletes may require up to 20% higher magnesium intake as compared to standard population guidelines [112]. Studies have revealed that a significant proportion of women

from a variety of sports have reported inadequate magnesium intake, with silhouette sports being among the most vulnerable due to restricted energy intake [78,112,113]. Deficiency in magnesium may elevate the oxidative costs associated with training, a factor relevant to endurance performance [78]. To maximise dietary Mg intake, foods such as cereals, legumes, nuts, cocoa, rennet cheeses, potatoes, and bananas are beneficial (Table 4) [60,83]. For optimal absorption, it is advised to avoid products containing phytic acid, Ca, and P [114–116]. Instead, magnesium should be consumed alongside food products rich in vitamin B_6 and substances that lower the pH of the digestive tract [115–117]. Magnesium citrate, aspartate, and lactate are recommended forms of Mg supplementation [114,118].

Zn is significant for the proper conduct of metabolic processes and muscle function. Although its deficiency is rare, in athletes, it is most commonly lost through sweat and skeletal muscle breakdown [68,119,120]. In addition, the combination of LEA and a vegan diet may exacerbate this condition, due to this diet's low content and low bioavailability of food products of plant origin. Iron-rich food products also serve as important sources of Zn [68,121]. It is important to note that during zinc supplementation, absorption of Fe and copper may be impaired. Eskici et al. [122] observed that supplementation of Zn at a dose of 220 mg/day for 4 weeks did not lead to increased urinary excretion of Mg, Ca, P, or copper. However, despite this observation, it is still advisable to measure those factors in the case of zinc administration. Dietary sources rich in Zn include meat products, especially liver, as well as eggs, buckwheat groats, and wholegrain bread [60,123]. The absorption of Zn may be impaired by the presence of oxalic and phytic acids, fibre, ethanol, and other minerals, such as Fe or cadmium (Table 4). Citric acid and a diet abundant in animal protein may facilitate Zn absorption in the intestines [99,121,123–125]. Gluconate, citrate, and picolinate constitute the most commonly utilised chemical forms for Zn supplementation [99,126].

Due to significantly increased loss of **Fe** among athletes (30–70%), deficiency is a common diagnosis. This deficiency may adversely affect athletes' capabilities, e.g., by reducing performance time, lowering VO_2 max (maximal oxygen consumption), diminishing energy efficiency, and impeding the ability to work out at an optimal level [127,128]. However, studies show that Fe supplementation may have a beneficial effect on iron status as well as athletic performance, particularly in individuals with reduced iron levels [129,130]. Meat products, particularly liver and kidney, are recognised for their high Fe content (Table 4) [60]. The haem Fe available in the diet is the most easily absorbed by the human body. For female athletes, it is suggested that Fe intake should be increased by up to 70% of the estimated average requirement [131]. Meanwhile, if a woman belongs to a risk group (e.g., vegetarians and distance runners), consumption higher than the recommended dietary allowance (>18 mg/day) should be considered [57,132]. Other well-absorbed forms of Fe include ferrous sulphate, gluconate, and citrate [133–135]. Intestinal absorption of this element may be readily impaired by phytic and oxalic acids, insoluble fibre fractions, polyphenols, and large intakes of Zn [136–138]. To facilitate Fe absorption, it is worth ensuring the presence of ascorbic or lactic acid in the diet [133,136,139–141].

Table 4. Dietary sources and bioavailability of micronutrients.

Micronutrient	Dietary Sources	Bioavailability Enhancement	Bioavailability Impairment	Best Bioavailable Forms
Vit. D [60,83–90,92]	fat-rich fish (salmon, herring, mackerel), eggs, milk, dairy products and cheese	fat-rich meals, vitamin E	polyunsaturated fatty acids, phytosterols, long-chain fatty acids	"exposure to sunlight", cholecalciferol
Ca [60,83,97–102]	milk, milk-based foods, kale, parsley leaves, spinach, bean seeds	lactose, vitamin D, phosphopeptides	oxalic acid (spinach, rhubarb, beans), phytic acid (seeds, nuts, grains, certain raw beans and soy isolates), insoluble fibre fractions, high phosphorus content	calcium carbonate, gluconate

Table 4. *Cont.*

Micronutrient	Dietary Sources	Bioavailability Enhancement	Bioavailability Impairment	Best Bioavailable Forms
P [83,107–111]	rennet cheese, buckwheat groats, fish, offal, meat, wholegrain bread, legumes, eggs	phosphorus from animal products, activation of phytases in plant products (sprouting process, soaking legumes, using sourdough to bake bread)	calcium, vitamin D deficiency	organic phosphate esters, ionised inorganic forms
Mg [60,68,83,114–118]	cereals, legumes, nuts, cocoa, dark chocolate, rennet cheese, potatoes, bananas, drinking water	fermentation of soluble fibre fractions, acidic pH, vitamin B6- pyridoxine	phytic acid, phosphates, calcium	magnesium carbonate, citrate, aspartate, hydroaspartate, lactate
Zn [60,99,121,123–126]	meat, liver, rennet cheese, dark bread, buckwheat groats, eggs	citric acid, animal protein	phytic acid, oxalic acid, insoluble fibre fractions, alcohol, iron, cadmium	zinc gluconate, citrate, picolinate
Fe [60,68,128,133–141]	meat, liver, kidney, parsley, legumes, eggs	ascorbic acid, lactic acid, fermented products	phytic acid, oxalic acid, insoluble fibre fractions, plant protein, polyphenols	haem iron, ferrous sulphate, gluconate, ferric citrate, sulphate

4. Conclusions

Disorders that occur among female athletes can be severe and affect an ever-growing number of women. The problem of inadequate EA and unmet requirements for protein and carbohydrates within the diet has been observed in various sporting disciplines among women. Furthermore, inadequate vitamin D intake was noted in all the groups of athletes studied. Deficiency was also reported for in average intake of Ca, Mg, Ca/P ratio, Zn and Fe. Low energy availability, low bone density, and menstrual dysfunctions require appropriate therapeutic management, with dietary strategies playing a pivotal role. Nutrition is also crucial as a preventive measure. Therefore, cooperation within an interdisciplinary team consisting not only of a physician but also a nutritionist, physiotherapist, and psychologist is imperative. Further scientific studies assessing this comprehensive approach are also required. This is vital for adapting nutritional interventions aimed at enhancing the physical performance of female athletes and effectively preventing TRIAD/REDs. However, it should not be forgotten that health complications caused by LEA may also affect men.

Author Contributions: Conceptualisation, M.G.; methodology, M.G.; investigation, M.G. and J.P.; writing—original draft preparation, M.G.; writing—review and editing, M.G. and J.P.; visualisation, M.G.; supervision, K.S. and R.M.-Ż.; project administration, M.G. All authors have read and agreed to the published version of the manuscript.

Funding: This research received no external funding.

Institutional Review Board Statement: Not applicable.

Informed Consent Statement: Not applicable.

Data Availability Statement: Not applicable.

Conflicts of Interest: The authors declare no conflicts of interest.

