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Sports Nutrition and Performance Enhancing Supplements

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Time of Day and Training Status Both Impact the Efficacy of Caffeine for Short Duration Cycling Performance

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Abstract: This project was designed to assess the effects of time of day and training status on the benefits of caffeine supplementation for cycling performance. Twenty male subjects (Age, 25 years; Peak oxygen consumption, 57 $mL \cdot kg^{-1} \cdot min^{-1}$) were divided into tertiles based on training levels, with top and bottom tertiles designated as 'trained' ($n = 7$) and 'untrained' ($n = 7$). Subjects completed two familiarization trials and four experimental trials consisting of a computer-simulated 3-km cycling time trial (TT). The trials were performed in randomized order for each combination of time of day (morning and evening) and treatment (6mg/kg of caffeine or placebo). Magnitude-based inferences were used to evaluate all treatment effects. For all subjects, caffeine enhanced TT performance in the morning (2.3% ± 1.7%, 'very likely') and evening (1.4% ± 1.1%, 'likely'). Both untrained and trained subjects improved performance with caffeine supplementation in the morning (5.5% ± 4.3%, 'likely'; 1.0% ± 1.7%, 'likely', respectively), but only untrained subjects rode faster in the evening (2.9% ± 2.6%, 'likely'). Altogether, our observations indicate that trained athletes are more likely to derive ergogenic effects from caffeine in the morning than the evening. Further, untrained individuals appear to receive larger gains from caffeine in the evening than their trained counterparts.

Keywords: exercise time of day; caffeine supplementation; training history; diurnal; training status

1. Introduction

Caffeine use in sport is widespread due to its reputed performance benefits. There is consistent evidence that caffeine enhances cycling performance in events lasting longer than a few minutes [1–5]. While not unanimous [6–8], caffeine intake can also improve peak anaerobic power and speed [9–11] as well as peak muscle function (strength, power, and endurance) under certain conditions [6,7,12,13]. Although caffeine has the capacity to improve physical performance, there are a number of unresolved factors that may impact the magnitude of the effect of caffeine, such as time of day and training status.

Only two studies have investigated the potential interaction between time of day and caffeine on performance outcomes, and both suggest that the value of caffeine is heightened in the morning. In the first study, caffeine increased peak squat power in the morning but not in the evening [13]. Caffeine appeared to compensate for underperformance in the morning placebo trial such that squat power was elevated to levels observed in both evening trials (caffeine and placebo). We recently investigated whether time of day influenced the effects of caffeine on cycling performance, using a post-hoc analysis in which cyclists who completed trials early in the day (prior to 10 a.m.) were

compared to those who performed later in the day (after 10 a.m.) [5]. In line with Mora-Rodríguez et al., caffeine ingestion improved performance among subjects that completed their trials early in the day but had an unclear effect on performance in those who performed later trials. Based on these preliminary results, the primary purpose of the present study was to use a crossover design to test the hypothesis that caffeine would elicit larger improvements in 3-km time trial (TT) performance in the morning compared to the evening.

Like time of day, training status may also mediate the magnitude of caffeine's ergogenic effect. A 2010 meta-analysis indicated that caffeine tended ($p = 0.08$) to enhance muscle endurance in untrained more so than trained subjects [14]. However, this conclusion was largely reached by comparing effect sizes derived from studies with trained subjects to other studies with untrained individuals. Regardless of the performance measure, we are aware of only four investigations that included both trained and untrained subjects in the same experimental design, the first of which reported that caffeine improved 100 m swim performance more so in trained than untrained swimmers [10]. Though this is in contrast to the meta-analysis, it may not be fair to use swimming as a model to determine the effects of training status, as the technical nature of swimming mechanics likely made it difficult for the untrained swimmers to take full advantage of potential improvements in whole muscle function. The only other study to compare trained and untrained subjects in the same design, that also observed caffeine-induced improvements in performance, reported that untrained and trained subjects experienced similar improvements in 10-km cycling performance [15], which again is in contrast to the prior mentioned meta analysis. The other two studies concluded that training status had no effect on time-to-fatigue [16] or peak strength [17], although there was no main effect of caffeine in either study. The lack of a significant ergogenic effect of caffeine in these studies (i.e., experimental models that did not detect a beneficial effect of caffeine) makes it impossible to tease out the impact of training levels. We recruited participants that were accustomed to cycling exercise and ultimately enrolled subjects that had a wide range of cycling experience and fitness levels. This allowed us to examine a separate factor (other than time of day) that may alter the magnitude of benefit conferred by caffeine ingestion. Specifically, in addition to time of day, we tested the hypothesis that untrained cyclists would receive more of a performance benefit from caffeine compared to their trained counterparts.

The outcomes of this investigation have marked practical relevance. Athletes and coaches make training/competition decisions based on risk and reward. It is therefore worthwhile to establish whether or not time trial performance is differentially impacted by time of day and/or training status, as this will instruct best practices for caffeine use as an ergogenic aid. There can be downsides to caffeine consumption, particularly in the evening. For instance, caffeine intake later in the day can interfere with quantity and quality of sleep [18], thereby possibly impairing recovery from heavy exercise [19] and subsequent performance [20]. Our collective hypothesis was that trained subjects supplementing with caffeine in the evening will experience the least improvement in performance and therefore should reconsider caffeine as an ergogenic aid late in the day.

2. Materials and Methods

2.1. Subjects

Twenty-two healthy male subjects from James Madison University and the surrounding area volunteered for the study. Two subjects withdrew for reasons unrelated to the study, resulting in complete data from eleven trained and nine untrained cyclists. Descriptive data are shown in Table 1. Subjects were required to have performed, at minimum, either "occasional" cycling (one day/month) for the untrained cyclists or "consistent" cycling (four days/week) in their weekly exercise routine over the past three months for trained cyclists. Cycling frequency and duration were self-reported. Trained and untrained cyclists were determined by the number of hours cycling per week, with comparison based on the top (trained) vs. bottom (untrained) tertiles. The categorization of untrained and trained subjects is generally supported by individual peak oxygen consumption

(VO_{2peak}) values (Table 1). The notable exception is that one 'untrained' subject possessed a VO_{2peak} of 61.3 $mL \cdot kg^{-1} \cdot min^{-1}$. However, this subject was only performing 1.5 h of weekly cycling. Subjects provided information about their resistance training routines and this information was used as a covariate for all analyses (data reported in Table 1). Subjects were informed of the experimental procedures and risks prior to giving written consent. The study was approved by the James Madison University Institutional Review Board (IRB #15-0559). We also implemented a questionnaire asking about caffeine habits (coffee, tea, soda, chocolate, etc.); daily caffeine intake was calculated by assigning typical caffeine values to each respective item. Caffeine levels are reported in Table 1. Only one subject regularly consumed >300 mg/day, the previously established benchmark for 'high' caffeine intake (400 mg/day). Therefore, any differences in caffeine intake between subjects likely had a negligible impact on our performance outcomes.

Table 1. Descriptive Data for All Subjects and the Upper and Lower Cycle Training Tertiles.

	All Subjects (n = 20)	**Trained (n = 7)**	**Untrained (n = 7)**
Height (m)	1.75 ± 0.07	1.75 ± 0.07	1.76 ± 0.08
Body Mass (kg)	73.6 ± 10.9	70.2 ± 10.7	76.0 ± 10.6
Age (year)	22 [18–44]	22 [18–39]	21 [19–44]
$\dot{V}O_{2peak}$ ($mL \cdot kg^{-1} \cdot min^{-1}$)	57.2 ± 9.3	64.8 ± 7.9	49.2 ± 5.6
Caffeine Intake (mg/day)	32 [0–407]	100 [8–407]	2 [0–204]
Cycle Training (h/week)	4.0 [1.5–10.0]	8.0 [5.0–10.0]	2.3 [1.5–3.5]
Resistance Training (h/week)	1.0 [0–22.5]	1.5 [0–22.5]	3.5 [0–9]

Age, caffeine intake, cycle training, and resistance training are expressed as medians [range] because data did not display a normal distribution. All other variables are expressed as means ± SD. VO_{2peak} and cycling volume were higher in Trained vs. Untrained ($p < 0.05$).

2.2. Cardiovascular Fitness Testing

Following height and body weight measurements, subjects performed an incremental exercise test to exhaustion on a bicycle ergometer (Velotron, Racermate, Inc., Seattle, WA, USA) to determine peak oxygen consumption (VO_{2peak}). The test began at a workload of 100 W (untrained) or 150 W (trained), and was increased by 25 W every minute until volitional fatigue. Metabolic measurements were assessed using a Moxus Modular Metabolic System (AEI Technologies, Pittsburgh, PA, USA) throughout the test and VO_{2peak} was determined by the highest 30-s mean oxygen uptake.

2.3. Experimental Design

A randomly counterbalanced, double blind, placebo controlled design was implemented to compare the effects of the four different treatment conditions. Subjects performed four trials: two morning trials starting between 6:00 a.m. and 10:00 a.m. (but with consistent starting times within each subject), and two evening trials starting between 4:00 p.m. and 8:00 p.m., with an eight-hour minimum separation between morning and evening start times for each subject. During the experimental trials, subjects ingested a capsule one hour prior to exercise containing either 6 mg/kg body weight anhydrous caffeine or all-purpose flour (placebo). Only ad libitum water consumption was permitted following capsule consumption. The four treatment conditions were designated as: 1 Morning placebo (AM_{PLA}); 2 Morning caffeine (AM_{CAF}); 3 Evening placebo (PM_{PLA}); and 4 Evening caffeine (PM_{CAF}).

2.4. Performance Trials

Each subject performed six exercise trials (two familiarization trials followed by four experimental trials) on both an isokinetic dynamometer (Biodex Multi-Joint System—PRO, Biodex Medical Systems, Inc., Shirley, NY, USA), and cycle ergometer, with 6 (2.5–17) days between each experimental trial. Venous blood samples were obtained immediately upon arrival to the laboratory and again prior to exercise (one-hour following capsule consumption). Subjects then began each trial with

a 5-min treadmill warm-up at 3.5 mph. Following the warm-up, subjects completed two sets of four leg extension repetitions on an isokinetic dynamometer (two warm up repetitions followed by two peak torque measurements) at 30 degrees/s with the right leg. Each set was separated by 60 s. This protocol was repeated at 120 degrees/s and 240 degrees/s, respectively (grand total of 24 repetitions; 12 total warm-up repetitions (4 at each speed) and 12 total maximum repetitions (4 at each speed)). After a ~3 min transition, subjects performed a flat 3-km time trial on the cycle ergometer. The familiarization trials were identical to the experimental trials, with the exception of the supplementation protocol. Cycling power output (and consequently cycling velocity) was self-controlled by adjusting both resistance on the flywheel using a simulated gear shifter and pedaling cadence. Subjects were instructed to treat each trial as a competition prior to the beginning of each trial, but subjects did not receive verbal feedback or encouragement from the investigators during testing. Further, no visual feedback from the time trial was provided, with the exception of elapsed distance. 3-km time trial time was used as the performance measure.

2.5. Serum Caffeine Levels

Blood samples were obtained from the antecubital vein. After 30 min of coagulations, samples were centrifuged at 2500 rpm for 15 min. Serum was stored at −80 °C until analysis. Serum caffeine levels were subsequently determined via mass spectrometry.

2.5.1. Sample Preparation for Liquid Chromatography/Mass Spectrometry Analysis

Serum samples were stored at −80 °C prior to extraction. 200 μL of serum was extracted by vortexing with 5 mL of ethyl acetate for 5 min. The extract was then centrifuged for 10 min at 4000× *g* to separate the organic and aqueous layers. The top ethyl acetate layer was transferred to a tube, the extraction repeated and the organic fractions combined. The extract was then lyophilized in a CentriVap (Labconco, Kansas City, MO, USA) and reconstituted in 200 μL of 96:4 water:methanol for quantitation by LC/MS.

2.5.2. LC/MS Analysis

An Agilent 1290 ultra-high performance liquid chromatograph (UHPLC) coupled to a 6224 time of flight mass spectrometer (TOF MS) (Agilent Technologies, Santa Clara, CA, USA) was used to separate caffeine from other metabolites and measure its concentration in the serum extracts. Gradient elution with an Agilent Zorbax Eclipse Plus C18 column (2.1 mm × 150 mm, 1.8 μm particles) held at 35 °C was performed with mobile phase A (water, 0.1% v/v formic acid) and B (acetonitrile, 0.1% v/v) at 0.45 mL/min. as follows: B was held at 4% for 7 min and increased to 70% by 12 min. At 14.5 min the gradient was returned to the initial conditions. Five microliters of serum extract were injected in duplicate. Caffeine was ionized by positive ion electrospray (ESI) as follows: capillary, +3500 V; drying gas, 350 °C and 10 L/min; nebulizer 30 psig. Mass spectral data was acquired in profile and centroid mode at 3 specta/s over 100–1700 m/z. TOF ion optics were: fragmentor, 115 V; skimmer, 65 V and octopole retardation factor $V_{p\text{-}p}$, 750 V. An internal reference mass (IRM) solution (purine and HP-921, Agilent Technologies, Santa Clara, CA, USA) was delivered to the ESI source to ensure high mass accuracy (<15 ppm).

A caffeine stock solution (1000 ppm, water) was serially diluted to yield a minimum of seven calibration levels that ranged from 0.01 to 20 ppm. Agilent's Mass Hunter Quantitative Analysis software (B.06) (Agilent, Santa Clara, CA, USA) was used to generate external calibration curves and calculate the concentrations of caffeine in ppm.

2.6. Dietary and Exercise Control

Subjects were provided with instructions for recording food intake so dietary intake could be replicated across trials. All subjects recorded food intake for 24 h prior to all experimental trials. Subjects were provided with a copy of food records from the 24 h preceding the initial experimental

trials to be used to facilitate dietary replication for the 24-h time period preceding subsequent trials. Subjects were also instructed to abstain from any alcohol (24 h), caffeine (12 h), and food intake (4 h; post-absorptive state) prior to each experimental trial. Our intent was to collect performance data in the morning and evening under similar feeding conditions. The most feasible way to accomplish this was to study subjects in a post-absorptive state, so as to avoid early waking and feeding prior to the morning trial. However, this leads to discrepancies in fasting duration prior to the morning and evening trials; the morning trials were conducted after an overnight fast (~7–10 h of fasting) whereas the evening trials were performed after a 4-h fast. While it is conceivable that this variance could impact performance, performance (both strength and 3-km TT) was virtually identical between the morning and evening under placebo conditions, suggesting that any error variance due to different fasting durations was likely negligible. Subjects were instructed to maintain consistent exercise habits between trials and to abstain from any heavy and/or unaccustomed exercise 48 h prior to each experimental trial. Subjects submitted physical activity logs for verification.

2.7. Statistical Analysis

All data were log transformed to diminish the effects of nonuniformity. Magnitude-based inferences about the data were derived using methods described by Hopkins and colleagues [21]. A previously established 'smallest worthwhile change' in performance was used as the threshold value for a substantial treatment effect (separate treatment conditions vs. placebo) [22]. The smallest worthwhile change in performance was defined as 0.3 × the within-subject variability of a similar group of cyclists previously studied in our laboratory [5] (Coefficient of Variation = 2.7% for time) which translates to a difference of 0.8% or 2.4 s in the current project [23]. As recommended by Hopkins, for the isokinetic data, 0.2 × SD of the AM_{PLA} trial was used to determine smallest worthwhile change [22]. The coefficient of variation for peak strength measurements (derived from placebo conditions) was: 3.9% at 30 degrees/s, 3.2% at 120 degrees/s, and 4.6% at 240 degrees/s. The coefficient of variation for 3-km TT performance was: 1.1% for all subjects, 1.1% for trained, and 0.8% for untrained.

A published spreadsheet [24] was then used to determine the likelihood of the true treatment effect (of the population) reaching the substantial change threshold (0.3 × CV); these were classified as <1% almost certainly no chance, 1%–5% = very unlikely, 5%–25% = unlikely, 25%–75% = possible, 75%–95% = likely, 95%–99% = very likely, and >99% = almost certain. If the percent chance of the effect reaching the substantial change threshold was <25% and the effect was clear, it was classified as a 'trivial' effect. If 90% confidence intervals included values exceeding the substantial change threshold for both a positive and negative effect, effects were classified as unclear (>5% chance of reaching the substantial threshold for both a positive and negative effect). To test the effects of time of day, the outcomes derived for each group using the spreadsheet mentioned above [24] were compared using a second spreadsheet [25]. Likewise, the effects of training status were compared using this same method. All data reported as mean ± 90% Confidence Interval unless noted otherwise.

We estimated the statistical power of our experimental design using a publicly available spreadsheet created for magnitude-based inferences [26]. Data derived from a subset of male subjects (n = 24) using a similar measurement protocol in our laboratory was used to estimate within-subject variability [5]. With a sample size of 20, the current design and statistical methods had the statistical power of 0.99 to detect changes in time trial performance of 1.5% and 0.7 to detect a performance change of 0.8%. For leg extension an effect of 4.05% (smallest meaningful effect derived from 0.2 × within subject standard deviation under placebo conditions) could be detected with a power of 0.96. The between subject comparisons (trained vs. untrained) were associated with low power thereby increasing the likelihood of making a type II error. However, we detected magnitude-based differences in 3-km TT performance (caffeine vs. placebo) between trained and untrained subjects and these data are reported; peak strength data specific to each training group are omitted because of the lack of power and lack of clear statistical outcomes.

3. Results

3.1. Serum Caffeine Levels

Serum caffeine levels in AM were: All Subjects—Pre 0.7 ± 1.3 ppm, Post 13.8 ± 2.4 ppm; Trained—Pre 0.6 ± 0.9 ppm, Post 13.1 ± 2.0; Untrained—Pre 0.2 ± 0.3 ppm, Post 13.6 ± 2.3 ppm. Caffeine levels in PM were: All Subjects—Pre 0.7 ± 0.8, Post 14.7 ± 3.1 PPM; Trained—Pre 0.6 ± 0.7 ppm, Post 13.1 ± 3.9 ppm; Untrained—Pre 0.6 ± 0.5 ppm, Post 15.0 ± 2.8 ppm. There were no differences between trained and untrained subjects, nor were there any differences between AM and PM caffeine levels following caffeine ingestion.

3.2. The 3-km Time Trial Performance

3.2.1. All Subjects

All 3-km performance data are displayed in Figure 1. Individual performance data are displayed in Figure 2. In all subjects, AM_{CAF} 3-km time trial performance (3-km TT) was 'very likely' better than AM_{PLA} (2.9% ± 1.7%), while PM_{CAF} 'possibly' improved performance vs. PM_{PLA} (1.1% ± 1.1%). AM_{CAF} 'likely' improved 3-km TT performance to a greater extent than PM_{CAF} (1.7% ± 2.0%) when compared to the respective placebo condition (PLA).

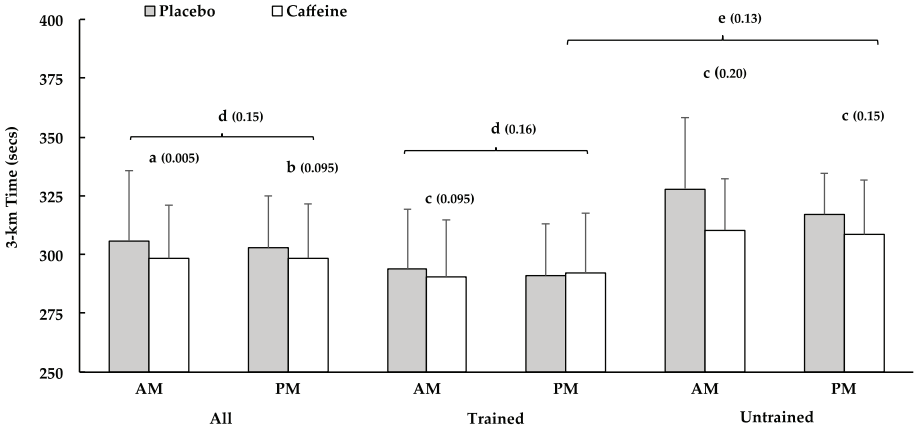

Figure 1. The 3-km Time Trial Performance. Bars depict mean finishing time in seconds (±SD). AM, morning; PM, afternoon; (**a**) 'very likely' faster than PLA; (**b**) 'possibly' faster than PLA; (**c**) 'likely' faster than PLA; (**d**) 'likely' different response to caffeine between AM and PM; (**e**) 'likely' different response to caffeine between Trained and Untrained in PM. *p*-values derived from pairwise comparisons are displayed in parentheses.

3.2.2. Trained Subjects

AM_{CAF} performance was 'likely' faster than AM_{PLA} (1.8% ± 1.9%), whereas caffeine's effect was 'unclear' in the evening (PM_{CAF} vs. PM_{PLA}: −1.0% ± 3.1%). Additionally, AM_{CAF} 'likely' improved performance more than PM_{CAF} (AM_{CAF} vs. PM_{CAF}: 2.8% ± 3.4%), when compared to PLA.

3.2.3. Untrained Subjects

AM_{CAF} and PM_{CAF} 'likely' improved time trial performance vs. AM_{PLA} (5.5% ± 8.0%) and PM_{PLA} (3.2% ± 3.8%), respectively. The time of day (AM vs. PM) comparison was 'unclear'.

3.2.4. Training Status

It was 'unclear' whether trained or untrained benefited more from caffeine in the AM condition, but untrained subjects 'likely' benefited more from caffeine supplementation than trained in the PM condition (trained: −1.0% ± 3.2%, untrained: 3.2% ± 3.8%, AM_{CAF} vs. PM_{CAF}: 4.2% ± 4.5%).

3.3. Peak Muscle Torque

All peak skeletal muscle torque data are presented in Table 2. Knee extension torque at 30 degrees/s (30EXT) was 'possibly' improved by caffeine in PM when compared to PM_{PLA}, but all other PM measures were 'likely' trivial. PM Caffeine 'possibly' increased PM_{CAF} torque more than AM_{CAF} torque in the 30EXT condition when compared to PLA. All other time of day comparisons were 'trivial' or 'unclear'.

Bars depict mean finishing time in seconds (±SD). (a) 'Very likely' faster than PLA; (b) 'possibly' faster than PLA; (c) 'likely' faster than PLA; (d) 'Likely' different response to caffeine between AM and PM; (e) 'Likely' different response to caffeine between Trained and Untrained in PM. *p*-Values derived from pairwise comparisons are displayed in parentheses.

Data are reported as individual 3-km finishing times under all four experimental conditions, grouped by training tertiles. Numbers below the horizontal axis (x-axis) represent each individual subject.

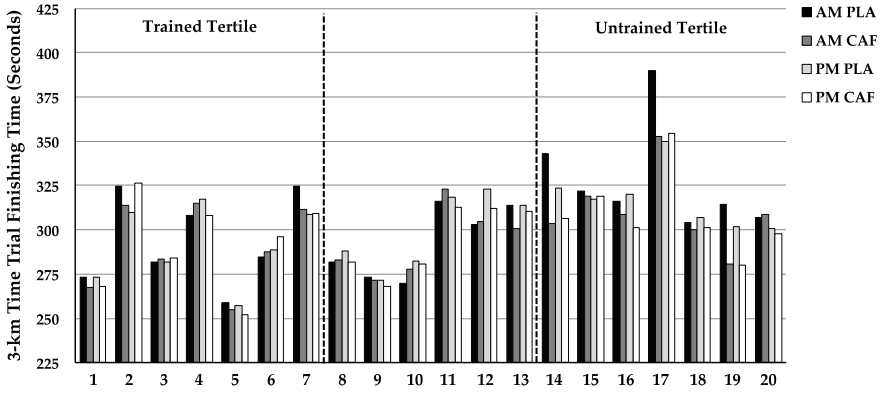

Figure 2. Individual 3-km Time Trial Performances. AM, morning; PM, afternoon; PLA, placebo; CAF, caffeine.

Table 2. Peak Muscle Strength Data.

Velocity	30 Degrees/s		120 Degrees/s		240 Degrees/s	
Time	**AM**	**PM**	**AM**	**PM**	**AM**	**PM**
PLA	192.7 ± 39.1	190.7 ± 38.7	171.3 ± 31.7	171.7 ± 29.5	154.6 ± 28.6	157.9 ± 29.9
CAF	194.1 ± 47.5	202.3 ± 41.8	171.3 ± 33.0	174.7 ± 29.2	158.4 ± 33.6	160.0 ± 26.1
PLA vs. CAF	0.9 ± 4.4 (−0.3 ± 4.3) 12/85/3 Likely Trivial	5.2 ± 3.6 (5.94 ± 3.5) 72/28/0 Possible; $p = 0.07$	−0.3 ± 3.5 (−0.1 ± 3.3) 4/91/6 Likely Trivial	1.3 ± 3.1 (1.9 ± 2.9) 10/90/1 Likely Trivial	2.0 ± 3.1 (2.0 ± 2.9) 18/81/0 Likely Trivial	0.8 ± 3.6 (1.8 ± 3.6) 9/89/2 Likely Trivial
AM vs. PM	−4.3 ± 5.5 (−6.19 ± 5.4) 1/46/53; Possible; $p = 0.06$		−1.6 ± 4.6 (−2.0 ± 4.3) 3/75/22; Likely Trivial		1.3 ± 4.6 (0.2 ± 4.5) 19/77/4; Likely Trivial	

Values for Placebo (PLA) and Caffeine (CAF) reported as Mean ± SD. AM, morning; PM, afternoon. Comparison values reported as adjusted (actual in parenthesis). Mean ± 90% CI for differences between change scores (i.e., AM vs. PM), % likelihoods of positive effect/trivial effect/negative effect and semantic inferences.

4. Discussion

The purpose of the current study was to investigate how the benefit of caffeine for 3-km cycling TT performance was influenced by time of day and training status. Caffeine enhanced 3-km TT performance more in the morning than in the evening (all subjects and trained subjects). Caffeine also improved cycling performance among untrained subjects in the morning and evening, whereas the benefit for trained subjects was 'likely' in the morning and 'unclear' in the evening. Further, caffeine intake enhanced 3-km performance more among untrained- than trained subjects, in the evening. Secondarily, we assessed peak muscle strength at three separate angular velocities prior to the time trials. Caffeine has been shown to increase peak strength [6,7,12,13,27] and there is some evidence that strength may contribute to the ergogenic properties of caffeine for cycling performance [28]. Therefore, we measured peak strength in an attempt to provide some physiological insight into the time trial outcomes. However, caffeine only increased strength at the slowest velocity (30 degrees/s) in the evening, which does not align with the TT performance results. This suggests that the gains in time trial performance were not mediated by improvements in strength.

Consistent with our general hypothesis, caffeine enhanced 3-km TT performance among trained subjects in the morning but not the evening. This supports results from a recent study, in which we reported that caffeine supplementation elicited the largest improvements in 3-km cycling TT performance among subjects that completed trials prior to 10:00 a.m. [5]. Importantly, prior observations made in strength-trained participants that caffeine elevates performance in the morning but not the evening [13] can now be extended to include longer sustained efforts. To our knowledge there are no other data from which to directly compare our findings.

The scant information on this topic also makes it difficult to provide a well-founded explanation for why caffeine appears to deliver a more pronounced benefit in the morning. We suspected that the time of day differences in performance could be related to varying rates of caffeine metabolism throughout the day. Cytochrome P450 1A2, the enzyme responsible for caffeine metabolism, has been shown to have higher activity levels during sleeping hours and directly after waking, when compared to the rest of day [29]. Considering that caffeine metabolites appear to be more potent than caffeine itself, faster caffeine metabolism could lead to a higher concentration of metabolites in the morning thereby delivering a stronger effect [30]. However, this was not the case in the current study. Caffeine levels were virtually identical between AM and PM trials (reported in Section 3.1). An alternative hypothesis is that the greater gains with caffeine in the morning are related to slower time trial performances in the morning compared to the evening, in the absence of caffeine. Though the physiology is largely unknown, there is good evidence that somatic control and physical performance (peak muscle strength, power, and swimming) can be impaired in the morning compared to the evening [20,31–33], perhaps providing an opportune time to utilize performance enhancing agents. This idea is supported by Mora-Rodriguez et al. where physical performance was worse in the morning compared to the evening, and caffeine raised morning performance to the levels achieved in the afternoon trials. The current data does not seem to support systematic somatic deficits in the morning, as only 9 of 20 subjects (2 of 7 trained tertile and 5 of 7 untrained tertile) performed slower in the AM_{PLA} than the PM_{PLA}. However, 5 of these 9 subjects (1 trained; 4 untrained) had much slower times under AM_{PLA} conditions, which had a large effect on the overall outcomes (i.e., larger gains in AM vs. PM). These slower times may represent a true time of day effect or may reflect individual circadian rhythms. Unfortunately, we do not have chronotype data from which to test this possibility.

While training status did not affect the response to caffeine in the morning, the untrained tertile did experience a more favorable response to caffeine than trained subjects in the evening. This aligns with a recent meta-analysis on this topic that concluded that caffeine tended to improve muscle endurance more in untrained than in trained subjects [14]. The current data are an important addition to our understanding since, as highlighted in the introduction, this conclusion was largely deduced by comparing effect sizes derived from separate studies conducted on trained vs. untrained cohorts. The differential impact that training status had on the caffeine benefits in the evening is a

function of both the lack of improvement among the trained subjects and a 'likely' beneficial effect among untrained subjects. The physiological mechanisms responsible for this result are unknown and beyond the scope of this investigation. However, the concentration of adenosine receptors (the presumed primary target of caffeine) do appear to be higher in trained compared to untrained individuals [34]. And though highly speculative, the higher concentration of adenosine receptors may increase tissue sensitivity to any given concentration of adenosine, thereby requiring larger doses of caffeine to elicit a desirable effect. This may especially be an issue when the effects of caffeine are expected to be relatively small (i.e., the evening).

The current project revealed that caffeine's effect on 3-km TT performance was partially mediated by time of day and training status. However, peak muscle torque was largely unaffected by caffeine except 'possibly' at the slowest speed of contraction (30 degrees/s). There is some precedent for null strength findings [35–37], but most of the literature suggests that peak muscle function is heightened with caffeine [6,7,12,13,27]. Interestingly, as angular velocity increases, so do the number of trivial outcomes, indicating that movement velocity may impact the effects of caffeine. This could possibly be related to caffeine's role as an adenosine antagonist, a mechanism responsible for its ergogenic effects [38]. Adenosine receptor density has been shown to be greater in slow-twitch muscle fibers [39]. However, higher movement velocities require a greater reliance on force output (and power) from fast twitch fibers due to reductions in slow twitch fiber power production secondary to shifting the velocity $\times$ power curve to the right [40]. Therefore, at the higher movement velocities, it is possible that the fiber type most responsive to caffeine supplementation (slow twitch fibers) would contribute a smaller proportion to whole muscle power output, resulting in a smaller measurable effect of caffeine. This would explain why no ergogenic effects of caffeine were observed for peak strength at speeds greater than 30 degrees/s. In support of this idea, Jacobson et al. [41] reported improvements in isokinetic knee extension strength with caffeine consumption which were greater at slower movement speeds.

5. Conclusions

The primary weaknesses of the current study include the relatively small sample size, the lack of mechanistic insight (RPE, muscle pain, etc.), and as discussed in Section 2.6, the markedly different fasting durations preceding the morning and evening trials. Specific to the latter, it seems possible that the different fasting durations preceding the morning and evening trials could have influenced performance in both placebo and caffeine conditions. However, performance was virtually identical across placebo trials (morning vs. evening). Further, despite evidence that feeding status can influence the pharmacokinetics of caffeine ingestion [42], caffeine levels were similar in both caffeine conditions, suggesting that the 4 h of fasting, regardless of duration, likely leads to similar rates of caffeine absorption/metabolism. Notwithstanding these potential issues, the findings of this study support the idea that time of day and training status influence caffeine ergogenics and that these are probably not mediated by peak strength. This suggests that caffeine may be a suitable supplement for use during morning competition, but with less noticeable results in the evening. The current results also indicate that trained subjects supplementing with caffeine in the evening did not benefit from caffeine. Because of the potential detrimental effects that evening caffeine consumption has on sleep, we recommend that athletes confirm that caffeine is effective on an individual basis before using in the evening. The research on external factors that may alter how an individual performs with caffeine supplementation is still sparse, and more information is needed before personalized prescription for optimal performance outcomes can be provided.

Author Contributions: James Boyett, Gabrielle Giersch, Michael Saunders, Christopher Womack and Nicholas Luden conceived and designed the experiments; James Boyett and Gabrielle Giersch performed the experiments; Gabrielle Giersch, Christine Hughey and Hannah Daley analyzed the blood samples; James Boyett and Nicholas Luden analyzed the data; Christopher Womack, Michael Saunders and Nicholas Luden contributed to reagents/materials/analysis tools; James Boyett wrote the paper; Michael Saunders, Christopher Womack, Nicholas Luden, Christine Hughey and Hannah Daley contributed to the major edits; James Boyett,

Gabrielle Giersch, Christopher Womack, Michael Saunders, Christine Hughey, Hannah Daley and Nicholas Luden gave their approval for the final version.

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2

Investigating the Cellular and Metabolic Responses of World-Class Canoeists Training: A Sportomics Approach

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Abstract: (1) Background: We have been using the Sportomics approach to evaluate biochemical and hematological changes in response to exercise. The aim of this study was to evaluate the metabolic and hematologic responses of world-class canoeists during a training session; (2) Methods: Blood samples were taken at different points and analyzed for their hematological properties, activities of selected enzymes, hormones, and metabolites; (3) Results: Muscle stress biomarkers were elevated in response to exercise which correlated with modifications in the profile of white blood cells, where a leukocyte rise was observed after the canoe session. These results were accompanied by an increase in other exercise intensity parameters such as lactatemia and ammonemia. Adrenocorticotropic hormone and cortisol increased during the exercise sessions. The acute rise in both erythrocytes and white blood profile were probably due to muscle cell damage, rather than hepatocyte integrity impairment; (4) Conclusion: The cellular and metabolic responses found here, together with effective nutrition support, are crucial to understanding the effects of exercise in order to assist in the creation of new training and recovery planning. Also we show that Sportomics is a primal tool for training management and performance improvement, as well as to the understanding of metabolic response to exercise.

Keywords: metabolism; biochemistry of exercise; ammonia; urate; exercise intensity biomarkers; physical stress response

1. Introduction

Physical stress response due to a sport challenge is implicated in many metabolic modifications which affect the equilibrium of the biochemical internal environment [1,2]. This includes changes in the amount and kinetics of diverse biomarkers that are correlated with exercise intensity and muscle damage [3,4]. Some of these changes in metabolism can be assessed using blood as a biological matrix. For more than one decade, our group has dedicated research efforts towards understanding changes in metabolism using exercise as an induced-stress metabolic model [3,5–16]. The Sportomics approach targets metabolic and signaling molecule evaluations during either mimicked or real conditions faced in sports situations; it combines "-omics" technique with classic clinical laboratory analyses in order to understand sport-induced modifications [16]. These approaches represent a powerful tool to understand changes in physical and metabolic stress [17–19] and allow researchers to propose interventions in order to optimize athletes' performance [5,20,21]. The approach

is also a useful investigation tool for studying the effects of nutrition supplementation on physical training in different physiological or clinical conditions, such as type 2 diabetes mellitus [22]. Therefore, the analysis of world-class athletes in a field perspective allows us the possibility of understanding metabolic and signaling responses during high metabolic stress. Similar to a personalized-medicine approach, the Sportomics method allows us to better understand individual changes and to propose individualized interventions.

Several recent investigations have focused on the ammonemia changes resulting from a physical effort, which may be modified due to different causes [23–25]. Amino acids play a central metabolic role as an energetic source during exercise, which requires their deamination in order to be transduced into chemical energy. Increased muscle contraction rate also can contribute to changes in ammonemia, through adenosine monophosphate (AMP) deamination [26–29]. During intense or prolonged exercise, the reduced ability to resynthesize ATP promotes accumulation of ammonia and inosine monophosphate (IMP) which is metabolized to urate [30]. An intensity relationship has been proposed between ammonemia and exercise, as ammonia rapidly increases at intensities greater than 50%–60% of VO_{2max} [31,32]. However, ammonia production and release is not solely restricted to intense exercise. During prolonged (>1 h) submaximal exercise (60%–75% VO_{2max}), ammonia could be produced through the breakdown of branched chain amino acid (BCAA) for additional energy provision [33–35].

Ammonia may cross the blood brain barrier causing neurotoxic effects including neuropsychiatric disorders, convulsion, and death [36], and may be implicated in central fatigue [25]. Therefore, ammonia accumulation may be avoided through a detoxification system. Humans convert ammonia to urea mainly in hepatocytes, and different cells can decrease ammonemia by synthesizing amino acids as a mechanism for further excretion of urea [9,11,37]. Therefore, an increase in urea levels reflects both AMP and amino acid deamination. On the other hand, urate is the final metabolite of the purine metabolism; hence, its measure can be stoichiometrically related to IMP deamination. Since urea and urate are, respectively, the final products of ammonia and purine metabolism, the study of the kinetics of those blood analytes leads to a better understanding of the metabolic pathways of ammonia origin and the response to exercise [37]. For this reason, our group has proposed nutritional and training interventions to promote metabolic adaptations in elite athletes to enhance their performance in training and competitions [3,10].

Canoeing has been featured as an Olympic sport since the Summer Olympic Games of 1936 in Berlin. Currently, men´s and women's competitions cover distances of 200 m, 500 m, and 1000 m either solo, in pairs, or in crews of four. Canoeing contests are sprint events requiring sustained bursts of speed and power, leading to intense mechanical and metabolic stress. Little is known about these athletes' metabolic responses during training sessions or competitions, therefore, the aim of this study was to evaluate four world-class canoeists during a training session through a Sportomics approach. As far as we know, this is the first metabolic investigation in the field, coming from our unique opportunity to investigate world-class athletes. This investigation will help enlighten us about the metabolism behavior in elite athletes.

2. Materials and Methods

This study assessed the metabolic response of four male world-class canoeists during a combined training session. All athletes were currently engaged in international elite competitions (including world championships, Pan-American, and Olympic games). During the trials, the athletes were instructed to maintain their typical hydration and food ingestion habits. Additionally, clinical evaluation, anthropometric measurements, and laboratory tests of collected blood samples were performed to assess health status. A Sportomics evaluation and analysis was performed to understand the metabolic effects of a training session. Subjects were fully instructed about the testing procedures and each signed a written informed consent. This study was conducted according to all procedures involving human subjects approved by the Ethics Committee for Human Research at the Federal University of the State of Rio de Janeiro (117/2007, renewed in 2011, 2013 and 2016) and met the requirements regulating

research on human subjects (Health National Council, Brazil, 1996) the proper written informed consent was read and signed by the athletes.

2.1. Experimental Designs

After a regular warm up, the athletes were subjected to a training protocol that consisted of several canoe sprint bouts, with three minute intervals between each bout, covering different distances and intensities. The total distance totaled 16 kilometers. This first part of the protocol had a duration of 210 min followed by a rest period of 20 min during which they ingested a 500 mL beverage consisting of about 20% carbohydrate (short and medium absorption); 2% lipids; 5% proteins (casein and whey proteins). Next, they performed a weight lifting training session for 50 min focusing on exercises that recruit large muscle groups for both upper and lower body, followed by a 70 min of recovery. See the experimental trial depicted (Figure 1).