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Systematic Review

Effects of Creatine Supplementation and Resistance Training on Muscle Strength Gains in Adults <50 Years of Age: A Systematic Review and Meta-Analysis

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Abstract: Background: Numerous meta-analyses have assessed the efficacy of creatine supplementation in increasing muscle strength. However, most have not considered the effect of the participants' age, training duration, or other confounding variables on strength outcomes. Therefore, the purpose of this study was to consider the effect of these variables on the potential efficacy of creatine supplementation and resistance training for improving measures of muscle strength. Methods: Four databases were searched (MEDLINE, Scopus, Embase, and SPORTDiscus) with a search end date of 22 May 2024. Twenty-three studies were included, with 20 studies involving males (447 male participants), 2 studies involving females (40 female participants), and 1 study involving both males and females (13 male participants and 9 female participants). Results: In comparison with a placebo, creatine supplementation combined with resistance training significantly increased upper-body (WMD = 4.43 kg, $p < 0.001$) and lower-body strength (WMD = 11.35 kg, $p < 0.001$). Subgroup analyses showed a trend for greater upper-body strength improvements for males on creatine compared with females on creatine ($p = 0.067$, Q = 3.366). Additionally, males who consumed creatine combined with resistance training significantly increased both upper- and lower-body strength, whereas females showed no significant gains. There was a trend indicating greater lower-body strength gains from high-dose creatine compared with lower doses ($p = 0.068$, Q = 3.341). No other variables influenced the effect of creatine supplementation. In conclusions, creatine supplementation with resistance training enhances upper- and lower-body muscle strength in adults aged < 50, with greater benefits likely to be seen in males than females.

Keywords: ergogenic aids; performance enhancement; sports nutrition; muscle performance; 1 RM

Citation: Wang, Z.; Qiu, B.; Li, R.; Han, Y.; Petersen, C.; Liu, S.; Zhang, Y.; Liu, C.; Candow, D.G.; Del Coso, J. Effects of Creatine Supplementation and Resistance Training on Muscle Strength Gains in Adults <50 Years of Age: A Systematic Review and Meta-Analysis. *Nutrients* **2024**, *16*, 3665. https://doi.org/10.3390/nu16213665

Academic Editor: Alaa El-Din A. Bekhit

Received: 25 September 2024
Revised: 18 October 2024
Accepted: 24 October 2024
Published: 28 October 2024

1. Introduction

Creatine supplementation is one of the most commonly used ergogenic aids by individuals involved in high-intensity and/or strength-based sports and activities (e.g., resistance training) [1,2]. It is well established that creatine supplementation, primarily when combined with resistance training, increases measures of muscle performance, specifically muscle strength, across a variety of populations [3]. In physiological and functional terms,

creatine supplementation may augment muscle strength by increasing intramuscular creatine stores (phosphocreatine (PCr) and free creatine), which may help resynthesize ATP during and following intense muscle contractions [4]. Creatine has also been shown to increase GLUT-4's transport kinetics, which could increase blood glucose disposal within the muscle and increase glycogen resynthesis [4]. Finally, creatine reduces blood acidosis and suppresses H^+ formation during exercise [5], which may allow an individual to perform more repetitions during each set, leading to greater muscle strength over time. These adaptations not only enhance muscle performance during acute high-intensity exercise but also result in greater strength gains when creatine is consumed over a prolonged period in conjunction with resistance training, compared with resistance training alone [3].

In recent years, several reviews and meta-analyses have summarized the wide number of randomized controlled trials (RCTs) involving creatine supplementation on muscle performance variables [6–10]. Collectively, the results have shown that creatine supplementation leads to greater improvements in muscle strength compared with a placebo. However, the generalizability and/or conclusions of these publications are limited in that they included various forms of exercise training (i.e., resistance training, aerobic, weight-bearing) involving participants across a wide age range with varying fitness levels. In this context, several reviews and meta-analyses have investigated the combined effects of creatine supplementation and resistance training—an effective intervention for increasing or maintaining muscle strength—on strength in the older population [11,12]. These studies have focused on the benefits of creatine for older adults, who are more susceptible to muscle mass and strength loss as a result of aging. Collectively, these studies have suggested that a combination of creatine supplementation and resistance training results in greater gains in muscle strength compared with resistance training and a placebo among older individuals. Moreover, it has been shown that creatine supplementation may be more effective in studies including participants > 50 years of age, as creatine supplementation may counteract the age-related reduction in muscle strength [3,11,12]. Interestingly, only one recent meta-analysis focused on the effect of creatine supplementation on fat mass in younger adults (under 50 years of age) has been published [13]. Consequently, the effect of creatine supplementation on strength gains from resistance training in adults under 50 years of age remains unknown to date.

Another limitation of the existing literature on this topic is the method of data pooling, which partially restricts the applicability and clarity of the results. The abovementioned meta-analyses have predominantly examined the pooled effect of creatine supplementation on muscle strength using standardized mean differences (SMD). However, interpreting the overall effect of creatine supplementation on muscle strength can be challenging, since SMD presents its effect size hierarchy in units of standard deviation rather than in more applicable units such as kilograms. Therefore, a more specific systematic review and meta-analysis is needed to update the outcomes of studies on the effect of creatine supplementation and resistance training on muscle strength performance, by using more severe filters to assess the magnitude of the effect on participants < 50 years of age. In line with this objective, a systematic review and meta-analysis were conducted of all RCTs that compared a group of healthy adults < 50 years of age receiving creatine supplementation during a controlled resistance training protocol vs. a comparable group of adults receiving placebo supplementation during the same resistance training protocol. Additionally, the presentation of the studies' pooled data focused on calculated improvements in muscle strength with creatine with respect to the placebo, measured in kilograms of gain. It was hypothesized that creatine supplementation combined with resistance training would enlarge muscle strength gains in comparison with the combination of a placebo and resistance training in adults < 50 years of age.

2. Methods

2.1. Search Strategy

This systematic review was conducted in accordance with the PRISMA guidelines for Exercise, Rehabilitation, Sports Medicine, and Sports Science (PERSiST) 2020 [14] and registered on the International Prospective Register of Systematic Reviews (PROSPERO) (ID: CRD42024464243). A comprehensive search was performed using relevant keywords related to creatine supplementation and resistance training, using both Medical Subject Headings (MeSH) and free text words. The databases used for the search included MEDLINE (via PubMed), Scopus, Embase, and SPORTDiscus (via EBSCO). The search was conducted from the inception of the database until 22 May 2024. The search strategy utilized key concepts: Concept 1 (strength training OR resistance training) AND Concept 2 (creatine monohydrate supplementation OR creatine supplementation). All titles and abstracts reported in these searchers were cross-referenced manually using Endnote 20 (Clarivate Analytics, London, UK). Subsequently, the titles and abstracts were carefully examined to detect duplicates and to narrow down the relevant studies for the full-text review. Two independent reviewers (Z.W. and B.Q.) conducted the searches, resolving any discrepancies through discussion and consensus. An a priori analysis of the inter-rater reliability showed that these reviewers had a kappa score of 0.83 regarding the number of studies found in each database.

2.2. Eligibility Criteria

The inclusion criteria for the reviewed studies were established on the basis of the PICOS principle (Population, Intervention, Comparison, Outcome, and Study design). The review was tailored to include only RCTs that compared the combination of creatine supplementation and resistance training with placebo supplementation and resistance training. Additionally, only RCTs focusing on outcomes related to muscle strength measured before and after resistance training were included. Only RCTs with healthy individuals and resistance-trained individuals < 50 years of age were included to help decrease the influence of biological aging on muscle strength (i.e., sarcopenia/dynapenia). Last, systematic reviews and meta-analyses were excluded, as well as studies that were not available in full text, acute interventions, non-peer-reviewed articles, opinion pieces, reviews, case reports, and editorials. Table 1 presents a detailed overview of the inclusion criteria.

Table 1. PICOS criteria for the inclusion of RCTs in which the supplementation of creatine was combined with a well-structured resistance training program and pre–post-training strength gain was compared with placebo supplementation with resistance training.

Parameters	Inclusion	Exclusion
Population	Healthy individuals under the age of 50	Individuals with diseases or those over the age of 50.
Intervention	Creatine supplementation with structured resistance training	Creatine supplementation with other types of training (e.g., aerobic training), with unstructured training or without any type of training
Comparison	Placebo with the same structured resistance training as the intervention group	Placebo without resistance training or resistance training without a placebo
Outcomes	Muscle strength gains	Any other form of performance or anthropometric assessments
Study design	Randomized controlled trials (RCTs)	Meta-analyses, articles without full text, acute interventions, non-peer-reviewed articles, opinion pieces, reviews, case reports, and editorials

2.3. Assessment of the Methodological Quality of the Included Studies

Two authors (Z.W. and B.Q.) independently assessed the methodological quality (i.e., Items 2–9) of the included studies using the Physiotherapy Evidence Database (PEDro) scale [15]. Any disagreement between researchers was resolved by consensus. Studies were scored as excellent (score: 9–10), good (score: 6–8), fair (score: 4–5), or poor (score: < 4) [16]. The score across all studies was 7.00 ± 0.95, indicating that the overall quality of the articles was categorized as "good". An a priori analysis of inter-rater reliability indicated that the reviewers achieved a kappa score of 0.91 for the scores of the included studies using the PEDro Scale. A more detailed explanation of the score obtained by each study is presented in the results section.

2.4. Data Extraction

The following characteristics of the included studies were extracted: (1) research characteristics (authors, year of publication, and country), (2) participants' characteristics (sample size; age, sex, and training status), (3) creatine supplementation characteristics (dose, duration of creatine loading (if any), and duration of creatine maintenance), and (4) resistance training characteristics (frequency and duration of the intervention). The primary outcome of this review was the effect of creatine supplementation on maximal muscle strength, and the data were extracted from tests that measured this variable before and after the resistance training program, mainly with one-repetition maximum tests (1 RM). For each of the groups included in each study, the sample size was included in the analysis, and the means and standard deviations were extracted for both the pre- and post-intervention measurements for the creatine and placebo groups. For studies that did not provide detailed information on the pre- and post-intervention measurements for the creatine and placebo groups but met the inclusion criteria for this systematic review, the authors were contacted by email to obtain such data.

2.5. Data Analysis

Comprehensive Meta-Analysis software (version 4; Biostat, Englewood, NJ, USA) was used as the data analysis processing software for this review. For the meta-analyses, weighted mean differences (WMD) were calculated, and 95% confidence interval (CI) estimates were obtained from studies by comparing pre–post-training changes in muscle strength in the creatine and placebo groups. WMD was used to provide the effect of creatine supplementation over the placebo in kilograms, as mentioned above. The I^2 statistic was used to measure the degree of heterogeneity, with values less than 50% indicating low heterogeneity, 50–75% indicating moderate heterogeneity, and values greater than 75% indicating high heterogeneity [17,18].

In the subgroup analyses, the effects of creatine supplementation on muscle strength were examined, depending on (1) the characteristics of creatine supplementation (low-dose: $\leq$5 g/day vs. high-dose: >5 g/day [19]; creatine loading phase vs. no creatine loading loading), (2) the characteristics of resistance training (training $\leq$3 times per week vs. >3 times per week; intervention period <8 weeks vs. $\geq$8 weeks), and (3) population characteristics (resistance-trained vs. untrained; males vs. females). To assess potential publication bias in the combined data from each study, funnel plots were visually examined, and Egger's linear regression tests were used. [20]. Moreover, Duval and Tweedie's trim and fill method was used to identify missing studies if there was a potential publication bias [21]. Statistical significance was considered at $p < 0.050$.

3. Results

3.1. Study Selection

After removing duplicates, 799 records remained from the initial 1586 found during the original search. Following title and abstract screening, 700 records were excluded, leaving 99 articles for full-text examination. Ultimately, according to the studies' characteristics,

23 papers [22–44] were selected for the current systematic review and meta-analysis, while the remaining 76 were discarded (Figure 1).

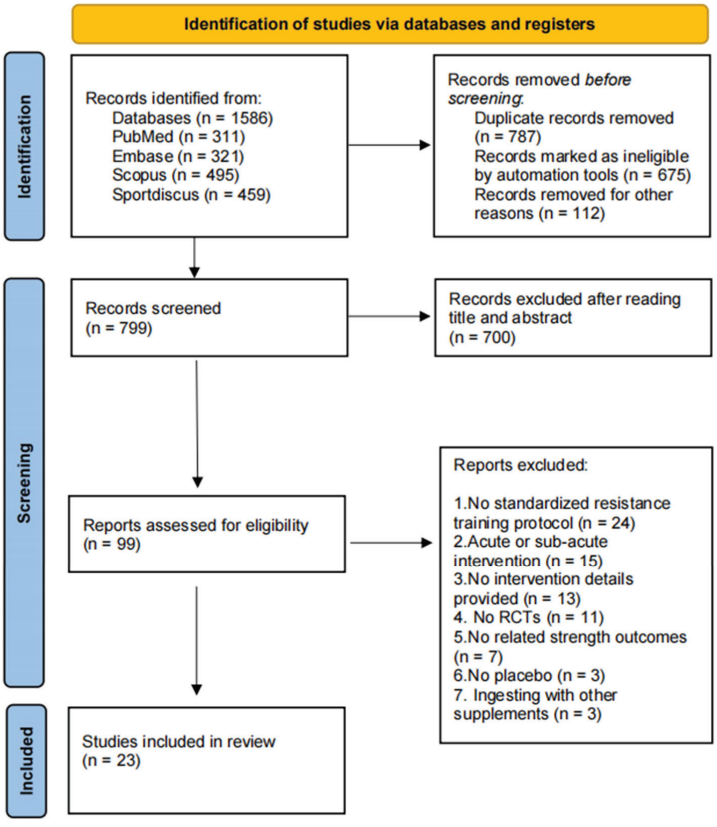

Figure 1. Literature search flowchart, following the PRISMA 2020 guidelines.

3.2. Methodological Quality of the Studies

Using the PEDro scale, it was determined that of the 23 studies [22–44] included, 2 articles [22,41] were rated as excellent, and the other 21 articles [23–40,42–44] were categorized as good quality (Table 2).

Table 2. Evaluation of the methodological quality of eligible studies (*n* = 23) utilizing the Physiotherapy Evidence Database (PEDro) scale.