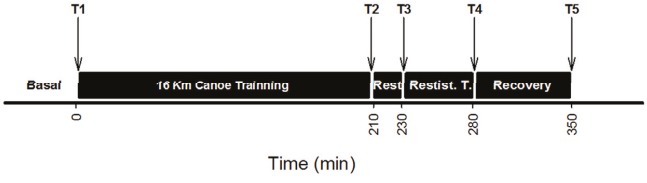

Figure 1. Experimental trial. Blood samples of the athletes were collected at the time points indicted in the Figure and as described in materials and methods.

2.2. Blood Collection

Blood samples were collected following an antecubital vein puncture before (T1) and after (T2) the 16 km canoe training session; before (T3) and after (T4) the resistance training; and after the recovery period (T5) (Figure 1). Samples for hematological analysis assays were collected into tubes with K_2-EDTA (Vacuette, Greiner Bio-One, Frickenhausen, Germany). White blood cell (total and differential), erythrocyte, and thrombocyte counts were measured in whole blood within a two-hour time frame after collection. Blood was immediately centrifuged to obtain either plasma or serum that was aliquoted, centrifuged ($3000\times$ *g*; 10 min; 4 °C), and stored in liquid nitrogen for later analysis (never more than eight hours). Samples were analyzed in duplicate or triplicate, when necessary, and measured against a standard curve with no less than five points.

2.3. Blood Analysis

A range of hematological and biochemical analyses was carried out totalizing around 100 analytes. The large amount of data generated was used in a non-target analysis linked to an ex-post facto study design. We chose near 20 analytes that could be relevant for our study of the athlete's performance. Among others, our data set included a broad spectrum of metabolites and biomarkers related to different cellular and systemic signaling processes like inflammation and both muscle and hepatic injury.

Alanine aminotransferase (ALT), aspartate aminotransferase (AST), alkaline phosphatase (ALP), lactate dehydrogenase (LDH), γ-glutamyltransferase (γGT), creatine phosphokinase muscle-brain fraction (CKMB), creatine phosphokinase (CK), ammonia, urea, blood urea nitrogen (BUN), creatinine, urate, glucose, lactate, and 2-hydroxybutyrate were measured by the enzymatic kinetic method [38] in an automatic analyzer (ADVIA 1200—SIEMENS, Erlangen, Germany/Autolab 18 Boehringer Mannheim, Ingelheim am Rhein, Germany). Myoglobin was evaluated by the Hybridization Signal Amplification Method [39]. Albumin and total protein were assessed by electrophoretic analysis [40]. High-density lipoprotein (HDL), low-density lipoprotein (LDL), very-low-density lipoprotein (VLDL), total lipids, triacylglycerols (TG), and total cholesterol were assessed by the Chabrol & Charonnat

method [41]. Amino acids were measured by high performance liquid chromatography (HPLC) [42]. CKMB-mass, insulin, adrenocorticotropic hormone (ACTH), and cortisol levels were assessed by chemiluminescence (Immulite 2000 Siemens, Erlangen, Germany) [43].

2.4. Statistical Analysis

Statistical analyses were performed using the software SigmaPlot 11.0 integrated with SigmaStat 3.5 packages (Systat, Santa Clara, CA, USA). Due to the nature of the experiment, including the similarity of subjects and the controlled experimental conditions (diet, sleep, training and major physical condition variables), the data were expressed as mean ± standard error (SEM). Data were normalized to pre-training results (T1) for clarity and analyzed by Analysis of Variance (ANOVA) using the condition and time as the repeated measured variables, which were confirmed using Tukey's post hoc test. $p < 0.05$ was defined as the limit for statistically different mean values.

3. Results

Anthropometric characteristics of the individuals are presented on Table 1. Approximate averages of the values measured were as follows: 1.77 m of height, 82.9 kg of weight, 9.8 kg of fat weight, 73 kg of fat-free mass, 11.5% of body fat percentage, indicating that all tested individuals presented typical body composition, fat distribution, and weight profiles. We assessed the lipid profiles and serum protein levels of the individuals to characterize their nutritional status. As observed in Table 2, the assessed lipid profiles were in accordance with the healthy status of the general population. Table 3 presents the results regarding serum protein levels. Despite the fact that these data are considered normal values for the general population, it is worth noting that the assessed albuminemia was low considering a world-class team of athletes. Due to the lack of knowledge of world-class biomarker levels we chose to show all the data as a reference for future studies [3,5,10,21].

Table 1. Anthropometric parameters of the athletes were measured and are shown here as mean ± standard error.

Anthropometry	
Height (m)	1.77 ± 0.02
Weight (Kg)	82.9 ± 5.0
Fat weight (Kg)	9.8 ± 2.4
Fat-free mass (Kg)	73.0 ± 2.6
Fat percentage (%)	11.5 ± 2.0
BMI (Kg/m^2)	26.2 ± 1.2

Table 2. Lipid panel values–high-density lipoprotein (HDL); low-density lipoprotein (LDL); very low-density lipoprotein (VLDL)-were assessed as described in materials and methods and are shown here as mean ± standard error.

Lipid Panel (mg/dL)	
Serum cholesterol	173.6 ± 24.2
Serum triacylglycerol	83.3 ± 20.0
HDL	55.3 ± 5.0
LDL	95.0 ± 26.5
VLDL	16.6 ± 3.8
Cholesterol/HDL ratio	3.2 ± 0.7
LDL/HDL ratio	1.9 ± 0.6
Non cholesterol lipids	118.3 ± 28.9

Table 3. Protein fractions were assessed as described in materials and methods and are shown here as mean ± standard error.

Protein Fractions (g/dL)	
Total proteins	6.7 ± 0.12
Albumin	2.7 ± 1.23
α-1 globulin	0.4 ± 0.14
α-2 globulin	0.5 ± 0.05
β-1 globulin	0.4 ± 0.02
β-2 globulin	0.2 ± 0.003
γ globulin	1.2 ± 0.12

3.1. Muscle Stress Biomarkers

Well-established metabolic stress biomarkers were assessed in order to characterize the training intensity of the proposed trial. Compared to basal levels, AST showed a statistically significant increase after the resistance training (T4) by 30%, and continued to increase by up to 40% after the recovery period. Other biomarkers, such as ALT, ALP, and γGT, did not change throughout the trial (Figure 2, panels A and B). CK activity in blood samples, a classic muscle injury marker, was significantly higher by approximately 60% at T4. Compared to the pre-exercise state, it kept increasing to nearly two-fold at T5 (Figure 3, panel A). Despite the fact that no significant change was observed in CKMB and LDH blood activity, the CKMB mass activity, a very specific muscle injury parameter, reached an increment of 170% at T4 and was up regulated by three-fold at T5 (Figure 3, panel A). Blood levels of myoglobin were significantly increased after the first session of exercise, with an increment of 170%, and kept increasing throughout the trial to reach six-fold values at T5 when compared to basal (Figure 3, panel B).

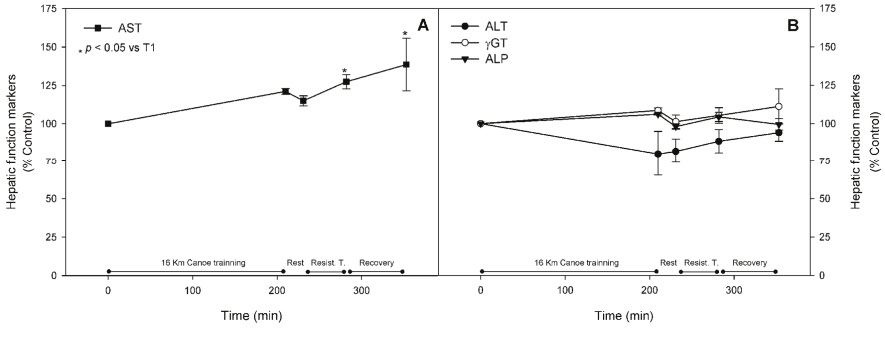

Figure 2. Hepatic injury biomarker. Aspartate aminotransferase (AST), alanine aminotransferase (ALT), γ-glutamyltransferase (γGT), and alkaline phosphatase (ALP) were measured as described in materials and methods and are represented as mean ± standard error of percentage values against control. * Indicates statistical difference against control values ($p < 0.05$). (**Panel A**) shows AST results and ALT, γGT, and ALP results are presented in (**Panel B**).

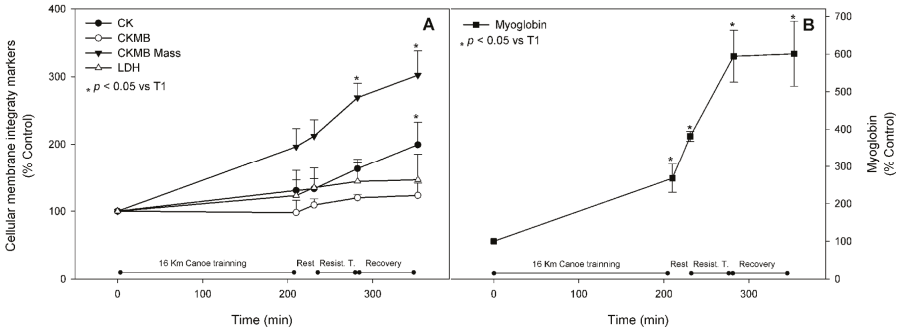

Figure 3. Cellular membrane integrity markers. Creatine phosphokinase (CK), creatine phosphokinase muscle-brain fraction (CKMB) activity and mass, lactate dehydrogenase (LDH) (**A**); and myoglobin (**B**) were measured as described in materials and methods and are represented as mean ± standard error of percentage values against control. * Indicates statistical difference against control values ($p < 0.05$).

3.2. White Blood Cells

During the trial, blood leukocytes rose by 40.0% ± 16.1% and 62.1% ± 26.8% after the canoe (T2) and weight lifting (T4) sessions, respectively, showing a discrete decrease after recovery and reaching levels of 43.2% ± 21.5% higher than basal. These results were mainly due to the increment in the neutrophil count, which showed a significant increase by 54.3% ± 22.3% and 166.2% ± 71.4% after T2 and T4, respectively, and was still 136.0 ± 58.2 higher than basal levels after the recovery. Despite the slight increase after the canoe training, the levels of lymphocytes showed a significant decrease of approximately 40% after the 20 min rest between the training sessions and remained significantly lower until the end of the protocol (Figure 4). Eosinophils measurements tended to accompany the lymphocytes pattern, presenting an increment of approximately 30% at T2 followed by an acute reduction that remained until the trial was terminated. Monocytes acutely responded to exercise stress and the recovery periods, increasing by about 30% after both exercise sessions with a rapid restoration of the original values. Thrombocyte levels responded positively and significantly to the canoe training sessions; they increased 30% compared to the control at T2, acutely returned to basal levels after the 20 min rest prior following resistance training, and remained similar to the original value for the rest of the trial (Figure 5).

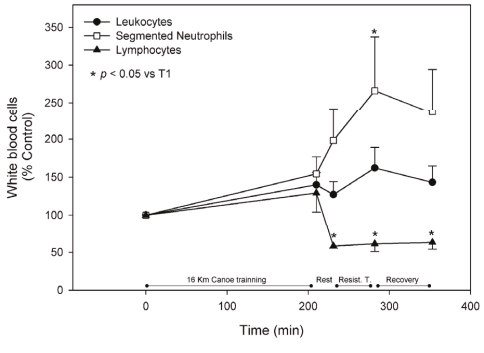

Figure 4. White blood cells. Leukocytes, segmented neuthrophils, and lymphocytes were measured as described in materials and methods and are represented as mean ± standard error of percentage values against control. * Indicates statistical difference against control values ($p < 0.05$).

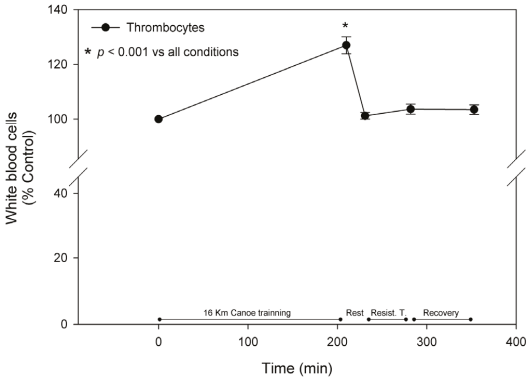

Figure 5. Thrombocytes. Thrombocyte levels were measured as described in materials and methods and are represented as mean ± standard error of percentage values against control. * Indicates statistical difference against any other condition ($p < 0.05$).

3.3. Branched Chain Amino Acids

Plasma branched chain amino acids (BCAA), which are important substrates either as metabolic fuel or as protein synthesis precursors, decreased right after both physical stimuli. Leucine showed the most prominent, significant decrease after the canoe training, reaching almost 50% of the basal value, and continued to decrease by approximately 22% after the resistance training. These decreases did not return to the original values, even after the recovery period. Both isoleucine and valine plasma concentrations seemed to be down regulated after canoe training, however, this decrease was not significantly different. After the 20 min rest between training sessions their levels returned to original values (Figure 6, panel A).

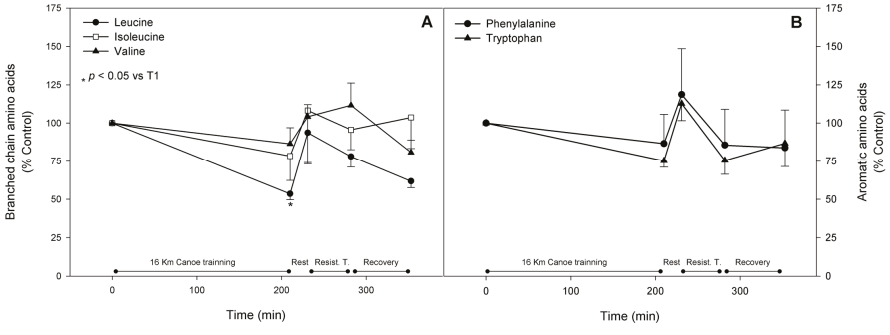

Figure 6. Branched chain (**A**) and aromatic (**B**) amino acids. Amino acid parameters were assessed as described in materials and methods and are represented as mean ± standard error of percentage values against control. * Indicates statistical difference against control values ($p < 0.05$).

3.4. Aromatic Amino Acids

The three amino acids comprising the aromatic amino acids (AAA), phenylalanine, tryptophan and tyrosine, are ketoglucogenic amino acids that may be deviated to the gluconeogenic pathway in hepatocytes. Our results showed that these amino acids decreased in the range of 15%–25% after both the canoe and the weight lifting exercises (Figure 6, panel B).

3.5. Gluconeogenic Amino Acids

Many amino acids may serve as both substrates in anaplerotic reactions replenishing intermediates of the tricarboxylic cycle and as gluconeogenic substrates. Hence, many amino acids serve as energy sources in metabolic pathways. Interestingly, the plasma concentration of some of these amino acids increased right after the first exercise bout. Alanine showed a two-fold increment after the canoe training and also showed a slighter increment of approximately 20% at T4, after resistance training. Glutamate was up regulated by 63% at T2, but regained the original levels at T4. Ornithine plasma levels were enhanced by approximately 20% at T2. Methionine was elevated by approximately 86% after the canoe training session and decreased for the remainder of the trial. Taurine followed the methionine response, which is one of its precursors, rising 56% at T2. Glycine showed a later increase, with levels elevated by about 35% at T3 and with measurements similar to control at all other time points. On the other hand, arginine blood levels were down regulated to 60% of control values at T2 and 69% at T4. Glutamine presented a similar but slighter response, reaching 85% of basal values at T2 and returning to control levels thereafter. Lysine showed an approximately 30% decremented level at T2 when compared to control. Other assessed amino acids, such as asparagine, aspartate, serine, and threonine, did not fluctuate throughout the trial.

3.6. Metabolic Pathway Substrates, Intermediates, and Products

The metabolism of amino acids results in the production of nitrogen compounds, including ammonia; the increase in the levels of these compounds in the blood is tightly related to exercise intensity and duration. Ammonemia was significantly up regulated after the canoe training session by 78% and remained significantly enhanced (by about 71%) even after the recovery period. Urea blood level was slightly higher by about 21% at T4 and tended to reach normal values after the recovery period. Urea concentration may reflect total ammonia excretion. IMP production is correlated to urate appearance in the blood, which is the final product of purine catabolism. Urate blood concentration rose significantly in blood by 24% and 20% at T4 and T5, respectively, when compared to the control. Blood levels of creatinine, a muscle damage indicator that may also suggest hemoconcentration alteration, responded acutely to both exercise sessions, augmenting significantly by 24% at T2. This was followed by a restoration of control values at T3, then a significant enhancement right after resistance training by 20% when compared to the control, and then a return to normal values at the end of the recovery period (Figure 7). BUN concentration remained unchanged throughout the trial.

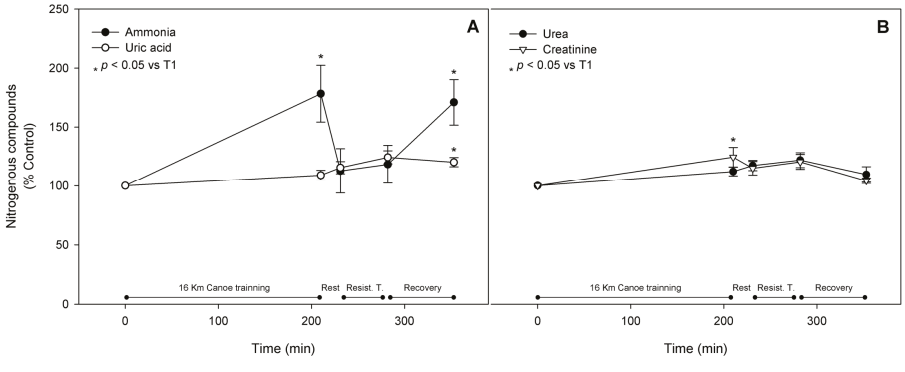

Figure 7. Nitrogenous compounds. Ammonia and uric acid are shown in (**Panel A**). Urea and creatinine fluctuations are presented in (**Panel B**). Nitrogenous compounds were evaluated as reported in materials and methods and are shown as mean ± standard error of percentage values against control. * Indicates statistical difference against control values ($p < 0.05$).

Serum glucose, insulin, ACTH, and cortisol fluctuations during exercise are also related to the destinations of amino acid metabolites and, therefore, were measured. Due to the stress caused by the canoe training session, the hypothalamus activated the production of Corticotropin Releasing Hormone (CRH), which in turn stimulated the anterior pituitary gland to produce ACTH, and then the adrenal gland to produce cortisol. While ACTH serum levels increased by 82% at T2, cortisol rose only 12% in comparison to initial levels. After exercise, the HPA axis was suppressed, and blood levels of both hormones diminished significantly when ACTH reached values of 36% at T4, and measured cortisol was 39% at T5 when compared to T2 (Figure 8).

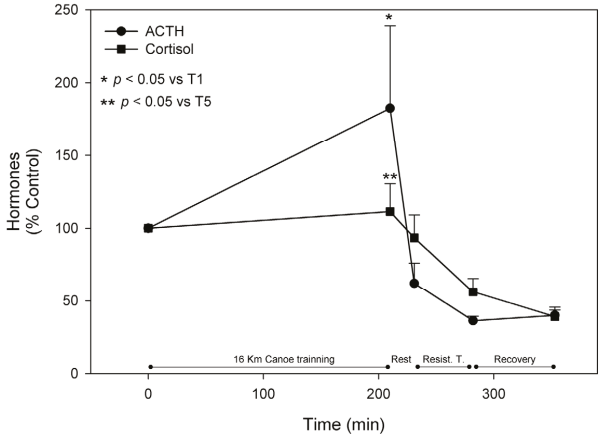

Figure 8. Hypothalamic-pituitary adrenal axis hormones. Adrenocorticotropic hormone (ACTH) and cortisol were measured as described in materials and methods and are represented as mean ± standard error of percentage values against control. * Indicates statistical difference against control values ($p < 0.05$). ** Indicates statistical significance when compared to T5 values ($p < 0.05$).

Insulin presented a slight decrease in response to the canoe exercise by about 10% and a significant increase of 137% after the 20 min of rest due to the food and hydro-electrolyte reposition; it returned to the approximate basal levels after the resistance training session and presented a drop by 50% of the original concentration. Glycemia was significantly enhanced by 78% after the canoe bout and tended to decrease progressively throughout the trial while maintaining its blood level slightly higher than basal. The ketone body 2-hydroxybutyrate blood levels were also significantly augmented after the canoe bout by 29%, followed by a slighter increment of 11% after the second exercise training session (Figure 9, panels A and B). It is well known that the lactate blood levels increase according to the exercise intensity. Our results showed a significant increment of 360% and 255% after the canoe and resistance training sessions, respectively (Figure 9, panel A).

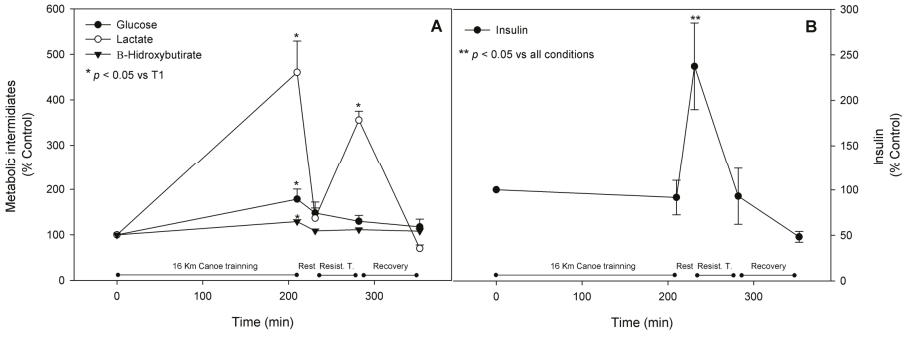

Figure 9. Metabolic intermediates and Insulin. Glucose, lactate, 2-hidroxybutirate (A), and insulin (B) were assessed as described in materials and methods and are represented as mean ± standard error of percentage values against control. * Indicates statistical difference against control values ($p < 0.05$). ** Indicates statistical significance when compared to all conditions ($p < 0.05$).

4. Discussion

Canoeing competitions are sprint events requiring sustained bursts of speed and power, leading to intense biochemical and metabolic stress. Many metabolites produced due to physical effort may be implicated in fatigue and impairment of physical performance, hence, the understanding of these responses is crucial to upgrade training sessions and optimize athlete performance. Here, we applied a Sportomics approach to study and evaluate different metabolic and cellular responses during a training session of world-class canoe athletes. Sportomics can help us in the understanding of the metabolism and signaling events that occur in response to exercise and allow us to perform interventions increasing both metabolic and sportive performances [10,16,19].

We measured serum levels of known exercise intensity biomarkers to characterize the applied training protocol magnitude and assure the possibility of correlating exercise intensity to the metabolic responses. Exercise intensity can be inferred by increases in plasma levels of CK, CKMB mass, CKMB, and LDH [3,44–46]. Several studies have investigated the increase in CK in response to exercise [3,5,10,21]. In this study, CK and CKMB mass blood levels increased continuously throughout the exercise trial, rising significantly at T4 and T5 when compared to basal levels. This suggests that the exercise stress and duration represented enough stimuli to cause such changes; a similar result was described by Siegel et al. [47]. Despite these observations, CKMB levels did not elevate significantly at any time point throughout the exercise session. However, it is worth noting that the blood basal levels of this enzyme were higher than expected due to exercise accumulation along the regular training season of the athletes, and this fact may have limited the furthest increment along the executed protocol. Additionally, immune assays show more analytical sensitivity when compared to enzymatic activity measurements. Therefore, it is necessary to separate immune assays from enzymatic assays. It is important to emphasize that most protocols measure the enzymatic activity of CK (also LDH and others) as a way to understand its increase or decline in response to exercise. These enzymes are also being subjected to blood environment changes that can lead to an increase in the specific activity (i.e., the ability to an enzyme to catalyze a reaction in a given unit of time), so it is our understanding that the preferable way to measure muscle cellular injury is to use a direct measurement of the enzyme content using immunological quantitative methods (such as ELISA or Western blot or mass spectrometry). For us, this is an important statement because we believe that researchers should carefully analyze the results of any increase of enzymes during exercise by the way of enzymatic activity.

Blood levels of LDH also remained constant throughout the exercise trial, and this result is in accordance with other studies which have shown a classic delayed response of LDH blood levels to

strenuous exercise [48–50]. Myoglobin release from muscle to blood is a well-known biochemical marker of muscle injury [51]. In our study, myoglobin significantly increased due to the exercise stress, confirming skeletal muscle damage induced by the exercise protocol. This confirms the CK findings and corroborates our interpretation concerning the difference in the CK and CKMB increase.

Classical biochemical hepatic damage markers such as AST, ALT, ALP, and γGT can be increased as a result of liver and/or muscle injury after strenuous exercise [52,53]. In the present study, the only enzyme that presented a significant increase throughout the protocol was AST, while the serum level of the other enzymes remained unchanged throughout the exercise. Since these enzymes are also present in muscles, the origin of this increase could not be differentiated between muscle or liver cell disruption. However, we have recently proposed that muscle damage can be distinguished from liver damage by using the more specific liver biomarkers such as ALT and γGT [5], which remained constant in the present study. These data together suggest that the increases of these proteins are more likely from muscle rather than liver cellular injury.

White blood cell counts increase after many types of exercise and the release of neutrophils is directly correlated with exercise intensity and duration [3,17,18]. In this study, we reported an increment in total leukocytes in response to both exercise sessions, which can be attributed to the release of neutrophils. Interestingly, after a discrete increment in lymphocyte levels at T2, it returned to basal values and continued decreasing reaching a significant reduction between the training sessions. Previous studies indicated that the response of white blood cells to exercise is dependent on both cytokine and myokines modulation [54–56]. Taken together these results may suggest that white blood cell mobilization is due not to a non-specific exercise-induced spleen release, but rather to a specific signal. Additionally, we reported an increase in platelet count without any change in erythrocyte count, indicating that this effect occurs in a spleen-independent manner. As previously suggested, these results may indicate that both thrombocytosis and leucocytosis observed during the exercise bout are induced by either muscle cell damage or differential cell signaling [3,18].

Muscle cell damage is known to stimulate immune cell mobilization to the bloodstream and migration to muscle tissue [5]. Exercise has been proposed to be a physiological way to modulate immunity; while acute severe exercise usually impedes immunity, chronic moderate exercise improves it [57,58]. Although the evidence to support these concepts is inconclusive, it supports the idea that exercise-induced immune suppression increases susceptibility to symptoms of infection, particularly around time of competition [59]. Moreover, metabolic stress is correlated with exercise induced white blood cell response, as carbohydrate supplementation and availability have been proposed to affect neutrophil count after intense exercise [60–62]. We previously described that a combination of training, rest and nutritional intervention could have an important impact in amino acid availability, muscle cellular injury, and immune response in another world-class athlete [10]. Therefore, the immune responses reported here may be directly correlated with the alterations in the nutritional status and metabolic availability as observed during the present experimental trial.

In this sense, it is important to maintain plasma level amino acids during training sessions, since many amino acids serve both as anabolic and energetic precursors. In addition, it has been proposed that blood fluctuations in the concentration of BCAAs may affect its ratio in the brain [63]. In our study, the levels of many amino acids presented a blood concentration decrease during the sport trial. Leucine showed the most important decrease after the canoe training session. Isoleucine and valine concentration also decreased in a smaller range. Aromatic amino acids, which are generally metabolized in the liver, were slightly consumed during the canoe exercise session; similar results were described before [10]. Glutamine levels presented a similar response; they decreased after the canoe trial and were restored after the recovery period. This could be the result of two processes: glutamine exportation from muscle to decrease its ammonia levels; and the use of glutamine as both a gluconeogenic substrate and a urea cycle feeder in the liver. On the other hand, alanine was up regulated after the first exercise bout, showing a two-fold increment at T2. This response may be attributed to a metabolic attempt to offer gluconeogenic substrates for further oxidation. The

depletion of glycogen storage is related to exercise intensity, duration, and nutritional status, which in turn may increase the use of amino acids as energy substrates, thereby increasing ammonia and the production of other nitrogen compounds [64]. Both glutamine and alanine are anaplerotic and gluconeogenic substrates and contribute to ATP and glucose synthesis. The ergogenic properties of glutamine have been extensively studied [6,62,65], and we have recently reported the metabolic effects of alanine in comparison to long-term glutamine supplementation during an intermittent exercise protocol. Long term administration of glutamine is capable of reducing ammonia production during intermittent exercise, hence, it is postulated to be a protector against an increase in blood ammonia in an exercise intensity-dependent manner [21].

Many studies have indicated that ammonia is a useful physiological marker of prolonged intense exercise, and its appearance in blood is positively correlated with exercise intensity [1,10,30]. High ammonemia can be toxic to both muscles and the central nervous system (CNS). Such changes are believed to contribute to the disturbances in neuropsychological function and motor control deficits and are also observed in patients with cirrhosis and, therefore, could induce central and peripheral fatigue [25,60,66]. Therefore, measuring ammonia production during a sport session may represent an important tool to control exercise intensity and to understand the metabolic response of a given athlete. The canoe athletes experienced an increase in their blood ammonia levels during the exercise trial due to both stimuli, which remained up regulated even after the recovery period. This effect was followed by an increase in other measured nitrogenous compounds, such as urea, urate and creatinine. These responses may have occurred as a result of an increased demand for ATP by muscle contraction, leading to adenosine monophosphate (AMP) deamination and, subsequently, the production of ammonia and urate [26–29]. Many studies have shown that ammonia production and release represents the exercise effort intensity, rapidly increasing in intensities greater than 50%–60% of VO_{2max} up to maximal exhaustion [31,32]. Ammonia plasma concentration is also up regulated during prolonged (greater than one hour) submaximal exercise (60%–75% VO_{2max}). In these conditions, ammonia could be produced in increasing amounts through the breakdown of branched chain amino acid (BCAA) prior to oxidation for additional energy provision [33–35].

The response of the other nitrogen metabolites may shed light on understanding the protein and amino acid oxidation response during exercise. Urea and urate blood concentration is indirectly correlated with the myokinase (adenylate kinase, ADK) contribution to ATP synthesis. Under a resting physiological state, approximately 90% of the skeletal muscle adenosine monophosphate deaminase (AMPD) is in a sarcoplasmic position and in an inactive form. However, a significant change occurs as intense muscle contraction begin, when approximately 50%–60% of AMPD becomes bound to the myofibrils [28]. Binding of the enzyme increases its activity causing an increased rate of degradation of AMP to IMP. This is correlated with the appearance of urate in the blood which is a final metabolite of purine metabolism [30]. This increased breakdown of AMP will affect the equilibrium of the ADK reaction by creating additional ATP from ADP to increase the cellular energy charge and maintain contractions under conditions of increasing stress [29]. During intense exercise, when AMP production and deamination are high, ADP levels also increase as utilization of ATP exceeds re-phosphorylation [67]. Therefore, any strategies, such as diet adequacies and supplementations, to protect against hyperammonemia or an increment of any nitrogenous compound could enhance physical performance or prevent CNS injuries, as previously reported by our group [6,10,21].

During the canoe trial, glycemia rose significantly, which may be a result of the HPA axis activation. Exercise is known to be a potent activator of this endocrine system, resulting in the release of ACTH, as confirmed here. This ultimately culminates with glucocorticoids production and release into blood circulation, which may lead to gluconeogenesis activation and promotion of an adrenergic stimulus, providing glucose to blood from hepatic glycogenolysis [68–70]. Nevertheless, afferent neural feedback signals from contracting muscle and feedback signals mediated via the blood stream can stimulate glucose production to maintain glycemia. Therefore, central mechanisms coupled with the degree of motor center activity can be responsible for part of the increase in glucose mobilization,

especially during intense exercise where hepatic glucose release exceeds peripheral glucose uptake, and plasma glucose rises [71]. Furthermore, cortisol is implicated in exercise induced lymphocyte apoptosis, via glucocorticoid dependent-pathways [72], which might affect immune function and protect the organism from an overreaction of the immune system in the face of exercise-induced muscle damage [73].

Hepatic glucose production increases during exercise, to cope with the augmented demand, as a product of liver glycogenolysis and gluconeogenesis. Whereas the former predominates during high intensity exercise, the latter contributes substantially with prolonged exercise and the concomitant decline in liver glycogen stores and with increased gluconeogenic precursor supply. In fact, it has been postulated that the increase in glucose production with exercise intensity in healthy subjects can be entirely attributed to increases in net hepatic glycogenolysis [74]. This pathway is also supported by our data. On the other hand, a decline in plasma insulin is important for the rise in glucose production during exercise [71], due to the fact that insulinemia tends to decrease in response to prolonged exercise, with a more pronounced effect on athletes than untrained individuals [75], which is in agreement with the results reported here.

5. Conclusions

The data presented here allow us to consider hormonal, metabolic, and signaling response together with the knowledge of nutrition and training environment. This combined information permits a better understanding of the individual responses of exercise and sport stress. Our group developed the concept of Sportomics with a focus on bridging the same existent gap between translational and personalized medicine [76]. As stated by Liebman et al. [77], the workflow bench to bedside approach is being refined in the face of a new bedside-bench-bedside approach. Sportomics is useful to evaluate the unprecedented kinetics of some metabolites [3,5,10,11,13,14,16,37,78] and to shed light on the importance of in-field metabolic analyses to the understanding of the inter-individual response to exercise. Besides an effective nutritional support, collecting physiological data during training and competition can provide important information about an athlete's clinical condition, bringing strategies to modify metabolism during exercise as well as supporting coaches to prescribe their sessions and recovery time. Therefore, and due to the uniqueness of this study, we believe Sportomics is a primal tool for training management and performance improvement, as well as for preserving health and increasing the quality of life of athletes.

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3

Exercise, Appetite and Weight Control: Are There Differences between Men and Women?

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Abstract: Recent years have witnessed significant research interest surrounding the interaction among exercise, appetite and energy balance, which has important implications for health. The majority of exercise and appetite regulation studies have been conducted in males. Consequently, opportunities to examine sex-based differences have been limited, but represent an interesting avenue of inquiry considering postulations that men experience greater weight loss after exercise interventions than women. This article reviews the scientific literature relating to the acute and chronic effects of exercise on appetite control in men and women. The consensus of evidence demonstrates that appetite, appetite-regulatory hormone and energy intake responses to acute exercise do not differ between the sexes, and there is little evidence indicating compensatory changes occur after acute exercise in either sex. Limited evidence suggests women respond to the initiation of exercise training with more robust compensatory alterations in appetite-regulatory hormones than men, but whether this translates to long-term differences is unknown. Current exercise training investigations do not support sex-based differences in appetite or objectively assessed energy intake, and increasing exercise energy expenditure elicits at most a partial energy intake compensation in both sexes. Future well-controlled acute and chronic exercise studies directly comparing men and women are required to expand this evidence base.

Keywords: appetite; appetite-regulatory hormones; compensation; energy balance; energy intake; exercise; sex-based differences; weight control

1. Introduction

Obesity is a major risk factor for several chronic diseases, including type 2 diabetes mellitus and cardiovascular disease, and remains a significant global burden from a public health and economic standpoint [1,2]. Weight loss as little as 3% of initial body mass is sufficient to promote favourable changes in several chronic disease risk markers and can be accomplished by increasing energy expenditure through exercise and/or reducing energy intake to achieve a sustained negative energy balance [3]. Recent years have witnessed significant research interest surrounding the interaction between exercise, appetite and energy balance, which has direct implications for the implementation of exercise as a weight management strategy [4].

Similar to many scientific fields, the majority of exercise and appetite regulation studies have traditionally focused research efforts on men. Consequently, much less is known about the interaction between exercise and appetite in women, and the opportunity to examine potential sex-based differences has been limited. A handful of exercise training studies have demonstrated that men experience greater reductions in body mass and body fat than women [5–7], although this is not a universal finding [8,9]. Authors supporting the concept of divergent weight loss outcomes have

suggested that women demonstrate greater compensatory responses to exercise by more accurately balancing energy intake and expenditure in order to defend body fat stores and preserve reproductive function [10–12].

Exercise-induced changes in hormones implicated in appetite control and energy balance (e.g., acylated ghrelin, peptide YY (PYY), glucagon-like peptide-1 (GLP-1), insulin, and leptin) may contribute to sex-based differences in body fat loss after exercise [13]. Although based on a limited number of studies, a previous review concluded that women exhibit compensatory changes in appetite ratings and hormones conducive to appetite stimulation; a response that is not seen in men [11]. However, this conclusion has not been supported by more recent experimental studies, which have documented similar appetite, appetite-regulatory hormone and energy intake responses to acute and chronic exercise-induced energy deficits in men and women [8,14,15].

The purpose of this article is to review recent developments regarding appetite, appetite-regulatory hormone and energy intake responses to single bouts of exercise (acute responses) and exercise training (chronic responses) in men and women. Furthermore, this review will consider the potential implications of these findings for health and highlight important areas for future research.

2. Appetite-Regulatory Hormones

Appetite and energy intake are regulated at the physiological level by the neuroendocrine system, which involves complex interactions between central and peripheral mediated pathways [16,17]. Appetite-regulatory hormones include episodic gut signals that are sensitive to short-term fluctuations in feeding behaviour and control hunger and satiety on a meal-to-meal basis (e.g., acylated ghrelin, PYY, and GLP-1), and tonic hormonal signals that regulate long-term changes in energy balance and body fat (e.g., insulin, and leptin). A brief introduction to these hormones is presented here, but the interested reader is directed to a number of comprehensive reviews documenting the precise role of these hormones in the homeostatic regulation of appetite and energy balance [16–19].

Of the short-acting appetite regulatory signals, ghrelin is unique as the only known gut peptide that is orexigenic, and is predominantly secreted into the circulation by the oxyntic glands of the stomach. Ghrelin exists in the circulation in two forms (acylated and unacylated) and, although only 10%–20% of circulating ghrelin is acylated ghrelin, this form is believed to be solely responsible for appetite stimulation [20]. Circulating ghrelin concentrations increase preprandially and are rapidly suppressed postprandially on a meal-to-meal basis. This temporal pattern of fluctuation is indicative of an important role in coordinating meal initiation [21].

Working in opposition to ghrelin, on a meal-to-meal basis, several appetite-inhibiting hormones serve to promote post-meal satiation and satiety (e.g., PYY, GLP-1, cholecystokinin, pancreatic polypeptide, and amylin). Of primary relevance to this review, PYY is predominantly synthesised and secreted from the intestinal L-cells and is present peripherally in two forms ($PYY_{1–36}$ and $PYY_{3–36}$), with $PYY_{3–36}$ representing the most abundant and biologically active form. Concentrations of PYY are low in the fasted state and increase rapidly after meal intake, which highlights a potential role in meal termination and sensations of fullness between meals. Glucagon-like peptide-1 is also secreted from the intestinal L-cells in response to nutrient intake and similarly contributes to meal termination and satiety. It exists as an active ($GLP_{7–36}$) and inactive ($GLP_{9–37}$) form, with the active form rapidly degraded to its inactive form upon secretion into the circulation. The appetite-inhibiting effect of these hormones is further supported by studies demonstrating that peripheral administration of $PYY_{3–36}$ [22] and GLP-1 [23] stimulates satiety and reduces ad libitum food intake in lean and obese individuals.

Leptin, secreted primarily from adipocytes, and insulin, released by the beta cells of the pancreas, are important regulators of energy balance, which are implicated in the long-term control of food intake and energy expenditure. Leptin and insulin are secreted in concentrations proportional to body fat mass, and act directly on the hypothalamus and other brain regions to exert anorexigenic effects. Circulating leptin and insulin concentrations are elevated in obese individuals, suggesting that

a degree of resistance to the anorexigenic effects of these hormones may occur with obesity. This is further supported by evidence that the accumulation of adipose tissue weakens the inhibitory effect of fat mass on energy intake [24,25].