	Assessment Criteria												
Study	**1**	**2**	**3**	**4**	**5**	**6**	**7**	**8**	**9**	**10**	**11**	**Total Score**	**Quality Assessment**
Almeida 2020 [23]	Y	1	0	1	1	1	0	1	0	1	1	7	Good
Arazi 2019 [22]	Y	1	1	1	1	1	1	1	0	1	1	9	Excellent
Arciero 2001 [24]	Y	1	0	1	1	1	0	1	0	1	1	7	Good
Bemben 2001[25]	Y	1	0	1	1	1	0	1	0	1	1	7	Good
Cribb 2007 [26]	Y	1	0	1	1	1	0	0	0	1	1	6	Good
Ferguson 2005 [27]	Y	1	0	1	1	1	0	0	0	1	1	6	Good
Hoffman 2006 [28]	Y	1	1	1	1	1	0	1	0	1	1	8	Good
Kaviani 2019 [29]	Y	1	0	1	1	1	0	0	1	1	1	7	Good
Kelly 1998 [30]	Y	1	0	1	1	1	0	1	0	1	1	7	Good
Larson 2000 [31]	Y	1	1	0	1	1	0	0	0	1	1	6	Good
Mills 2020 [32]	Y	1	1	1	1	1	0	0	1	1	1	8	Good
Noonan 1998 [33]	Y	1	1	1	1	1	0	0	1	1	1	8	Good
Pearson 1999 [34]	Y	1	0	1	1	1	0	1	0	1	1	7	Good
Peeters 1999 [35]	N	1	0	1	1	1	0	0	0	1	1	6	Good
Sandro 2012 [36]	Y	0	1	0	1	1	0	1	0	1	1	6	Good
Saremi 2010 [37]	Y	1	0	1	1	1	0	0	0	1	1	6	Good

Table 2. *Cont.*

	Assessment Criteria												
Study	**1**	**2**	**3**	**4**	**5**	**6**	**7**	**8**	**9**	**10**	**11**	**Total Score**	**Quality Assessment**
Stone 1999 [38]	Y	1	0	1	1	1	0	1	0	1	1	7	Good
Stout 1999 [39]	Y	1	0	1	1	1	0	0	0	1	1	6	Good
Syrotuik 2000 [40]	Y	1	0	1	1	1	0	0	0	1	1	6	Good
Taylor 2011 [41]	Y	1	1	1	1	1	0	1	1	1	1	9	Excellent
Volek 1999 [42]	Y	1	0	1	1	1	0	1	0	1	1	7	Good
Wang 2018 [43]	Y	1	1	1	1	1	0	1	0	1	1	8	Good
Wilder 2002 [44]	Y	1	0	1	1	0	0	1	1	1	1	7	Good

1, eligibility criteria; 2, random allocation; 3, concealed allocation; 4, baseline comparability; 5, blinded subjects; 6, blinded therapists; 7, blinded assessors; 8, adequate follow-up; 9, intention-to-treat analysis; 10, between-group comparisons; 11, point estimates and variability. The total score represents the score of the PEDro scale. Item 1 was not scored. Y, yes; N, no.

3.3. The Characteristics of the Included Studies

Out of the 23 studies [22–44] included in this systematic review, 20 studies [22–26,28–30,33–44] involved males, with a total of 447 male participants, while 2 studies [27,31] involved females, with a total of 40 female participants, and 1 study [32] involved both males and females, including 13 male participants and 9 female participants. Participants in 18 studies [25–28,30–36,38–44] had experience with resistance training, while the remaining 5 studies [22–24,29,37] included participants with no experience of resistance training.

The doses of creatine used ranged from 15 to 25 g/day or 0.3 g/kg/day during the creatine loading period (i.e., the first $\leq$ 7 days of the creatine intervention) and from 2 to 10 g/day or from 0.03 to 0.22 g/kg/day during the remainder of the creatine intervention. The duration of the resistance training ranged from 4 to 12 weeks, with weekly frequencies ranging between two and five sessions.

Twenty-one studies provided data on upper-body muscle strength (n = 433) and nineteen studies provided data on lower-body muscle strength (n = 395), all of which were measurements of maximal muscle strength. Detailed information on the controlled trials included in this systematic review is presented in Table 3.

Table 3. Information on the studies that were included in the systematic review (n = 23).

Author, Year, Country	**Participants, Total N, Age**	**Duration, Sessions/ Week**	**Creatine, Loading Protocol**	**Creatine, Maintenance Protocol**	**Muscle Group Location**
Almeida et al. [23], 2020, Brazil	Untrained males, 34, 23.45 ± 3.17	4 weeks, 3/week	0.3 g/kg/day for 7 days	0.03 g/kg/day for 21 days	Upper body, lower body
Arazi et al. [22], 2019, Iran	Untrained males, 16, 18 ± 3	6 weeks, 3/week	20 g/day for 5 days	5 g/day for 32 days	Upper body, lower body
Arciero et al. [24], 2001, USA	Untrained males, 20, 22 ± 2.99	4 weeks 3/week	20 g/day for 5 days	5 g/day for 23 days	upper body, lower body
Bemben et al. [25], 2001, USA	Trained males, 17, 19.35 ± 0.34	9 weeks 4/week	20 g/day for 5 days	5 g/day for 44 days	Upper body, lower body
Cribb et al. [26], 2007, Australia	Trained males, 15, 24.53 ± 6.27	11 weeks 4/week	0.3 g/kg/day for 7 days	0.1 g/kg/day for 70 days	Upper body, lower body
Ferguson et al. [27], 2005, Canada	Trained females, 26, 24.6 ± 3.68	9.5 weeks 4/week	0.3 g/kg/day for 7 days	0.03 g/kg/day for 58 days	Upper body, lower body
Hoffman et al. [28], 2006, USA	Trained males, 33, NR	10 weeks 4/week	No loading	10.5 g/day for 70 days	Upper body, lower body
Kaviani et al. [29], 2019, Canada	Untrained males, 18, 23 ± 3	8 weeks 3/week	No loading	0.07 g/kg/day for 56 days	Upper body, lower body