3. Exercise and Weight Loss

Exercise is an important component of weight management [3], and promotes a myriad of health benefits independent of weight loss [26]. It is well documented that exercise typically results in modest weight loss that can be enhanced when exercise is combined with dietary modifications [27,28]. However, the efficacy of exercise as a successful strategy for weight management varies markedly between individuals [29]. Interestingly, it has been suggested that sex may be a primary factor that affects the ability of structured exercise to promote weight loss and/or facilitate weight management [30].

The strongest evidence of a sex-based difference in the weight loss response to exercise was provided in the Midwest Exercise Trial by Donnelly and colleagues [6]. This study involved a 16-month supervised exercise training program at a set intensity and duration (five days per week, 20–45 min per session at 55%–70% peak oxygen uptake ($\dot{V}O_{2peak}$)) with ad libitum diet in previously sedentary men and women. After the exercise intervention, men lost an average of 5.2 kg in body weight and 4.9 kg in fat mass, whereas women maintained body weight and fat mass. Other studies have also demonstrated that men experience greater weight loss than women in response to a supervised program of exercise when exercise is prescribed at a similar duration and relative exercise intensity across the sexes [5,31,32].

However, in many of these studies, the exercise-induced energy expenditure was substantially greater in men than women. This has been suggested as a potential reason for the reported sex-based differences in exercise-induced weight loss [33], in accordance with evidence that the energy expenditure of exercise is the strongest predictor of fat loss during an exercise program [34,35]. Exercise training studies prescribing exercise based on energy expenditure have reported comparable body composition changes in response to the training stimulus in men and women [8,9,36]. Specifically, Donnelly and colleagues [9] have published findings from a subsequent randomised controlled trial as a follow-up to the Midwest Exercise Trial in which the exercise-induced energy expenditure was matched between men and women over a 10-month supervised aerobic exercise training intervention. In contrast to their earlier study [6], when the energy expenditure was equivalent between the sexes, similar reductions in body weight and body fat were seen between men and women [9].

A common finding in the literature is the degree of individual variation in the weight loss response to exercise training in both sexes [8,9,29,35,37,38]. It has been suggested that individual differences in compensatory behaviours that negate the exercise-induced energy deficit may be responsible for this variability [29]. Specifically, evidence of increased hunger and energy intake have been reported in individuals who experience a lower than expected weight loss after a period of exercise training [29,37,38]. Consequently, studies investigating the effect of exercise on appetite regulation (appetite perceptions, appetite-regulatory hormones, energy intake) in men and women are important and will be discussed in the following sections of this review.

4. Acute Effects of Exercise on Appetite, Appetite-Regulatory Hormones and Energy Intake

A plethora of studies have been conducted examining the appetite, appetite-regulatory hormone and energy intake responses to acute exercise in men, and to a much lesser extent, women. This research has been reviewed in detail elsewhere [4,39–43], but a brief synopsis of the most pertinent studies is presented in this article to frame the research literature which has examined sex-based differences.

4.1. Appetite and Appetite-Regulatory Hormones

The consensus of evidence in healthy, normal weight men suggests that acylated ghrelin concentrations are transiently suppressed, and satiety hormones, most notably PYY and GLP-1, are elevated during and immediately after an acute bout of exercise. Such hormonal changes often coincide with a transient reduction in subjective appetite responses, which has been described as "exercise-induced anorexia" [44]. These responses become apparent when acute exercise is performed $\geq$60% of $\dot{V}O_{2peak}$ typically [45–49], and have been replicated during a variety of exercise modes including running [45,46,48], cycling [47,50–53], swimming [54], resistance exercise [46,55] and high-intensity interval exercise [52,53,56]. Circulating appetite-regulatory hormones and appetite ratings typically return to control values within 30 to 60 min of exercise completion [39,46,48]; however, compensatory increases in appetite have been reported in some studies [52,54,57]. Furthermore, current evidence suggests that acute exercise elicits similar appetite and appetite-regulatory hormone responses in lean and overweight men [47], and does not stimulate compensatory changes in those who are overweight or obese [47,58].

Despite postulations that sex-based differences in appetite regulation may exist to enable women to preserve energy balance and reproductive function [10–12], several acute studies conducted in women suggest that they respond similarly to men. Specifically, transient alterations in appetite and appetite-regulatory hormone concentrations (acylated ghrelin, $PYY_{3–36}$, and GLP-1) have been reported in a direction expected to suppress appetite in healthy, recreationally active [15], endurance-trained [59] and overweight and obese [60] women. Furthermore, the majority of studies report no evidence of compensatory increases in appetite perceptions and appetite-regulatory hormones up to 7.5 h after a single bout of exercise in women [15,59–62].

However, exceptions have been observed in the literature with some studies demonstrating that women do not exhibit an acute exercise-induced suppression of appetite [62–64] or changes in appetite-regulatory hormones [61,62]. Furthermore, in contrast to the aforementioned studies in men and women, Larson-Meyer and colleagues [64] reported an increase in acylated ghrelin concentrations during the 2 h period after 60 min running at 70% $\dot{V}O_{2peak}$. Such discrepancies are likely related to differences in the exercise intensity, training status of participants, completion of exercise in the fasted or postprandial state, timing of meal intake and analytical methods used to quantify hormone concentrations.

Sex-based differences in the regulation of appetite in response to acute exercise have been examined directly in four studies [14,15,65,66]. The first acute exercise and appetite study that compared men and women was published by Kawano and colleagues [65]. The authors reported that 20 min of rope skipping exercise increased ratings of subjective hunger 30 min after exercise in women but not men; however, the absence of a control condition in this study and the somewhat unusual mode of exercise make this finding difficult to interpret. Furthermore, this study did not control for the potential confounding effects of the menstrual cycle, which represents an important consideration for acute exercise studies comparing men and women. In this regard, recent evidence suggests that compared with untailored programs, synchronising diet and exercise training interventions around the hormonal changes that occur during the menstrual cycle elicits greater weight loss [67] and improvements in muscle strength [68]. In addition, cyclical fluctuations in sex hormones (estrogen and progesterone) have been shown to alter appetite-regulatory hormone concentrations and energy intake in women across the menstrual cycle [69,70]. However, whether appetite responses to exercise in women are influenced by the menstrual cycle phase is not known and represents a research avenue to consider in the future.

Subsequent studies directly comparing men and women have also incorporated measures of appetite-regulatory hormones and energy intake (discussed below) alongside subjective appetite perceptions to provide a more comprehensive picture of potential sex-based differences in appetite regulation. In this regard, Hagobian and colleagues [14] examined the appetite and hormonal responses to a single bout of cycling performed at 70% $\dot{V}O_{2peak}$ until 30% of total daily energy expenditure was expended in healthy men and women matched for age and cardiorespiratory fitness. Importantly,

the female participants were all studied during the early follicular phase of the menstrual cycle. The authors reported that appetite perceptions and appetite-regulatory hormone concentrations (acylated ghrelin and $PYY_{3–36}$) were not different during the 40 min after exercise in either sex. Similarly, in another acute study, breaking up prolonged sitting with light- or moderate-intensity walking did not alter appetite or concentrations of acylated ghrelin and total PYY over the 5 h observation period in either sex [66]. The walking interventions adopted in this study comprised a total of 28 min walking performed in 2 min bouts every 20 min. This intermittent pattern of exercise contrasts with the vast majority of acute exercise and appetite studies, which have reported transient perturbations in appetite and appetite-regulatory hormones in response to continuous, moderate- to high-intensity exercise protocols. Indeed, the authors recognise that the exercise stimulus may have been insufficient (in intensity and duration) to provoke transient changes in appetite and appetite-regulatory hormones.

Recently, Alajmi and colleagues [15] examined the effect of 60 min treadmill running at 70% $\dot{V}O_{2peak}$ on appetite and acylated ghrelin concentrations over 7 h in healthy men and women (studied during the follicular phase of the menstrual cycle). Despite the greater net energy expenditure during exercise in the men (3971 vs. 2536 kJ in men and women, respectively), both men and women exhibited an equivalent suppression in appetite and acylated ghrelin concentrations in response to acute exercise (Figure 1), with no evidence of compensatory responses to exercise in the 7 h observation period in either sex. Interestingly, the female participants in this study exhibited significantly greater acylated ghrelin concentrations compared with men. However, the relevance of this difference is unclear given subjective appetite ratings were greater in men than women. Furthermore, despite the greater appetite and lower acylated ghrelin concentrations in men than women, the appetite and acylated ghrelin responses to exercise were similar between the sexes.

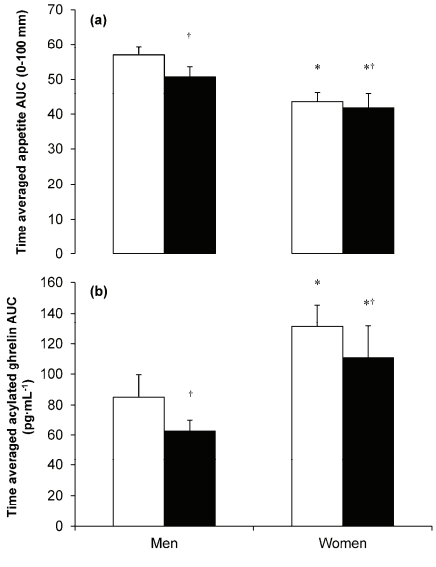

Figure 1. Time averaged total area under the curve (AUC) for appetite ratings (**a**); and plasma acylated ghrelin concentrations (**b**) in the control (□) and exercise (■) conditions. Each condition was 7 h and a single bout of exercise was performed between 0 to 1 h in the exercise condition (60 min running at 70% peak oxygen uptake). † Significant difference between exercise and control $p \leq 0.05$; * Significant difference between women and men $p \leq 0.05$. Values are mean (SEM), appetite ratings: $n = 10$ men, $n = 10$ women; acylated ghrelin: $n = 8$ men, $n = 8$ women. Data reproduced from reference [15]. © Wolters Kluwer Health, Inc. Reproduced with permission.

4.2. Energy Intake

Many of the studies highlighted above included an ad libitum meal in the post-exercise period to assess potential changes in energy intake after a single exercise stimulus. The majority of studies in men report no change in absolute energy intake after acute exercise when a single or multiple ad libitum meals are provided 30 min to 7.5 h after the cessation of exercise [48,49,52,53,55,71–73]; however, some studies have reported increases [50,74] or decreases [47,58,75] in energy intake after acute exercise. Nevertheless, two studies have demonstrated that 24 h energy intake is unchanged after acute exercise in healthy men quantified from laboratory-based ad libitum meals and overnight food bags [48,52].

Similarly, evidence suggests that ad libitum energy intake remains unchanged in response to acute exercise in healthy women [64,76–78] and overweight and obese women [61,62,76]. As an exception, Larson-Meyer and colleagues [64] reported that absolute energy intake (ad libitum meal provided 120 min after exercise) was unchanged after 60 min running at 70% $\dot{V}O_{2peak}$, but was increased after 60 min walking performed at the same relative intensity in a different group of women. The strength of this evidence is limited however by the between-measures design and the stark differences in body composition and cardiorespiratory fitness between the two groups. In another study, Pomerleau and colleagues [79] reported that ad libitum energy intake was increased 1 h after brisk walking at 70% $\dot{V}O_{2peak}$ in healthy, young women. However, this change did not translate to altered energy intake over the remainder of the day after the provision of an ad libitum meal 6.5 h after exercise and an overnight snack bag. This highlights the importance of monitoring feeding behaviour over longer time periods.

Regardless of whether absolute energy intake remained unchanged, increased or decreased in response to acute exercise in the studies cited thus far, relative energy intake (total energy intake minus net energy expenditure of exercise) is invariably lower after exercise compared with control in men and women. Whilst this suggests that the exercise-induced energy deficit is maintained after exercise, which may have significant implications for weight management, it should be noted that the short-term follow up in these studies prevents us from drawing conclusions about behavioural and physiological responses over a greater period of time.

Studies directly comparing men and women have demonstrated that total energy intake is greater in men compared with women [14,15], but this difference disappears after adjustment for lean body mass [15]. These findings coupled with the higher appetite ratings reported in men in the study conducted by Alajmi and colleagues [15] lend support to the theory that lean body mass, as the largest contributor to resting metabolic rate, is a primary determinant of appetite control and energy intake [24,25].

In addition to the appetite and hormone responses discussed in the previous section, Hagobian and colleagues [14] reported that absolute energy intake was unchanged in response to a single bout of cycling inducing a similar energy expenditure (30% of total daily energy expenditure) in men and women (energy expenditure: men, 975 kcal; women, 713 kcal) (Figure 2). The authors observed large variability in the energy intake responses (note large SDs on Figure 2 especially for men) with evidence of both higher and lower energy intake after exercise compared with a resting control condition in both men and women, which supports previous acute exercise and appetite regulation studies in healthy weight [78] and overweight and obese [62] women. Although the authors reported no significant change in energy intake after acute exercise in men or women, it is worth noting that mean ad libitum energy intake was higher in men after exercise (Figure 2) [14]. A closer examination of the mean differences and estimated standardised effect sizes revealed that energy intake after the exercise bout was 432 kcal higher than control in men (effect size = 0.68 indicating a moderate to large effect) compared with a 1 kcal increase after exercise in women (effect size = 0.004 indicating a trivial effect) (Figure 2). While this opposes the hypothesis that women are more likely to compensate for acute exercise-induced energy deficits by increasing energy intake, the conclusion that energy intake was unchanged in men should perhaps be interpreted with caution.

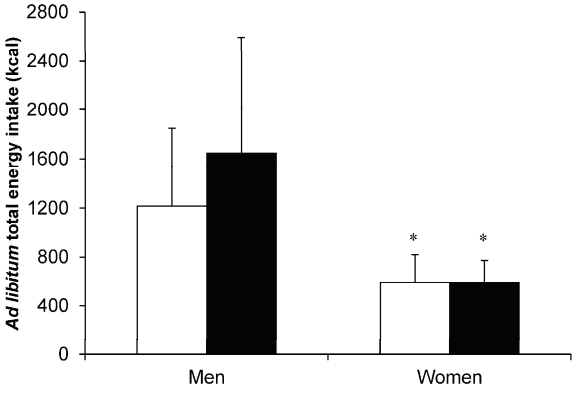

Figure 2. Total ad libitum energy intake during a single laboratory-based buffet meal in the control (□) and exercise (■) conditions in 11 men and 10 women. Exercise involved a single bout of cycling performed at 70% peak oxygen uptake until 30% of total daily energy expenditure was expended. * Significant difference between women and men $p \leq 0.05$. Values are mean (SD). Data from reference [14]. © 2008 Canadian Science Publishing or its licensors. Reproduced with permission.

Subsequent studies investigating potential sex-based differences have reported no change in absolute energy intake in response to a single bout of running [15] and accumulating short bouts of walking to break up sedentary time [66]. Furthermore, these studies have consistently reported a lower relative energy intake after acute exercise compared with control in both sexes, suggesting that acute exercise suppressed relative energy intake independent of sex [14,15,66].

5. Chronic Effects of Exercise on Appetite, Appetite-Regulatory Hormones and Energy Intake

Although acute exercise studies provide important information regarding appetite regulation, exercise training studies are required to discern the long-term effects of exercise on energy balance and weight control. Exercise training studies are now reviewed with continued focus on appetite sensations, appetite-regulatory hormones and energy intake responses between men and women. It should be noted that few well-controlled exercise training studies have been conducted with many studies inherently limited by methodological constraints such as unsupervised exercise, self-reported energy intake, low exercise-induced energy expenditure and a lack of objective measures to quantify exercise energy expenditure.

5.1. Appetite and Appetite-Regulatory Hormones

Alterations in ghrelin concentrations after chronic exercise have been reported in conjunction with favourable changes in body weight. Specifically, weight loss in response to an exercise intervention has been shown to elevate total ghrelin concentrations in healthy weight and overweight and obese women in the fasted state and postprandially (reviewed by [40]). In contrast, Guelfi and colleagues [80] reported no effect of 12 weeks of aerobic or resistance exercise on fasting and postprandial hunger and concentrations of acylated ghrelin and PYY in overweight and obese men, despite a reduction in body fat mass in both exercise interventions. However, the authors reported lower fasting and postprandial leptin concentrations after exercise, which has been observed in other studies with men and women after exercise-induced weight loss [81,82]. Furthermore, chronic exercise studies resulting in weight loss in women appear to reduce fasting insulin concentrations [83,84], but have little effect on fasting total PYY and GLP-$1_{7–36}$ concentrations [84,85].

Several exercise and appetite training studies recruiting both men and women have presented findings with the data for men and women combined [36,86,87]. Although these studies are informative,

it is not possible to elucidate the direct effect of sex on the observed responses. Nevertheless, short-term exercise training without weight loss (1 h of daily walking at 70% $\dot{V}O_{2peak}$ for 15 days) resulted in no changes in appetite or circulating concentrations of total PYY and insulin in obese men and women [87]. Martins and colleagues [36,86] have performed two studies investigating appetite and appetite-regulatory hormone responses to standardised meals in overweight and obese men and women undertaking 12 weeks of supervised aerobic exercise resulting in weight loss. The exercise intervention reduced fasting insulin concentrations but resulted in an increase in fasting acylated ghrelin concentrations and hunger perceptions [36]. In the postprandial state, circulating insulin was reduced along with a greater suppression in acylated ghrelin and a tendency for increased PYY and GLP-1 concentrations compared with levels before the intervention [36]. Furthermore, fasting and postprandial concentrations of leptin were reduced after the exercise intervention [86]. These findings led the authors to conclude that in response to chronic exercise, overweight individuals may balance the increased drive to eat with a concomitant increase in the satiety response to a meal, which supports previous findings in overweight and obese men [38].

In many of the studies discussed thus far, participants maintained their usual diet and subsequently lost body mass and fat mass by the end of the chronic exercise intervention. Therefore, it is difficult to determine whether the reported exercise-induced changes in appetite control are attributable to weight loss or to exercise training *per se*. In this regard, the study by Kanaley and colleagues [87] discussed previously did not report changes in appetite or appetite-regulatory hormones (total PYY, and insulin) in response to short-term exercise training without weight loss. Furthermore, total ghrelin concentrations were unchanged after exercise training in women who did not experience weight loss [85,88], and a study conducted in overweight adolescents observed no changes in fasting acylated ghrelin when body weight remained stable during the eight-month supervised exercise intervention [89]. Therefore, it is likely that alterations in appetite-regulatory hormones arise as a secondary consequence to changes in body mass.

Early evidence of exercise-induced sex differences in appetite hormones was provided by Hickey and colleagues [90]. In this study, 12 weeks of aerobic exercise training, without a change in body mass or body fat, significantly reduced fasting insulin and leptin concentrations in women but not in men. Subsequently, Hagobian and colleagues [13] examined appetite hormone responses to meal intake before and after four consecutive days of exercise in previously sedentary overweight and obese men and women. Daily aerobic exercise was performed on a treadmill at 50%–65% $\dot{V}O_{2peak}$ resulting in an energy expenditure equivalent to ~30% of total daily energy expenditure and was completed with and without dietary replacement of the exercise-induced energy deficit. The authors reported that acylated ghrelin concentrations were higher and insulin concentrations were lower after both exercise interventions in women (Figure 3). In contrast, although men demonstrated lower insulin concentrations in the energy deficit condition, this effect was eliminated with energy replacement and acylated ghrelin was not different after exercise regardless of energy status (Figure 3). These findings suggest that women experience perturbations in appetite-regulatory hormones conducive to appetite stimulation in response to the initiation of exercise training. This is consistent with the hypothesis that the mechanisms governing energy balance are more tightly regulated in women than men.

However, in the Midwest Exercise Trial, lower insulin concentrations were observed in men but not women after the 16-month exercise training intervention [32]. This was accompanied by a divergent weight loss response to exercise training (discussed previously) which, coupled with the greater exercise energy expenditure in men, is likely to explain the differential insulin findings between this investigation and that of Hagobian and colleagues [13].

Although replacing the exercise-induced energy deficit suppressed appetite perceptions in men but not women, appetite was not altered when the energy deficit was maintained in either sex [13]. This supports a previous study reporting no change in postprandial appetite in response to 14 days of moderate- or high-intensity exercise training in lean men and women [91]. In another study, sex-based differences in body weight and appetite were examined in response to a 12-week supervised

aerobic exercise intervention in overweight and obese men and women [8]. The 12-week exercise program resulted in similar reductions in body mass and body fat in the male and female participants. Furthermore, although fasting hunger ratings were elevated after the exercise training intervention, the magnitude of change was similar between the sexes and this difference did not translate to altered hunger responses in the postprandial period.

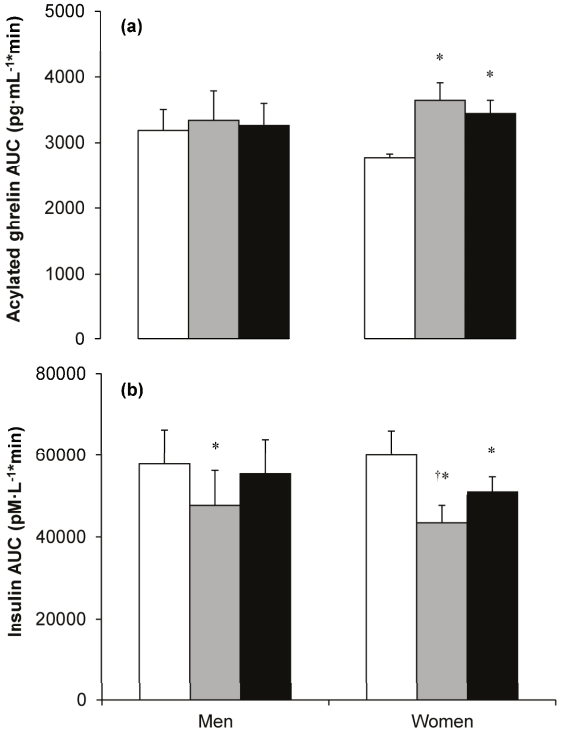

Figure 3. Total area under the curve (AUC) for plasma acylated ghrelin (**a**) and insulin (**b**) concentrations in the control (□), exercise with energy deficit (■) and exercise with energy balance (■) conditions in nine men and nine women. Exercise involved four consecutive days of treadmill exercise at 50%–65% peak oxygen uptake until 30% of total daily energy expenditure was expended. * Significant difference between exercise intervention and control; † Significant difference between exercise with energy deficit and exercise with energy balance. Values are mean (error bars not stated in original article). Data reproduced from reference [13]. © The American Physiological Society. Reproduced with permission.

5.2. Energy Intake

Current evidence suggests that increasing energy expenditure during short-term exercise training (3 to 14 days) elicits partial compensations in energy intake [91–94]. Furthermore, a recent systematic review concluded that longer term exercise training studies (>2 weeks to 18 months) typically observe no change in energy intake across the training intervention [95]. However, the authors recognised that the available literature is prone to various methodological shortcomings as highlighted previously (e.g., unsupervised exercise, self-reported energy intake) which makes it difficult to interpret the findings with confidence.

A recent study directly comparing isoenergetic three-day energy deficits imposed by diet or exercise reported that dietary restriction stimulated a compensatory increase in ad libitum energy

intake that was not observed in response to exercise [96]. This supports the findings from acute studies demonstrating rapid compensatory changes (appetite, appetite-regulatory hormones, energy intake) in response to diet-, but not exercise-induced energy deficits in men [71] and women [15]. These findings suggest that dietary restriction may represent a greater challenge to appetite regulation and energy balance than exercise, highlighting the importance of exercise to facilitate weight management in men and women [3].

A potential sex difference in energy intake responses during short-term exercise training was uncovered in two separate studies by Stubbs and colleagues [93,97]. Specifically, the authors reported that increasing energy expenditure through exercise training (seven days daily moderate- or high-intensity exercise) resulted in a partial compensation in energy intake in healthy women that equated to ~33% of the additional exercise-induced energy expenditure [93]. In contrast, there was no compensation in the energy intake response to an identical training stimulus in healthy men [97]. However, it is worth noting that energy intake was self-recorded in these studies through subjective dietary records and self-weighed intakes. This method of recording energy intake is particularly susceptible to participant bias, which makes it challenging to reconcile self-reported food intake with actual intake [98].

In a subsequent study adopting objective measures of both energy intake and energy expenditure, evidence of partial compensations in energy intake emerged after 14 days of supervised daily exercise which was equivalent to ~30% of the exercise-induced energy deficit [91]. This response was observed when energy intake data were combined for men and women, but only reached significance in men when analysed independently by sex [91]. This is consistent with an early study demonstrating that men, but not women, increase energy intake in response to five days of daily exercise, yet neither sex fully compensated for the imposed exercise energy expenditure [92]. Consequently, these findings refute the hypothesis that women compensate for chronic exercise-induced energy deficits by increasing energy intake.

When data is combined for men and women, studies investigating the effect of 12 weeks of supervised exercise (five days·week^{-1}, 500 kcal·session^{-1}) on body composition and appetite control have reported no exercise-induced change in energy intake assessed using self-reported food diaries [36] or laboratory-based test meals [99]. Furthermore, Westerterp and colleagues [5] reported no significant change in energy intake assessed using a self-reported seven-day weighed diary in men or women after 40 weeks of endurance training. In the Midwest Exercise Trials, energy intake was assessed using a combination of ad libitum meals in the University cafeteria and 24 h recall in overweight and obese men and women undergoing a supervised aerobic exercise program for 10 [9] or 16 months [6]. Similarly, no difference in energy intake was reported after the exercise training interventions in either sex [6,9].

When exercise is supervised and energy intake is quantified objectively using laboratory-based ad libitum meals, no changes in daily energy intake were observed in overweight and obese men or women after a 12-week aerobic exercise intervention (Figure 4) [8]. The authors of this study also highlighted the large variability in individual weight loss responses, both in magnitude and direction, which may afford some insight into why many individuals do not achieve their predicted changes in body composition with chronic exercise. Such heterogeneity in response to alterations in energy balance has been recognised previously [8,9,29,35,37,38]. Interestingly and pertinent to this review, overweight and obese men and women typically demonstrate a similar degree of individual variability when the exercise-induced energy expenditure is equivalent between the sexes [8,38]. For example, Caudwell and colleagues [8] reported body mass changes ranging from −14.7 to 2.0 kg in men and −10.0 to 4 kg in women. Furthermore, when participants are retrospectively classified as “responders” or “non-responders” (based on their actual weight loss relative to their predicted weight loss), there is some evidence supporting higher ad libitum energy intake in individuals experiencing lower than their predicted weight loss [29,37,38].

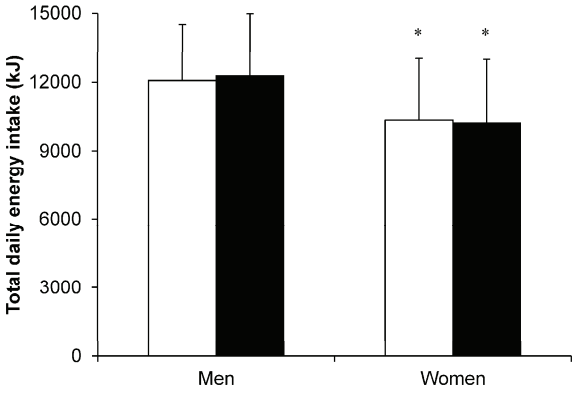

Figure 4. Total daily energy intake before (□) and after (■) a 12-week aerobic exercise training intervention in overweight and obese men (n = 35) and women (n = 72). Total daily energy intake was quantified objectively using laboratory-based test meal days at Weeks 0 and 12. On each day, participants were provided with an individualised fixed-energy breakfast (ad libitum at Week 0), fixed-energy lunch, ad libitum dinner and evening snack box. * Significant difference between women and men $p \leq 0.05$. Values are mean (SD). Data from reference [8]. © Wolters Kluwer Health, Inc. Reproduced with permission.

6. Implications and Future Directions

Scientific interest in potential sex-based differences in appetite regulation stems from initial evidence suggesting that men experience greater body mass and body fat reductions after exercise training than women [5,6]. Furthermore, evidence from an evolutionary biology perspective suggesting that women have evolved to store body fat to preserve energy balance and reproductive function has also driven research endeavour in this regard [10,12]. However, more recent experimental work has questioned the prevailing view that exercise is less effective for inducing weight loss in women, with several studies showing equivalent effects of exercise training on body composition in both sexes when the exercise-induced energy expenditure is matched [8,9]. Collectively, the balance of findings' presented in this review suggest that men and women do not exhibit different responses (appetite, appetite-regulatory hormones, energy intake) to acute or chronic exercise-induced energy deficits. This has important implications for men and women engaging in exercise for health, and supports the promotion of exercise as a weight management tool for all. However, it is likely that women will need to exercise for a longer duration and/or at a higher intensity to achieve a similar exercise energy expenditure as men.

The findings provided within the current literature from experiments focussing on weight loss can be useful in informing individuals about exercise and dietary approaches for health. In this regard, it has been established that exercise-induced energy deficits stimulate smaller changes in appetite, appetite-regulatory hormones and energy intake compared with dietary restriction in both men [71,96] and women [15]. This may assist in informing an individuals' decision regarding their preferred method of inducing an energy deficit for weight loss and also provide information regarding the anticipated homeostatic responses (i.e., greater appetite stimulation with food restriction).

Considering that exercise training appears to elicit at most a partial, but incomplete, compensation in energy intake in both sexes, it seems that men and women can endure prolonged periods in an exercise-induced energy deficit which may facilitate the development of a negative energy balance. Such incomplete compensation also supports evidence that larger exercise-induced energy deficits promote greater weight loss during an exercise intervention [34,35]. However, it is worth reiterating the considerable variability in responses (albeit to a similar degree in men and women), with some

individuals appearing susceptible to increased hunger and energy intake with exercise training that attenuates the degree of weight loss [29,37,38]. Nevertheless, exercise training triggers marked improvements in other important outcomes in the absence of weight loss (e.g., cardiorespiratory fitness, body composition, insulin sensitivity) [26], which is important for those undertaking exercise for health benefits.

Although a considerable body of literature has developed understanding of the relationship between exercise, appetite and weight control, there are only a few studies which have directly focused on sex-based differences. Additional research is required to expand the evidence base before definitive conclusions can be drawn. This should include different types of exercise and insights into the mechanisms governing appetite control, both of which appear sparse in the current literature. Studies investigating a wider array of appetite parameters, particularly appetite hormones beyond the initiation of exercise training, between men and women would also be welcomed. Furthermore, considering potential sex-based differences in non-homeostatic factors governing energy balance (e.g., neuronal responses [100], and cognitive/behavioural cues [101]) is another important line of scientific inquiry and will provide a more holistic insight into appetite regulation in men and women. Future research into the appetite, appetite-regulatory hormone and energy intake responses of elite athletes to exercise and dietary interventions also represents an important future research direction to better understand energy balance and the consequences of energy manipulation in this population. It is imperative that acute and chronic investigations adopt mixed-measures designs and utilise objective measures of energy balance components when examining interactions among appetite, appetite-regulatory hormones and energy intake between men and women.

7. Conclusions

This review has demonstrated that appetite, appetite-regulatory hormone and energy intake responses to acute exercise-induced energy deficits are similar between men and women. Specifically, the consensus of evidence suggests that acute exercise transiently suppresses appetite, and does not stimulate compensatory changes in appetite, appetite-regulatory hormones or energy intake in the hours after acute exercise in either sex. Evidence derived from exercise training studies appear less conclusive, with limited evidence that women, but not men, respond to the initiation of exercise training with compensatory changes in appetite-regulatory hormones conducive to appetite stimulation. However, it is not known whether this change translates into long-term differences after a more sustained period of exercise training. Furthermore, evidence does not support a sex dimorphism in appetite or energy intake when assessed objectively, and increasing energy expenditure through exercise elicits at most a partial energy compensation in both sexes. Few studies have directly compared appetite, appetite-regulatory hormone and energy intake responses to acute and chronic exercise interventions between men and women. Therefore, these conclusions are supported by evidence drawn from the limited studies directly comparing the sexes and supplemented by those conducted in men and women separately. A better understanding of whether appetite, appetite-regulatory hormone and energy intake responses to exercise-induced energy deficits differ by sex may contribute to the development of more effective weight management strategies.

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4

Cardiorespiratory Fitness and Peak Torque Differences between Vegetarian and Omnivore Endurance Athletes: A Cross-Sectional Study

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Abstract: In spite of well-documented health benefits of vegetarian diets, less is known regarding the effects of these diets on athletic performance. In this cross-sectional study, we compared elite vegetarian and omnivore adult endurance athletes for maximal oxygen uptake (VO2 max) and strength. Twenty-seven vegetarian (VEG) and 43 omnivore (OMN) athletes were evaluated using VO2 max testing on the treadmill, and strength assessment using a dynamometer to determine peak torque for leg extensions. Dietary data were assessed using detailed seven-day food logs. Although total protein intake was lower among vegetarians in comparison to omnivores, protein intake as a function of body mass did not differ by group (1.2 ± 0.3 and 1.4 ± 0.5 g/kg body mass for VEG and OMN respectively, $p = 0.220$). VO2 max differed for females by diet group (53.0 ± 6.9 and 47.1 ± 8.6 mL/kg/min for VEG and OMN respectively, $p < 0.05$) but not for males (62.6 ± 15.4 and 55.7 ± 8.4 mL/kg/min respectively). Peak torque did not differ significantly between diet groups. Results from this study indicate that vegetarian endurance athletes' cardiorespiratory fitness was greater than that for their omnivorous counterparts, but that peak torque did not differ between diet groups. These data suggest that vegetarian diets do not compromise performance outcomes and may facilitate aerobic capacity in athletes.

Keywords: vegetarian; endurance; VO2 max; dynamometer; protein; sustainability; torque; body composition; Dual X-ray Absorptiometry (DXA)

1. Introduction

Vegetarian diets are increasingly being adopted for a variety of reasons including health, sustainability, and ethics-related concerns. Adherence to a vegetarian diet has been associated with a reduced risk of developing coronary heart disease [1], breast cancer [2], colorectal cancers [3], prostate cancer [4], type 2 diabetes [5], insulin resistance [6], hypertension [7], cataracts [8] and dementia [9]. Vegetarians also typically have a lower body mass index (BMI) [10] and an improved lipid profile [11]. In addition to promoting physical health, reducing or eliminating meat from the diet is environmentally advantageous since producing meat requires more land, water, and energy resources than growing plants for food [12], and producing meat creates more greenhouse gases compared to a plant-based diet [13,14].

In spite of the many health aspects of vegetarian diets some concern has been raised pertaining to the nutrient adequacy of vegetarian diets for supporting athletic performance. Vegetarian diets are typically lower in vitamin B12, protein, creatine, and carnitine [15,16], and iron and zinc from plant sources are less bioavailable than from meat sources [17]. However, vegetarian diets are typically higher in carbohydrate and antioxidants [18,19], which may be advantageous for athletic performance, particularly for endurance activities [20].

Despite these issues, little research directly examining vegetarian diets and athletic performance is available. There have been mixed results regarding hypertrophic potential when comparing vegetarian diets with omnivore diets during resistive exercise training; however, in all cases these differences did not translate to differential strength gains at the completion of the trials [21–24]. Adoption of a lacto-ovo vegetarian (LOV) diet for six weeks did not significantly affect endurance performance among a group of trained, male endurance athletes, in spite of a decrease in total testosterone while on the vegetarian diet [25]. There were also no group differences between 20 participants adopting an LOV diet compared to maintaining their usual omnivorous diet in terms of muscle buffering capacity in conjunction with sprint training for five weeks [26]. These studies provide some insight into the effect of a vegetarian diet on athletic performance. However, a considerable limitation in many of these studies is the inclusion of participants who typically consume meat but subsequently adopt a vegetarian diet only for the duration of the study rather than comparing participants who have adhered to a vegetarian or meat-containing diet long-term.

In a 1986 observational trial, Hanne and colleagues compared athletes who had maintained either an LOV or omnivore diet for at least two years and found no group differences for aerobic or anaerobic capacity [27]. However, aerobic capacity was estimated using cycle ergometry and predicted VO2 max, and strength or torque were not measured. Moreover, body adiposity was estimated using skinfold thickness. Given the current interest in vegetarian diets, in terms of both long-term health and environmental benefits, it is important to reaffirm, using leading-edge technology, that high-level athletic performance is supported by these diets.

The purpose of the present cross-sectional study was to examine body composition and performance measures in vegetarian and omnivore adult endurance athletes who had adhered to their respective diet plans for at least three months. Body composition, including visceral adiposity, was measured using dual-energy X-ray absorptiometry (DXA), leg strength was measured using a dynamometer, and aerobic capacity was determined using the Bruce protocol treadmill test. It was hypothesized that there would be no differences between groups on any parameters.

2. Materials and Methods

2.1. Participant Recruitment

Healthy men and women, both vegetarians and omnivores, were recruited through advertisements on Stevebay.org (a popular website for endurance athletes), Facebook, and through word of mouth. Participants were either on a competitive club sports team at a National Collegiate Athletic Association (NCAA) Division 1 university or training for a major endurance race (such as a marathon, triathlon, cycling race, or other ultra-endurance event). An equal number of omnivore and vegetarian athletes were enrolled in the study between the ages of 21–58 years (35 per group); however, answers to diet questions indicated that eight of the vegetarians ate meat on occasion, and these subjects were reclassified as omnivores. Participants completed a health history questionnaire and were excluded if they had any chronic disease. All participants had the study verbally explained to them and provided their written consent; this study was approved by the Institutional Review Board at Arizona State University, number HS1211008557. Study recruitment and all study measurements took place between August and November 2015.

2.2. Experimental Approach

In this cross-sectional investigation participants completed all study measurements in a single visit. Prior to the visit, participants completed a seven-day food log. Fifty-seven out of seventy participants returned completed food logs, all of which were used in dietary analysis using Food Processor SQL Nutrition and Fitness Software by ESHA Research, Inc. (version 10.11.0, Salem, OR, USA). Height and body mass were measured using a SECA directprint 284 digital measuring station when participants were wearing light clothing and no shoes. Participants also completed a full-body DXA

scan (Lunar iDXA, General Electric Company, East Cleavland, OH, USA), which was conducted by a certified radiology technologist.

Maximal oxygen uptake was determined by following the Bruce protocol [28] on a Trackmaster TMX425C treadmill using the Parvo Medics TrueOne 2400 (Sandy, UT, USA) metabolic measurement system. Prior to beginning the test, participants were instructed how to report their fatigue level using the Borg rating of perceived exertion (RPE) scale [29]. When asked by a research assistant, they reported their RPE at the end of each minute of the test by pointing to a printed Borg RPE chart being held by a research assistant. Participants were verbally encouraged by the research team to push as long as they could and to try to reach a true maximal effort. Handrail support was not allowed during the test. Maximal respiratory exchange ratio (RER) was recorded to help determine whether subjects had reached a "true" maximal effort during the test. Maximal RER values of $\geq$1.1 were considered indicative of true maximal oxygen uptake [30,31]. Peak oxygen uptake reported is the highest oxygen uptake measured during the test.