Table 3. *Cont.*

Author, Year, Country	Participants, Total N, Age	Duration, Sessions/ Week	Creatine, Loading Protocol	Creatine, Maintenance Protocol	Muscle Group Location
Kelly et al. [30], 1998, Australia	Trianed males, 18, 26.8 ± 5.78	4 weeks 2/week	20 g/day for 5 days	5 g/day for 21 days	Upper body
Larson et al. [31], 2000, USA	Trained females, 14, 19.15 ± 1.40	12 weeks 3/week	15 g/day for 5 days	5 g/day for 12 weeks	Upper body, lower body
Mills et al. [32], 2020, USA	Trained males and females, 22, 26 ± 4.32	6 weeks 5/week	No loading	0.1 g/kg/day for 6 weeks	Upper body, lower body
Noonan et al. [33], 1998, USA	Trained males, 39, 19.83 ± 1.3	8 weeks 4/week	20 g/day for 5 days	Low dose: 0.1 g/kg/day for 51 days High dose: 0.3 g/kg/day for 51 days	Upper body
Pearson et al. [34], 1999, USA	Trained males, 16, 20.7	10 weeks 4/week	No loading	5 g/day for 70 days	Upper body, lower body
Peeters et al. [35], 1999, USA	Trianed males, 34, 21.2 ± 2.6	6 weeks 4/week	20 g/day for 3 days	10 g/day for 39 days	Upper body, lower body
Sandro et al. [36], 2012, Brazil	Trained males 18, 17.10 ± 1.63	4 weeks 3/week	20 g/day for 5 days	5 g/day for 27 days	Upper body
Saremi et al. [37], 2010, Iran	Untrained males, 16, 23.06 ± 2.65	8 weeks 3/weeks	0.3 g/kg/day for 7 days	0.05 g/kg/day for 35 days	Upper body, lower body
Stone et al. [38], 1999, USA	Trained males, 20, 18.48 ± 0.74	5 weeks 3/week	No loading	0.22 g/kg/day for 35 days	Upper body, lower body
Stout et al. [39], 1999, USA	Trained males, 16, 19.6 ± 1.0	8 week 4/week	21 g/day for 5 days	10.5 g/day for 51 days	Upper body
Syrotuik et al. [40], 2000, Canada	Trained males, 14, 23.15 ± 0.89	5 weeks 4/week	0.3 g/kg/day for 5 days	0.03 g/kg/day for 32 days	Upper body, lower body
Taylor et al. [41], 2011, USA	Trained males, 29, 20.38 ± 2.88	8 week 4/week	No loading	5 g/day for 8 weeks	Upper body, lower body
Volek et al. [42], 1999, USA	Trained males, 19, 25.51 ± 5.2	12 weeks 4/week	25 g/day for 7 days	5 g/day for 77 days	Upper body, lower body
Wang et al. [43], 2018, China	Trained males, 30, 20 ± 1.55	4 week 3/week	20 g/day for 6 days	2 g/day for 22 days	Lower body
Wilder et al. [44], 2002, USA	Trained males, 25, 19 ± 1.02	10 week 4/week	20 g/day for 1 week	Low dose: 3 g/day for 10 weeks High dose: 5 g/day for 9 weeks	Lower body

3.4. Meta-Analyses

3.4.1. Upper-Body Muscle Strength

Creatine supplementation combined with resistance training resulted in greater increases in upper-body strength compared with a placebo, with a very high probability (WMD = 4.43 kg, 95% CI [3.12,5.75], $p < 0.001$) (Figure 2). The test for heterogeneity showed that there was no statistically significant heterogeneity among studies for this outcome ($p = 0.926$, $I^2 = 0\%$).

Six subgroup meta-analyses were performed (Figure 3) according to the characteristics of the creatine supplementation protocol (the existence of creatine loading and creatine dose), the characteristics of the resistance training protocol (duration and frequency of training), and the characteristics of the participants (training status and sex).

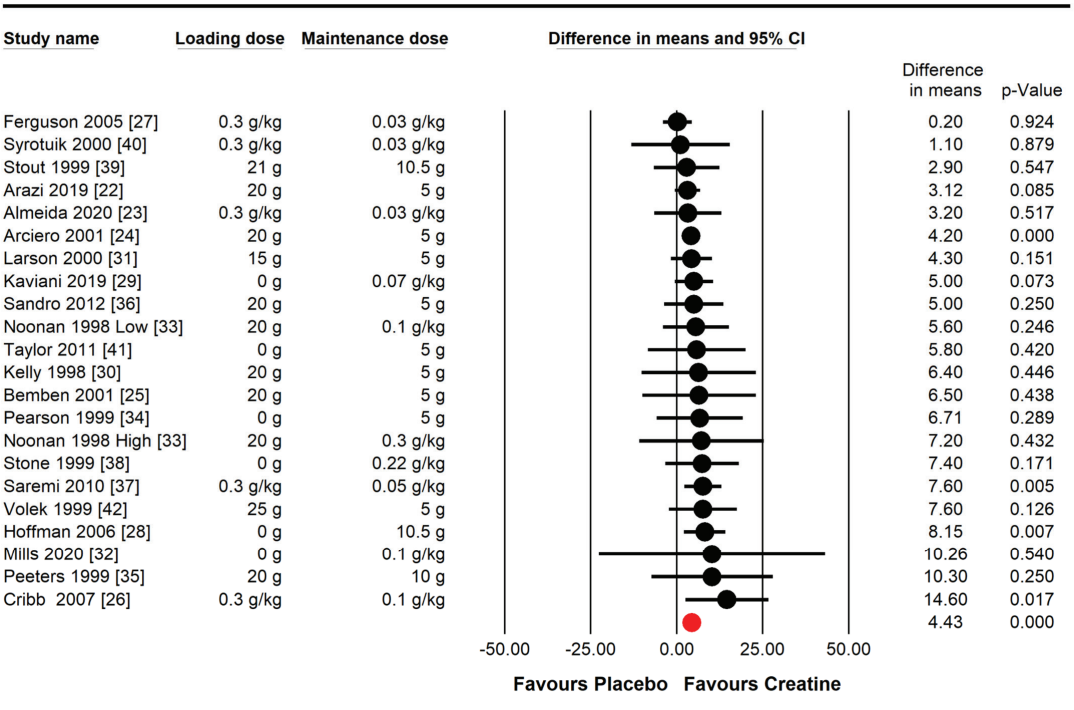

Figure 2. Effect of creatine supplementation and resistance training compared with a placebo and resistance training on upper-body strength. The red circle represents the pooled weighted mean difference following a random effect meta-analysis, expressed in kg.

Regarding biological sex differences, males on creatine experienced a greater improvement in upper-body strength compared with males on a placebo, with a very high probability (WMD = 4.95 kg, 95% CI [3.52, 6.38], $p < 0.001$). However, the effects of creatine did not reach statistical significance over a placebo in females (WMD = 1.54 kg, 95% CI [−1.81, 4.89], $p = 0.368$). There was a trend for greater upper-body strength gains from creatine in males vs. females ($p = 0.067$, Q = 3.366).

Creatine dosage (≤5 g/day vs. >5 g/day) and type of ingestion protocol, training status and duration and frequency of the training program had no influence on the upper-body strength gains obtained with creatine.

3.4.2. Lower-Body Muscle Strength

Creatine supplementation combined with resistance training produced greater increases in maximal lower-body strength compared with a placebo, with a very high probability (WMD = 11.35 kg, 95% CI [8.44,14.25], $p < 0.001$; Figure 4). Heterogeneity tests showed no statistically significant heterogeneity among studies for lower-body muscle strength gains ($p = 0.897$, $I^2 = 0\%$).

Similar to upper-body strength, six subgroup meta-analyses were analyzed for lower-body strength, including the same categories (Figure 5).