Finally, participants completed a series of leg extensions and flexions on the HumacNorm isokinetic dynamometer (Computer Sports Medicine Inc. (CSMi, Stoughton, MA, USA) at 60 degrees per second (d/s), 180 d/s, and 240 d/s. Participants were familiarized with the protocol and conducted one practice repetition at each speed prior to performing three maximal effort repetitions at each speed. All sets, including practice repetitions, were performed on both legs, and self-reported dominant side was recorded. Participants moved from the VO2 max test immediately into the dynamometer testing, and there were 30 s of rest between sets on the dynamometer.

2.3. Statistical Analyses

Based on the data of Hanne et al. [27], at 80% power and an alpha level of 5%, 15 participants per group would be needed to detect a 10% difference in strength and 80 participants per group would be needed to detect a 10% change in aerobic capacity between groups. Data were analyzed for normality and log transformed if necessary, and outliers (values > 3 standard deviations (SD) from the mean) were removed prior to data analyses. Data reported are the mean $\pm$ SD, and participant characteristics are displayed by gender and diet group. A 2-way analysis of variance (ANOVA) analysis was used to determine differences between diet groups for participant characteristics followed by an independent *t*-test for post-hoc examination by diet within gender if indicated. Dietary data are reported by group, and a general linear model analysis was used to examine differences between groups controlling for gender. Data were analyzed using the Statistical Package for Social Sciences (SPSS) 23.0 for Mac (SPSS, Inc., Chicago, IL, USA).

3. Results

In the vegetarian group, 24 of the 27 participants (89%) had adhered to a vegetarian diet for >2 years. Of the remaining three participants, the diet had been followed for three, six, or eleven months. Fifteen of the vegetarians were vegans (nine men and six women), and twelve were lacto-ovo vegetarians (five men and seven women).

There were no significant age or gender differences between groups (Table 1). Significant differences were noted between diet groups for body mass and for lean body mass (LBM): female vegetarians tended to have a lower total body mass and LBM compared to the female omnivores ($-$11% and $-$7% respectively). Adiposity, however, did not differ between diet groups. Physical activity levels, recorded as kcal·kg^{-1}·week^{-1}, were 20% higher for vegetarians compared to omnivores (p = 0.018) (Table 1). Maximal oxygen uptake (mL/kg/min) differed significantly between diet groups, and post-hoc analyses revealed a significantly greater aerobic capacity in the female vegetarians in comparison to the female omnivores (+13%, $p < 0.05$) (Table 1); however, absolute maximal oxygen uptake (L/min) did not differ between diet groups. Peak torque when doing leg extensions was not different between diet groups. The 7-day diet records revealed several differences in nutrient intake between diet groups. Although total energy intakes were similar between the diet

groups, the vegetarians consumed more carbohydrate, fiber, and iron daily compared to omnivores (Table 2). However, daily intakes for protein, saturated fat, cholesterol, vitamin B12, and selenium were lower among the vegetarians in comparison to the omnivores.

Table 1. Participant characteristics by diet group (vegetarian, VEG; omnivorous, OMN) [1].

	VEG		OMN		*p*
Measure	**Male (14)**	**Female (13)**	**Male (26)**	**Female (17)**	
Age, year	36.1 ± 10.2	36.7 ± 7.7	38.0 ± 10.0	37.1 ± 8.7	0.608
Body mass, kg	73.3 ± 14.8	58.3 ± 7.6 **	78.0 ± 11.0	65.4 ± 11.6	**0.043**
BMI, kg/m^2	24.0 ± 4.4	21.8 ± 2.5	24.8 ± 2.6	23.5 ± 3.8	0.123
Lean mass, kg	56.3 ± 7.4	42.0 ± 4.9 **	60.2 ± 7.3	45.4 ± 5.1	**0.026**
Waist, cm	81.6 ± 10.7	69.0 ± 14.8	85.2 ± 7.4	73.8 ± 8.2	0.093
Body fat, %	19.2 ± 6.5	25.5 ± 4.2	19.2 ± 6.4	26.9 ± 8.1	0.659
Visceral fat, cm^3	447.4 ± 419.8	110.4 ± 123.0	538.5 ± 404.3	206.4 ± 254.6	0.656
METS, kcal·kg^{-1}·week^{-1}	108.8 ± 32.9	106.1 ± 36.6 **	91.7 ± 33.2	85.6 ± 20.8	**0.018**
VO2 max, mL/kg/min	62.6 ± 15.4	53.0 ± 6.9 *	55.7 ± 8.4	47.1 ± 8.6	**0.011**
VO2 max, L/min	4.44 ± 0.81	3.21 ± 0.67	4.29 ± 0.59	3.03 ± 0.49	0.295
Peak torque, ft-lbs	114.4 ± 26.2	65.5 ± 12.8	124.2 ± 24.5	73.6 ± 18.6	0.104

[1] Data are the mean ± SD; n in parentheses; gender distribution did not differ by diet group ($p = 0.460$; Chi Square analysis). *p* for 2-way ANOVA analyses by diet (non-normal data transformed prior to analysis (visceral fat)). The single asterisk (*) indicates significant difference within gender by diet group ($p < 0.05$); the double asterisk (**) indicates a trend for difference within gender by diet group ($0.05 < p < 0.10$).

Table 2. Nutrient differences by diet group (vegetarian, VEG; omnivorous, OMN) [1].

	VEG (22)	OMN (35)	*p*	Reference Range [2]
Total kilocalories (kcal)	2443 ± 535	2266 ± 612	0.072	-
Carbohydrate (CHO) (g)	328 ± 70	248 ± 101	**0.001**	-
CHO (% energy)	53 ± 6	48 ± 7	**0.010**	45%–65%
Fiber (g)	38 ± 13	24 ± 9	**<0.001**	38/25 g [M/F]
Protein (g)	78 ± 19	101 ± 35	**0.006**	-
Protein (% energy)	12 ± 2	17 ± 4	**<0.001**	10%–35%
Protein (g/kg body mass)	1.2 ± 0.3	1.4 ± 0.5	0.220	0.8 g/kg
Fat (g)	90 ± 26	83 ± 33	0.901	-
Fat (% energy)	32 ± 5	32 ± 6	0.952	20%–35%
Saturated fat (g)	22.8 ± 11.2	25.7 ± 10.1	0.207	-
Saturated fat (% energy)	8.3 ± 3.1	11.6 ± 6.3	**0.002**	<10%
Cholesterol (mg)	102.8 ± 119.5	301.2 ± 165.6	**<0.001**	-
Vitamin C (mg)	117.0 ± 64.0	83.0 ± 46.5	0.076	90/75 mg [M/F]
Vitamin D (IU)	115.4 ± 111.4	129.0 ± 115.5	0.201	600 IU
Vitamin B12 (mcg)	3.0 ± 3	4.8 ± 4.6	**0.006**	2.4 mcg
Selenium (mcg)	41.8 ± 36.0	62.6 ± 33.6	**0.002**	55 mcg
Sodium (mg)	2931.2 ± 783.1	2972.8 ± 887.5	0.794	<2300 mg
Iron (mg)	19.4 ± 7.8	15.4 ± 5.4	**0.017**	8/18 mg [M/F]
Zinc (mg)	8.5 ± 9.1	8.9 ± 4.9	0.149	11/8 mg [M/F]
Calcium (mg)	971.0 ± 401.6	878.1 ± 314.9	0.378	1000 mg
Phosphorus (mg)	782.0 ± 378.0	831.2 ± 336.4	0.507	700 mg
Omega-3 fatty acid (g)	1.6 ± 2.5	0.9 ± 0.7	0.326	-
Omega-3 fatty acid (% energy)	0.004 ± 0.005	0.004 ± 0.003	0.613	0.6%–1.2%
Omega-6 fatty acid (g)	7.7 ± 5.4	6.1 ± 4.4	0.145	-
Omega-6 fatty acid (% energy)	2.8 ± 1.6	2.4 ± 1.3	0.358	5%–10%

[1] Data are the mean ± SD; sample size in parentheses. *p* for general linear model analyses (non-normal data transformed prior to analysis (all variables except carbohydrate variables and fat percentage) and 2 outliers (VEG group) removed prior to analysis for saturated fat); [2] Reference ranges are the Recommended Dietary Allowance or the Acceptable Macronutrient Distribution Range; note the American College of Sports Medicine recommends that athletes consume 1.2–2.0 g protein/kg body mass.

4. Discussion

Results from this study indicate that compared to their omnivore counterparts, vegetarian endurance athletes have comparable strength as indicated by leg extension peak torque, and possibly a greater degree of aerobic capacity, particularly in females, as indicted by a progressive maximal treadmill test to exhaustion. Dietary intake on several key nutrients differed considerably between groups. Some, but not all, results are consistent with previous reports.

Our study is significant for its increased rigor in measurement assessments compared to previous comparisons of vegetarian and omnivore athletes. We determined maximal oxygen uptake by a graded test to exhaustion on a treadmill instead of predicting VO2 max using a cycle ergometer, as recommended by Shepard and colleagues [32]. Additionally, we measured body composition using a DXA scan, currently regarded as the clinical gold standard for body composition assessment, instead of skinfolds [33]. Finally, we assessed both athletic performance and nutrient intake differences between vegetarians and omnivores, whereas most previously published studies focus exclusively on one of these areas.

4.1. Body Mass and BMI

Like other studies of vegetarians in the general population, vegetarian participants in the present study had significantly lower body mass compared to omnivores [10,34]. This is in spite of the fact that our study included participants engaged in considerable endurance activities, which could be very different in multiple ways from the general population. One prior study in athletes, conducted by Hanne et al. compared vegetarians and omnivores anthropometrically and found no significant differences between groups for weight [27]. It is noteworthy that the athletes in the Hanne et al. study included football, basketball, and water polo players in addition to endurance athletes.

4.2. Lean Body Mass

LBM was significantly lower for the vegetarian athletes compared to their omnivore counterparts, a difference which was most prominent among the female participants with female vegetarian athletes possessing 7% less LBM as compared to the female omnivore athletes. In spite of this, there were no significant differences in body fat percentage or BMI between groups. To our knowledge, this is the first study to examine lean body mass differences between vegetarian and omnivore athletes. It is important to note, however, that this difference in lean body mass did not translate into differential peak torque on the leg extension.

Although other studies have not assessed lean body mass of vegetarian athletes specifically, Campbell and colleagues compared resistance-training induced changes in lean body mass and strength between groups assigned to either an omnivorous diet or a lacto-ovo-vegetarian diet for the duration of the study and found that, in spite of differential lean body mass gains, the two groups increased strength similarly [21]. Conversely, a 12-week training study by Haub and colleagues showed no significant differences in strength, body composition, or muscle cross-sectional area between groups assigned to either a lacto-ovo-vegetarian or beef-containing diet.

4.3. Body Fat Percent and Visceral Adipose Tissue (VAT)

Contrary to the female vegetarian athletes in Hanne's group, no significant differences in body fat percentage were found between vegetarian and omnivore athletes in this study. Additionally, there were no significant differences between groups for visceral adipose tissue (VAT). Participants in the present study had VAT values above those reported for similar aged healthy lean sedentary adults (~250 cm^3), both omnivores and vegetarians [35,36], but lower than those noted for older adults (1000–1560 cm^3) [37]. Although there are no standard reference ranges for VAT, values near 1000 cm^3 were associated with BMI values near 25 kg/m^2 and values > 300 cm^3 have been suggested as predictive of risk for metabolic syndrome in young adults [36,37]. As technology

permitting quantification of visceral adipose tissue is relatively new for research purposes, this study contributes to the emerging literature by providing VAT values for athletes. VAT and BMI is strongly correlated in this study ($p = 0.742$), a factor that may be important for estimating VAT inexpensively without a DXA scan.

4.4. VO2 Max

Unlike athletes in Hanne's study, vegetarians in the present study had significantly higher maximal oxygen uptake than their omnivore counterparts [27]. This difference was most predominant in the female participants with a 13% greater VO2 max score for the female vegetarians as compared to the female omnivores, but this difference was not observed for absolute VO2 max (L/min), which suggests that body weight factored into this difference. This gender difference is intriguing and merits further investigation in future studies. One potential reason that athletes in the present study had higher VO2 max values than those in Hanne's study may be due to the difference between cycle ergometry and treadmill testing methods. However, it is possible that the athletes in our study simply were more trained and that diet effects on differences in VO2 potential emerge only at higher levels of fitness.

Other work that contributes to our understanding of aerobic and anaerobic performance differences by diet include the study of Hietavala et al. that found no significant difference in time to exhaustion (albeit a higher oxygen uptake at a given percent of maximal oxygen consumption) between participants following a low-protein vegetarian diet compared to a mixed diet [38]. Subjects in this study adhered to the low protein vegetarian diet (0.80 ± 0.11 g of protein per kilogram of body mass (g/kg) vs. 1.59 ± 0.28 g/kg on their normal diet) for four days before being tested on a cycle ergometer. As this study did not use participants who practiced vegetarianism outside of the study, and the amount of protein that subjects were allowed to consume on the vegetarian diet was restricted, true differences between vegetarians and omnivores may not be evident. Baguet et al. found no differences in repeated sprint ability between participants following a vegetarian or mixed diet for five weeks; again, these subjects were not following a vegetarian diet long-term [26]. Raben et al. found no differences in maximal oxygen uptake among subjects after adoption of a lacto-ovo vegetarian diet for six weeks [25]. However, the major disadvantage of interpreting results of these studies for vegetarian athletes is that participants in these studies only adhered to a vegetarian diet briefly for the duration of the study.

4.5. Peak Torque

Similar to the Hanne et al. study that compared the power output of vegetarian and omnivore athletes [27], we found no significant differences by diet in terms of peak torque using leg extensions. Other studies in untrained older men that have examined strength development over time in response to a training program have found mixed results when comparing participants following a vegetarian or mixed diet [21,24]. This is noteworthy, particularly since strength and lean body mass were strongly correlated ($r = 0.764$) in the present study, as well as the fact that omnivores had significantly more lean body mass vs. the vegetarians. A nonsignificant trend for omnivores to produce higher peak torque is observed, however. It is conceivable that the omnivore diet pattern may be preferred for sports that rely on greater lean mass, and subsequently peak torque. To further investigate this, future work ought to examine if strength can be increased similarly by vegetarian and omnivore athletes engaged in strength training (not just by participants following a vegetarian diet for a few weeks).

4.6. Nutrient Intake

Nutrient intake was calculated from food and beverage intakes only and did not include any supplements. There were no significant differences in caloric intake or total fat intake between vegetarians and omnivores. However, vegetarians reported significantly more dietary carbohydrate (both in terms of absolute intake and as a percent of daily calories), fiber, and iron intake.

Omnivores consumed more dietary protein (both in terms of absolute intake and as a percent of daily calories), saturated fat, cholesterol, and vitamin B12. However, when expressed relative to body mass, there were no differences in dietary protein intake.

That vegetarians and omnivores in the present study did not differ in terms of caloric intake is consistent with findings by Janelle and Barr from their comparison of 45 vegetarian and omnivore women [16], yet it is in contrast to results from Calkins and colleagues who compared 50 vegetarian, vegan, and omnivores. They found vegetarians consumed about 200 fewer kcal than omnivores [19]. These studies were both in the general population, not specifically with athletes. Calkins et al. also reported that omnivores consumed more fat than vegetarians, a fact that partially contributed to the higher caloric intake. This too is in contrast to the findings in the present study which found no significant difference either in grams of fat consumed or the percent contribution of fat to the daily calorie intake, even though saturated fat was significantly higher in omnivorous diets. Other studies involving the general population have also reported omnivores eating more energy and total fat than vegetarians [10,39–41].

Higher carbohydrate (when expressed either as an absolute amount or as a percent of total daily calories) and fiber intake among vegetarians in comparison to omnivores in the present study is consistent with findings in other studies [10,39,41–44]. As these studies have been conducted in the general population, the present study contributes to the literature by demonstrating that this dietary pattern can be extended to endurance athletes as well. One study by Janelle and Barr stands in contrast to these findings, as they did not find significant differences in carbohydrate or fiber intake between vegetarian and omnivore women; those participants were not athletes [16]. That vegetarians in the present study consumed more carbohydrates than omnivores is notable since they are all athletes, and the importance of carbohydrates for exercise is well-established [45–47].

Like the present study, other studies have also reported that vegetarians consume less protein (both absolute intake and as a percent of the daily calories) [10,16,39,42] and vitamin B12 [40,48] than omnivores. Our study contributes to the literature since other reports have been in the general population instead of within athletic groups. Of note, though, differences in dietary protein intake are not significant when expressed relative to body mass, which is typically the preferred method for recommending protein for athletes [47]. Nonetheless, dietary protein intake was weakly correlated with peak torque ($r = 0.359$, $p = 0.006$) in the present study, and dietary protein intake was moderately correlated with lean body mass ($r = 0.415$, $p = 0.001$). Expectantly, lean body mass was strongly correlated with peak torque ($r = 0.764$, $p < 0.001$). Hence, it is conceivable that protein intake could influence strength if intakes had been inadequate. In the present evaluation, protein intakes in the vegetarian participants averaged 1.2 g/kg body mass, which falls in the recommended range for athletes [47,49].

There are conflicting findings in the rest of the literature regarding whether omnivores or vegetarians consume more iron. The Wilson et al. study of vegetarian men found that vegetarians consumed more iron [41], but Ball and Bartlett reported no difference in dietary iron intake between female vegetarian and omnivores [50]. Clary et al. compared 1475 vegans, vegetarians, semi-vegetarians, pescetarians, and omnivores and also showed that vegetarians consume more iron than omnivores [39]. Although vegetarians consumed more iron than omnivores in the present study, iron bioavailability was likely reduced as has been shown in other trials [17]. Dietary intakes of zinc did not vary by diet group herein, but generally the literature suggests that vegetarians consume somewhat less dietary zinc than omnivores [16,51–53]. The lower intakes of selenium by vegetarians in comparison to omnivores has also been reported by others and reflects the low levels of selenium in plant foods relative to flesh foods [54,55].

4.7. Limitations

In addition to the small sample size, limitations to the study include the variable level of experience of the athletes for their respective sports, and related fitness levels. Although most

participants were training for and competing in races such as marathons, Ironman-distance triathlons, and competitive cycling, there were a few participants who were training for shorter distance races. However, this variation makes results more generalizable to athletes of various fitness levels.

4.8. Future Directions

Future work is needed to compare vegetarian and omnivore endurance athletes' performance on events more similar to actual sporting events (such as time trials or peak power on a cycle ergometer) and probe differences by type of vegetarian diet (lacto-ovo vegetarian or vegan). Additional work is needed to explore the adequacy of long-term adherence to vegetarian and vegan diets for supporting development of lean body mass.

5. Conclusions

Our cross-sectional comparison of vegetarian and omnivore adult endurance athletes shows higher maximal oxygen uptake values among vegetarians and comparable strength, in spite of anthropometric and dietary differences. This study suggests that following a vegetarian diet may adequately support strength and cardiorespiratory fitness development, and may even be advantageous for supporting cardiorespiratory fitness. Certainly many factors affect an athlete's sports performance, and there is no dietary substitute for quality training. However, our study contributes to the literature about cardiorespiratory and strength comparisons between vegetarian and omnivore endurance athletes, and may provide a rationale about the adequacy of vegetarian diets for sport performance. As this was a small cross-sectional study using endurance athletes, larger intervention trials are necessary to bolster conclusions about adequacy of vegetarian diets to support performance in strength and power-focused sports.

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5

Effect of 12-Week Vitamin D Supplementation on 25[OH]D Status and Performance in Athletes with a Spinal Cord Injury

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Abstract: (1) Background: studies with able-bodied athletes showed that performance might possibly be influenced by vitamin D status. Vitamin D seems to have a direct impact on neuromuscular function by docking on vitamin D receptors in the muscle tissue. Additionally, a high prevalence of vitamin D deficiency was shown not only in infants and in the elderly but also in healthy adults and spinal cord injured individuals. Therefore, the aim of our study was to investigate whether a vitamin D dose of 6000 IU daily over 12 weeks would be sufficient to increase vitamin D status in indoor wheelchair athletes to a normal or optimal vitamin D level and whether vitamin D deficiency is associated with an impairment in muscle performance in these individuals; (2) Methods: vitamin D status was assessed in indoor elite wheelchair athletes in order to have a baseline measurement. If vitamin D status was below 75 nmol/L, athletes were supplemented with 6000 IU of vitamin D daily over 12 weeks. A vitamin D status over 75 nmol/L was supplemented with a placebo supplement. Vitamin D status, as well as a Wingate test and an isokinetic dynamometer test, were performed at baseline and after six and 12 weeks; (3) Results: 20 indoor elite wheelchair athletes participated in this double-blind study. All of these athletes showed an insufficient vitamin D status at baseline and were, therefore, supplemented with vitamin D. All athletes increased vitamin D status significantly over 12 weeks and reached an optimal level. Wingate performance was not significantly increased. Isokinetic dynamometer strength was significantly increased but only in the non-dominant arm in isometric and concentric elbow flexion; (4) Conclusion: a dose of 6000 IU of vitamin D daily over a duration of 12 weeks seems to be sufficient to increase vitamin D status to an optimal level in indoor wheelchair athletes. It remains unclear, whether upper body performance or muscle strength and vitamin D status are associated with each other.

Keywords: 25[OH]D; spinal cord injuries; anaerobic performance test; dynamometer test

1. Introduction

A high prevalence of vitamin D deficiency was shown not only in infants [1,2] and in the elderly [3], but also in young and healthy adults [4,5]. As vitamin D is primarily produced by ultraviolet radiation through sunlight exposure, a deficiency can possibly develop in healthy people as well. Such a deficiency may not only increase the risk for several different diseases, such as cancer [6,7], cardiovascular disease [8,9], and dementia [10], but also decrease neuromuscular function [11]. Such a neuromuscular impairment might be explained by the existence of vitamin D receptors (VDR) in human muscle tissue [12]. Thus, vitamin D deficiency might also lead to muscle weakness and pain. Further, vitamin D seems to influence not only muscle growth and cell differentiation, but also

increase sarcoplasmic calcium uptake resulting in a higher muscle contractility [13]. Therefore, it is not surprising that positive effects of vitamin D supplementation on muscle function were found [14–18]. Studies showed a reduction in falls and a beneficial effects on muscle strength, balance, and gait performance in the elderly [19]. Other studies found an increase in upper and lower body muscle strength after vitamin D supplementation [18]. Nonetheless, the impact of vitamin D supplementation on muscular performance in athletes remains controversial. Some studies found no effect on performance [20,21], whereas others found a significantly increased isometric quadriceps strength, vertical jump, and sprint time after vitamin D supplementation [22,23].

Similar to the studies with able-bodied individuals, a high prevalence of vitamin D deficiency or insufficiency was found in patients [24] and athletes [25,26] with a spinal cord injury. Due to the impairment of the spinal cord, muscle strength might already be decreased and an additional impairment through vitamin D deficiency needs to be avoided. Only one study investigated the effect of vitamin D supplementation in athletes with a spinal cord injury on vitamin D status [27]. In this study, a vitamin D supplementation with 5000 IU daily increased vitamin D status over wintertime.

Therefore, the aim of our study was to investigate the effect of vitamin D supplementation on muscle strength and performance in indoor wheelchair athletes. Firstly, the objective was to detect whether a dose of 6000 IU daily is sufficient to increase vitamin D status to a normal level over 12 weeks in athletes suffering from a deficiency. Another goal was to investigate the relationship between vitamin D status and muscle strength.

2. Materials and Methods

2.1. Study Participants

Swiss male elite wheelchair indoor athletes, 18 to 60 years old and physically active for at least 45 min twice a week were recruited for this study. They had to perform their sport for more than two years and suffer from a chronic spinal cord injury or from cerebral palsy. The intervention study took place during the winter months (November–April) and the follow-up during spring (April–June) in Nottwil, Switzerland (47° north latitude). Any participant being abroad below the 37th parallel during the study phase or shortly before the start of the study was withdrawn from participating. Participants already supplementing with a vitamin D dose higher than 400 IU daily were also excluded from the study. Other exclusion criteria were suffering from a respiratory or cardiovascular disease, kidney insufficiency, or parathyroid gland ailment. All participants were asked to sign written informed consent and had to maintain their regular training schedule as well as to refrain from taking any additional supplements. The study was approved by the local ethics committee (Ethikkommission Nordwest-und Zentralschweiz (EKNZ), Basel, Switzerland) (Project #2015-344, clinicaltrials.gov NCT02621320).

2.2. Study Design

The double-blind, non-randomized intervention study took place at the Institute of Sports Medicine in Nottwil, Switzerland. Participants visited the institute on five different occasions during the intervention phase and on two additional occasions for those participating in the follow up. On the first visit, the screening questionnaire was completed and the medical history was checked to ensure that all criteria were fulfilled. The second visit was conducted in order to familiarize the participants with the performance tests. All participants performed and isokinetic dynamometer test (see Section 2.4) followed by a fifteen minute recovery break. Subsequently, a 30 s Wingate test on an arm crank ergometer (see Section 2.5) was performed.

Each participant replicated this test procedure on three occasions during the intervention phase and on two additional occasions during the follow up phase (only vitamin D concentration and the Wingate test). The tests took place at the same time of the day and were separated by six weeks. Before each session, the fulfillment of the test requirements was checked (i.e., no exercise twelve hours and no

intense exercise 48 h before testing, at least seven hours of sleep during the previous night, no caffeine intake and replicated food intake prior to each session). After completion of this checklist, two venous blood samples were drawn in order to analyze the vitamin D and the calcium status. All participants with an insufficient vitamin D status (<75 nmol/L) received a vitamin D supplement during the intervention phase and all participants with a sufficient vitamin D status (>75 nmol/L) received a placebo supplement during the intervention phase (see Section 2.3). After the blood withdrawal, a Disabilities of the Arm, Shoulder and Hand (DASH) questionnaire [28] was completed.

2.3. Vitamin D Supplementation

Vitamin D3 (cholecalciferol) supplement (Vi-De 3®, Wild and Co. AG, Muttenz, Switzerland) was given in a dose of 6000 IU daily over twelve weeks (intervention phase). The tolerable upper limit intake level of the Endocrine Practice Guidelines Committee of 10,000 IU daily was not exceeded and, therefore, no side effects were expected [29]. The placebo supplement was based on the same alcohol solution (65% ethanol, Dr. Wild and Co. AG, Muttenz, Switzerland). The supplements were handed over in identical bottles and were ingested dropwise (either 60 drops or 1.3 mL daily). Bottles and solutions were not distinguishable for the participants in smell and color.

Self-reported compliance was assessed by regularly asking the frequency of taking vitamin D or placebo supplementation over the last two weeks. To achieve a high compliance, each participant installed a mobile app (Medisafe, Medisafe Inc., Boston, MA, USA) and set a daily reminder.

To assess tolerance, participants were asked every two weeks how they tolerate the supplement.

2.4. The Isokinetic Dynamometer Test

An isokinetic dynamometer (Cybex Norm II, Lumex Inc., Ronkomkoma, NY, USA) was used to measure peak torque of elbow flexion strength at different velocities for isometric (0°/s) and concentric (60°/s and 180°/s) exercise. The device was connected to the software (Humac 2015, CSMi, Stroughton, MA, USA) and calibration was performed in monthly intervals as proposed by the manufacturer. Participants were placed in a supine position fixed with straps and with a pillow under their knees. The shoulder joint was abducted in 45° and the wrist was strapped proximal with the hand in a neutral position to the lever arm of the dynamometer. The axis of rotation was aligned with the lateral epicondyle. Testing was limited between 20° and 120° of elbow flexion. Strong verbal encouragement was used during maximal effort.

A standardized warm-up with 10 repetitions at 120°/s was performed before the data collection. Subsequently, data collection started with the measurement of concentric work at 60 and 180°/s followed by isometric work.

Test-re-test reliability was checked prior to the start of the study in 10 able-bodied participants. Isometric, as well as both concentric measurements, showed "high" to "very high" reliability according to Munro's classification of the intra-class correlation coefficient (ICC) [30]. The ICC ranged between 0.843 and 0.925 for the different test settings (Table S1).

2.5. The Wingate Test

Participants performed a Wingate test using a rotational speed-dependent arm crank ergometer (Angio V2, Lode B.V., Groningen, The Netherlands) which was connected to the software (Wingate, Lode B.V., Groningen, The Netherlands). This Wingate test on the arm crank ergometer was shown to be highly reliable in individuals with a paraplegia [31] and tetraplegia [32]. Participants were seated in an adapted office chair, which was positioned to allow a slight bend of the elbows. The height of the crank and the distance between the chair and the crank were recorded to replicate the conditions in the next test session. In some participants hand fixations and chest straps were needed to fix them to the crank or the chair. A resistance load of 1%–3% was applied in individuals with a tetraplegia [32]. In individuals with paraplegia, a resistance load of 4% was used. These settings were tested during the familiarization trials and adjusted for the intervention sessions where needed.

Five minutes of a standardized warm-up at 20 W and 60 rpm was performed before the start of the test. Subsequently, the resistance load was applied and the Wingate test was started. After the test, participants stopped to crank immediately and blood lactate concentrations were measured at 0, 2, 4, 6, 8, and 10 min after the end of the test using an enzymatic amperometric chip sensor system (Biosen C-Line Clinic, EKF diagnostic GmbH, Cardiff, UK). Blood samples were taken from the earlobe. A heart rate monitor (S610i, Polar Electro Oy, Kempele, Finland) was used to measure maximal heart rate during the test. These data were analyzed with the Polar Pro Trainer 5 software (Polar Electro Oy, Kempele, Finland). Rated perceived exertion (RPE) was assessed during warm-up and at the end of the test by using a Borg scale ranging from 6 to 20 [33]. Maximal power (P_{peak}), average power (P_{mean}) and fatigue index (FI) during the Wingate test were analyzed.

2.6. Blood Parameters

Blood samples were drawn from the antecubital vein using a blood collection system (S-Monovette® 4.9 mL Z, Sarestedt, Nümbrecht, Germany). Samples were immediately packed into an opaque plastic tube to protect them from any ultraviolet radiation. The samples were then immediately centrifuged at 20 °C at 3000 rpm for 10 min (Rotina 380, Hettich GmbH, Tuttlingen, Germany) in the in-house laboratory of the Swiss Paraplegic Centre, Nottwil, Switzerland. After centrifugation, the samples were stored at −25 °C for later analysis.

Serum 25-hydroxyvitamin D (25[OH]D) was analyzed with an automated benchtop immunoanalyzer (Vidas®, bioMérieux, Marcy l'Etoile, France) using enzyme-linked fluorescent assay (ELFA). Serum calcium concentration was assayed with a photometric technique method (Cobas c501, Roche Diagnostic GmbH, Mannheim, Germany).

2.7. DASH Questionnaire

The Disabilities of the Arm, Shoulder and Hand (DASH) questionnaire was used to assess upper extremity function and symptoms (non-specific for wheelchair users) [28]. The DASH questionnaire was completed at the laboratory previously to the start of the Wingate test.

2.8. Data Analysis

Statistical analysis was performed using the software IBM SPSS Statistics Version 23.0 for Windows (IBM, Armonk, NY, USA). Statistical significance was set at an α-level of 0.05. Distribution of our data was tested by using the Kolmogorov-Smirnov, the Shapiro-Wilk test and the Q-Q plot. The results indicated, that all of your data was normally distributed except for the isokinetic dynamometer test and for the analysis of the follow up phase. For normally distributed data mean ± standard deviation (SD) was used. Not normally distributed data is presented as median [minimum; maximum]. To analyze differences in the mean of the outcome parameters between different time points, a one-way repeated-measurement ANOVA was performed for normally distributed data and the Brunner model [34] was applied for nonparametric data. Pairwise *t*-tests and Wilcoxon post hoc test were performed as post hoc analysis in normal and nonparametric data, respectively. In the case of multiple testing, Bonferroni corrections were applied. Tests six weeks after supplementation were called "intermediate" whereas tests after 12 weeks were called "post". The first measurement during the follow up after six weeks is called "follow up 1" and the second follow up tests after 12-week placebo supplementation are called "follow up 2". Spearman correlation was used to correlate the increase in vitamin D with the difference of peak elbow flexion at baseline and post. Pearson correlation was used to correlate the increase in vitamin D with the difference in peak power and mean power from baseline to post.

3. Results

Twenty-one healthy, male Swiss elite wheelchair indoor athletes participated in this study. Athletes were competing in wheelchair rugby (n = 15), basketball (n = 4), or table tennis (n = 2). One participant had to be excluded from data analysis due to non-compliancy. Therefore, twenty participants were included into data analysis. Ten out of these twenty participants agreed to take part in the follow up study (Table 1).

Table 1. Participants' characteristics.

Participant	Training (h/Week)	Age (Years)	Height (cm)	Weight (kg)	Lesion Level	AIS	Sport	Classification	Follow up
1	3.5	37	185	103	C7	D	WR	2.0	Yes
2	11.0	20	170	54	C6	A	WR	1.5	No
3	4.0	24	179	58	C6	B	WR	0.5	Yes
4	3.5	35	187	97	T4	A	WB	1.0	No
5	4.0	47	185	63	T1	A	WR	2.5	No
6	2.5	27	180	70	T4	A	WB	1.0	Yes
7	3.5	27	181	61	C6	B	WR	2.5	No
8	5.0	48	186	67	C6	B	WR	1.0	Yes
9	5.0	44	176	80	C6	A	WR	0.5	Yes
10	12.0	35	193	92	T1	C	WB	2.5	Yes
11	8.0	38	172	70	C7	D	WR	2.0	Yes
12	4.0	26	188	90	C6	A	WR	0.5	No
13	5.0	30	182	65	C6	B	WR	0.5	No
14	8.0	34	180	85	C5	C	WR	1.5	No
15	6.5	50	185	83	L3	A	WB	3.0	Yes
16	13.5	21	184	63	C7	D	WR	2.5	Yes
17	3.5	65	175	65	C6	C	WR	1.5	No
18	15.0	33	180	60	C6	A	PT	class 1	No
19	4.0	26	155	52	CP	-	WR	1.5	Yes
20	5.0	57	170	72	T5	D	PT	class 4	No
Mean ± SD	6.3 ± 3.7	36 ± 12	180 ± 8	72 ± 15	-	-	-	-	-

AIS = American Spinal Injury Association Impairment Scale; T = thoracic; L, lumbar; C = cervical, CP = cerebral palsy, WB = wheelchair basketball, WR = wheelchair rugby, PT = para table tennis.

3.1. Vitamin D and Calcium Status

All participants enrolled into the study showed an insufficient or deficient vitamin D status at the baseline measurement (Figure 1). Therefore, no placebo group could be formed. Nineteen out of twenty athletes reached an optimal vitamin D status (100 to 220 nmol/L) after six weeks, and no one showed a toxic level (>375 nmol/L). Vitamin D status for the participants taking part in the follow up is shown in Figure 2. Significant differences were found between all different time points ($p < 0.05$). Calcium concentration was not significantly different between the three time points in the intervention study ($p = 0.16$) nor in the five time points, including the follow up data ($p = 0.39$). All calcium concentrations were within the normal physiological range (2.15 to 2.55 mmol/L).

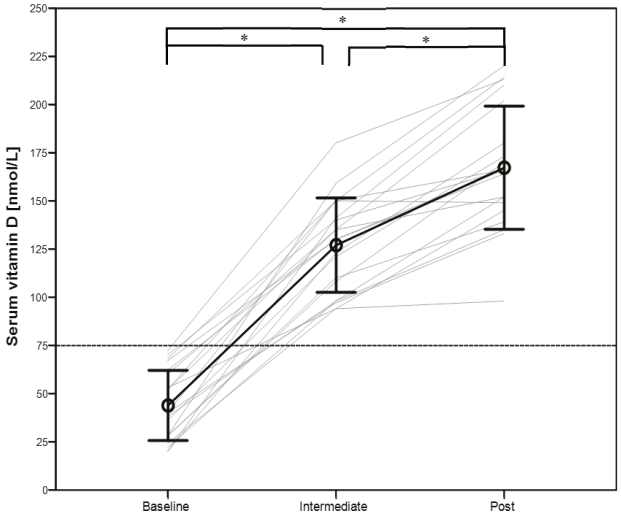

Figure 1. Serum vitamin D concentration at baseline, after six (intermediate) and 12 (post) weeks following vitamin D supplementation. * = significant difference ($p < 0.05$), data presented as mean and standard deviation, grey lines represent individual data.

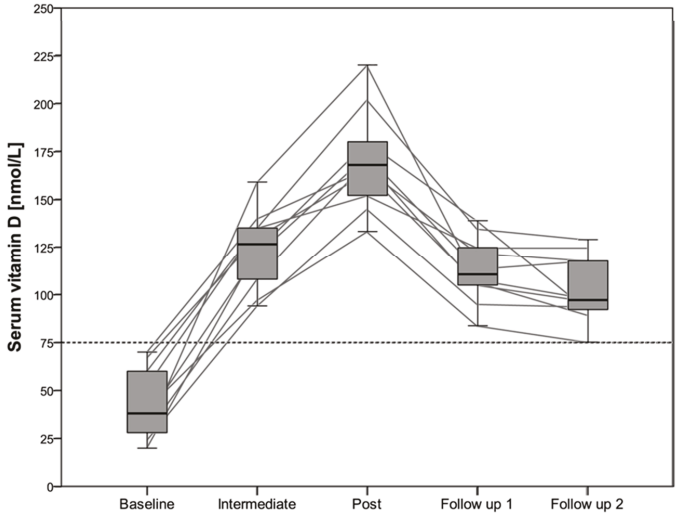

Figure 2. Serum vitamin D concentrations during the intervention and the follow up in 10 participants. Grey lines represents individuals' data, data presented as median with interquartile range.

3.2. Performance Tests

Significant improvements in the non-dominant arm were shown in isometric and 180°/s concentric exercise (Table 2).

Table 2. Peak elbow flexion [Nm] reached during isokinetic dynamometer measurements.

Mode	Arm	Baseline	Intermediate	Post	*p*-Value
isometric	dominant	65 [46; 96]	71 [46; 98]	72 [50; 100]	0.071
	non-dominant	64 [49; 104]	68 [46; 106] *	71 [49; 106] *	0.019
concentric 60°/s	dominant	46 [30; 77]	47 [35; 71]	47 [37; 73]	0.197
	non-dominant	47 [30; 73]	50 [33; 71]	49 [37; 75]	0.078
concentric 180°/s	dominant	34 [24; 61]	31 [24; 58]	33 [27; 61]	0.269
	non-dominant	34 [24; 53]	37 [24; 54] **	35 [26; 53] **	0.001

Data presented as median [minimum; maximum] from 20 participants. Significant differences compared to baseline measurement * $p < 0.05$ and ** $p < 0.01$.

No significant differences in peak power were found over the three measurements during the intervention study ($p = 0.09$), nor during the follow-up study ($p = 0.53$). The same findings were shown for average power in the intervention ($p = 0.13$) and in the follow-up ($p = 0.71$) study. No significant differences were found in fatigue index ($p = 0.15$), maximal heart rate ($p = 0.92$), RPE ($p = 0.76$), and maximal lactate concentrations ($p = 0.58$) for the intervention study at the different time points (Table S2). Individual absolute and relative changes in peak power from baseline to post measurement in the intervention study are shown in Figure 3. The data for peak power in the follow up study is shown in Figure 4. Spearman correlation showed a significant correlation between the difference in the non-dominant arm at 60°/s and the increase of vitamin D from baseline to post ($p = 0.01$; $r_s = 0.564$). This correlation coefficient (r_s) reflects only "moderate" correlation. No other correlation for the dominant arm or the other exercise velocities showed any significant correlation. The increase in vitamin D status was significantly correlated with the difference in peak power from baseline to post ($p = 0.044$, $r = 0.455$). Again Pearson's r reflects medium correlation. No significant correlation between the increase of vitamin D and mean power from baseline to post was found ($p = 0.27$; $r = 0.258$).

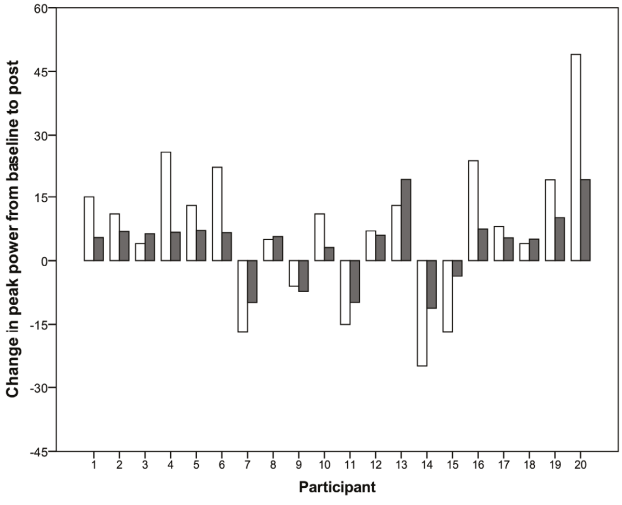

Figure 3. Individual changes in absolute (white bars in [W]) and relative (grey bars in [%]) of peak power from baseline to post measurement in the intervention study.

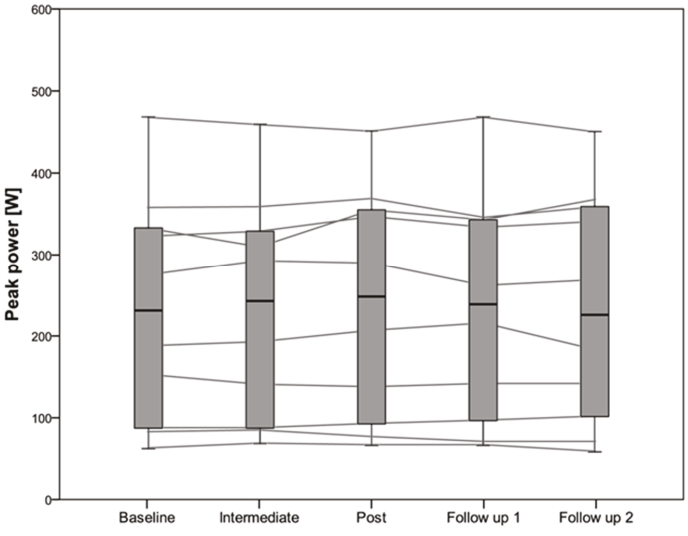

Figure 4. Peak power in 10 participants during the five measurements including the follow up study.

3.3. Other Outcome Parameters

No significant changes over time were found in the DASH score (p = 0.20) nor in the DASH sport score (p = 0.94) during the intervention phase. The participants reported a compliance of 97.3% over twelve weeks of vitamin D supplementation. Three out of twenty participants reported gastrointestinal side effects, such as a higher frequency of bowel movement and loose stool during vitamin D supplementation. Two out of the three showed the lowest compliance (75% and 82.1%) of all participants, and only one had a positive change in peak power from baseline to post measurement.

4. Discussion

A daily supplementation dosage of 6000 IU vitamin D seems to be sufficient to reach an optimal vitamin D status after 12 weeks in indoor athletes with an insufficient vitamin D status at baseline (Figure 1). After a 12 weeks follow up period with placebo supplementation, vitamin D status still was over 75 nmol/L but decreased significantly compared to the value at the end of the 12-week vitamin D supplementation period (Figure 2). The real effect of vitamin D supplementation on upper body exercise performance in athletes with a spinal cord injury still remains unclear due to a lack of a placebo group in our study.

4.1. Vitamin D Status

It is already well-known that a high amount of able-bodied and disabled Swiss athletes suffer from vitamin D deficiency or insufficiency during winter months [25,35]. The prevalence of vitamin D deficiency was even higher in indoor wheelchair athletes compared to outdoor athletes [25]. Nevertheless, it was surprising that all recruited athletes in the present study showed a deficient vitamin D status at baseline (44 $\pm$ 18 nmol/L). Oral vitamin D supplementation of 6000 IU daily over a 12-week time period was sufficient to increase vitamin D status to an optimal level (167 $\pm$ 24 nmol/L). The calculated slope of the relation between the vitamin D change and the supplementation was 2.05 nmol/L per 100 IU in the present study. This is slightly higher than the proposed slopes of 1.48 nmol/L per 100 IU [36] or 1.75 nmol/L per 100 IU [37]. The lower baseline vitamin D status in our study might explain this slight discrepancy.

Only a few other studies investigated the effect of vitamin D supplementation on vitamin D status in individuals with a spinal cord injury [27,38,39]. Most of these studies failed to achieve a sufficient vitamin D status after the supplementation period [39,40]. In one study [39], over 80% of the participants remained insufficient after vitamin D supplementation with 2000 IU daily over a two-week period. Similar findings were shown with a supplementation of 800 IU daily over a duration of one year [39], and in a study where the compliance for taking vitamin D supplements was only at 72% [40]. Our study showed not only a high compliance but also that a dosage of 6000 IU vitamin D daily over six weeks would be sufficient to increase vitamin D status to a normal level. Nevertheless, an individual approach needs to be applied, as a large individual variability occurred in the intermediate and post measurement of vitamin D status. Concerns whether such a high dose of vitamin D might be problematic were eliminated by the fact that calcium concentration remained in the normal range in our study. In addition, Heaney, Davies, Chen, Holick, and Barger-Lux [37] showed that a dose of 10,000 IU daily over five months was a safe intervention. Therefore, the Endocrine Society set their tolerable upper limit of 10,000 IU daily [29].

The dose of 6000 IU daily over 12 weeks seems to be sufficient and safe for its application in athletes with a spinal cord injury. Due to the fact that vitamin D status decreased during the 12-week follow up under placebo supplementation, we recommend to re-evaluate vitamin D status after a certain time period (e.g., 18 week after the end of the supplementation period).

4.2. Vitamin D and Muscle Performance

The present study did not find any significant increase in anaerobic Wingate test performance, although vitamin D status significantly increased over these 12 weeks. Fifteen out of twenty athletes showed an increase in peak power over this period (Figure 3). A significant increase in isokinetic strength in the non-dominant arm was found comparing baseline and post supplementation measurement (Table 2). Even though this increase was significant, it is not obvious, whether it was due to a training adaptation or due to the increased vitamin D status. The lack of a placebo or control group prevents drawing further conclusions. Similar results were shown by Pritchett, Pritchett, Ogan, Bishop, Broad, and LaCroix [26], whereas vitamin D status did not correlate with 20 m sprint or handgrip strength in wheelchair athletes. A recent meta-analysis [18] found a significant increase in upper limb strength in able-bodied participants favoring the supplementation compared to the placebo intervention. Two of these studies applied a relatively high dose (60,000 and 14,000 IU vitamin D per week) over four to six months [23,41]. A single bolus of 150,000 IU vitamin D increased quadriceps muscle strength of elite judokas significantly after eight days [42]. In contrast, a dose of 2000 IU vitamin D daily over 12 weeks did not significantly increase swimming performance as well as arm-grip strength and one-legged balance in adolescent swimmers [20]. Thus, no conclusive results were found for upper body muscle strength after vitamin D supplementation.

Similar to our results, Hamilton, et al. [43] found a significantly increased peak torque in the non-dominant leg in professional soccer players, but no increase in the dominant leg. Overall, no consistent association between vitamin D and isokinetic strength was found in this study. It remains unclear why, in the non-dominant leg in this study, and the non-dominant arm in our study, muscle strength improved in contrast to the dominant one. Further studies are needed to elucidate this issue as well as to investigate the effects of vitamin D on upper body muscle strength and handgrip function.

In addition, it is known that vitamin D binds to vitamin D receptors in the muscle tissue in order to turn up gene expression of type II muscle fibers [13]. A study performed with an elderly vitamin D deficient population showed a decreased atrophy of type II muscle fibers under vitamin D supplementation [44]. The relative number and size of type II muscle fibers was increased and muscle strength was improved. In addition, a reduction of falls and hip fractures occurred in the intervention group. This study showed, that the type II muscle fibers are possibly more affected compared to type I fibers. Knowing that, in individuals with a spinal cord injury type I muscle fibers

are more predominant in the upper body [45], a smaller impact of vitamin D supplementation on these muscles might be expected. Again, such a speculation has to be further investigated in the future by means of muscle biopsies in order to determine the change in muscle fiber size and number after vitamin D supplementation.

4.3. Other Parameters

Our study did not find any significant change in the DASH score outcome over the supplementation period. This result suggests that the upper body impairment did not change over time and no additional injury occurred. Of course, due to the lack of a placebo group, this finding cannot be compared. A recent systematic review performed in healthy adults [46] revealed a small, but positive, effect on reducing the incidence of injuries when supplemented with vitamin D. Wyon, Koutedakis, Wolman, Nevill, and Allen [23] found a similar result in elite ballet dancers, who sustained significantly fewer injuries compared to the placebo group. Much more data is needed to finally conclude the impact of vitamin D supplementation on injury rate and severity of the injury. It is yet not clear whether there exists a positive association in general.

4.4. Limitations

The aim was to conduct a placebo-controlled intervention study. Unfortunately, all recruited participants showed an insufficient vitamin D status and were enrolled into the vitamin D supplementation group. The number of participants was limited due to the lack of further elite wheelchair athletes and due to the stringent inclusion or exclusion criteria. Even though an a priori power analysis showed a high power with 10 participants, this study might have been slightly underpowered due to a high variability in muscle force in athletes with a paraplegia or a tetraplegia. Therefore, it seems very difficult to draw final conclusions on how an insufficient vitamin D status impairs upper body performance or how a vitamin D supplementation might help to improve neuromuscular function. Nevertheless, this study showed clearly how vitamin D status increased over 12 weeks after supplementation and to which extent it decreased during the follow up. Further research is needed to investigate the relationship of vitamin D deficiency and neuromuscular performance in athletes with a spinal cord injury.

5. Conclusions

The present finding show, that a dose of 6000 IU vitamin D daily over 12 weeks is safe and sufficient to reach an optimal vitamin D level in indoor wheelchair athletes. Due to the lack of a placebo group, no final conclusion can be drawn whether vitamin D influences neuromuscular performance in athletes with a spinal cord injury. Isokinetic strength seems to have improved in the non-dominant arm over 12 weeks, but this finding might also result from continuous training.

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6

An Exploratory Investigation of Endotoxin Levels in Novice Long Distance Triathletes, and the Effects of a Multi-Strain Probiotic/Prebiotic, Antioxidant Intervention

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Abstract: Gastrointestinal (GI) ischemia during exercise is associated with luminal permeability and increased systemic lipopolysaccharides (LPS). This study aimed to assess the impact of a multistrain pro/prebiotic/antioxidant intervention on endotoxin unit levels and GI permeability in recreational athletes. Thirty healthy participants (25 males, 5 females) were randomly assigned either a multistrain pro/prebiotic/antioxidant ($LAB^4{}_{ANTI}$; 30 billion CFU·day^{-1} containing 10 billion CFU·day^{-1} *Lactobacillus acidophilus* CUL-60 (NCIMB 30157), 10 billion CFU·day^{-1} *Lactobacillus acidophillus* CUL-21 (NCIMB 30156), 9.5 billion CFU·day^{-1} *Bifidobacterium bifidum* CUL-20 (NCIMB 30172) and 0.5 billion CFU·day^{-1} *Bifidobacterium animalis* subspecies *lactis* CUL-34 (NCIMB 30153)/55.8 mg·day^{-1} fructooligosaccharides/ 400 mg·day^{-1} α-lipoic acid, 600 mg·day^{-1} *N*-acetyl-carnitine); matched pro/prebiotic (LAB^4) or placebo (PL) for 12 weeks preceding a long-distance triathlon. Plasma endotoxin units (via *Limulus* amebocyte lysate chromogenic quantification) and GI permeability (via 5 h urinary lactulose (L): mannitol (M) recovery) were assessed at baseline, pre-race and six days post-race. Endotoxin unit levels were not significantly different between groups at baseline ($LAB^4{}_{ANTI}$: 8.20 ± 1.60 pg·mL^{-1}; LAB^4: 8.92 ± 1.20 pg·mL^{-1}; PL: 9.72 ± 2.42 pg·mL^{-1}). The use of a 12-week $LAB^4{}_{ANTI}$ intervention significantly reduced endotoxin units both pre-race (4.37 ± 0.51 pg·mL^{-1}) and six days post-race (5.18 ± 0.57 pg·mL^{-1}; $p = 0.03$, $\eta p^2 = 0.35$), but only six days post-race with LAB^4 (5.01 ± 0.28 pg·mL^{-1}; $p = 0.01$, $\eta p^2 = 0.43$). In contrast, endotoxin units remained unchanged with PL. L:M significantly increased from 0.01 ± 0.01 at baseline to 0.06 ± 0.01 with PL only ($p = 0.004$, $\eta p^2 = 0.51$). Mean race times (h:min:s) were not statistically different between groups despite faster times with both pro/prebiotoic groups ($LAB^4{}_{ANTI}$: 13:17:07 ± 0:34:48; LAB^4: 12:47:13 ± 0:25:06; PL: 14:12:51 ± 0:29:54; $p > 0.05$). Combined multistrain pro/prebiotic use may reduce endotoxin unit levels, with $LAB^4{}_{ANTI}$ potentially conferring an additive effect via combined GI modulation and antioxidant protection.

Keywords: endotoxemia; probiotics; prebiotics; antioxidants; triathlon

1. Introduction

Participation trends, including "recreational athletes", in multi-sport and ultra-endurance events have increased in recent years [1,2]. Symptoms associated with gastrointestinal (GI) distress (e.g., cramping, diarrhoea, nausea, and abdominal pain) are estimated to occur in 25%–90% of endurance athletes, and are often cited as reasons for non-completion [3–5]. In preparation for such events, exercise-related GI symptoms may go unreported, which could impact on training efficiency and race completion. It has been shown that exercise induced GI hypoperfusion may provoke transient damage to the gut epithelium [6], with one study demonstrating that 30 min of running at 80% of peak oxygen uptake (VO_2peak) significantly increased luminal permeability in healthy volunteers [7].

Mechanistically, prolonged or strenuous exercise may increase key phosphorylation enzymes [8], disrupting the tight junction proteins claudin (influenced by protein kinase A) and occludin (influenced by both protein kinase C and tyrosine kinase). Acute changes in tight junction permeability and paracellular transport may lead to a greater prevalence of systemic lipopolysaccharides (LPS). LPS from gram-negative intestinal bacteria may provoke immune responses and endotoxin-associated symptoms characteristic of GI complaints often experienced in runners [8]. Despite this, research is relatively sparse on whether prolonged training or ultra-endurance events actually result in elevated LPS, particularly in more "recreationally active" athletes; or whether targeted nutrition strategies offer beneficial support.

In one study, 68% of highly trained athletes taking part in a long-distance triathlon reported with endotoxin levels of 5–15 pg·mL^{-1} in the first 16 h post-event, corresponding with elevated cytokine responses in the same period [9]. In contrast, 81% of runners requiring medical attention at the end of an ultra-marathon were found to have LPS concentrations >100 pg·mL^{-1} [10], with 80.6% of these athletes reporting GI symptoms (nausea, diarrhoea, and vomiting). LPS concentrations at or above these levels have been more commonly associated in patients with Crohn's disease [11] and sepsis [12].

The term "mild endotoxemia" has been used to depict an acute elevation in LPS from endurance exercise by several authors [9,13,14], but may well reflect normal or transient levels of circulatory LPS. It has also been shown that LPS responses to exertional heat stress may be significantly higher in less trained individuals [13], but still within normal limits. Conversely, one study reported an average increase in resting LPS levels of 60 pg·mL^{-1} across a five-stage ultra-run, with daily (pre–post stage) average LPS changes of 30 pg·mL^{-1} [15]. Despite such diversity, the potential for exercise related endotoxin-mediated cytokinemia may explain individual susceptibility to GI symptoms and recovery from endurance exercise. If prevalent, the presence of, and repeated exposure to, "low grade" LPS (ranging from ~10 to 50 pg·mL^{-1} or higher [16]) may promote a mild inflammatory state which could be detrimental to the longer term health of recreational athletes who regularly engage in exercise.

Probiotic bacteria, particularly the gram-positive genera *Lactobacillus* and *Bifidobacterium* species, are known to modify GI microbiota [17–19], and have been shown to reduce GI episode severity [20] and respiratory tract infections commonly associated with training [21]. However, therapeutic benefits of probiotics are highly strain specific. As example, the use of *Lactobacillus casei* strain Shirota in one study, significantly increased natural killer cell cytolytic activity in healthy volunteers [22], whereas combined *Streptococcus thermophilus* FP4/*Bifidobacterium breve* BR03 was recently shown to reduce circulating IL-6 in response to muscle damaging exercise [23] elsewhere. In clinical trials, a multistrain high dose probiotic (LAB^4—containing *Lactobacillus acidophilus* CUL60 and CUL21, *Bifidobacterium lactis* CUL34 and *Bifidobacterium bifidum* CUL20), resulted in significant improvements in irritable bowel syndrome responses [24] and prevented an increase in antibiotic resistant enterococci [25]. Chronic multistrain interventions have also been shown to reduce faecal zonulin levels by ~25% in endurance trained athletes, demonstrating improved GI barrier integrity [26]. The inclusion of *Bifidobacterium species* and prebiotics (e.g., fructo-oligosaccharides, inulin, pectin) in such formulas may also play an important role in short-chain fatty acid production, which may also support epithelial integrity [27].

Antioxidants nutrients such as α-lipoic acid, *N*-acetyl-carnitine, vitamin C, quercetin, resveratrol, and curcumin may also provide important roles in minimizing epithelial disruption [28–31], associated with elevated oxidative stress from GI hypoperfusion. Alpha lipoic acid in particular is proposed to act as a multi-functional antioxidant, regenerating endogenous glutathione, and minimising GI mucosal injury [31–33]. The aims of this exploratory study were therefore: (i) to assess endotoxin levels and GI permeability in recreational athletes training for and taking part in their first long distance triathlon; and (ii) to assess the potential benefits of a 12-week multistrain pro/prebiotic/antioxidant strategy on GI symptoms, endotoxin levels and race time compared to a control group.

2. Materials and Methods

2.1. Participants

Following study approval from the University of Hertfordshire Life and Medical Sciences Ethics Committee (LMS/SF/UH/00011), and power calculation assessment for sample size (G*power3, Dusseldorf [34]; using $\alpha = 0.05$; $1 - \beta = 0.80$; based on observed data [9,14]), thirty recreationally active participants (25 males, 5 females; M ± SE: age 35 ± 1 years; weight: 76.52 ± 2.20 kg; initial VO_2max: 48.93 ± 0.99 mL·kg^{-1}·min^{-1}) were randomly invited to take part in an intervention study which took place in the final 12 weeks of a nine month progressive training programme. All participants provided written, informed consent, and satisfactorily completed a general health screen prior to study inclusion. Participant characteristics are displayed in Table 1, with no observed differences between intervention groups for age, height, weight, bodyfat or VO_2max.

Table 1. Pre-screening (Month 0) and baseline (Month 6) characteristics for intervention groups.

Variable	LAB^4_{ANTI}		LAB^4		PL	
Distribution	(*n* = 10; 7 male, 3 female)		(*n* = 10; 9 male, 1 female)		(*n* = 10; 9 male, 1 female)	
Age (years)	33 ± 2		35 ± 2		35 ± 3	
Height (m)	1.74 ± 0.34		1.79 ± 0.27		1.76 ± 0.16	
	Pre-screening	Baseline	Pre-screening	Baseline	Pre-screening	Baseline
Weight (kg)	75.21 ± 4.12	73.61 ± 3.96 *	83.77 ± 4.71	81.94 ± 4.44 *	77.42 ± 3.03	74.56 ± 2.76
Body fat (%)	22.56 ± 1.67	19.36 ± 2.23 *	21.88 ± 1.68	20.93 ± 1.52	21.28 ± 2.38	18.64 ± 1.93 *
VO_2max (L·min^{-1})	3.26 ± 0.20	3.57 ± 0.19 *	3.78 ± 0.28	3.94 ± 0.27	3.30 ± 0.14	3.70 ± 0.10 *
VO_2max (mL·kg^{-1}·min^{-1})	42.90 ± 1.59	48.60 ± 1.80 *	43.89 ± 1.75	47.56 ± 1.69 *	43.40 ± 2.53	50.50 ± 1.71 *

Data presented as mean ± SE No significant differences reported between groups. * denotes significant difference ($p < 0.05$) to pre-screening only within group.

Pre-screening: At the start of the nine month training programme, all participants underwent full screening including suitability assessment from their General Practitioner, a 12-lead electrocardiogram to assess for potential underlying cardiac abnormalities, and completion of a standard incremental maximal stress test (using a Computrainer erogometer system, RaceMate Inc., Seattle, WC, USA) for the assessment of maximal oxygen consumption (using a Metalyser 3B automated gas-analyser; Cortex Biophysik, Leipzig, Germany). In addition, routine assessment of height (Seca 200 stadiometer, Hamburg, Germany), body mass (Seca 780, Hamburg, Germany) and body composition (Tanita Body Segmental Analyser 418-BC, Tokyo, Japan) was undertaken. Participants were required to have no previous experience of long distance triathlons, be recreationally active (defined as general exercise activity 1–3 times per week) and have basic proficiency in swimming, cycling and running disciplines. As a means to further quantify "recreationally active", participants were required to have a relative maximum oxygen uptake of 30–50 mL·kg^{-1}·min^{-1} for women, and 35–55 mL·kg^{-1}·min^{-1} for men during pre-screening testing. Participants were excluded if there was any history (including familial) of cardiovascular abnormalities (including coronary heart disease) and diabetes; or any known blood related disorders.

2.2. Experimental Design and Procedures

In a randomized, repeated-measures, double-blind, placebo controlled study design, participants attended the Human Physiology Laboratory, University of Hertfordshire 12 weeks prior to undertaking a long distance triathlon (Barcelona Challenge Triathlon) comprising a 3.8 km sea swim, 180.0 km road cycle course and a 42.2 km marathon run. Although participants had no prior experience to this triathlon distance, they had all adhered to a standardized training programme for the previous six months as part of a larger training cohort. General training progression ("recreationally trained") from the previous six months was assessed prior to the intervention study using the same incremental test procedure and equipment (including anthropometrical measures) as for pre-screening (see Table 1). Thereafter, participants attended the laboratory on three occasions: baseline (Week 0), pre-race (Week 12) and post-race (six days post) for blood and urine sampling as described below. Due to constraints with field based sampling, and varying participant travel arrangements, the post-race timepoint (six days) was selected for consistency and to assess whether any previous patterns were still evident during the longer term recovery period.

Blood sampling: Participants were requested to rest the day before all test sessions. Upon arrival, a fasted, venous wholeblood sample was collected from participants by a qualified phlebotomist into duplicate 4 mL K_3EDTA vacutainers (Greiner Bio-One GmbH, Kremsmunster, Austria). Samples were centrifuged for 10 min at 3000 rpm, with aliquotted plasma pipetted into sterile, nonpyrogenic, polypropylene cyrovials (Fisherbrand, Fisher Scientific, Loughborough, UK) and immediately frozen at −80 °C for later assessment of resting endotoxin units and IgG endotoxin-core antibodies.

Urine sampling: Assessment of GI permeability was assessed via 5 h recovery of urinary lactulose and mannitol via a standard sugar absorption test [35]. Briefly, upon arrival, participants provided a urine sample with total volume assessed, and then (following blood sampling) consumed a standardized 100 mL test drink containing 5 g lactulose solution (Sandoz Ltd., Camberley, Surrey, UK), 2 g mannitol (Mannitol powder: 99.86% pure certified, Blackburn Distributions Ltd., Nelson, Lancashire, UK) and 40 g of sucrose (Tate and Lyle, London, UK). For the first two hours post consumption, participants were not allowed to eat or drink, and thereafter could eat/ drink as normal (with the exception of refined/sugary products or drinks). Over a five hour period, participants collected total urine output into 3 L polyethylene opaque beakers (Sarstedt, Numbrecht, Germany). With total sample volume assessed, duplicate urine samples were aliquotted into sterile cryovials and immediately frozen at −80 °C for later assessment of saccharide recovery.

2.3. Biochemical Assays

Endotoxin unit assessment: Quantification of endotoxin units was derived from plasma samples using an established endpoint chromogenic assay method (Pierce® LAL Chromogenic Endotoxin Quantitation Kit, Thermo Fisher Scientific, Waltham, MA, US). After thawing to room temperature and sample preparation, 50 µL aliquots were added to an endotoxin-free microtitre plate and incubated at 37 °C for 5 min. Following this, 50 µL aliquots of *Limulus* amoebocyte lysate (LAL) were added to each well, the plate gently shaken for 10 s, and re-incubated at 37 °C for 10 min. At exactly 10 min, 100 µL aliquots of chromogenic substrate solution was added to each well, the plate gently shaken for 10 s, and then further re-incubated at 37 °C for 6 min. At this point, 50 µL aliquots of stop reagent (25% acetic acid) was added to each well, and the plate gently shaken for 10 s. Samples were read on a spectrophotometer at an absorbance of 405 nm (Victor 3 multilabel plate reader, PerkinElmer Inc., Llantrisant, UK) and referenced against a calibration curve based on dilutions of an *Escherichia coli* (*E. coli*) endotoxin standard (011:B4; vial concentration 26 EU·mL^{-1}) with non-incubated mock reaction controls taken into consideration. Values of quantified endotoxin units (EU·mL^{-1}) were then converted to pg·mL^{-1}.

IgG Endotoxin-core Antibody Assessment: IgG endotoxin-core antibodies (IgG anti-EU) were measured from plasma samples via solid-phase ELISA (EndoCab® IgG, Hycult Biotech, Uden, The Netherlands). Reagents were prepared in accordance with the manufacturer's instructions at

room temperature. Plasma samples were thawed to room temperature and diluted 200-fold using the supplied dilution buffer. Following this, 100 μL aliquots of the standard or prepared sample were carefully pipetted into microtitre wells coated with endotoxin rough-lipopolysaccharides, the microtitre plate then covered and incubated at 37 °C for 60 min. The plate was then washed four times manually, with 200 μL of supplied washer buffer added to each microtitre well during each wash cycle. Following this, 100 μL of diluted conjugate (streptavidin-peroxidase) was added to each well to bind the captured endotoxin core-antibodies. The plate was then covered and incubated at 37 °C for 60 min, before being manually washed a further four times with washer buffer. Then, 100 μL aliquots of tetramethylbenzidine (TMB) were added to each microtitre well, the plate covered and incubated at room temperature for 30 min avoiding exposure to sunlight. The reaction was then stopped by addition of 100 μL aliquots of oxalic acid to each well. Samples were read on a spectrophotometer at an absorbance of 450 nm (Victor 3 multilabel plate reader, PerkinElmer Inc., Llantrisant, UK) and referenced against a calibration curve (logarithmic scale) based on dilutions of a reconstituted human EndoCab IgG standard. Values are presented in standard median units ($MU \cdot mL^{-1}$).

GI Permeability Assessment: Following sample thawing and preparation, assessment of saccharide recovery was performed via enzymatic method assays for lactulose and mannitol using a Randox RX Monza semi-automated, flow cell based clinical chemistry analyser (Randox Ltd., Country Antrim, UK). Briefly, for lactulose, reagents were prepared in accordance with the manufacturer's instructions at room temperature (INstruchemie BV, Delfzil, Netherlands). Sample preparation involved 50 μL urine aliquots being mixed with 50 μL dissociation buffer and 5 μL galactosidase reagent, incubated at 37 °C overnight, and centrifuged at 2000 rpm for 5 min. Additionally, a non-incubated control was also prepared to account for NADPH already present in the sample. Thereafter, 200 μL of lactulose buffer reagent was carefully pipetted into centrifuge tubes and mixed with 5 μL sample, incubated at 37 °C for 5 min, and read at an absorbance of 340 nm (reading A1). Following this, 50 μL start reagent was added to the sample, mixed and incubated at 37 °C for 10 min and read at an absorbance of 340 nm (reading A2). Recovered lactulose ($mmol \cdot L^{-1}$) was calculated taking into consideration pre-incubated and non-incubated samples against standard, blank and quality control samples.

For mannitol assessment, reagents were prepared in accordance with the manufacturer's instructions at room temperature (INstruchemie BV, Delfzil, The Netherlands); and a reference curve generated from dilutions of a 20 $mmol \cdot L^{-1}$ mannitol standard. Samples were prepared by mixing 3 μL urine aliquots with 200 μL NAD buffer reagent and incubating at 37 °C for 5 min. From this 60 μL diluted start reagent was added to the sample, mixed and incubated for a further 37 °C for 10 min. Samples were read at an absorbance of 340 nm, and recovered mannitol ($mmol \cdot L^{-1}$) calculated against a standard reference taking into consideration blank and quality control samples.

2.4. Nutritional Interventions and Diaries

Nutritional interventions: Following baseline assessment, participants were allocated, in a double-blinded manner, to one of three intervention groups using a random number generator approach. Participants were provided with a 90-day supply of either: capsulated (hydroxypropyl methylcellulose) multistrain probiotic/prebiotic/antioxidant ($LAB^4{}_{ANTI}$), matched pro/prebiotic (LAB^4) or placebo (PL) in opaque, sealed pots with instructions for daily ingestion timing. This allocation covered the 12-week pre-race period and the six-day post-race period. Intervention supplementation was provided by Biocare Ltd. (Birmingham, UK) for commercial use, with products pre-capsulated by the manufacturer. Placebo supplements were prepared within our laboratory using the same size hydroxypropyl methylcellulose capsules.

For both LAB^4 and $LAB^4{}_{ANTI}$, participants were instructed to consume one multistrain pro/prebiotic capsule daily in the evening with food. Each multistrain capsule contained 150 $mg \cdot day^{-1}$ *Lactobacillus acidophilus* (10 billion $CFU \cdot day^{-1}$, *Lactobacillus acidophilus* CUL-60 [NCIMB 30157] and 10 billion $CFU \cdot day^{-1}$ *Lactobacillus acidophillus* CUL-21 [NCIMB 30156]), 16.8 $mg \cdot day^{-1}$ *Bifidobacterium bifidum* and *lactis* (9.5 billion $CFU \cdot day^{-1}$, *Bifidobacterium bifidum* CUL-20 [NCIMB

30172] and 0.5 billion CFU·day^{-1} *Bifidobacterium animalis subspecies lactis* CUL-34 [NCIMB 30153]), and 55.8 mg·day^{-1} fructooligosaccharides (Bio-Acidophilus Forte, Biocare Ltd., Birmingham, UK). For those assigned to PL, participants were instructed to consume one placebo capsule daily in the evening with food, containing 200 mg cornflour.

For $LAB^4{}_{ANTI}$, participants additionally consumed two capsules in the morning with breakfast (each capsule contained 200 mg α-lipoic-acid and 300 mg of *N*-acetyl-carnitine hydrochloride; Acetyl Carnitine and Alpha Lipoic Acid formulation, Biocare Ltd., Birmingham, UK). For control consistency between groups, those assigned to LAB^4 and PL were instructed to additionally consume matched cornflour placebo capsules with breakfast. Throughout the study, participants were required to not be consuming any other nutritional supplements other than glucose drinks/gels as part of endurance training. Adherence was checked via nutrition diaries and monthly briefings with all participants.

Nutrition diaries: Participants were requested to maintain habitual dietary intake throughout the intervention and record via weekly food diaries at the beginning and end of each month. Participants were provided with example diaries and individually instructed in diary completion, with emphasis on meal breakdown, portion size and weight, fluid intake and consumption of prescribed supplementation. Dietary analyses were undertaken using Dietplan 6.50 (Forestfield Software Ltd., West Sussex, UK) based on a seven-day representation from each month.

2.5. Training Monitoring, GI Questionnaires and Assessment of Race Times

Training programme: Over the course of the 12-week intervention, participants continued with a triathlon training programme, prescribed by an accredited Sport and Exercise Physiologist, focusing on swimming, cycling and running disciplines, as well as functional training. Training was designed to be flexible around daily activities with a requirement to achieve a minimum of 80% of the total training volume set. Training was monitored via weekly training diaries in which participants recorded exercise duration and overall session rating of perceived exertion (sRPE). Training load, training monotony and training strain were determined from a modified training method previously described (duration × sRPE [36,37]).

GI response questionnaire: Participants completed an overall monthly training GI response questionnaire, adapted from symptoms previously reported [4,9,38]. Participants were asked to subjectively rate their responses across four subsections (general training, endurance training (>3 h), acute (<24 h) and longer term (<72 h) recovery periods). For the training subsections the following symptoms were evaluated: urge to urinate, urge to defecate, bloating, belching, flatulence, nausea, stomach/intestinal pain or discomfort, stomach/intestinal cramping, headaches, and dizziness. For the recovery subsections, the following symptoms were evaluated: constipation and/or diarrhoea, stomach/intestinal pain or discomfort, bloating, flatulence, nausea, stomach/intestinal cramping, headaches, dizziness, mental fatigue, excessive and sweating. Collectively, this resulted in a maximum symptom count of 40. Symptoms were graded for severity according to a category scale (0 = none; 1 = low severity; 2 = moderate severity; 3 = high severity). From this, mean symptom count and symptom severity scores were assessed to evaluate the subjective impact of each intervention.

Race times: All participants, as entrants of the Barcelona Challenge Triathlon, were required to wear official timing chips throughout the race. Overall race times, including triathlon specific stage times (swim, bike, and run) were provided by the race director following confirmation of official final times.

2.6. Statistical Analyses

Statistical analyses were performed using SPSS (v22, IBM, Armonk, NY, USA). Following assessment of normality via a Shapiro–Wilk test, a 3 × 3 factorial design analysis of variance (Anova) was employed to assess treatment and time interactions, using least significant difference (LSD) post hoc evaluation. Where pertinent, within group assessment was undertaken

using a general linear repeated measures Anova, with LSD post hoc evaluation. For assessment of race times between groups only, a between-group Anova was performed, with LSD post hoc analysis. GI questionnaires were assessed via chi-squared analysis. An alpha level of ≤ 0.05 was employed for statistical significance. Data are reported as means ± SE.

3. Results

3.1. Nutrition and Training Data

Dietary analysis comparisons for each month are shown in Table 2. No significant differences were reported between or within groups across the 12-week intervention period for energy, carbohydrate, fat or protein intake ($p > 0.05$). On average across the 12-week intervention, daily energy intake for $LAB^4{}_{ANTI}$ was 35.10 ± 1.31 kcal·kg^{-1}·day^{-1} compared with 33.97 ± 1.73 kcal·kg^{-1}·day^{-1} for LAB^4 and 35.53 ± 1.66 kcal·kg^{-1}·day^{-1} for PL. Macronutrient intake was also comparable, with an average fat intake of 1.45 ± 0.08 g·kg^{-1}·day^{-1} for $LAB^4{}_{ANTI}$ compared 1.27 ± 0.08 g·kg^{-1}·day^{-1} for LAB^4 and 1.30 ± 0.07 g·kg^{-1}·day^{-1} for PL. Likewise, average carbohydrate intake was 4.06 ± 0.22 g·kg^{-1}·day^{-1} for $LAB^4{}_{ANTI}$ compared with 4.02 ± 0.24 g·kg^{-1}·day^{-1} for LAB^4 and 4.37 ± 0.31 g·kg^{-1}·day^{-1} for PL. Similarly, average protein intake was comparable between pro/prebiotic groups at 1.55 ± 0.07 g·kg^{-1}·day^{-1} for $LAB^4{}_{ANTI}$, 1.50 ± 0.11 g·kg^{-1}·day^{-1} for LAB^4, and non-significantly higher for PL at 1.72 ± 0.10 g·kg^{-1}·day^{-1}.

Table 2. Dietary analysis comparisons between groups.

Variable	$LAB^4{}_{ANTI}$	LAB^4	PL
Energy intake (kcal·kg^{-1}·day^{-1})			
T1	35.96 ± 2.16	33.13 ± 1.16	35.57 ± 2.88
T2	33.88 ± 2.06	33.03 ± 4.66	34.57 ± 2.98
T3	35.42 ± 2.57	35.76 ± 2.43	36.60 ± 2.85
Fat (g·kg^{-1}·day^{-1})			
T1	1.45 ± 0.13	1.28 ± 0.10	1.24 ± 0.14
T2	1.37 ± 0.12	1.27 ± 0.21	1.19 ± 0.08
T3	1.52 ± 0.18	1.26 ± 0.12	1.47 ± 0.11
Carbohydrate (g·kg^{-1}·day^{-1})			
T1	4.29 ± 0.33	3.80 ± 0.30	4.46 ± 0.47
T2	3.95 ± 0.43	3.90 ± 0.59	4.32 ± 0.58
T3	3.93 ± 0.39	4.36 ± 0.36	4.34 ± 0.57
Protein (g·kg^{-1}·day^{-1})			
T1	1.51 ± 0.12	1.43 ± 0.14	1.81 ± 0.18
T2	1.56 ± 0.07	1.39 ± 0.17	1.70 ± 0.14
T3	1.57 ± 0.15	1.68 ± 0.28	1.67 ± 0.19

Data represent average daily intake (mean ± SE). T1–3 represent Months 1–3 respectively. No significant differences reported between or within groups ($p > 0.05$).

Training load comparisons are shown in Table 3. No significant differences were reported between groups across the intervention for training load, monotony or strain ($p > 0.05$). There was, however, a significant time interaction effect for training load ($F = 16.30$, $p < 0.0001$, $\eta p^2 = 0.38$) and training strain ($F = 4.88$, $p = 0.011$, $\eta p^2 = 0.16$). The training programme was designed to progressively build over the 12 weeks, with a peak training load in Month 2, and an increased training strain in Month 3 leading to a final taper period prior to the race. The target range (particular for training load) represents the generic range set for all participants i.e., to meet a minimum of 80% training load.

Across the intervention, all groups satisfactorily met the minimum training load. Average training loads at Month 2 were all significantly higher than Months 1 and 3, as expected ($p < 0.0001$).

Interestingly, however, peak training loads at Month 2 were all greater than the high end target set at 3278 AU (arbitrary units). For LAB^4 in particular, training load was noted at 4311 ± 348 AU ($F = 8.21$, $p = 0.006$, $\eta p^2 = 0.58$ compared to Month 1), further reflecting the increased strain (4065 ± 381 AU) in this month ($F = 6.79$, $p = 0.011$, $\eta p^2 = 0.53$). Training strain in Month 3 was noted as being lower in all groups ($p = 0.003$) compared to Month 2 and in direct comparison to the target range.

Table 3. Training load comparisons.