Subgroup	Creatine	Placebo	MD with 95%CI	P1	P2
All participants	224	209	4.43 (3.12 to 5.75)	<0.001	NA
Creatine loading					0.192
No loading	64	63	6.57 (3.10 to 10.04)	<0.001	
Yes loading	160	146	4.07 (2.65 to 5.50)	<0.001	
Creatine dose					0.322
Low dose(≤5g/d)	157	142	4.17 (2.75 to 5.58)	<0.001	
High dose(>5g/d)	67	67	6.11 (2.54 to 9.68)	0.001	
Intervention week					0.543
Short intervention(<8w)	93	94	4.08 (2.35 to 5.81)	<0.001	
Long intervention(≥8w)	131	115	4.91 (2.89 to 6.93)	<0.001	
Training frequency					0.987
Low frequency(≤3/pw)	86	88	4.44 (2.90 to 5.98)	<0.001	
High Frequency(>3/pw)	138	121	4.42 (1.88 to 6.95)	0.001	
Training status					0.844
No trained	60	59	4.53 (2.89 to 6.17)	<0.001	
Trained	164	150	4.25 (2.04 to 6.47)	<0.001	
Gender					0.067
Female	20	20	1.54 (-1.81 to 4.89)	0.368	
Male	191	180	4.95 (3.52 to 6.38)	<0.001	

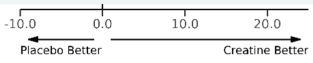

Figure 3. Subgroup analyses for creatine supplementation combined with resistance training on upper-body muscle strength compared with placebo supplementation combined with resistance training. MD, mean difference (kg); P1, *p*-value for the within-subgroup comparison (i.e., pre–post-intervention changes within each subgroup); P2, *p*-value for the between-subgroup comparison (i.e., comparison of the pre–post-intervention changes between subgroups); NA, Not Available.

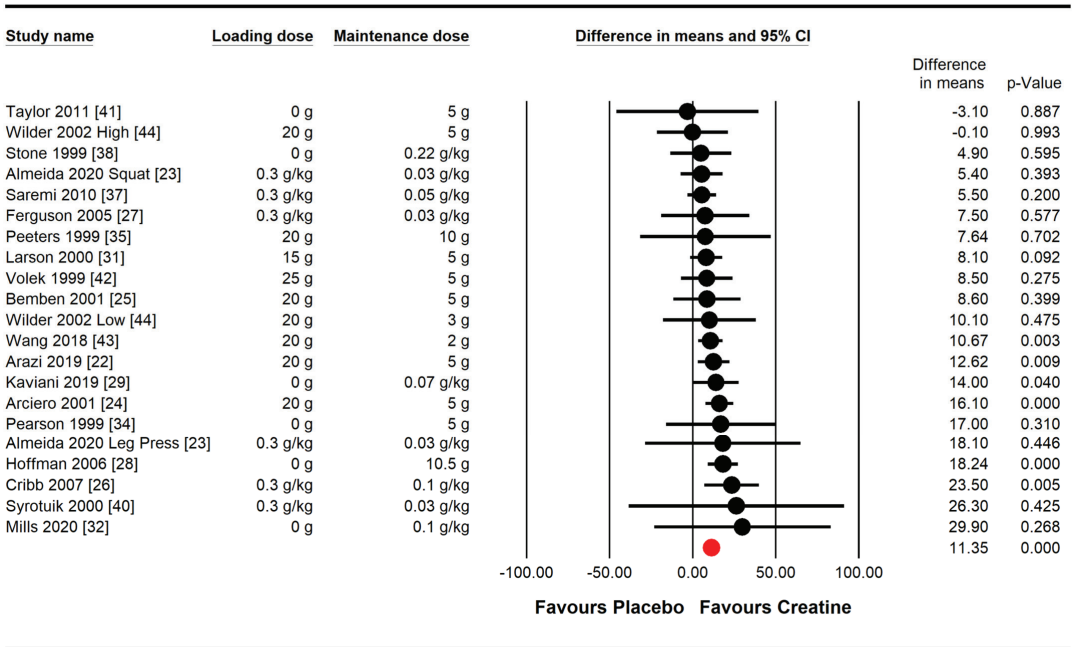

Figure 4. Effect of creatine supplementation and resistance training compared with a placebo and resistance training on lower-body muscle strength. The red circle represents the pooled weighted mean difference following a random effect meta-analysis, expressed in kg.

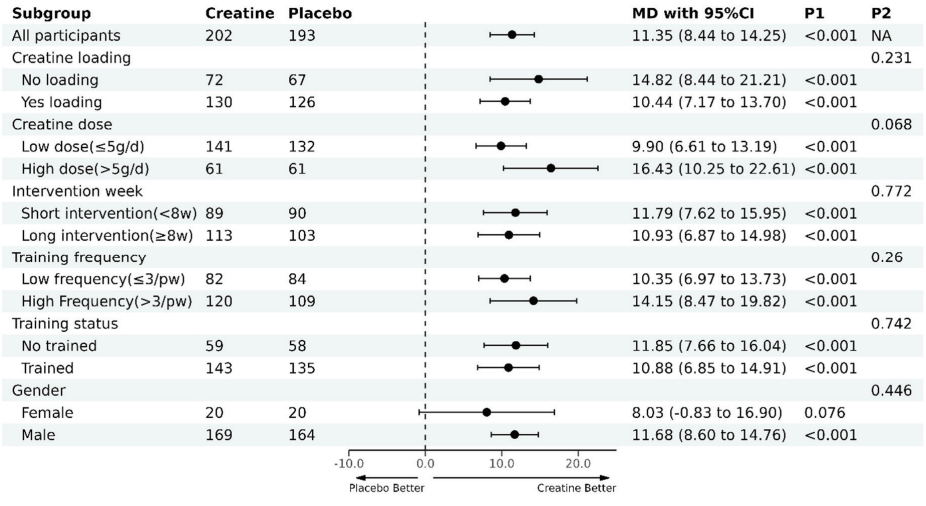

Figure 5. Subgroup analyses for creatine supplementation and resistance training compared with a placebo and resistance training on lower-body strength. MD, mean difference(kg); P1, *p*-value for the within-subgroup comparison (i.e., pre–post-intervention changes within each subgroup); P2, *p*-value for the between-subgroup comparison (i.e., comparison of the pre–post intervention changes between subgroups); NA, Not Available.

Regarding biological sex differences, males on creatine experienced a greater improvement in lower-body strength compared with males on a placebo, with very high probability (WMD = 11.68 kg, 95% CI [8.60,14.76], $p < 0.001$). However, creatine did not result in greater strength improvements compared with the placebo in females (WMD = 8.03 kg, 95% CI [−0.83,16.90], $p = 0.076$). Strength improvements from creatine were similar for males and females over time ($p = 0.446$, Q = 1.056).

With regard to dose–response differences, it is noteworthy that there was a trend ($p = 0.068$, Q = 3.341) indicating greater strength gains with high-dose creatine (>5 g/day) compared with lower-dose creatine (≤5 g/day) for lower-body strength gains. Creatine dosage (≤5 g/day vs. >5 g/day), ingestion protocol, training status, and the duration and frequency of training had no influence on strength gains between creatine and the placebo.

3.5. Publication Bias

Funnel plots were used to detect publication bias. The funnel plot for the gains obtained with creatine over the placebo on upper-body muscle strength was asymmetric, suggesting possible publication bias. This was confirmed by Egger's linear regression test ($t = 2.102$, $p = 0.048$). However, there was no significant change in upper-body muscle strength (WMD = 3.60 kg, 95% CI [2.39, 4.81]) after adjustment for Duval and Tweedie's trim-and-fill test (Figure 6).

The funnel plot for the gains obtained with creatine over the placebo on lower-body muscle strength was symmetrical (Figure 7), indicating no significant publication bias, which was confirmed by Egger's linear regression test ($t = 0.122$, $p = 0.90$).