Variable	Target Range	$LAB^4{}_{ANTI}$	LAB^4	PL
Weekly training load (AU)				
T1	2173–2716	2410 ± 242	2851 ± 279	2807 ± 368
T2	2622–3278	3885 ± 558 #	4311 ± 348 *#	3915 ± 516 #
T3	2231–2789	2232 ± 148	2768 ± 498	2263 ± 180
Training monotony (AU)				
T1	1.07–1.33	0.94 ± 0.11	0.98 ± 0.08	1.11 ± 0.08
T2	0.97–1.21	0.88 ± 0.07	0.96 ± 0.08	0.88 ± 0.08
T3	1.27–1.58	0.90 ± 0.12	0.87 ± 0.09	0.72 ± 0.05
Training strain (AU)				
T1	2951–3688	2755 ± 562	2945 ± 450	3224 ± 566
T2	3350–4187	3430 ± 620	4065 ± 381 *#	3293 ± 552
T3	3352–4440	2281 ± 370	2681 ± 650	1946 ± 186

Data represent arbitrary units (AU) and presented as mean ± SE Target range indicates 80%–100% of training programme. No significant differences reported between groups ($p > 0.05$). * denotes significant difference from T1 within group ($p \leq 0.006$). # denotes significant difference from T3 within group ($p \leq 0.04$).

3.2. Endotoxin Unit (EU) Assessment

Data for endotoxin units (EU) and IgG endotoxin-core antibody assessment are shown in Figure 1a–c for $LAB^4{}_{ANTI}$, LAB^4 and PL respectively. A significant interaction effect was reported for endotoxin units over time ($F = 4.21$, $p = 0.019$, $\eta p^2 = 0.11$) and group ($F = 3.50$, $p = 0.036$, $\eta p^2 = 0.09$) only. At baseline, whilst EU levels were highest with PL (9.72 ± 2.42 pg·mL^{-1}), no significance was found in comparison to either $LAB^4{}_{ANTI}$ (8.20 ± 1.60 pg·mL^{-1}) or LAB^4 (8.92 ± 1.20 pg·mL^{-1}, $p > 0.05$). EU concentrations ranged from 3.03 to 27.75 pg·mL^{-1}. Within group, $LAB^4{}_{ANTI}$ resulted in a significant reduction in endotoxin units both pre-race (4.37 ± 0.51 pg·mL^{-1}) and six days post-race (5.18 ± 0.57 pg·mL^{-1}; $F = 4.27$, $p = 0.033$, $\eta p^2 = 0.35$). For LAB^4, there was a significant reduction in endotoxin units over time ($F = 6.04$, $p = 0.011$, $\eta p^2 = 0.43$), with post-hoc analysis indicating EU levels of 5.01 ± 0.28 pg·mL^{-1} six days post-race being significantly lower than baseline ($p = 0.047$) only. Endotoxin unit levels for PL did not significantly differ across the intervention period or six days post-race ($p > 0.05$).

3.3. IgG Endotoxin-Core Antibody (Anti-EU) Assessment

Overall, a significant group interaction effect was reported for IgG anti-EU, with $LAB^4{}_{ANTI}$ demonstrating lower concentrations of IgG endotoxin core-antibodies in comparison to both LAB^4 and PL ($F = 10.82$, $p < 0.0001$, $\eta p^2 = 0.25$) at baseline. Whilst IgG anti-EU levels remained significantly lower pre-race with $LAB^4{}_{ANTI}$ compared to LAB^4 ($p = 0.003$), there was no statistical difference in comparison to PL. By post-race, no significant differences were reported between groups ($p > 0.05$). Within group, IgG anti-EU levels for $LAB^4{}_{ANTI}$ increased from 40.42 ± 12.39 MU·mL^{-1} at baseline, to 58.83 ± 22.94 MU·mL^{-1} pre-race in contrast to the decrease in endotoxin unit levels observed. However, the overall increase in IgG anti-EU to 77.93 ± 26.03 MU·mL^{-1} post-race was not deemed significant ($F = 1.01$, $p = 0.387$, $\eta p^2 = 0.11$) overall.

For LAB^4, IgG anti-EU also increased from 209.23 ± 59.73 MU·mL^{-1} at baseline to 251.73 ± 60.72 MU·mL^{-1} pre-race in relative contrast to the decrease in endotoxin unit levels observed for this group. Post-race IgG anti-EU concentrations decreased to 161.61 ± 50.16 MU·mL^{-1}, but overall changes were not deemed statistically significant ($F = 1.95$, $p = 0.174$, $\eta p^2 = 0.20$) for LAB^4. In a converse

manner, average plasma IgG anti-EU concentrations decreased from 223.98 ± 51.46 MU·mL^{-1} at baseline to 181.56 ± 58.19 MU·mL^{-1} pre-race with PL, returning to 207.94 ± 31.96 MU·mL^{-1} six days post-race; however, overall changes in IgG anti-EU for PL were not statistically significant (F = 0.30, p = 0.746, ηp^2 = 0.04).

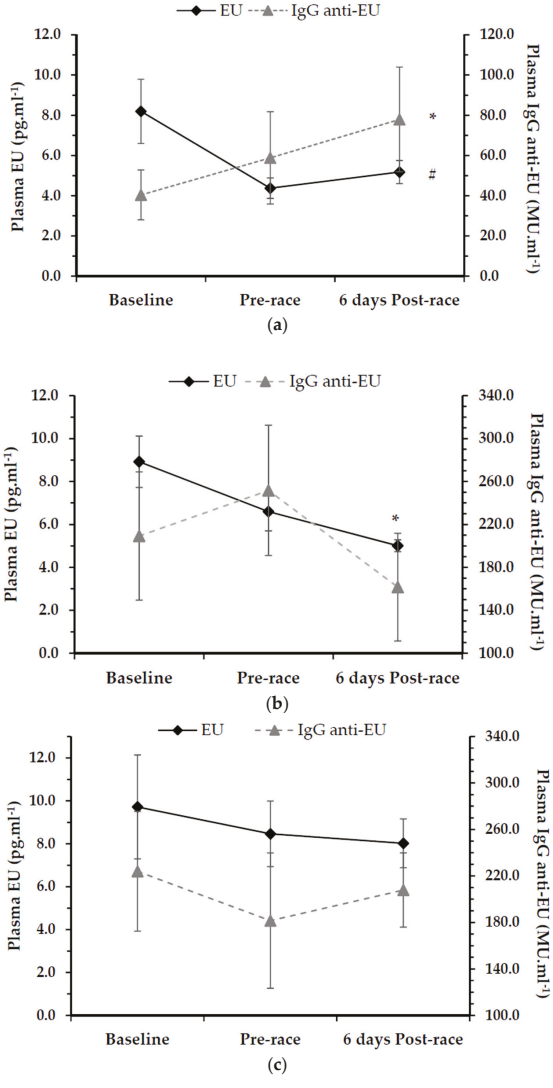

Figure 1. (**a**) Plasma endotoxin unit (EU) concentrations (pg·mL^{-1}) and IgG endotoxin antibodies (anti-EU; MU·mL^{-1}) for LAB$^4_{ANTI}$ group (Mean ± SE). * denotes lower IgG anti-EU values overall than both LAB^4 and PL conditions (p < 0.001); # denotes significant reduction in endotoxin units over time within group (p = 0.03); (**b**) Plasma endotoxin unit (EU) concentrations (pg·mL^{-1}) and IgG endotoxin antibodies (anti-EU; MU·mL^{-1}) for LAB^4 group (Mean ± SE). * denotes significant difference from baseline for endotoxin units within group (p = 0.047); (**c**) Plasma endotoxin unit (EU) concentrations (pg·mL^{-1}) and IgG endotoxin antibodies (anti-LPS; MU·mL^{-1}) for PL group (Mean ± SE). No significant differences reported (p > 0.05).

3.4. Intestinal Permeability

Assessment of intestinal permeability from urinary lactulose:mannitol (L:M) ratio measurement is shown in Figure 2. GI permeability generally increased in all groups from baseline to six days post-race ($F = 9.66$, $p < 0.0001$, $\eta p^2 = 0.21$). No significant differences were reported between groups ($p > 0.05$). Within group, L:M increased marginally from 0.032 ± 0.006 at baseline, to 0.037 ± 0.010 pre-race and 0.054 ± 0.007 six days post-race with $LAB^4{}_{ANTI}$ ($p > 0.05$) Similarly, there was a non-significant increase in L:M with LAB^4 from 0.028 ± 0.005 at baseline, to 0.039 ± 0.007 and 0.044 ± 0.012 both pre- and six days post-race respectively ($p > 0.05$).

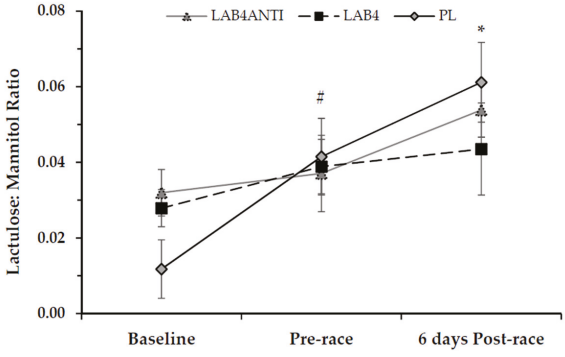

Figure 2. Assessment of intestinal permeability via urinary lactulose:mannitol ratio (Mean ± SE). Values measured in $mmol \cdot L^{-1}$. # denotes significant increase from baseline for PL only ($p = 0.05$); * denotes significant increase from baseline for PL only ($p = 0.002$).

However, for PL, L:M significantly increased over the intervention ($F = 8.16$, $p = 0.004$, $\eta p^2 = 0.51$) from 0.012 ± 0.008 at baseline to 0.041 ± 0.010 pre-race ($p \leq 0.05$). L:M further increased in PL to 0.061 ± 0.011 six days post-race ($p = 0.002$). A similar interaction effect for time was also observed with the per cent recovery of lactulose ($F = 5.66$, $p = 0.005$, $\eta p^2 = 0.14$), as shown in Table 4. Within group, for PL only, the per cent recovery of lactulose increased from $0.35\% \pm 0.18\%$ at baseline to $0.94\% \pm 0.12\%$ six days post-race ($p = 0.01$). No significant differences were found for per cent recovery of mannitol either between or within groups ($p > 0.05$).

Table 4. Recovery of urinary lactulose and mannitol (%).

Variable	$LAB^4{}_{ANTI}$	LAB^4	PL
% recovery of lactulose			
Baseline	0.71 ± 0.13	0.52 ± 0.07	0.35 ± 0.18
Pre-race	0.55 ± 0.14	0.74 ± 0.10	0.72 ± 0.17
6 days Post-race	0.83 ± 0.11	0.90 ± 0.24	0.94 ± 0.12 *
% recovery of mannitol			
Baseline	29.99 ± 2.87	25.57 ± 2.22	30.61 ± 3.96
Pre-race	23.31 ± 3.60	27.42 ± 3.33	23.51 ± 2.18
6 days Post-race	22.42 ± 3.27	25.01 ± 1.89	23.48 ± 2.64

Data presented as mean ± SE. * denotes significant difference to baseline within group only ($p = 0.01$).

3.5. GI Questionnaire

Overall symptom counts for training related GI issues were significantly lower in both LAB^4 groups at the end of Month 1 (7.80 ± 2.20 for $LAB^4{}_{ANTI}$ and 6.78 ± 1.31 for LAB^4) compared with PL

(11.90 ± 2.02; $p \leq 0.013$). However, by Month 2, only symptom counts for LAB^4 were significantly lower (8.11 ± 2.18) than PL (13.20 ± 2.72; $p < 0.001$). At Month 2, there was a significant increase in symptom counts for LAB$^4{}_{ANTI}$ (10.70 ± 2.88, $p = 0.015$ within group), which was also greater than LAB^4 ($p = 0.036$). By the end of the intervention, there was a similar pattern to Month 1, with both LAB^4 groups reporting lower symptom counts to training GI issues (8.80 ± 2.70 for LAB$^4{}_{ANTI}$ and 7.00 ± 2.16 for LAB^4) compared with PL (13.90 ± 2.42; $p < 0.001$).

Average symptom severity was significantly lower with both LAB^4 groups at Month 1 (9.80 ± 3.05 for LAB$^4{}_{ANTI}$ and 7.56 ± 1.56 for LAB^4) compared to PL (15.50 ± 2.97; $p < 0.001$). This pattern continued throughout the intervention, with severity scores for LAB^4 groups remaining lower than PL (16.70 ± 3.64) at Month 3 (10.10 ± 3.27 for LAB$^4{}_{ANTI}$ and 8.00 ± 2.50 for LAB^4; $p < 0.001$). No differences were reported for average symptom severity within group across the intervention ($p > 0.05$) or between LAB^4 groups ($p > 0.05$).

3.6. Race Times

Mean race finishing times are shown in Figure 3. Overall, no significant differences were found between groups for overall finishing times (F = 2.12, $p = 0.149$), despite faster completion times for both LAB$^4{}_{ANTI}$ (13 h 17 min 07 s ± 34 min 48 s) and LAB^4 (12 h 47 min 13 s ± 25 min 06 s) compared with PL (14 h 12 min 51 s ± 29 min 54 s). Faster swim and cycle stage times were also recorded on average for LAB^4 (93.7 ± 4.4 min and 370.7 ± 10.4 min respectively) compared with both LAB$^4{}_{ANTI}$ (99.8 ± 6.5 min and 392.5 ± 16.9 min) and PL (103.6 ± 9.9 min and 405.1 ± 14.3 min). However, average stage times were not significantly different between groups for either swim (F = 0.45, $p = 0.642$) or cycle (F = 2.30, $p = 0.129$) stages.

This was further reflected in the marathon stage, despite both LAB^4 groups completing the marathon course in similar times (285.8 ± 13.1 min for LAB$^4{}_{ANTI}$ vs. 287.41 ± 16.04 min for LAB^4) in contrast to PL (320.8 ± 21.1 min; F = 1.06, $p = 0.368$). It was however noted that despite a non-significant interaction effect, post-hoc comparisons indicated a significant difference for the bike stage between LAB^4 and PL groups only ($p = 0.046$), and a strong trend for a significant differences in overall finishing times between these two groups ($p = 0.058$).

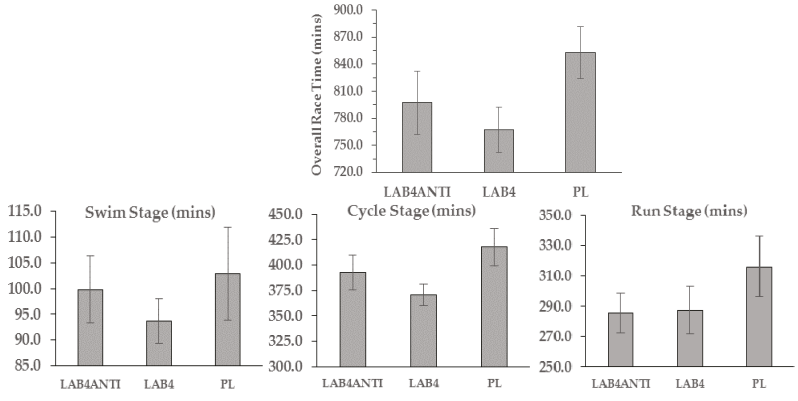

Figure 3. Race time comparisons in minutes (Mean ± SE), including triathlon stage disciplines. No significant differences reported between groups ($p > 0.05$). Converted overall times: LAB$^4{}_{ANTI}$ = 13 h 17 min 07 s (±34 min 48 s); LAB^4 = 12 h 47 min 47 s (±25 min 31 s); PL= 14 h 01 min 40 s (±31 min 32 s).

4. Discussion

The concept of exercise-mediated endotoxemia remains contentious, with varying terminology reported in the literature, including methodologies used to assess endotoxin units. Whereas some authors have referred to the term "mild" endotoxemia to reflect relatively small changes in endotoxin levels (5–15 pg·mL^{-1}) along with acute cytokinemia following sustained endurance exercise [9], others have suggested that values ranging from 10 to 50 pg·mL^{-1} are indicative of normal, yet sustained "low grade" endotoxin levels which may modulate a systemic inflammatory response [13,16]. Clinical states of endotoxemia reflect much higher endotoxin concentrations (>80–300 pg·mL^{-1} [11,12]), with only a handful of studies demonstrating that strenuous ultra-endurance exercise actually elicits these levels at the point of exhaustion or during acute recovery [10,15]. Less is known whether repetitive GI provocation from repeated training elevates resting endotoxin levels, and what impact this may have on individuals preparing for, or recovering from, long distance events.

Average resting endotoxin units in the current study remained within normal limits (<10 pg·mL^{-1}), and were comparable to values observed (~11.0 $\pm$ 5.0 pg·mL^{-1}) for healthy volunteers with similar fitness levels [14,39,40]. This is in contrast to our hypothesis that plasma endotoxin units would be raised following repetitive endurance exercise as evidenced elsewhere [13,15]. However, the range for endotoxin units was 3.03–27.75 pg·mL^{-1}, indicating that if "low grade endotoxemia" does occur at values >10–50 pg·mL^{-1}, then some individuals may be susceptible to repeated exposure.

LPS translocation across the GI tract is known to provoke systemic immune reactions with varied consequences [41]. Specifically, LPS attachment to LPS-binding protein and its transference to an MD-2/toll-like receptor (TLR) 4/CD14 complex activates NF-kappa-B and various inflammatory modulators (TNF-α, IL-1β, IL-6 and CRP). This is considered a protective mechanism acting to minimise bacterial entry across the GI tract. Under normal physiological conditions, endotoxins from gram negative bacteria are usually contained locally, with only relatively small quantities entering the systemic circulation. However, when GI defences are either disrupted (i.e., luminal damage from exercise) or LPS "sensing" is "overloaded" a heightened inflammatory response may result which could, in part, relate to GI symptoms associated with exercise [42]. This could have implications to daily recovery mechanisms throughout prolonged training periods, and in the days following ultra-endurance events.

The use of a 12-week LAB^4 strategy reduced average endotoxin units by 26.0%, but was not statistically significant. In contrast, the LAB$^4{}_{\text{ANTI}}$ intervention resulted in a significant 46.6% reduction in endotoxin units, with pre-race levels in this group reducing to 4.37$\pm$ 0.51 pg·mL^{-1}. These levels are comparable to resting values observed in trained athletes elsewhere at ~3.8 $\pm$ 2.0 pg·mL^{-1} [13], and could have important implications for those individuals with previously raised endotoxin levels (e.g., >20 pg·mL^{-1}) or who are more susceptible to training related GI symptoms. Whilst the general trend in IgG anti-EU supported these findings, the inter-individual variability observed resulted in non-significant findings. It was noted that average IgG anti-EU levels for LAB$^4{}_{\text{ANTI}}$ were, however, significantly lower than both LAB^4 and PL. Although this possibly indicates an adaptive response in this group, IgG anti-EU ranges observed were comparable to those reported elsewhere [43] and most likely reflect variance in relation to individual gut microbiota profiles.

Altered GI permeability was only observed in the PL condition, which whilst not reaching clinical significance (i.e., L:M $\geq$ 0.09; [44,45]), represented a 4.2 fold increase over the intervention and recovery periods (compared to a 0.7 fold increase in the L:M ratio for LAB$^4{}_{\text{ANTI}}$ and 0.6 fold increase for LAB^4). Additionally, both GI symptom count and severity were significantly lower in both LAB^4 interventions compared with PL by the end of the training period, observations similar to those reported elsewhere employing probiotic strategies [21,46,47]. Collectively, these results support the contention that a multistrain pro/prebiotic intervention maintains tight junction stability, potentially through interference with phosphorylation processes. Although this supports previous findings [26,48], such strategies may only apply to chronic interventions, as recent research has demonstrated no impact

of acute (7 days) probiotic use on endotoxin levels following endurance exercise at 60%VO_2max under ambient or heat-stressed conditions [49].

Studies have demonstrated that regular use of probiotics can improve epithelial resistance by establishing competitive "biofilm" activity. Indeed, as LPS types vary across gram-negative bacteria species, some LPS are poorly sensed by TLR4 and may have more direct impact on NF-κ-B activation [50]. Therefore, direct exclusion of LPS translocation through maintained epithelial integrity and/or increased preponderance of gram-positive genera may offer potential therapeutic benefit [51]. Specifically, the provision of *Lactobacillus* genus may work by activating TLR2 and hence more favourable innate immune responses [52–54]. Additionally the use of a 14 week multistrain probiotic strategy significantly decreased faecal zonulin levels elsewhere, supporting improved tight junction stability [26].

However, effects of probiotics are strain specific. The product used in the current study does not appear to have been used in a training context previously. Clinical trials, however, have demonstrated that the inclusion of the *Lactobacillus* strains CUL-60 and CUL-21 modulated the facultative anaerobes (*Enterobacteriaceae*, *Enteroccus/Streptococcus* and *Staphylococcus* species) during antibiotic therapy [24,55]. Two other papers utilizing similar dosages to the current study also indicate that the CUL-60 and CUL-21 strains prevented an increase in antibiotic resistant *Enterococci* and reduced the incidence of *Clostridium difficle* toxins [25,56]. Future research should address strain specific colonization and impact on gut microbiota, which may explain inter-individual differences particularly in athletes.

The inclusion of *Bifidobacterium* and prebiotics (e.g., inulin, galacto- or fructo-oligosaccharides [FOS]) in such formulas may also provide additional benefits. Studies have demonstrated a significant increase in short-chain fatty acids (SCFA), with prebiotic use additionally supporting increased *Bifidobacteria* growth, and decreased levels of bacteriodes and *Fermicutes* phyla [57–61]. Additionally, prebiotic use has been shown to improve mucosal dendritic cell function associated with TLR2 activity [62], and increase the expression of glucagon-like peptide 2, associated with GI barrier regulation [59]. Although low dose FOS was employed in the current study, the "synbiotic" effect with a multistrain probiotic formula has been shown to confer improvements in gastrointestinal well-being elsewhere [63].

In the current study, a combined antioxidant in conjunction with a multistrain pro/prebiotic strategy appeared to confer an additive effect through reduced endotoxin unit levels at the end of the 12-week training period, as well as six days post-race. Specifically, alpha lipoic acid acts as a multi-functional antioxidant through rapid regeneration of glutathione [64,65]. GI epithelial damage may be directly associated with oxidative stress from GI ischemia (particularly hydrogen peroxide), and in extreme cases may lead to ischemic colitis or infarct tissue [66]. Endogenous glutathione peroxidase may be a crucial enzyme in the protection of the intestinal lumen from repetitive damage [67]. Alpha lipoic acid, along with *N*-acetyl-carnitine, may therefore act in a local antioxidant manner, and via phosphoinositide 3-kinase/Akt signalling may down-regulate LPS stimulation of NF-κ-B [68–70]. Other dietary antioxidants such as ascorbic acid have been shown to blunt the endotoxin response to exercise, but with secondary effects on ascorbate radical production [28]. Various flavonoids (e.g., quercetin found in onions) and isoflavones (e.g., genistein found in soybeans) have been shown to inhibit protein kinase C and protein tyrosine kinases respectively, although the use of 2 g·day^{-1} quercetin for seven days was also shown to block the rise in heat shock protein 70, potentially restricting thermotolerant adaptation [71].

To date, only two studies appear to have assessed endotoxin levels in the hours/days following ultra-endurance events. Subclinical symptoms associated with exercise-mediated endotoxemia may vary in both severity and duration (possibly lasting several days). One study demonstrated raised (but effectively normal) endotoxin levels at 16 h following an ironman triathlon, but did not assess return to baseline levels [9]. A further study demonstrated that endotoxin levels had returned to baseline 1–3 weeks post event, reflecting the exhaustive nature of the event [10]. A limitation of the current study was the logistical difficulty of collecting samples immediately or 24

h post event. With varying individual travel plans, participants were instructed to rest in the 5 days post-race. At six days post-race, endotoxin units remained unchanged with PL (8.02 ± 1.14 pg·mL^{-1}), but were significantly lower for both intervention groups (5.18 ± 0.57 pg·mL^{-1} for LAB^4$_{ANTI}$, and 5.01 ± 0.28 pg·mL^{-1} for LAB^4). This represented an overall reduction in endotoxin units from baseline of 36.8% for LAB^4$_{ANTI}$ and 43.9% for LAB^4 strategies. Although cytokine profiles were not assessed in the current study, a general reduction in endotoxin levels via pro/prebiotic/antioxidant combinations may have important benefits in minimizing low grade cytokinemia from endurance exercise [72,73].

The use of LAB^4$_{ANTI}$ or LAB^4 did not significantly improve times within-race only in direct comparison to PL. This is despite a 6.5% (~56 min) faster overall time for LAB^4$_{ANTI}$ compared to PL, and 10.0% (~86 min) faster for LAB^4 compared to PL. This did not reflect our original hypothesis, and likely reflects the wider variance in capabilities observed with "recreationally trained" individuals. However, it was noted that faster times were reported for LAB^4 during both swim and cycle stages, with a trend towards an overall difference compared to PL ($p = 0.058$). It is acknowledged that exercise performance was not assessed in the current study as baseline measures could not be ascertained. As participants were entering their first long distance triathlon, comparison between groups only provided an insight into whether either intervention strategy offered potential race benefits. Future research should focus on whether combined pro/prebiotic/antioxidant strategies offer direct performance benefits in controlled settings, particularly in individuals more susceptible to GI related issues.

5. Conclusions

Chronic multistrain pro/prebiotic supplementation during periods of endurance training may provide individual support to minimise GI symptoms through maintenance of intestinal permeability. The inclusion of an antioxidant strategy (e.g., α-lipoic acid/*N*-acetyl carnitine) may confer additive benefits via reductions in training-related endotoxin unit levels. In a recreationally trained cohort, LAB^4$_{ANTI}$ or LAB^4 strategies did not influence race times in direct comparison to a control group also undertaking their first long distance triathlon. Combined pro/prebiotic/antioxidant strategies may have important implications for individuals undertaking endurance training, particularly those more susceptible to GI symptoms.

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7

Dietary Intake of Athletes Seeking Nutrition Advice at a Major International Competition

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Abstract: International travel and short-term residence overseas is now a common feature of an elite athlete's competition schedule, however, food choice away from home may be challenging and potentially impact on performance. Guidelines for dietary intake specific to competition exist for athletes, however, there is little evidence available to ascertain if athletes meet these recommendations during competition periods, particularly when food is provided in-house. During the Delhi 2010 Commonwealth Games, dietitians based in the dining hall recorded 24 h dietary recalls with all athletes who visited the nutrition kiosk. Analysis of dietary intake was conducted with FoodWorks (Xyris Pty Ltd., Brisbane, Australia). Overall, athletes reported consuming a median total daily energy intake of 8674 kJ (range 2384–18,009 kJ), with carbohydrate within the range of 1.0–9.0 g per kg of bodyweight (g/kg) (median = 3.8) and contributing to 50% total energy (TE) (range 14%–79%). Protein and fat intake ranged from 0.3–4.0 g/kg (median = 1.7) to 10–138 g (median = 67 g), and contributed to 21% TE (range 8%–48%) and 24% TE (range 8%–44%), respectively. Athletes reported consuming between 4 and 29 different food items (median = 15) in the previous 24 h period, with predominately discretionary, grains/cereals, meats, poultry, fish, eggs, and meat alternative items. This suggests that dairy, fruit, and vegetable intake may be suboptimal and intake of the micronutrients iron, zinc, calcium, and vitamins A and C may be of concern for a number of athletes.

Keywords: dietary intake; athlete; international competition

1. Introduction

International travel and short-term residence overseas is now a common feature of an athlete's competition schedule, however, differing eating arrangements and food options when away from home may influence an athlete's food choice, and potentially their performance. An athlete needs to consume suitable food and fluid prior to, during, and after competition in order to maximise performance [1,2]. While sport-specific recommendations exist for athletes to ensure that they consume sufficient total energy (TE) to meet requirements, carbohydrate (CHO) to replenish glycogen stores, protein to aid in muscle repair and growth, as well as fluid to stay adequately hydrated [1,3–9], very little evidence is available to ascertain if athletes meet these recommendations in residence during major international competitions.

While appropriate nutrition is important for performance, investigation into the dietary intake of high performance athletes is limited. Although there appears to be considerable individual variability in dietary intake, the majority of studies to date show that athletes tend on average to meet current evidence-based recommendations for protein, but not CHO [10–14]. This is particularly evident in females [15]. A number of studies have reported that TE intake may also be below expected requirements [14,16,17]. However, these studies are limited by the difficulties experienced when

attempting to accurately measure dietary intake. Discrepancies exist between methods of data collection (for example a 24 h recall vs. 7 days weighed food diaries), whether the athlete is in a competition or training phase (or a combination of both), if the athlete is living at home or in a training camp, differing physiological requirements (e.g., strength and power athletes vs. endurance athletes), level of competition, and specific behaviours that may be associated with a particular sport (e.g., methods to make weight in weight-category sports). Assessing dietary intake is further made difficult by the practice of underreporting, where individuals report consuming less food than actual intake [18].

Additionally, the majority of research on dietary intake in athletic populations primarily focuses on quantifying dietary intake in regards to energy and macronutrient content; however, this does not guarantee that the athlete is selecting foods that contribute to a high quality diet. Diet quality is a concept based on the variety and type of foods in an entire diet, the relationship between health status and food groups, and is usually assessed by comparison to national dietary guidelines or similar, and the diversity of choices apparent within the diet [19]. To date, very little research on diet quality within the athletic population exists, with the exception of a comparison of the diets of a select group of Polish athletes to the Swiss Food Pyramid. This study found that athletes did not meet the recommendations for a number of food groups within the food pyramid guide [20]. Sufficient variety of foods from all core food groups is not only important for sports performance; it is often indicative of micronutrient intake and thus linked to prevention of deficiency and decreased risk of chronic disease [21], and therefore warrants investigation.

While literature to date provides some information on dietary intake of athletes during both training and competition phases, limited data is available regarding intake while in residence at international competition events. At major events such as the Olympic (OG) and Commonwealth Games (CG), the majority of athletes and their support team live in a village residence and dine in a large communal dining hall. An extensive range of food is provided free of charge, 24 h a day, for the duration of the competition. While recent data shows that athletes attending these events are generally satisfied with the food provided [22,23], data on actual dietary intake is limited. Only one study has investigated the types of diets (regimens) that are followed by these athletes [24]. Apart from records collected in 1949 [25] and 1964 [26], the most recent and relevant data on dietary intake in this type of environment was collected at the Sydney 2000 OG [23]. This data on apparent consumption within the dining hall suggested that athletes were consuming on average 592 g of carbohydrate (46% TE and on average 7–10 g/kg BM), 202 g (16% TE) of protein and 197 g (35% TE) of fat daily [23], however, no data on individual consumption was collected. Additionally, no data is available on the variety of foods consumed. Therefore, the aim of this research was to describe the self-reported food and dietary intake of athletes who sought professional guidance in regards to their competition diet immediately prior to or during competition at a major international competition.

2. Materials and Methods

2.1. Data Collection

Australian dietitians (n = 4) based at the main dining hall nutrition kiosk at the Delhi 2010 Commonwealth Games recorded consultations with athletes who requested assistance with their competition dietary intake from 23 September to 14 October 2010. Athletes were asked to provide demographic details including gender, sport (and event if appropriate), country representing, country of birth, and highest level of education (no schooling, primary/middle school, senior school, or University or other tertiary institution). Information about past experience at similar events, stage of competition (more than 2 days before event, day before event, day of event, between events, or event completed) and previous nutrition support was also collected. Athletes were asked to report if they had a nutrition competition plan to follow specifically for this event. Dietitians also recorded the purpose of the athletes visit to the kiosk (e.g., weight loss/making weight, weight gain, training or performance nutrition, and clinical issues such as food intolerance/allergy).

The dietitian then collected a recall of quantity and timing of all food, fluids and supplements consumed by the athlete over the previous 24 h period on a standard proforma, and verified intake of all food groups from a provided checklist as per the USDA five-step multiple pass method [27]. General questions about usual intake as per standard diet history were collected. The 24 h recall template was piloted and reviewed by a panel of expert dietitians prior to use at this competition. This was based on a similar template used in other settings [28]. Upon completion of the interview, dietitians were asked to subjectively rate the athlete regarding their expert opinion of the athletes' dietary intake and nutrition knowledge on a Likert scale of 1 (very poor) to 5 (very good).

2.2. Data Analysis

Participants were classified into a sport category (power/sprint, weight category, endurance, racquet, skill, and team) based on the physiological requirements of their sport, and a region/country (western: Including Australia and the British Isles, and non-western: Africa, Caribbean, India and Sri Lanka, and Southeast Asia and the Pacific Islands) based on location and cultural style of eating [22]. Athletes were also grouped based on the reason for requesting advice at the kiosk including: General weight loss and making weight, weight gain, performance/training nutrition, and clinical issues (for example, food allergy/intolerance, gastrointestinal issues).

The 24 h recall data was coded and input into FoodWorks Premium Edition (Version 7, Xyris software, Brisbane, Australia 2013) (FoodWorks) by the primary researcher. Foods consumed within the dining hall were matched to the nutritional analysis for the specific menu items that had previously been coded in FoodWorks. If not consumed from the menu, the item was coded against the most appropriate matching food. As FoodWorks is a database of Australian and New Zealand foods, items were coded into the 2013 Australian Dietary Guidelines (ADG) five core food groups (1) Grains—grain and cereal based foods; (2) Vegetables—vegetables and legumes/beans; (3) Fruit; (4) Dairy—milk, yoghurt, cheese and/or alternatives; (5) Meat and alternatives—lean meats, poultry, fish, eggs, tofu, nuts and seeds) and discretionary foods [21] for the analysis of diet variety (number of choices from each group). Discretionary foods were defined as per the ADG as containing high amounts of saturated fat, added sugars and/or salt, and alcohol (for example, potato chips, biscuits, pizza and fried foods) [21]. Some items were coded as both discretionary and a core food due to the contribution of macro- and micronutrients to the athlete's diet. For example, a number of discretionary foods were included in the calculation of the main food groups. Cakes/biscuits ($n = 11$), pizza ($n = 6$), Coco-pops™ ($n = 2$), and a muffin ($n = 1$) were included in the calculation of the grains group, as the predominant ingredient is a cereal grain and thus are a source of CHO. All data were cross-checked to ensure consistency and accuracy of coding.

Data were further coded and input into IBM SPSS Statistics (Version 21, IBM Corp., Armonk, NY, USA, 2012) for analysis. Data associations were calculated with the Kruskal–Wallis test, Mann–Whitney U test, independent t test or ANOVA, depending on normality of data. Statistical significance was considered to be $p \leq 0.05$ a priori. Results on nutrient intake are presented as median and range of g per kg of bodyweight (g/kg) and as a percentage of total energy intake (% TE). Micronutrient results were compared to the estimated average requirement (EAR), which is the "daily nutrient level estimated to meet the requirements of half the healthy individuals in a particular life stage and gender group" [29] or adequate intake (AI) which is the average daily nutrient intake level based on observed or experimentally-determined approximations or estimates of nutrient intake by a group (or groups) of apparently healthy people that are assumed to be adequate [29]. Each athlete's 24 h recall data was also compared to recommendations [3–7,9,30] for CHO, protein, and fat intake, specific to type of sport and demographic information (for example, height, weight, age, and gender). Diet variety and dietitians' rating of dietary intake and nutrition knowledge is presented as a median score and range.

2.3. Ethical Approval

Ethical approval was granted by the University of the Sunshine Coast Human Ethics Committee (A/10/253). Participation was voluntary and participants were considered to have given consent to participate by taking part in a consultation.

3. Results

3.1. Participant Characteristics

A total of 44 athletes completed a 24 h dietary recall at the nutrition kiosk, representing 1% of the total number of athletes who competed at this event (n = 4352). However, not all athletes reside in the village, eat within the dining hall, and thus have access to the nutrition kiosk. This cohort was the entire sample of athletes that sought dietary advice. Athletes were predominately from non-western regions and reported competing in 13 specific sports (Table 1). Over half of the athletes reported being in a precompetition stage (n = 30, 68%), with the majority of these athletes greater than 2 days away from competition (n = 28, 82%). The mean self-reported body weight of the male and female athletes was 74 kg (range 56–113 kg) and 65 kg (range 49–101 kg), respectively. Six athletes (14%) reported that they had received nutrition education prior to attending this event. While four athletes (10%) reported having a competition plan to follow, only two of these reported nutrition education prior to this event.

3.2. Dietary Intake and Eating Behaviours

Overall, athletes reported consuming a median total daily energy intake of 8674 kJ (range 2384–18,009 kJ), with CHO within the range of 1.0–9.0 g/kg (median = 3.8 g/kg) and contributing to 50% TE (range 14%–79%). Protein intake ranged from 0.3 to 4.0 g/kg (median = 1.7 g/kg) and contributed to 21% TE (range 8%–48%), while total fat intake ranged from 10 to 138 g (median = 67 g) and contributed to 24% TE (range 8%–44%). Dietary intake varied according to reason for requesting assistance at the kiosk and gender of athletes (Table 2). Those competing in racquet (n = 6) and power/sprint sports (n = 9) reported consuming the greatest energy (median = 11,298 kJ, range 7032–13,485 kJ and 10,149 kJ, range 2472–18,009 kJ, respectively). Athletes competing in skill and power/sprint sports reported consuming the highest protein intake (median = 1.8 g/kg, range 1–3 g/kg, and median = 1.7 g/kg, range 0.5–4 g/kg, respectively), while athletes in team sports (n = 2) reported the lowest energy intake (median = 7274 kJ, range 5953–8596 kJ). Athletes in team (n = 2) and weight category sports (n = 13) reported the lowest contribution of energy from CHO (median = 2.5 g/kg, range 1.7–3.3 g/kg, 39% TE, and median = 3.0 g/kg, range 1.0–6.0 g/kg, 46% TE), while athletes competing in endurance sports (n = 4) reported consuming the lowest amount of fat (median = 51 g, range 35–72 g, 21% TE). There was no significant difference in nutrient intake between those in the pre-versus postcompetition phases.

Three main meals were consumed by the majority of athletes (n = 23, 77%). Overall, the greatest median contribution to total energy intake was from breakfast (29%) to lunch (31%). Carbohydrate contributed a greater proportion to total energy at breakfast (56%) and to snacks (69%) than lunch and dinner. Protein contribution was predominately from meals consumed at lunch and dinner. Fourteen athletes reported consuming sports drinks as a snack, with these drinks contributing to over half of the TE consumed between meals. Five athletes reported not consuming any snacks in the precompetition period. The average contribution of macronutrients to the total daily intake was similar between genders (Table 3).

Dietary analysis showed that 80% of all athletes did not meet the estimated average requirement (EAR) for at least one micronutrient in the previous 24 h, with 25% not meeting the EAR/AI for 5 or more nutrients. Greater than 80% of both genders did not meet the EAR for iron and phosphorus, and vitamin B1–B3 and vitamin C. A greater proportion of women and men did not meet the EAR for vitamin A and magnesium, and zinc respectively (Figure 1). Over two-thirds (n = 39, 90.5%) did not meet the EAR for iron (female M = 13.66 mg, range 3.8–28; male M = 12.32 mg, range 4.2–25 mg). Overall, n = 22 (50%) reported using at least one type of supplement, with almost half (n = 19, 43%) of all athletes reporting the use of a vitamin or multivitamin supplement. Multivitamin supplements were not disclosed in the 24 h recall by any athletes, and therefore were not included in the calculation of dietary intake. Five athletes (11%) reported that they had been previously diagnosed with a nutrient deficiency (one from each of team, skill, power/sprint, weight, and endurance), of which 4 of these reported as iron deficiency anaemia (n = 1, unknown).

Table 1. Demographic information of all athletes who took part in a 24 h recall.

Demographic	TOTAL *n* = 44	Gender		Reason for Consultation [@]			
		Female *n* = 18 (41%)	Male *n* = 26 (59%)	Making Weight or Weight Loss *n* = 26 (59%)	Weight Gain *n* = 4 (9%)	Performance *n* = 9 (21%)	Clinical [#] *n* = 5 (11%)
Age (years) (M ± SD)	26.6 (8)	27.6 (10)	25.9 (7)	25.9 (7)	24 (9)	30.9 (9)	24 (9)
Region * (*n*, %)							
Non-Western [^]	36 (86)	14 (78)	24 (92)	26	4	9	2
Western [$]	8 (14)	4 (22)	2 (8)	-	-	-	3
Sport (*n*, %) [~]							
Endurance	4 (9)	2 (11)	2 (8)	2	-	1	1
Power/Sprint	9 (21)	7 (39)	2 (8)	6	-	1	2
Racquet	6 (14)	2 (11)	4 (15)	1	1	3	1
Skill	10 (23)	4 (22)	6 (23)	6	-	3	1
Team	2 (5)	2 (11)	-	1	-	1	-
Weight	13 (30)	1 (6)	12 (46)	10	3	-	-
Education (*n*, %)							
Middle/Senior School	7 (17)	2 (12)	5 (20)	4	1	-	2
Completed Senior School	17 (40)	6 (35)	11 (44)	12	1	4	-
Attended University	18 (43)	9 (53)	9 (36)	9	1	5	3
Experience (*n*, %)							
First CG/OG	34 (79)	15 (83)	19 (73)	21	3	5	5
First athletes village	30 (70)	14 (78)	16 (62)	18	2	5	5
Previous nutrition assistance (*n*, %)							
	6 (14)	2 (12)	4 (16)	5 (20)	0	0	1 (20)

* 2 responses unknown for region and level of education; [#] Clinical consultations included Coeliac disease, corn allergy, nut allergy and reflux; [^] Bahamas, Bangladesh, Belize, Gambia, India, Kenya, Malawi, Sierra Leone, Sri Lanka, St Vincent, Tanzania, Tonga, Trinidad and Tobago; [$] Australia, England, Falkland Islands, Guernsey; [@] Proportions not calculated for region, sport, education or assistance as numbers in most categories are <10; [~] Endurance includes; athletic events 800 m and over, cycling and swimming distance events; Power/Sprint includes; athletic events under 400 m, athletic field events and swimming sprint events; Racket includes; badminton, table tennis and squash; Skill includes; archery and shooting; Team includes hockey; Weight includes; boxing, weight lifting and wrestling.

Table 2. Energy and macronutrient intake of athletes.

Energy and Macronutrients	TOTAL (All Athletes) Median, Range	Gender Median, Range		Reason for Consultation Median, Range			
		Female (n = 18)	Male (n = 26)	Making Weight or Weight Loss (n = 26)	Weight Gain (n = 4)	Performance (n = 9)	Clinical # (n = 5)
Energy							
kilojoules/day	8674, 2384–18,009	8484, 2473–18,008	9369, 2384–15,175	8632, 2384–18,009	11542, 9622–14,560 *	10798, 5953–14,908	7795, 2473–8091 *
Carbohydrate							
g/day	241, 68–576	244, 81–576	230, 68–512	239, 68–576	342, 208–512	317, 133–478	198, 81–258
g/kg **	3.8, 1.0–9.0	4.2, 1–9	3.5, 0.1–7	3.6, 0.1–9.0	4.3, 4.3–6.2	4.8, 1.7–7.4	3, 1.3–4.6
% TE	50, 14–79	49, 35–64	51, 14–79	50, 14–79	49, 36–57	52, 36–62	49, 38–53
Protein							
g/day	121, 20–276	109, 30–276	127, 20–231	115, 20–276	164, 129–232	120, 55–169	109, 30–163
g/kg **	1.7, 0.3–4	1.7, 0.5–4	1.6, 0.3–3.0	1.6, 0.3–4	1.8, 1.7–2.1	1.6, 0.8–2.1	1.7, 0.5–1.8
% TE	21, 8–48	20, 8–40	22, 9–48	21, 8–48	23, 17–41	18, 10–38	24, 19–40
Fat							
g/day	67, 10–138	65, 13–129	67, 10–138	70, 10–138	79, 57–91	74, 28–115	36, 13–56
% TE	24, 8–44	25, 17–43	22, 8–44	25, 8–44	22, 21–31	25, 16–39	20, 17–27

* Energy, kilojoules/day and reason for consultation (Kruskal–Wallis test, $p = 0.047$); ** $n = 41$ as weight unknown for three athletes; # Clinical consultations included coeliac disease, corn allergy, nut allergy, and reflux.

Table 3. Distribution of energy and macronutrient contribution at meal periods.

Distribution of Energy at Meal Time	TOTAL (All Athletes) Median, Range	Gender Median, Range		Reason for Consultation Median, Range			
		Female (n = 18)	Male (n = 26)	Making Weight or Weight Loss (n = 26)	Weight Gain (n = 4)	Performance (n = 9)	Clinical [#] (n = 5)
Breakfast							
% TE	29, 8–84	26, 8–55	33, 8–84	33, 8–84	34, 16–35	27, 18–40	19, 15–38
% E CHO	56, 22–87	63, 36–87	54, 22–82	55, 22–87	54, 41–59	55, 28–70	71, 63–83
% E PRO	16, 5–39	15, 6–37	16, 5–39	14, 5–39	18, 12–24	17, 13–23	13, 6–18
% E FAT	23, 2–54	22, 3–46	25, 2–54	23, 2–54	32, 12–35	27, 9–50	11, 3–13
Lunch							
% TE	31, 0–72	32, 2–45	30, 0–72	30, 0–72	28, 4–33	32, 18–51	36, 11–45
% E CHO	48, 4–94	45,–81	51, 5–94	46, 5–81	52, 17–94	48, 14–74	35, 4–62
% E PR	26, 2–66	22, 4–55	26, 2–66	28, 4–58	29, 2–66	21, 8–51	34, 7–55
% E FAT	22, 2–48	26, 5–48	17, 2–46	23, 5–45	15, 12–35	17, 11–46	25, 7–48
Dinner							
% TE	26, 0–49	28, 0–49	24, 0–44	25, 0–48	22, 0–42	28, 0–40	28, 0–49
% E CHO	46, 12–85	43, 15–76	49, 12–85	45, 12–85	23, 21–70	50, 15–61	33, 26–76
% E PR	22, 5–62	25, 8–55	22, 5–62	22, 5–49	58, 17–62	19, 15–55	30, 8–44
% E FAT	25, 2–53	28, 5–53	18, 2–50	26, 2–53	16, 7–17	27, 10–43	25, 5–38
Snacks							
% TE	9, 0–54	10, 0–39	9, 0–54	9, 0–39	21, 9–54	7, 0–50	17, 0–31
% E CHO	69, 35–100	72, 35–100	66, 35–100	69, 35–100	54, 41–80	69, 36–100	71, 57–73
% E PR	8, 0–30	6, 0–20	8, 0–30	6, 0–30	10, 5–15	7, 0–13	9, 6–14
% E FAT	18, 0–56	18, 0–53	18, 0–56	18, 0–56	32, 9–41	18, 0–50	14, 8–30

[#] Clinical consultations included coeliac disease, corn allergy, nut allergy and reflux.

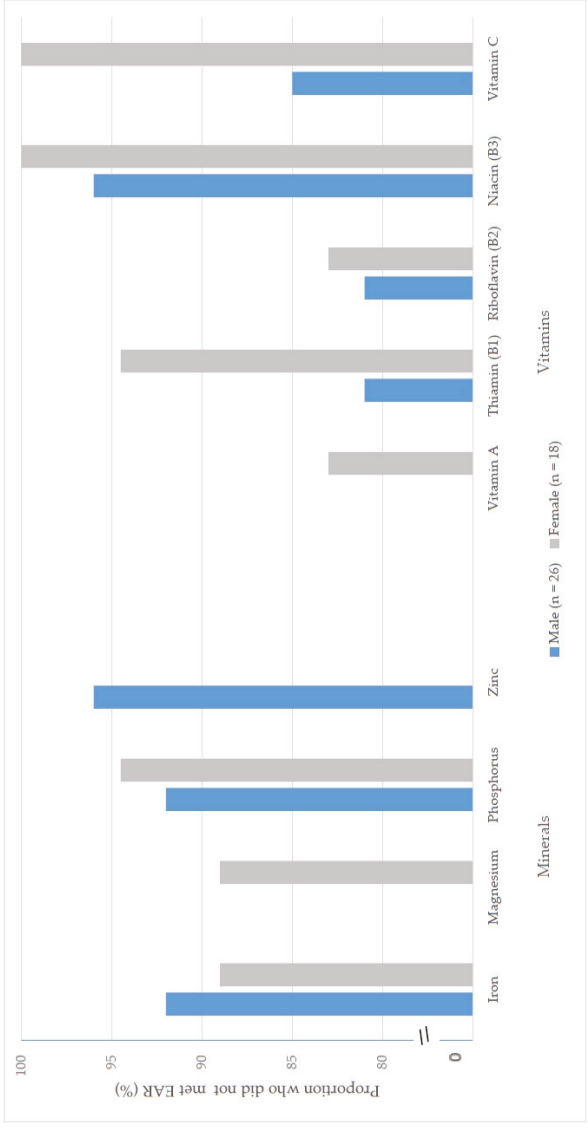

Figure 1. Proportion of male and female athletes who did not meet the micronutrient Estimated Average Requirement (EAR) [#]. [#] Micronutrients displayed are those where greater than 80% of the cohort were below the EAR. The following EAR for 19–50 years old were used: iron male = 6 mg/day , iron female = 8 mg/day; magnesium male 330 mg/day (19–30 years of age), 350 mg/day (31–50 years of age), female 255 mg/day (19–30 years of age), 265 mg/day (31–50 years of age); phosphorus male = 580 mg/day, female = 580 mg/day; zinc male = 12.5 mg/day, female = 6.5 mg/day; vitamin A male = 625 µg/day, female = 500 µg/day; thiamine (B1) male = 1.0 mg/day, female = 0.9 mg/day; riboflavin (B2) male = 1.1 mg/day, female = 0.9 mg/day; niacin (B3) male = 12 mg/day, female = 11 mg/day; vitamin C male = 30 mg/day, female = 30 mg/day. 2 athletes missing data to calculate EAR.

3.3. Food Variety

3.3.1. Number of Total Food Items Consumed

Overall, athletes reported consuming between 4 and 29 different food items (median = 15, range 4–29) in the previous 24 h period, with a broad range of items chosen from the discretionary (median = 4, range 0–10), grain (median = 3.5, range 0–7), and meat (median = 3, range 0–7) groups. Athletes from racquet (median = 20, range 15–24) and team sports (median = 18, range 16–20) reported consuming a greater number of food items than other sports in the previous 24 h (Kruskal–Wallis test, $p = 0.034$).

3.3.2. Number and Variety of Items from Each Food Group

Females reported consuming a greater number (median 3.5 vs. 2.0) and variety (3 vs. 1.5) of fruit choices than males ($p = 0.005$ and $p = 0.001$, respectively). Athletes requesting advice for weight gain reported consuming significantly less variety of fruit items than athletes requesting advice for clinical issues ($p = 0.038$) (Table 4). In addition, athletes competing in weight category sports reported consuming less items from the grains group (median 1.0, range 0–5, $p = 0.028$), as well as a lower variety of grains (median 1.0, range 0–3, Kruskal–Wallis test, $p = 0.017$) compared to other sports. There was no significant difference between athletes from western and non–western regions.

Table 4. Variety of items consumed from each food group and dietitians rating of diet and nutrition knowledge based on gender and reason for requesting assistance.

		Gender Median (Range)		Reason for Consultation Median (Range)			
Variety of Items from Each Food Group [&]	TOTAL Median (Range)	Female (n = 18)	Male (n = 26)	Making Weight or Weight Loss (n = 26)	Weight Gain (n = 4)	Performance (n = 9)	Clinical [#] (n = 5)
Grains	2 (0–6)	2 (0–5)	2 (0–6)	2 (0–5)	2.5 (2–5)	3 (1–6)	2 (0–3)
Vegetables	2 (0–9)	2 (0–9)	2 (0–8)	2 (0–9)	4 (2–8)	4 (0–5)	2 (0–8)
Fruit *	3 (0–8)	3 (0–8)	1.5 (0–4)	2 (0–7)	1.5 (0–2)	2 (0–8)	4 (3–7)
Dairy	1 (0–2)	1 (0–2)	1 (0–2)	1 (0–2)	1 (1–2)	1 (0–2)	1 (0–2)
Meats	2 (0–7)	2 (0–6)	2 (0–7)	2 (0–7)	2.5 (2–4)	3 (1–6)	2 (1–2)
Discretionary [^]	3 (0–7)	3 (1–7)	3 (0–6)	3 (0–7)	4.5 (3–6)	2 (0–6)	3 (1–3)
TOTAL	15 (4–49)	16.5 (7–29)	13.5 (4–24)	13 (4–21)	15 .5 (14–24)	16 (8–29)	15 (9–22)

[#] Clinical consultations included coeliac disease, corn allergy, nut allergy, and reflux; [^] Discretionary foods included: cakes/biscuits/muffin/pastries, pizza, coco-pops™, soft drinks, ice cream, desserts, and chocolate spread; * Significant difference in fruit variety between genders (Mann–Whitney U test $p = 0.001$) and reason for consultation (Kruskal–Wallis test, $p = 0.038$).

3.3.3. Dietitians Rating of Dietary Intake and Nutrition Knowledge

Overall, dietitians rated the dietary intake and nutrition knowledge of athletes as "average" (median 3, range "very poor–good") and "poor" (median 2, range "very poor–good"), respectively (Table 4). There were no significant differences in international regions or gender. Differences in rating were observed depending on reason for requesting assistance (Table 4). Athletes who requested assistance for weight gain and making weight/weight loss (both median 3 "average", range "very poor–good") were rated as having a significantly poorer dietary intake than those requesting performance nutrition (median 3.5 "average–good", range "poor–good") and clinical advice (median 3 "average", range "very poor–good") (Kruskal–Wallis test, $p = 0.03$).

4. Discussion

This research provides a unique insight into the food selection and dietary intake of athletes at a major competition. Although this is based on the results of a small selection of athletes, this is the entire sample of those who actively sought expert advice from dietitians at this event. Our results indicate that there is considerable variability in the dietary intake of these athletes, and despite representing a single day of intake, many report consuming inadequate total energy and CHO for both basic health requirements and athletic performance. The large variation in intake seen in our results

may have to do with differing cultural background, sport category, gender, stage of competition and previous professional advice on dietary intake. In addition, our results indicate that many athletes did not consume a varied diet, with some athletes consuming as little as four or five different items in the previous 24 h period, with many not eating items from a range of food groups. While an athlete can appear to meet macronutrient recommendations based on quantifying intake, the variety of food that is consumed may not provide adequate intake of the micronutrients, fibre, and other food components. Inadequate dietary intake was conferred by the dietitians' general subjective perception of the nutrition knowledge of athletes as poor and dietary intake as average.

In general, we found that the CHO intake of athletes in this study was similar to that observed previously in similar samples [10,11,31], and supports other literature reporting that athletes from varying sports may not consume enough CHO to meet current recommendations for performance [10–14]. While our results are based on one 24 h recall, and we recognise that this may not be indicative of actual habitual intake, there are a number of potential implications on an athlete's performance, as inadequate CHO may compromise storage of muscle and liver glycogen, and may in turn affect physical and/or mental performance. Athletes in this study appeared to consume an adequate amount of protein based on a g/kg BW measure, however as we did not investigate the quantity and timing of protein intake in relation to competition, and this would be worth further investigation. The average contribution of fat to dietary intake varied considerably, but can be considered acceptable for general health [1]. It is also similar to that seen in other athletic populations [10,15,31–33]. The high consumption of discretionary food items appears to have contributed to fat intake, with foods such as pizza, ice cream, biscuits, cake, and muffin commonly consumed by these athletes. The main gender differences appeared to be around the consumption of a variety of fruit, which is not surprising as it is recognised that males typically consume less servings of fruits and vegetables per day than females [34].

We found those athletes that were attempting to make or lose weight were the predominant group to seek advice on their dietary intake. These athletes reported the lowest TE intake of all sports groups and, while not significantly different, appeared to consume less variety of foods with some individuals reporting no foods consumed from a range of food groups. These athletes are required to weigh in at a certain weight in order to participate in their event, and have been reported to reduce their CHO, protein, and fluid intake before competition in a similar environment [23]. A number of these athletes specifically requested assistance for making weight, suggesting that they were under pressure to lose weight within a short time period, and may therefore have already limited their consumption of food. Athletes, particularly those attempting to make weight, may also have inadequate intramuscular stores of CHO after restricting intake, and an insufficient time period in which to try and restore these before competition for maximal performance. It is possible that this may be linked to a lack of professional guidance on dietary intake, as we found that only 5 of the 26 athletes requesting assistance for making weight had reported having dietary advice prior to this event and a nutrition plan to follow. Furthermore, nutrition knowledge of these athletes was rated as poor by the dietitians.

Given the varied intake of TE and macronutrients, and the poor variety of foods consumed in our sample, it is not surprising that a large number of athletes did not meet the EAR/AI for various micronutrients based on their intake in the previous 24 h period. Previous research suggests that athletes generally tend to consume sufficient food to have an adequate intake of most micronutrients with some exceptions in specific athletic groups for vitamin E [11,33], zinc [14], vitamin A, and iron [17]. Interestingly, we found that iron, phosphorus, vitamin C, and the B vitamins were below the EAR in most athletes in this sample. It is likely that our sample had a poorer intake than the rest of the athlete population, as suggested by their reason for seeking a consultation and the dietitians' subjective assessment of their intake. No athlete reported consuming supplements in the previous 24 h, however, half of the athletes reported generally consuming a multivitamin (MV) supplement as part of their normal routine. Clearly, some athletes had poor dietary intake and nutrition knowledge, and a

lack of variety in their diet. This may place the athletes at increased risk of illness, particularly when combined with the stress of travel and competition in a foreign country.

While we did not detect any differences in athletes' diet pre- and postcompetition, the phase of competition may actually change eating behaviours. It is plausible that an athlete will be more focused on meeting nutritional goals prior to, as compared to after, competition [35]. Anecdotally, athletes have been known to "relax" their attitude to eating for performance once their event is over, and have been seen to indulge, or consume foods that they may have avoided prior to competition. While this was not apparent in our sample, this may be due to the characteristics of the athletes who participated in this study.

The unique environment of the athlete's village and the location of training/competition venues at this event may have also influenced our results. The majority of athletes must leave the village and travel to different locations to train or compete. Anecdotally, we noted that a number of comments were made about unsuitable food and snack items being available at various training and competition venues. This may have also led athletes to consume more food before travelling away from the village. There may also be differences in the TE consumed at the dinner meal, as a number of competition events were held at night and this may have influenced when, or if, an athlete could eat this meal.

Additionally, while we asked athletes about stage of competition, we did not ask how long the athlete had been based in the athlete's village. If the athlete had recently arrived in the athlete's village, they may have been experiencing jet lag or a loss of appetite on arrival [36]. Conversely, an athlete who has been based in the athlete's village for a longer period of time may be experiencing menu boredom [22] which may influence food choice. Athletes may also vary from their usual intake as the novelty of attending an elite competition event and living in an athlete's village may distract them from focusing on nutritional goals [36]. A large proportion of athletes (79%) reported that this was their first experience at this type of event. It is possible that athletes with previous experience in this environment may find it easier to locate appropriate items and deal with the challenges of eating in a communal setting. Another important characteristic of the dining hall is the influence of other individuals on the athletes food choice [35,37,38]. Research shows individuals who are dining with strangers will consume less food than usual, while those who dine with familiar individuals tend to consume more [38]. Further research on the influences on food choice in this environment would provide valuable information to those who work with athletes competing at these events.

It is important to note that this sample of athletes were recruited when they approached the nutrition kiosk for assistance, thus may not have known how to choose appropriate foods for their particular sport. A large proportion of the athletes who took part in this study were from less westernised countries. Previous research at this [39,40], and similar events [23,41] has demonstrated that athletes from these regions are more likely to seek nutritional support in this environment. We noted that only six athletes had received professional nutrition advice prior to this event, so a lack of sports-specific nutrition knowledge may also be a reason for the variability in dietary intake. Interestingly, the dietitians did not rate the dietary intake of athletes from western and non-western regions differently.

There are a number of limitations in this research that need to be acknowledged. Our results are specifically focused on athletes who approached the kiosk for a consultation and therefore do not represent every athlete present at this event. While the dietitians used a predesigned form to conduct the 24 h recall, there may have been differences in recording methods, and the subjective rating given for both the assessment of dietary intake and nutrition knowledge. As with any collection of dietary intake data, there are limitations with the use of the 24 h recall method. While this method is quick and can provide in-depth information about dietary intake [42], it is only a measure of a single day, and does not represent usual intake [18,43]. There is also the potential for underreporting, as some individuals may report consuming less than their actual intake [18]. It is also feasible that the menu items did not reflect the original recipe, resulting in inaccurate nutritional analysis. Due to the nature of the data that was collected, we were not able to score diet quality nor determine exact servings

of each food group, but were able to provide an indication of variety of items consumed in the diet, and variety of items from within each food group.

Future Directions

Based on the results of this study, further research on the dietary intake of athletes in this type of environment is warranted. It would be of interest to investigate what athletes consume over a longer period (both before and during competition) with more detailed methods of data collection. Further research with a larger sample would be beneficial, particularly regarding dietary intake and eating behaviours (e.g., snacking, use of sports drinks, diet quality). As food choice is complex, further research on the factors which influence food choice in this environment would provide insight for caterers and dietitians working with individual athlete.

5. Conclusions

Athletes who requested assistance at the nutrition kiosk at a major international competition generally had a poor variety of foods, distribution of, and in some cases inadequate intake of, energy, macro-, and micronutrients. Of particular concern was the dietary intake of athletes who were attempting to make weight or lose weight in the days prior to competition. While this data is limited in that it is only a measure of one day's intake and is based on the athletes recall, this highlights that these athletes may not be consuming a diet that will assist with maximising performance, and if the same dietary habits are followed over a prolonged period of time, health may also be compromised. Further research is required to examine the dietary habits of athletes in this unique environment.

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Author Contributions: For research articles with several authors, a short paragraph specifying their individual contributions must be provided. The following statements should be used "F.E. Pelly and S.J. Burkhart conceived and designed the experiments; performed the experiments; S.J. Burkhart analyzed the data; S.J. Burkhart and F.E. Pelly interpreted the data and wrote the paper". Authorship must be limited to those who have contributed substantially to the work reported.

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8

Dietary Intake, Body Composition, and Menstrual Cycle Changes during Competition Preparation and Recovery in a Drug-Free Figure Competitor: A Case Study

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Abstract: Physique competitions are events in which competitors are judged on muscular appearance and symmetry. The purpose of this retrospective case study was to describe changes in dietary intake, body mass/composition, and the menstrual cycle during the 20-week competition preparation (PREP) and 20-week post competition recovery (REC) periods of a drug-free amateur female figure competitor (age = 26–27, BMI = 19.5 kg/m^2). Dietary intake (via weighed food records) and body mass were assessed daily and averaged weekly. Body composition was estimated via Dual-energy X-ray absorptiometry (DXA) and 7-site skinfold measurements. Energy intake, body mass and composition, and energy availability decreased during the 20-week PREP period (changes of ~298 kcals, 5.1 kg, 6.5% body fat, and 5.4 kcal/kg fat free mass, respectively) and returned to baseline values by end of the 20-week REC period. Menstrual cycle irregularity was reported within the first month of PREP and the last menstruation was reported at week 11 of PREP. Given the potentially adverse health outcomes associated with caloric restriction, future, prospective cohort studies on the physiological response to PREP and REC are warranted in drug-free, female physique competitors.

Keywords: bodybuilders; physique athletes; competition preparation; competition recovery; dieting; energy availability; amenorrhea

1. Introduction

Physique competitions (bodybuilding, figure, and bikini) are unique athletic events in which competitors are judged on muscular appearance and symmetry rather than physical performance. In preparation for these contests competitors aim to decrease fat mass while maintaining lean mass through a combination of prolonged ($\geq$12 weeks) caloric restriction, resistance training, and aerobic exercise [1,2]. Currently, no evidence-based dietary guidelines exist for physique athletes to achieve body mass/composition goals for competition, or to re-gain appropriate levels of fat mass following competition, particularly in a manner that preserves (or at least minimizes risks to) overall health [1,3]. This may contribute to the large number of preparation strategies implemented by coaches and athletes, some of which may be dangerous (extremely low caloric intakes, reliance on un-tested supplements, extreme dehydration, etc.) [4–9]. Healthcare professionals working with these understudied athletes will need to understand the culture and associated constraints of the sport in order to assist competitors in developing nutrition strategies to support their training and competition goals.

Previous research on physique athletes is limited and has mainly focused on male competitors, female competitors using anabolic steroids, and/or the competition preparation (PREP) phase only [1,3,10–17]. Furthermore, the published literature on female physique competitors is limited by: (1) low methodological quality; (2) inadequate description of competition phase; and (3) being dated (e.g., published in the 1980s and 1990s when top-level competitors had lower body masses, and fewer competition categories existed [1,3]. Thus the data may be less applicable to current day physique competitors). Recent case studies of male physique competitors [11,18–20] have provided empirical evidence on the nutritional and exercise regimens, and the associated metabolic and physiological responses of these athletes. To our knowledge, no studies have provided a detailed account of both the PREP and competition recovery (REC) phases in drug-free female competitors. Given the potential health implications (e.g., female athlete triad) of obtaining a low level of fat mass through caloric restriction and exercise [21,22], evaluation of these athletes is warranted. To address gaps in the literature, the purpose of this case study was to describe changes in dietary intake, body mass and composition, and the menstrual cycle in a drug-free, female, figure competitor during both the PREP and REC periods.

2. Materials and Methods

This case study was considered exempt from Institutional Review Board review and approval. It was conducted and prepared in accordance with the Health Insurance Portability and Accountability Act.

2.1. Subject and Timeline Overview

The subject (26–27 years; BMI: 19.4 kg/m^2; body fat: 15%) was a Caucasian, drug-free, amateur figure competitor preparing for her first competition. The subject did not take any medications, including oral contraceptives, during the PREP or REC periods. The 20-week competition PREP and 20-week REC timeline for this competitor, including nutrition and exercise training programs were developed in collaboration with a contest preparation coach who is a certified personal trainer and professional male natural bodybuilder with 20 years of competition and coaching experience. Alterations to the program were determined based on body composition changes and subjective assessment of physique during posing practices. An overview of the timeline for study measurements is presented in Figure 1. In addition, the subject returned for assessment of body mass and composition (DXA) 32 weeks after the competition (i.e., 1 year since the initiation of PREP) and when menses resumed, 71 weeks post competition.

2.2. Dietary Intake

The subject electronically tracked dietary intake via weighed food records, using a commercially available digital food scale (Soehnle Optica®) to the nearest gram throughout PREP. Following the competition, the subject was less motivated to maintain a rigid diet and track intake as diligently. Thus, the 20-week REC period contains estimates of weekly macronutrient and caloric intake from a combination of weighed records and food diary estimates. Nutrient information was obtained from the USDA National Nutrient Database [23] or product-specific nutrition facts panels. Daily nutrient intake information (total kcals, macronutrient (g and %), and fiber (g)) was averaged each week.

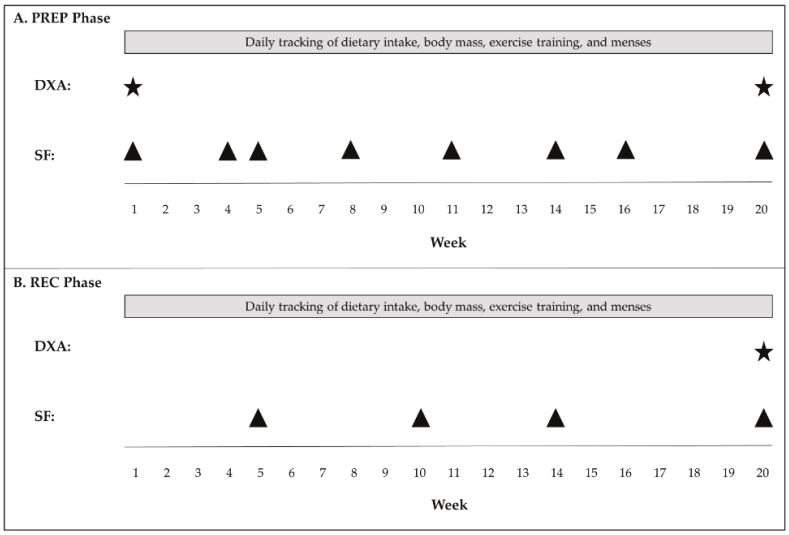

Figure 1. Timeline of Study Measurements (**A**) PREP and (**B**) REC phases. PREP: Competition preparation; REC: Competition recovery; DXA: Dual-energy X-ray absorptiometry; SF: Skinfolds.

2.3. Body Mass and Composition

The subject electronically tracked body mass daily on a commercially-available home scale (Health O Meter Professional®) throughout PREP and REC. Daily body masses were averaged each week. Body composition was assessed just before the PREP period began, the week of the competition (week 20 PREP), and at week 20 of the REC period via dual-energy X-ray absorptiometry (DXA; pre-PREP using Lunar Prodigy Advance, GE Medical Systems, software version 8.10e, Madison, WI, USA; and remainder of scans using Lunar iDXA, GE Medical Systems, software version enCORE 15, Madison, WI, USA model due to equipment upgrading in our laboratory) performed by a trained research technician licensed as a Radiologic Technologist-Limited in the state of Virginia. Skinfold thickness was measured 8 times during the 20-week PREP period and 4 times during the 20-week REC period via 7-site skinfold measurements according to ACSM guidelines [24] and using Jackson-Pollock generalized skinfold equation for body density [25] and the Siri equation for estimating body fat [24] by the subject's contest preparation coach.

2.4. Exercise Training

The subject recorded (paper/pen) exercise training daily for the duration of PREP and REC periods. Exercise Energy Expenditure (EEE) was estimated using the 2011 Compendium on Physical Activity [26].

2.5. Energy Availability

Before and at weeks, 1, 10, and 20 of PREP and weeks 10 and 20 of REC energy availability (EA) ((energy intake (kcals)-EEE (kcals))/fat-free mass (FFM) (kg)) [27] was calculated from the dietary intake record, exercise training record, and estimated FFM of the corresponding week. The established threshold of 30 kcal/kg was used as the reference level for comparing this subject's EA to the level below which adverse health outcomes have been detected [21].

2.6. Menstrual Cycle

Menses was tracked (paper/pen calendar) and reported by the subject for PREP and REC phases.

3. Results

3.1. Dietary Intake

The subject's diet during PREP and REC consisted of 2 days of high carbohydrate intake (~180–230 g/day), 3 days of moderate carbohydrate intake (~150–180 g/day), and 2 days of low carbohydrate intake (~100–150 g/day) each week. High carbohydrate intake days occurred on lower body resistance training days and low carbohydrate intake days occurred on off or low-intensity cardio training days. A sample daily weighed food record is presented in Table 1, representing typical food choices and portions consumed during both PREP and REC. Dietary supplement intake included: whey and casein protein powders, which were calculated into daily caloric and protein intake totals; and 5 g/day of creatine monohydrate from weeks 11 to 20 of PREP.

Table 1. Sample Weighed Food Record Representative of a Typical Day During both PREP and REC.

Food [1]	Preparation and/or Description	Weight (g) (Approximate Volume)
Breakfast:		
Oatmeal	With water	40 (dry) (1/2 cup, dry)
Whey Protein Isolate (De Novo Nutrition)	N/A	20 (2/3 scoop)
Blueberries	Frozen, no sugar added	70 (1/3 cup)
Peanut Butter	Natural, no added oil, sugar, or salt	15 (1 Tbsp)
Morning Snacks:		
Greek Yogurt (Chobani)	Plain, non-fat	150 (1 single-serve container)
Apple	Raw, with peel	180 (1 medium, 3-inch diameter)
Almonds	Raw, unsalted	12 (12 almonds)
Lunch:		
Broccoli	Steamed from fresh or frozen	142 (1 small stalk)
Black Beans	Canned, drained and rinsed	120 (1/2 cup)
Brown Rice, Jasmine	With water, no added oil or salt	98 (prepared) (1/2 cup, prepared)
Hummus (Sabra)	Classic flavor	28 (2 Tbsp)
Whey Protein Isolate (De Novo Nutrition)	N/A	20 (2/3 scoop)

Table 1. *Cont.*

Food [1]	Preparation and/or Description	Weight (g) (Approximate Volume)
Afternoon Snacks:		
Greek Yogurt (Chobani)	Plain, non-fat	150 (1 single-serve container)
Blueberries	Frozen, no sugar added	70 (1/3 cup)
Oatmeal	With water	40 (dry) (1/2 cup, dry)
Whey Protein Isolate (De Novo Nutrition)	N/A	20 (2/3 scoop)
Green Bell Pepper	Raw	164 (1 large, 3-inch diameter)
Dinner:		
Tilapia fillet	Baked, from frozen	114 (1.3 fillets)
Green Bell Pepper	Raw	164 (1 large, 3-inch diameter)
Kale	Raw	100 (6 cups, loosely packed)
Carrot	Raw	100 (0.9 cups, grated)
Red Cabbage	Raw	100 (1.1 cups, chopped)
Extra Virgin Olive Oil	Dressing for kale salad	10 (2 tsp)
Sesame Seed Oil	Dressing for kale salad	5 (1 tsp)
Rice Vinegar	Dressing for kale salad	15 (1 Tbsp)
Sesame Seeds	Whole, dry, dressing for kale salad	5 (1/2 Tbsp)
Brown Rice, Jasmine	With water, no added oil or salt	80 (prepared) (2/5 cup, prepared)
Evening Snacks:		
Almond Butter	Natural, no added oils, sugar, or salt	18 (1 Tbsp)
Beverage Intake [2]:		
Water	N/A	24–48 fl. oz.
Diet Soda	N/A	24–36 fl. oz.
Coffee/tea	Black, unsweetened	24–48 fl. oz.

PREP: Competition Preparation; REC: Competition recovery; Tbsp: Tablespoon. [1] Use of seasonings (e.g., salt) was not weighed or tracked; [2] Beverage intake was not weighed or rigidly tracked. It was reported by participant as typical consumption.

Changes in averaged weekly caloric intake are presented in Figure 2 for PREP and REC. Habitual energy and macronutrient intake at baseline (i.e., before PREP), weeks 1, 10, and 20 of PREP, and weeks 10 and 20 of REC are listed in Table 2.

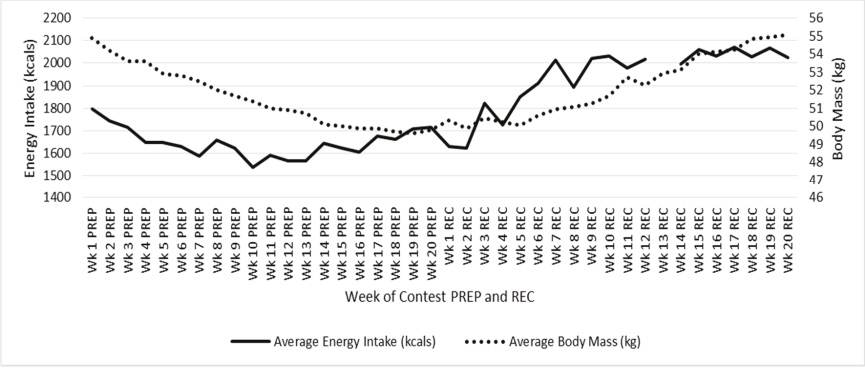

Figure 2. Changes in Energy Intake and Body Mass during Competition Preparation and Recovery. PREP: Competition preparation; REC: Competition recovery.

Table 2. Energy and Macronutrient Intake.

	Energy (kcals/Day)	CHO (g) (% Total kcals) (g/kg BM)	Protein (g) (% Total kcals) (g/kg BM)	Fat (g) (% Total kcals)	Fiber (g)
Baseline	2010	225 45% (4.1 g/kg)	120 24% (2.2 g/kg)	70 31%	48
Week 1 PREP	1798	187 42% (3.4 g/kg)	150 33% (2.7 g/kg)	50 25%	40
Week 10 PREP	1541	143 37% (2.7 g/kg)	150 39% (2.9 g/kg)	41 24%	24
Week 20 PREP	1712	188 44% (3.8 g/kg)	150 35% (3.0g/kg)	40 21%	34
Week 10 REC	2032	219 43% (4.2 g/kg)	146 29% (2.8 g/kg)	63 28%	49
Week 20 REC	2023	233 46% (4.2g/kg)	133 26% (2.4 g/kg)	62 28%	47

PREP: Competition preparation; REC: Competition recovery; CHO: carbohydrate; BM: body mass.

3.2. Body Mass and Composition

Changes in average weekly body mass are presented in Figure 2 for PREP and REC. Body mass decreased from 54.9 kg at Week 1 of PREP to 49.8 kg by Week 20 of PREP, and then increased to 55.1 kg by Week 20 REC. Body fat (assessed via DXA) decreased from 15.1% (8.3 kg) at baseline to 8.6% (4.3 kg) by Week 20 of PREP. Lean mass was maintained at 44.3 kg pre and post PREP (80.7% and 89% lean mass, respectively). By Week 20 REC, percent body fat had returned to baseline at 14.8%. By Week 32 of REC (e.g., 1 year since initiation of PREP), body mass had increased to 57.3 kg and body fat to 20%. By Week 71 REC (when menses resumed) body mass was 56.1 kg and body fat had been maintained at 20%.

Total and site-specific skinfold thickness changes during PREP and REC are presented in Figure 3. Total skinfold thickness decreased from 66.5 mm at Baseline to 30 mm by the week of competition

(Week 20 PREP) (corresponding to a decrease from 14.8% to 8.3% body fat, indicating concordance with the DXA results). Skinfold thickness steadily increased in the REC period and returned to baseline (64 mm) by Week 20 REC.

3.3. Exercise Training

Exercise training during PREP consisted of a high-volume resistance training program 4–5 days/ week (training all major muscle groups of the upper and lower body 2–3 days/week), brief (e.g., 10–30 min) high-intensity interval training 1–2 day(s)/week, and longer (e.g., 45–120 min) aerobic exercise session 1 day/week. This training regimen resulted in an EEE of 484, 459, and 440 kcal/day at weeks 1, 10, and 20 of PREP, respectively. Exercise training during REC consisted of a high-volume resistance training program 3–4 days/week, brief (10–30 min) high-intensity interval training 1–2 day(s)/week, and a longer (45–60 min) aerobic exercise session 1 day/week. This training regimen resulted in an EEE of 355 and 378 kcal/day at weeks 10 and 20 of REC, respectively.

3.4. Energy Availability and Menstrual Cycle

Prior to PREP and at weeks 1, 10, and 20 of PREP, EA was 32.7, 28.2, 23.2, and 27.3 kcal/kg FFM, respectively. At weeks 10 and 20 of REC, EA was estimated to have increased to 36.5 kcal/kg and 35.1 kcal/kg FFM, respectively. Our subject reported a habitual cycle length of ~42 days without use of hormonal birth control for several years prior to engaging in competition preparation. Menstrual cycle irregularity (spotting between typical menses) was reported within the first month of PREP and the last menstruation was reported at week 11 of PREP. Menses did not resume until 71 weeks following the competition.