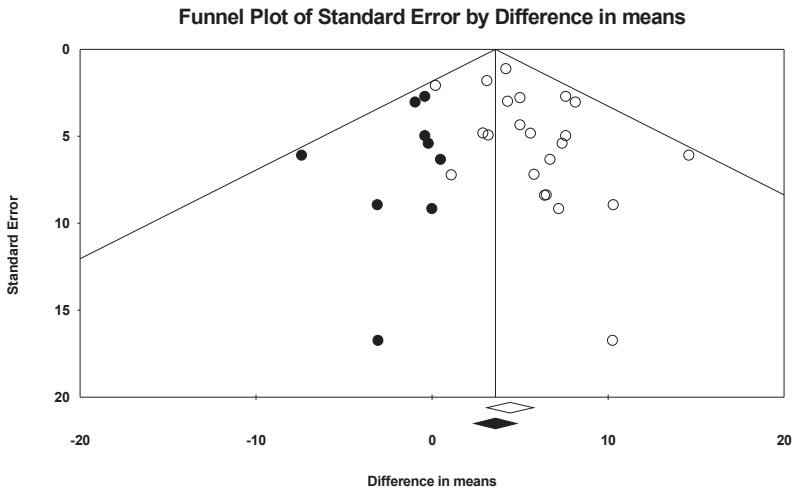

Figure 6. Observed and imputed funnel plot for upper-body muscle strength. The funnel plot displays the distribution of studies included in this meta-analysis, with white circles representing the original observed studies and black circles indicating the imputed studies added to account for potential publication bias using the trim and fill method. At the bottom of the funnel plot, the white and black diamonds represent the combined effect sizes, with the white diamond indicating the overall effect from the observed studies, and the black diamond showing the adjusted effect after imputation.

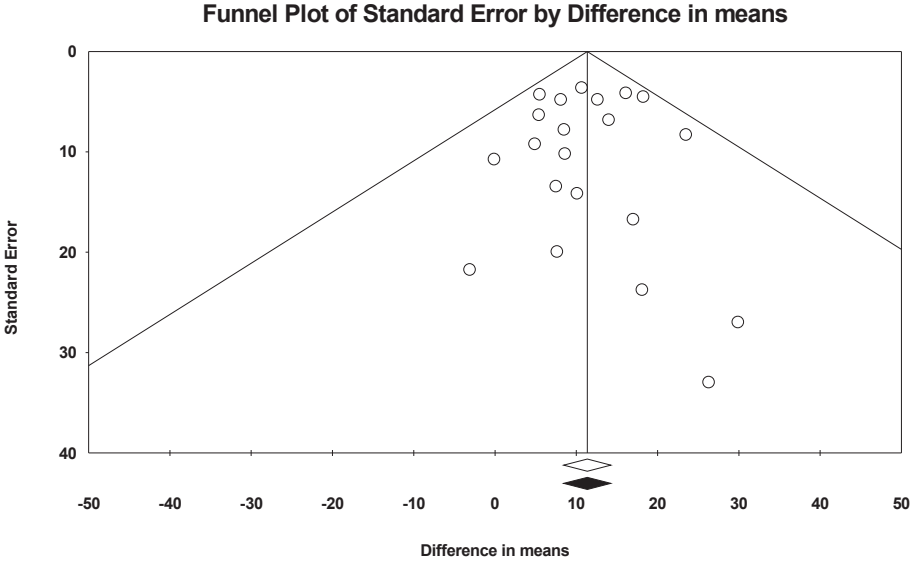

Figure 7. Observed and imputed funnel plot for lower-body muscle strength. The funnel plot displays the distribution of studies included in this meta-analysis, with white circles representing the original observed studies. At the bottom of the funnel plot, the white and black diamonds represent the combined effect sizes, with the white diamond indicating the overall effect from the observed studies and the black diamond showing the adjusted effect after imputation.

4. Discussion

The most important findings from the meta-analyses were that: (1) the combination of creatine supplementation and resistance training results in greater strength gains in both the upper and lower body compared with resistance training alone in adults < 50 years of age, (2) males on creatine improved muscle strength compared with males on a placebo for both the upper and lower body, and (3) creatine had no significant effect on strength in females. Collectively, these results suggest that the addition of creatine supplementation to a resistance training program improves training adaptations for upper- and lower-body maximal muscle strength in healthy individuals under 50 years of age, and these effects are likely driven by the inclusion of biological males.

4.1. Effects of Creatine Supplementation on Muscle Strength

In the present meta-analysis, creatine supplementation during resistance training induced higher gains in maximal strength than placebo, with a 4.43 kg benefit for upper-body strength and an 11.35 kg benefit for lower-body strength. These results support previous meta-analyses [9–11,45] which showed that creatine supplementation increased measures of upper- and lower-body maximal strength. While no mechanisms were determined in any of these meta-analyses, it is possible that the greater improvements in muscle strength from creatine supplementation are related to creatine augmenting intramuscular creatine stores (i.e., PCr and free creatine), which would expedite ATP resynthesis and/or PCr recovery during and after exercise [46–48]. Additionally, creatine has also been shown to increase GLUT-4 transport kinetics, which could increase blood glucose disposal within the muscle and increase glycogen resynthesis [4,49,50]. Finally, while creatine is suggested to reduce blood lactate levels and enhance exercise performance [5], this study did not directly measure blood acidosis or H^+ formation. The reduced lactate may indicate improved lactate utilization by muscle, potentially allowing individuals to perform more repetitions in each set, leading to greater muscle strength over time.

4.2. Characteristics of Creatine Supplementation

The most rapid way to increase muscle creatine stores is to ingest high doses (~20 g/day for 5–7 days; the creatine-loading phase) [51]. Once intramuscular creatine stores are fully saturated, muscle creatine stores can usually be maintained by ingesting small doses (~2–5 g/day) [3]. Another supplementation strategy is to refrain from the creatine loading phase and ingest lower daily doses (~3–5 g/day) of creatine monohydrate for longer periods of time (i.e., ≥28 days) [52]. The results showed no significant difference in strength gains between lower-dose (≤5 g/d) vs. high-dose(>5 g/d) creatine, with gains of 4.17 kg vs. 6.11 kg for upper-body strength, respectively. Although the lower-dose supplementation approach may delay the saturation of intramuscular creatine levels [52], the intervention cycles of the RCTs analyzed were all ≥ 4 weeks, which may account for the lack of significance between the lower- and high-dose creatine protocols. Therefore, it is likely that the presence of a creatine loading phase may not be needed if the objective is to increase muscle strength when performing resistance training for ≥ 4 weeks. Specifically, the results of this study show that for upper-body strength, the subgroup of studies including a loading phase had an average increase of 6.57 kg, while the subgroup of studies without a loading phase had an increase of 4.07 kg; for lower body strength, the loading subgroup increased by 14.82 kg compared with 10.44 kg for the non-loading subgroup. Although the comparison between the two methods of supplementation with creatine did not report statistically significant differences, the slightly higher muscle strength gains in those studies with loading vs. non-loading phases (2.50 kg for the upper body and 4.38 kg for lower-body strength) may be considered as potentially beneficial by some strength training practitioners, especially if seeking fast strength gains with resistance training programs shorter than 4 weeks.

Regarding possible creatine dose–response differences, the results showed that high-dose creatine supplementation (>5 g/day) had a favorable but not significantly larger effect

(9.90 kg vs. 16.43 kg for the lower body, $p = 0.068$) on lower-limb muscle strength, compared with lower-dose creatine ($\leq$5 g/day). This is similar to another previous meta-analysis with a similar approach [45]. This may be due to the fact that individuals with a heavier body weight may need to consume as much as 5–10 g/day of creatine in order to maintain stores [53,54], making the enhancement effect of higher doses of creatine supplementation (>5 g/day) significant. Collectively, all this information suggests that the loading phase and the dose are not key factors to obtain further strength gains with creatine supplementation combined with resistance training. However, these factors (i.e., including a loading phase or the use of doses $\leq$ 5 g/day) may make valuable contributions for shorter resistance training programs or for those focused on the lower limbs.

4.3. Population Characteristics

Regarding biological sex differences, the present meta-analysis showed that creatine supplementation + resistance training demonstrated superior efficacy for improving muscle strength compared with a placebo and resistance training in males only, with average increases of 4.95 kg in upper-body strength and 11.68 kg in lower-body strength. In contrast, the analysis found that creatine supplementation combined with resistance training did not result in a significant improvement in muscle strength for females, with average increases of 1.54 kg in upper-body strength and 8.03 kg in lower-body strength, indicating no notable differences compared with the placebo and resistance training alone. Mechanistically, the blunted response in females may be related to higher pre-supplementation intramuscular creatine levels [55], which may attenuate the responsiveness to creatine supplementation over time [56]. There is also some evidence that females do not experience a reduction in measures of amino acid catabolism to the same extent as males [57]. Further, menstrual cycle fluctuations and estrogen cessation cannot be ruled out [56].

4.4. Characteristics of Resistance Training

Subgroup analyses showed that the duration of training did not influence strength adaptations over time, with average increases of 4.08 kg for upper-body strength in the short-duration subgroup compared with 4.91 kg in the long-duration subgroup, and 11.79 kg in the short-duration subgroup compared with 10.93 kg in the long-duration subgroup for lower-body strength. These results are in contrast to a previous review [11], which showed that interventions of $\geq$24 weeks appeared to be more effective in resistance-trained older females. Unfortunately, the studies included in this investigation with healthy participants < 50 years or age included resistance training protocols with a duration ranging from 4 to 12 weeks. Therefore, it was not possible to determine the optimal duration of an RT for younger populations aiming to maximize the strength gains obtained with creatine supplementation. This study reveals the need to experiment with the effects of creatine supplementation during RT for more than 12 weeks in young and healthy adults, as has been carried out in other populations. Additionally, different resistance training frequencies seemed to not affect the adaptation of creatine supplementation combined with resistance training to muscle strength, with average increases of 4.44 kg for upper-body strength in the low-frequency subgroup compared with 4.42 kg in the high-frequency subgroup, and 10.35 kg in the low-frequency subgroup compared with 10.93 kg in the high-frequency subgroup for lower-body strength. These data should be interpreted cautiously, as the studies had different durations and frequencies, and even the subgroups created to normalize training frequency presented studies with a certain variation.

4.5. Limitations, Practical Applications, and Future Research Directions

Several limitations must be acknowledged in this study.

Firstly, despite applying selective search and inclusion filters, the included studies utilized varying doses of creatine and differing durations of supplementation, and some included creatine-loading phases, while others did not. In addition, nine studies adjusted the doses based on the participants' body mass, whereas others used absolute doses of

creatine. These variations likely introduced differences in the magnitude of creatine's effects compared with a placebo [58,59] and may have led to publication bias regarding creatine's impact on upper-body muscle strength. Secondly, the intensity, volume, and types of resistance training protocols varied across included studies, preventing the determination of which type of resistance training optimally enhances muscle strength. Thirdly, the effects of creatine supplementation combined with resistance training on different sexes should be interpreted with caution, as only two studies included female participants. Lastly, other factors, such as the timing of creatine supplementation and its combination with substances such as CHO were not analyzed, though these factors could influence muscle creatine uptake and, consequently, the benefits obtained from supplementation.

Despite its limitations, this research provides valuable insights for resistance training enthusiasts and offers a direction for future studies. The findings suggest that creatine supplementation, with or without a loading phase, can significantly enhance the strength gains from resistance training. This indicates that resistance training enthusiasts may experience faster and greater strength improvements with creatine supplementation compared with training without it. Additionally, this investigation also suggests that, overall, all doses of creatine lead to benefits, but higher doses of creatine (greater than 5 g/day) may lead to more substantial improvements in lower-body strength. Therefore, it is advisable to adjust the creatine dosage on the basis of individual body weight during resistance training, while some athletes focused on lower-body strength may consider the use of higher doses of creatine. The current findings suggest that the periodization of resistance training and training frequency have minimal effects on strength adaptations related to creatine supplementation. This outcome may be a conclusion affected by the different training protocols used and the relatively small number of studies that used comparable strength training protocols. Future investigations should aim to study the effect of creatine supplementation with modifications of the intensity, frequency, time, and type of resistance training. These future investigations should focus on resolving which strength training protocol maximizes the benefits that can be obtained with creatine. Finally, it is crucial to explore potential sex differences in these responses, as there is a limited number of studies focused on female participants.

5. Conclusions

Creatine supplementation combined with resistance training significantly improves upper- and lower-body muscle strength compared with resistance training alone in healthy individuals < 50 years of age. Specifically, the data on this systematic review and meta-analysis suggest that 4–12 weeks of 2 to 10 g/day or from 0.03 to 0.22 g/kg/day of creatine supplementation combined with resistance training significantly improves upper-body muscle strength by 4.43 kg and lower-body muscle strength by 11.35 kg compared with resistance training alone in healthy individuals < 50 years of age. The benefits of creatine supplementation combined with resistance training are likely of higher magnitude in males than in females. Future research should prioritize investigating different resistance training protocols to determine which approach maximizes the benefits of creatine supplementation. Additionally, addressing the imbalance in male and female representation is crucial for developing more generalizable supplementation strategies.

Author Contributions: Conceptualization, Z.W., B.Q., Y.Z., C.L. and J.D.C.; methodology, Z.W. and B.Q.; software, Z.W.; validation, B.Q., R.L. and Y.H.; formal analysis, Z.W. and B.Q.; writing—original draft preparation, Z.W., B.Q., R.L., Y.H., C.P., S.L., Y.Z., C.L., D.G.C. and J.D.C.; writing—review and editing, Z.W., B.Q., R.L., Y.H., C.P., S.L., Y.Z., C.L., D.G.C. and J.D.C. All authors have read and agreed to the published version of the manuscript.

Funding: This research received no external funding.

Data Availability Statement: The raw data supporting the conclusions of this article will be made available by the authors on request. The data are not publicly available due to concerns regarding data integrity.

Conflicts of Interest: D.G.C. has conducted industry-sponsored research involving creatine supplementation and received creatine donations for scientific studies and travel support for presentations involving creatine supplementation at scientific conferences. In addition, D.G.C. serves on the Scientific Advisory Board for Alzchem and Create (companies that manufacture creatine products) and as an expert witness/consultant in legal cases involving creatine supplementation. The authors declare no conflict of interest.

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ISBN 978-3-7258-2816-6