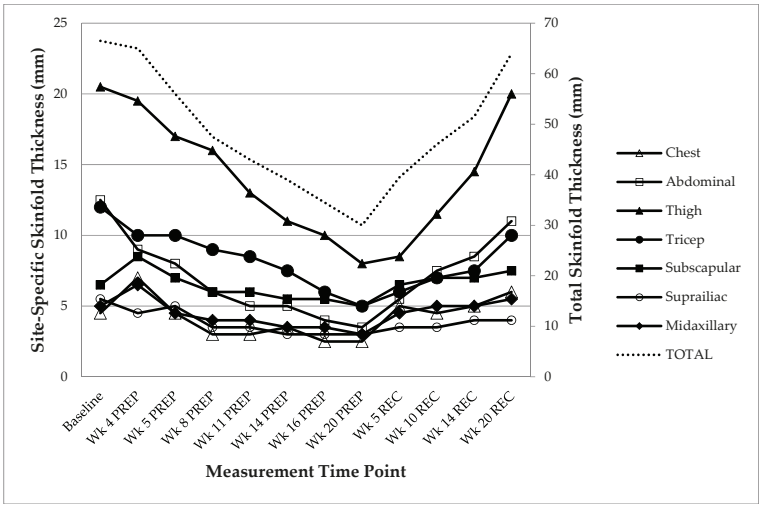

Figure 3. Total and Site-Specific Skinfold Thickness Changes. Wk: Week; PREP: Competition Preparation; REC: Competition recovery.

4. Discussion

This case study provides a detailed and comprehensive examination of the dietary and exercise habits, and the associated alterations in body mass, body composition, EA, and menses during both the PREP and REC phases for a drug free, female figure competitor. The major finding from this investigation was that caloric restriction, low EA, and decreased fat mass led to loss of menses early in the PREP phase. Despite a return to baseline levels of energy intake, EA, and fat mass during the

REC phase, resumption of menses was delayed. In addition, this investigation provides insight on the diet-related culture of the sport which is vital for healthcare professionals working with these clients to be familiar with.

4.1. Dietary Intake

4.1.1. Energy Intake

Competition PREP for our subject consisted of a gradual decrease in energy intake for the initial 10-weeks and then a gradual increase back to week 1 PREP energy intake by week 20 PREP (i.e., week of competition). Our subject's habitual (2010 kcals/day) and PREP energy intake (low of 1541 kcals/day at week 10) were greater than those previously reported in female physique competitors (average of 1636 and 1214 kcals/day, respectively) [1]. Energy intake at competition was similar for our subject and previous reports (1712 vs. 1739 kcals/day) [1]. However, prior investigations only monitored dietary intake for short periods of time, and many did not include information on dietary supplement use [1], thus limiting our ability to compare our more detailed analysis to previous reports.

Following the competition, our subject slowly increased energy intake. This was in an effort to limit a rapid increase in fat mass due to the known propensity for fat accumulation following energy restriction [28]. To our knowledge only one previous study assessed dietary intake following competition in female physique athletes. Walberg-Rankin et al. instructed female bodybuilders to keep 3-day food records the day of until 2 days following the competition and 19 to 21 days following the competition [14]. Compared with their participants' energy intakes in the 1 month prior to the competition (1536–1839 kcals/day), energy intake immediately post competition (3237 kcals) and 3 weeks post was significantly greater (2790 kcals). This was associated with a rapid increase in body mass that was 1.2 kg above their initially reported body mass 1 month before the competition. Our subject was more cautious in the REC period than the athletes previously studied by diligently increasing energy intake slowly and limiting days 'off' the diet.

4.1.2. Macronutrient Intake

During PREP, carbohydrate and fat intake decreased and protein intake increased compared to the subject's baseline dietary habits. Carbohydrate intake fell below the Acceptable Macronutrient Distribution Range (AMDR) of 45%–65% of total caloric intake and protein intake rose above the AMDR of 10%–35%. Our subject's macronutrient intake is consistent with previous reports in male and female physique competitors [1,11,14,20] which show carbohydrate intake below sports nutrition recommendations (e.g., 3–12 g/kg/day depending on training volume/intensity and body composition goals) [29] and protein intake above recommendations for strength training athletes (e.g., 1.2–2.0 g/kg/day) [29]. The elevated protein intake is presumably in an effort to maintain muscle mass (which our subject was successful at doing) during a period of weight loss, and is in line with recent findings demonstrating the efficacy of an increased protein intake during periods of energy restriction [30–32]. Following the competition, increases in energy intake were due to higher carbohydrate and fat intake. Protein intake decreased slightly, but still remained above 2.0 g/kg.

4.1.3. Fiber Intake

Fiber intake decreased during PREP, but remained above the Dietary Reference Intake of 14 g/1000 kcals [33], and increased during REC. This was due to a reliance on nutrient-dense, low-energy foods such as fruits and vegetables [34]. High intake of fibrous foods likely promoted feelings of fullness and enabled this competitor to adhere to the energy restriction [35–39]. In addition, high fruit and vegetable intake is beneficial in ensuring that micronutrient needs are met while energy intake is reduced, and therefore should be included in the development of nutrition recommendations for competition PREP [34,36]. Prior studies in physique competitors have not quantified fiber

consumption or tracked dietary intake as accurately so it is unknown if this is typical practice amongst physique competitors.

4.2. Exercise Training and Body Mass and Composition

Exercise training consisted of a high-volume resistance training regimen and modest amounts of aerobic exercise. Contrary to previous reports from the 1990s [14,16,40], but in agreement with more recent data [1,11] alterations in body mass and composition occurred mainly by reduced energy intake and not increased aerobic exercise. This may be indicative of an overall shift in preparation strategy over time. However, anecdotally we have observed that some current day physique athletes do rely on high levels of aerobic exercise to decrease fat mass before competitions.

Unsurprisingly, 20-weeks of caloric restriction during PREP resulted in reduced body mass and fat mass, which was reversed when caloric intake increased during the 20-week REC phase. Lean mass was maintained during the PREP phase, likely due to a combination of high protein intake and the intensive resistance training program [30,31]. Our subject was lean (~15% body fat) at the start of competition preparation and achieved a body composition (~8%) similar to female bodybuilders previously studied [1,14,16]. While these reported body composition values are less than recommendations for essential fat for women [24], it is likely necessary in order to be competitive in this sport. As noted in the dietary intake section, the REC period has not been well studied in female physique competitors. Given the rapid increase in body mass (which overshot initial body mass) seen in the Walberg-Rankin et al. study [14], and an even more pronounced increase of 8.6 kg gain in body mass by 4 weeks post competition detected by Lamar-Hildebrand et al. in college-aged female bodybuilders [41] compared with the more controlled return to habitual body mass and composition in our subject, the REC period is deserving of additional investigation and likely requires unique dietary intake recommendations.

4.3. Energy Availability and Menstrual Cycle

The EA of our subject fell below recommended levels of 30 kcal/kg of FFM [27] upon initiation of PREP and remained below this level for the entire 20 weeks. In addition, disruption to normal menstruation was reported early and amenorrhea occurred by the end of PREP. Due to an increase in energy intake and a decline in exercise energy expenditure during REC, EA increased to >35 kcal/kg FFM by week 10 of REC. Despite the return of caloric intake and body composition and mass to baseline levels during the 20-week REC period, menses did not resume until 71 weeks following the competition. Interestingly, the subject had returned to our laboratory for assessment of body composition (via DXA) 32 weeks post competition (i.e., 1 year since the initiation of PREP) and at week 71 post competition when menses resumed. Body composition and mass at those two assessment time points (20% body fat, 57.3 kg and 20% body fat, 56.1 kg, respectively) were higher than the habitual body composition and mass (~15% body fat, 54.9 kg) our subject had maintained for years leading up to the competition.

This finding suggest that the reductions to EA and body composition which occur with preparation for physique competitions may have prolonged, detrimental effects on normal reproductive hormonal profiles. Resumption of menses may require that EA and body composition exceed baseline values for a prolonged period of time. Since our subject was concerned about the health implications of amenorrhea, she did not have plans to compete again. However, for competitors who plan to complete yearly, they may not have adequate time in between PREP cycles for menstrual cycle recovery. Prior investigations have noted that both resistance training and energy restriction are associated with alterations to reproductive hormones [14,42–44] leading to menstrual disruption (e.g., increases in estradiol and beta-endorphin which then reduce gonadotropin releasing hormone and luteinizing hormone pulsatility). Therefore, since physique athletes subject themselves to both intensive resistance training regimens and prolonged caloric restriction, they are at greater risk for menstrual disturbance and the associated insults to bone and metabolic health [21,22]. This has

important ethical implications for the advice and treatment provided by coaches and health care professionals who work with these athletes.

Previous research on female athletes has established that those in lean build sports are more likely to have menstrual dysfunction than those in non-lean build sports [45]. However, while more common in this group of athletes than non-athletes, participation in physique competitions does not always lead to menstrual dysfunction [14,46]. Therefore, investigation of individual factors contributing to disruptions of normal menses, as well as analysis of alterations in reproductive hormones during PREP and REC warrant investigation in this at-risk group of athletes.

4.4. Strengths and Limitations

The current study has several strengths. Most notably this is the first to provide detailed, weighed dietary intake analysis over an extended time period (e.g., 40 weeks). This overcomes limitations of previous reports that rely solely on 1–3-day food records or food frequency questionnaires kept for a limited duration leading up to competitions [1]. Second, we accounted for intake of dietary supplementation, which has not been consistently reported in many earlier papers describing the dietary habits of physique competitors [1]. Third, we utilized DXA technology to evaluate changes in body composition. While we did rely on two separate DXA machines due to unavoidable equipment upgrades in our laboratory, the estimates tracked similarly with skinfold estimates, giving us greater confidence in our measurements. Fourth, this study is the first to estimate EA and track menses during PREP and REC. These are important considerations for the development of sports nutrition recommendations that will also support the long-term health of physique athletes. Finally, the case study approach is also a strength since longer-term, detailed information was obtained which would be challenging with a larger cohort [47]. These findings can be utilized to inform future studies in this athletic population.

Despite these strengths, we acknowledge limitations of this study. First, we did not collect biochemical or clinical data (aside from body composition). Future investigations should be done prospectively and plan to obtain blood and urine samples to evaluate alterations in hormonal and metabolite values related to weight loss/gain and menstrual function as well as relevant clinical outcomes (e.g., metabolic rate, blood pressure, heart rate, etc.). Second, we did not include psychological measures. Questionnaires related to dietary restraint, disinhibition, and disordered eating would be valuable to include in future longitudinal research on physique competitors. Third, our participant did not track timing of dietary intake and supplement use throughout the day, or in relation to workouts. While nutrient timing is an important and interesting sports nutrition consideration, this level of detail would likely be unrealistic in investigations of similar duration. Nonetheless, future trials and interventions focusing on this topic may provide important information on nutrient timing strategies to assist physique athletes in achieving their body composition and/or strength goals before and after competitions.

4.5. Future Directions

The popularity of physique competitions is increasing, with a greater number of organizations created and competitions held each year [48]. Therefore, research is needed in order to establish evidence-based nutrition and exercise recommendations related to improving performance, while minimizing potential adverse health outcomes of caloric restriction in these athletes. Randomized- controlled trials will likely be unfeasible in this population. Instead, long-term observational trials which track competitors during PREP and REC phases will be needed. Specific questions to answer include: What is the typical degree of caloric restriction competitors subject themselves to? What is the prevalence of the female athlete triad? What best predicts both performance and maintenance of health in these athletes (e.g., baseline caloric intake, degree of restriction employed, age, dietary composition, exercise energy expenditure, etc.)? What is the time course for recovery of physiological, metabolic, and menstrual responses to competition preparation? What is the long-term impact of several cycles of

competition preparation and recovery on health outcomes? What are the psychological ramifications (e.g., eating attitudes, mood disturbance, and sleep habits) of physique competition participation? Overall, these competitors are a unique and understudied group of athletes whom much can be learned from in regards to the metabolic adaptations to caloric restriction during competition preparation and metabolic recovery during refeeding following competitions.

5. Conclusions

This case study provides the first long-term assessment of dietary intake, body mass/composition, and menstrual cycle changes associated with competition PREP and REC in a drug-free, female figure competitor. As expected caloric restriction and decreased EA led to a decline in fat and body mass and cessation of menses. Energy intake, body mass and fat mass returned to baseline levels by the end of the 20-week REC period. However, return of menstruation was delayed, not resuming until over a year following the competition. Our case study adds long-term, detailed information to the limited literature available on this population. Future studies should build upon this approach in order to lead to the creation of evidence-based dietary intake and exercise recommendations for physique competitors across the competitive cycle that aims to increase 'performance' (e.g., subjectively rated appearance) while maintaining the health of the competitors.

Author Contributions: T.M.H. and J.P.L. conceived and designed the study; T.M.H. collected the data and T.M.H., J.P.L. and B.M.D. analyzed and interpreted the data. T.M.H. was primarily responsible for writing the manuscript with input from J.P.L. and B.M.D. All authors read and approved the final manuscript.

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The Effect of a 20 km Run on Appetite Regulation in Long Distance Runners

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Abstract: The purpose of the present study was to investigate appetite-related hormonal responses and energy intake after a 20 km run in trained long distance runners. Twenty-three male long-distance runners completed two trials: either an exercise trial consisting of a 20 km outdoor run (EX) or a control trial with an identical period of rest (CON). Blood samples were collected to determine plasma acylated ghrelin, peptide YY_{3-36} (PYY_{3-36}) and other hormonal and metabolite concentrations. Energy intake during a buffet test meal was also measured 30 min after the exercise or rest periods. Although plasma acylated ghrelin concentrations were significantly decreased after the 20 km run ($p < 0.05$), plasma PYY_{3-36} did not change significantly following exercise. Absolute energy intake during the buffet test meal in EX (1325 $\pm$ 55 kcal) was significantly lower than that in CON (1529 $\pm$ 55 kcal), and there was a relatively large degree of individual variability for exercise-induced changes in energy intake (−40.2% to 12.8%). However, exercise-induced changes in energy intake were not associated with plasma acylated ghrelin or PYY_{3-36} responses. The results demonstrated that a 20 km run significantly decreased plasma acylated ghrelin concentrations and absolute energy intake among well-trained long distance runners.

Keywords: appetite-related hormones; energy intake; long distance run; athletes

1. Introduction

Appetite regulation is closely associated with circulating hormones secreted from digestive organs. Plasma ghrelin, secreted from the stomach, is known to be the only hormone that promotes hunger and food intake [1]. In contrast, peptide YY_{3-36} (PYY_{3-36}) and glucagon-like peptide-1 (GLP-1) are produced in the gastrointestinal tract. These hormones have the opposite role of ghrelin, resulting in attenuation of appetite [2–5]. Recently, attention to the influence of acute exercise on feeding behavior and its related endocrine regulations has increased. King et al. [6] showed that plasma ghrelin concentrations and subjective feelings of hunger were significantly impaired by 90 min of running at 70% of maximal oxygen uptake ($\dot{V}O_{2max}$). Moreover, Martins et al. [7] demonstrated that plasma GLP-1 and PYY_{3-36} concentrations were significantly increased by 60 min of endurance exercise at 65% of $\dot{V}O_{2max}$. Jokisch et al. [8] suggested that energy intake during a buffet test meal was reduced significantly after exercise compared with rest conditions for sedentary males. Although some inconsistent results still exist [9,10], the attenuating effect of exercise on hunger and energy intake is well established [6,7,11,12]. These findings could contribute to the design of optimal exercise prescriptions for health promotion and protection against obesity.

In contrast to the abundant studies on untrained individuals, appetite regulation after high-intensity (above 80% of $\dot{V}O_{2max}$) and prolonged (>60 min) exercise, which is commonly incorporated into the daily training program of trained athletes, remains under exploration. Sim et al. [13] showed that

high-intensity interval training (HIIT, 15 s sprint at 170% of $\dot{V}O_{2max}$ with 60 s active recovery at 32% of $\dot{V}O_{2max}$) suppressed subsequent ad libitum energy intake and ghrelin concentrations in obese individuals. Deighton et al. [14] investigated the influence of HIIT (30 s all-out sprint with 4 min active recovery at 30 W) on appetite regulation in young untrained males, with the results suggesting that subjective feelings of appetite, as well as ghrelin concentrations, were markedly suppressed following HIIT. However, previous studies which investigated the effects of prolonged exercise (>60 min) on appetite regulation are limited. In particular, the majority of previous studies were conducted in a laboratory setting. To our knowledge, no study found any influence from prolonged exercise among well-trained athletes during actual training in the field. Since trained athletes experience greater exercise-induced metabolic and endocrine responses compared with individuals with lower fitness levels [15], their levels of exercise-induced appetite suppression may be more profound. Reduction of energy intake by exercise (exercise-induced anorexia) may be beneficial for weight management. However, athletes are commonly required to facilitate recovery of energy substrates (e.g., muscle glycogen, intramyocellular lipid) and promote muscle protein synthesis after training. Impaired energy intake after strenuous exercise is thought to delay recovery of exercise capacity and to promote accumulated fatigue. Levenhagent et al. [16] demonstrated that nutrient intake immediately after exercise enhanced glucose uptake and protein synthesis in the leg and whole body muscles when compared with consuming the same meal 3 h after exercise. Considering the importance of nutrient intake during the early phase of the post-exercise period, elucidation of appetite regulation during the early phase of prolonged high-intensity exercise in athletes is valuable.

In the present study, we investigated the time course of changes in appetite-related hormonal responses and spontaneous energy intake after a 20 km outdoor run (approximately 78 min in duration) in trained long distance runners. We hypothesized that the run would result in decreased spontaneous energy intake during a subsequent meal, with lowered plasma acylated ghrelin concentrations and elevated plasma $PYY_{3\text{-}36}$ concentrations.

2. Materials and Methods

2.1. Subjects

Twenty-three male, college endurance runners (age, 20.0 ± 0.3 (mean ± standard error) years; height, 171.2 ± 1.9 cm; weight, 56.3 ± 1.0 kg; BMI, 19.3 ± 0.4 kg/m^2; and $\dot{V}O_{2max}$, 67.1 ± 1.0 mL/kg/min) participated in this study. All subjects belonged to the same running team, which specialized in long-distance running and maintained regular practice (2.5 h/day) 6 times a week. Subjects were informed of the purpose, experimental procedures, and risks of the study, and written informed consent was obtained from all participants. The study was approved by the Ethics Committee for Human Experiments of Ritsumeikan University (BKC-IRB-2014-015), Japan.

2.2. Experimental Design

Prior to conducting experiments, $\dot{V}O_{2max}$ was determined using incremental running test. Subjects started running at 14 km/h and running velocity was increased by 2 km/h every 4 min until 18 km/h. Once the running velocity reached 18 km/h, it was increased by 0.6 km/h every 1 min until exhaustion. Respiratory gases were collected and analyzed using an automatic gas analyzer (AE310S, Minato Medical Science Co., Ltd., Tokyo, Japan). The collected data were averaged every 30 s.

All subjects completed two trials on different days. The first visit was designed as an exercise trial (EX), and the second visit consisted of a trial without exercise (CON). Each trial was separated by 1 week. Due to experimental setting with performing 20 km outdoor run, the present study was conducted without crossover-design to match environmental factor within subjects during a 20 km run. We selected a 20 km run because it was actually incorporated into the training program in long distance runners. Exercise-induced metabolic and hormonal responses, subjective appetite, and energy intake after exercise or rest were compared between the two trials. On the day prior to the trials, the content

of regular practice and calories consumed during dinner were matched to avoid any influence on metabolic and appetitive responses on the following day. Dinner was provided between 8:00 p.m. and 9:00 p.m. and consisted of regular Japanese food. The total calories consumed (1331 $\pm$ 50 kcal) were identical in each trial. Subjects stayed in accommodations at the university, and their scheduled sleep time was set from 11:00 p.m. to 6:30 a.m.

On the measurement days, the subjects arrived at the laboratory at 7:00 a.m. following an overnight fast, and they rested for at least 20 min before blood collection. On the EX day, all subjects completed a 20 km outdoor run between 7:30 a.m. and 10:30 a.m. They were instructed to run at a prescribed pace and their elapsed time was monitored. Heart rate was recorded continuously every 15 s during exercise using a heart rate monitor (Polar RCX5, Polar Electro Oy, Kempele, Finland). Subjects were allowed to consume a total of 400 mL of water during the exercise period. The ambient temperature during the run was 12.1 °C. On the CON day, the subjects did not engage in exercise, and instead rested in the laboratory for a period of time identical to that taken to complete the run. During this period, they were allowed to read books and were required to consume the same amount of water (400 mL) they had consumed on the day of EX. The room temperature was set at 19 °C.

Blood sampling, evaluation of subjective feelings of appetite using a visual analog scale (VAS), and respiratory gas sampling were conducted several times before and after exercise or rest, and 30 min following the 20 km outdoor run or rest period. Thirty min after the run or the rest period, energy and macronutrient intake during a buffet test meal were evaluated.

2.3. Blood Parameters

On the experimental trial days, subjects arrived at the laboratory at 7:00 a.m. following an overnight fast. After resting for 20 min, a baseline blood sample was obtained. A series of blood samples were subsequently collected immediately after exercise or rest, and 30 min following the exercise or rest period. Serum and plasma samples were obtained by centrifugation (10 min, 4 °C) and stored at −80 °C until analysis. From the obtained samples, plasma acylated ghrelin and $PYY_{3\text{-}36}$, serum growth hormone (GH), free fatty acids (FFA), creatine kinase (CK), and myoglobin (Mb) concentrations were measured. Blood glucose and lactate concentrations were measured immediately after blood collection using a glucose analyzer (Free Style, Nipro Co., Osaka, Japan) and a lactate analyzer (Lactate Pro, ARKRAY Co., Kyoto, Japan), respectively. Blood glucose measurements were performed in duplicate and the average values were used. Serum GH concentration was measured using electrochemiluminescence immunoassay. Serum FFA concentration was measured using enzymatic methods. Serum CK and Mb concentrations were measured at a clinical laboratory (SRL Inc., Tokyo, Japan). The intra-assay coefficients of variation (CV) were 1.9% for GH, 1.3% for FFA, 2.8% for CK, and 2.4% for Mb.

For the measurement of plasma acylated ghrelin and $PYY_{3\text{-}36}$ concentrations, blood was drawn into a chilled tube containing EDTA, dipeptidyl peptidase-4 (DPP-IV), protease, and esterase inhibitors. After obtaining plasma by centrifugation at 4 °C, hydrochloric acid (1 mmol/L) was immediately added to the micro tube for acylated ghrelin analysis, following the manufacturer's instructions. Plasma acylated ghrelin concentration was measured using an enzyme-linked immunosorbent assay (ELISA) kit (Mitsubishi Chemical Medicine Corp., Tokyo, Japan). The intra-assay CV was 4.6%. The plasma $PYY_{3\text{-}36}$ concentration was measured using an ELISA kit (Phoenix Pharmaceuticals, Inc., Burlingame, CA, USA) and the intra-assay CV was 6.1%. All ELISAs were performed in duplicate.

2.4. Subjective Feelings of Hunger, Appetite, Perceived Food Consumption, Satiety, and Fatigue

Ratings of subjective hunger, appetite, perceived food consumption, satiety, and fatigue were evaluated using a 100 mm VAS [17] before exercise (or rest), immediately after exercise (or rest), at 15 and 30 min after exercise (or rest), and after the buffet test meal.

2.5. Respiratory Parameters

A resting expired gas sample was collected 20 min after completing the 20 km run or rest. The subjects sat on a comfortable chair, and a respiratory gas sample was collected for 3 min and analyzed using an automatic gas analyzer (AE310S, Minato Medical Science Co., Ltd., Tokyo, Japan) to evaluate oxygen uptake ($\dot{V}O_2$), carbon dioxide output ($\dot{V}CO_2$), ventilatory volume ($\dot{V}E$), and the respiratory exchange ratio (RER). The values were averaged every 30 s. Appropriate calibrations of O_2 and CO_2 sensors and the volume transducer were performed using calibration gases and a 2 L syringe immediately before measurements were taken.

2.6. Ad Libitum Buffet Meal

A buffet test meal was started 30 min after the 20 km run or rest to evaluate energy and macronutrient intake. The meal lasted 30 min; however, the participants were not informed of the elapsed time during the test. All subjects were instructed to "eat until they felt comfortable satiety" in a separate environment from other subjects. The buffet meal consisted of abundant food items eaten regularly in standard Japanese breakfasts and included rice balls, bread, jam, grilled salmon, boiled beef, ham, sausages, boiled eggs, potato salad, natto (fermented soybean), boiled spinach, miso soup, milk, yogurt, cheese, oranges, apples, bananas, green tea, orange juice, and vegetable juice. The energy intake was determined by counting number of plates (calorie for each plate is already known) and by weighting remaining foods after eating. A dietary analysis program (Excel Eiyou-kun version 6.0, Kenpakusha, Tokyo, Japan) was also used to calculate energy intake and macronutrient content.

2.7. Statistical Analysis

Data are expressed as means ± SE. For all variables, normal distribution was confirmed using Kolmogorov-Smirnov test. Time courses of changes in blood parameters and subjective feelings of appetite were compared using a two-way repeated-measures analysis of variance (ANOVA) to determine interaction (trial × time) and main effects (trial, time). When ANOVA revealed a significant interaction or main effect, a Tukey-Kramer post hoc test was performed. Energy intake and respiratory gas parameters were compared between the two conditions using a paired *t*-test. The relationship between the exercise-induced relative change in energy intake and each blood parameter was determined using Pearson correlation coefficients. Statistical significance was accepted as a p-value < 0.05.

3. Results

3.1. Exercise Duration and Heart Rate Response during the 20 km Run

The average time taken to complete the 20 km run was 77.9 ± 0.3 min. The average heart rate (HR) during exercise was 157 ± 3 beats/min. The estimated percentage for maximum HR was 78.0% ± 1.3%.

3.2. Scores for Subjective Appetite and Fatigue

Table 1 shows the time-course changes in subjective scores for appetite and fatigue. A significant interaction (trial × time), as well as main effects of trial and time, were observed for hunger and appetite ($p < 0.05$). Hunger scores were significantly lower in EX than in CON immediately and 15 min after exercise ($p < 0.05$); however, this significant difference was not observed between the trials 30 min after exercise. Similarly, scores of appetite were significantly lower in EX compared with CON immediately and 15 min after exercise ($p < 0.05$). A two-way ANOVA revealed a significant interaction (trial × time) effect, and a main effect of time for perceived food consumption ($p < 0.05$). Perceived food consumption was significantly lower in EX compared with CON immediately after exercise ($p < 0.05$). Significant main effects of trial and time for satiety were observed ($p < 0.05$). Satiety

scores were significantly higher in EX than in CON immediately, 15 min and 30 min after the exercise period ($p < 0.05$). Two-way ANOVA revealed a significant interaction effect (trial × time), as well as main effects of trial and time, for fatigue. In EX, scores for fatigue were significantly increased immediately and 15 min after exercise ($p < 0.05$). In addition, fatigue scores were significantly higher in EX than in CON at all time points after the exercise period ($p < 0.05$).

Table 1. Change in scores of subjective feeling of appetite and fatigue.

		Pre	Post			After Meal
			0 min	15 min	30 min	
Hunger (mm)	EX	57 ± 4	51 ± 6 †	60 ± 5 †	68 ± 4	11 ± 1 *
	CON	60 ± 3	70 ± 3 *	71 ± 3 *	72 ± 3 *	16 ± 3 *
Appetite (mm)	EX	59 ± 5	52 ± 7 †	62 ± 5 †	68 ± 5	18 ± 4 *
	CON	58 ± 4	70 ± 3 *	72 ± 3 *	72 ± 3 *	23 ± 4 *
Prospective food consumption (mm)	EX	63 ± 4	54 ± 6 †	61 ± 5	69 ± 4 *	20 ± 4 *
	CON	58 ± 4	67 ± 3 *	70 ± 2 *	68 ± 3	22 ±3 *
Satiety (mm)	EX	30 ± 4	29 ± 5 †	32 ± 5 †	31 ± 5 †	82 ± 4 *,†
	CON	26 ± 3	20 ± 3	18 ± 5	18 ± 3	70 ± 5 *
Fatigue (mm)	EX	43 ± 4 †	58 ± 4 *,†	55 ± 4 *,†	52 ± 4 †	41 ± 4 †
	CON	29 ± 3	26 ± 3	23 ± 3 *	24 ± 4	26 ± 4

Values are means ± SE. *: $p < 0.05$ vs. pre, †: $p < 0.05$ vs. CON.

3.3. Blood Parameters

Table 2 shows the time-course of changes in blood glucose, lactate, serum GH, FFA, Mb, and CK concentrations. No significant differences between the trials were observed at baseline (before exercise or rest) for any blood parameters, expect for blood glucose concentrations. A significant interaction (trial × time) and a main effect of time were observed. Blood glucose concentrations were significantly increased immediately after the exercise period compared with CON ($p < 0.05$). However, blood glucose concentrations were significantly lower in EX compared with those in CON 30 min after exercise ($p < 0.05$). No significant interaction (trial × time), or main effects of time or trial were observed for blood lactate concentrations ($p < 0.05$). Blood lactate concentrations did not significantly change from baseline values in either trial. Two-way ANOVA revealed a significant interaction (trial × time), as well as main effects of time and trial, for serum GH, FFA, and Mb concentrations. Serum GH concentrations were significantly increased after exercise in EX ($p < 0.05$). Thirty min after exercise, serum GH concentrations remained significantly higher in EX compared with CON ($p < 0.05$). Serum FFA concentrations were markedly increased after exercise ($p < 0.05$), and were significantly different to those in CON ($p < 0.05$). Serum Mb concentrations were significantly increased immediately and 30 min after exercise ($p < 0.05$), and were also significantly different between EX and CON ($p < 0.05$). Lastly, a significant interaction (trial × time) as well as main effects of time for serum CK concentrations were observed. Although serum CK increased significantly with exercise ($p < 0.05$), there was no significant difference between the trials at any point (main effect of trial; $p > 0.05$).

Figure 1 shows the changes in plasma acylated ghrelin concentrations. Significant main effects of time and trial were observed for plasma acylated ghrelin ($p < 0.05$). Plasma acylated ghrelin concentrations at baseline were significantly lower in EX compared with CON ($p < 0.05$) and exercise significantly decreased plasma acylated ghrelin concentrations immediately after the exercise period (before exercise, 20.2 ± 1.4 fmol/mL; immediately after exercise, 17.3 ± 1.7 fmol/mL, $p < 0.05$) with a significant reduction relative to CON ($p < 0.05$). Thirty minutes after exercise, plasma acylated ghrelin concentrations remained significantly lower in EX than in CON ($p < 0.05$). In contrast, the CON trial did not show significant change in acylated ghrelin concentration over time.

Figure 2 shows the time-course change of plasma $PYY_{3\text{-}36}$ concentrations. Two-way ANOVA revealed a significant main effect of the trial for plasma $PYY_{3\text{-}36}$ concentration. Although plasma $PYY_{3\text{-}36}$ concentrations at baseline were significantly lower in EX than in CON ($p < 0.05$), there was no

significant difference between the trials after exercise. Furthermore, plasma PYY_{3-36} concentration did not change significantly from baseline values in either EX or CON.

Table 2. Change in blood variables.

		Pre	Post	
			0 min	30 min
Glucose (mmol/L)	EX	4.92 ± 0.05 †	5.30 ± 0.12 *,†	4.64 ± 0.08 *,†
	CON	4.78 ± 0.05	4.84 ± 0.05	4.93 ± 0.04 *
Lactate (mmol/L)	EX	1.6 ± 0.2	1.6 ± 0.2	1.6 ± 0.1
	CON	1.4 ± 0.1	1.5 ± 0.1	1.4 ± 0.1
GH (ng/mL)	EX	1.8 ± 0.5	8.9 ± 1.8 *,†	4.1 ± 0.8 †
	CON	2.5 ± 0.6	2.1 ± 0.4	1.3 ± 0.3
FFA (mmol/L)	EX	0.42 ± 0.05	1.22 ± 0.08 *,†	0.90 ± 0.08 *,†
	CON	0.38 ± 0.03	0.35 ± 0.03	0.55 ± 0.04 *
Mb (ng/mL)	EX	36 ± 2	136 ± 26 *,†	140 ± 22 *,†
	CON	37 ± 3	36 ± 2	35 ± 2
CK	EX	349 ± 27	457 ± 30 *	436 ± 29 *
(U/L)	CON	402 ± 74	389 ± 68 *	385 ± 70 *

Values are means ± SE. *: $p < 0.05$ vs. pre, †: $p < 0.05$ vs. CON.

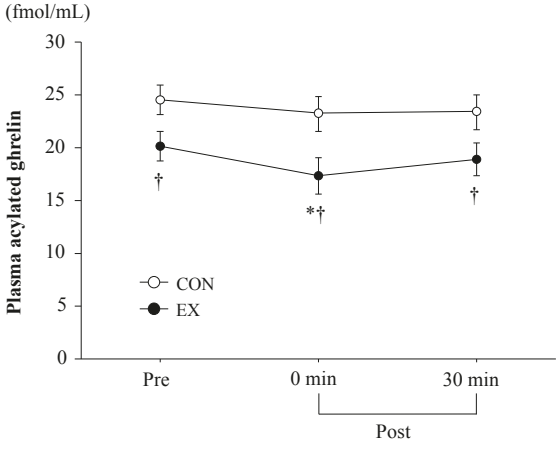

Figure 1. Change in plasma acylated ghrelin concentrations. Values are means ± SE. * $p < 0.05$ vs. pre, † $p < 0.05$ vs. CON.

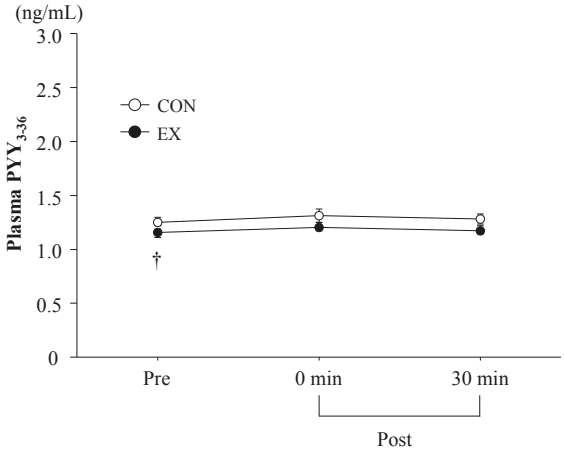

Figure 2. Change in plasma PYY_{3-36} concentrations. Values are means ± SE. † $p < 0.05$ vs. CON.

3.4. Respiratory Parameters

Resting $\dot{V}O_2$ and $\dot{V}CO_2$ after exercise were significantly higher in EX than in CON ($\dot{V}O_2$, 271 ± 5 mL/min in EX vs. 233 ± 8 mL/min in CON; $\dot{V}CO_2$, 202 ± 6 mL/min in EX vs. 182 ± 6 mL/min in CON, $p < 0.05$). Moreover, there was a trend toward lower RER in EX than in CON (0.75 ± 0.02 in EX vs. 0.78 ± 0.02 in CON, $p = 0.056$) and toward higher $\dot{V}E$ in EX than in CON (8.7 ± 0.4 L/min in EX vs. 7.9 ± 0.3 L/min in CON, $p = 0.057$).

3.5. Energy and Macronutrient Intake

Table 3 shows the energy intake, macronutrient intake ratios, and types of menu selected during the buffet test meal. The time required to finish eating was not significantly different between EX and CON. Energy intake was significantly lower in EX (1325 ± 55 kcal) compared to CON (1529 ± 55 kcal, $p < 0.05$); the exercise-induced relative change in energy intake was −12.9% ± 2.8%. With regard to macronutrient distribution, fat intake was significantly lower in EX than in CON ($p < 0.05$), while carbohydrate intake was significantly higher in EX than in CON ($p < 0.05$). Moreover, comparing the caloric intake within four categories of food (staple foods, others, fruits, and drinks) among the 21 different menus indicated that calories consumed from staple foods, including carbohydrates (e.g., rice, bread) and others (e.g., fish, meat) were significantly lower in EX ($p < 0.05$). In contrast, calories consumed from drinks including tea, juice, milk, and soups were slightly but significantly greater in EX ($p < 0.05$).

Table 3. Energy intake, macronutrient intake ratio and categories of selected menus.

		EX	CON
General information			
Duration of eating	(min)	22 ± 1	23 ± 1
Energy intake	(kcal)	1325 ± 55 †	1529 ± 55
Detailed information			
Macronutrient intake			
Protein	(%)	14.6 ± 0.5	15.3 ± 0.5
	(g)	49 ± 3 †	58 ± 2
Fat	(%)	26.2 ± 1.4 †	29.8 ± 1.2
	(g)	39 ± 3	51 ± 3
Carbohydrate	(%)	59.2 ± 1.9 †	54.9 ± 1.5
	(g)	190 ± 9	202 ± 10
Categories of selected menus			
Staple food (rice and bread)	(kcal)	545 ± 34 †	659 ± 45
Others	(kcal)	553 ± 38 †	694 ± 28
Fruits	(kcal)	101 ± 15	75 ± 12
Drinks (tea, juice milk and soup)	(kcal)	126 ± 11 †	101 ± 16

Values are means ± SE. †: $p < 0.05$ vs. CON.

3.6. Inter-Individual Variability in Exercise-Induced Changes in Energy Intake

Figure 3 shows the individual data of exercise-induced relative changes in energy intake [(energy intake in EX − energy intake in CON)/energy intake in CON × 100]. A relatively large individual difference in energy intake was observed (ranging from −40.2% to 12.8%). In total, 3 of 23 subjects (13.0%) had increased energy intake after the exercise period compared with rest, while there were no differences in intake between the trials in 2 subjects (8.7%). However, 18 subjects (78.3%) had reduced energy intake after exercise compared with rest.

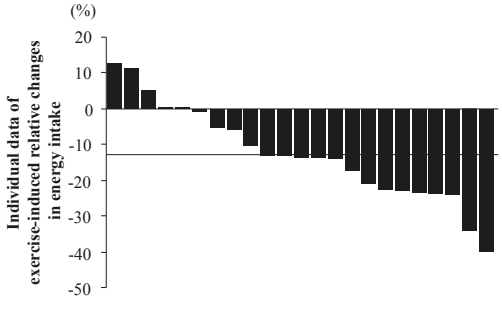

Figure 3. Individual data of exercise-induced relative change in energy intake. The line indicates the average value of relative change in exercise-induced energy intake (−12.9% ± 2.8%).

3.7. Correlation between Exercise-Induced Relative Changes in Energy Intake and Blood Variables

When the relationship between the exercise-induced relative change in energy intake and blood variables was determined, changes in energy intake showed a significant inverse correlation with serum Mb concentrations 30 min ($r = -0.477$, $p < 0.05$) after exercise. Moreover, there was an inverse trend of correlation between exercise-induced relative change in energy intake and serum Mb concentrations immediately after exercise ($r = -0.372$, $p = 0.08$). Exercise-induced absolute change in energy intake showed an inverse trend of correlation with that in area under the curve (AUC) of serum Mb ($r = -0.393$, $p = 0.06$). However, plasma acylated ghrelin concentrations immediately ($r = 0.11$, $p = 0.61$) and 30 min

($r = 0.20$, $p = 0.37$) after exercise did not correlate significantly with exercise-induced relative changes in energy intake. Similarly, plasma PYY_{3-36} concentrations immediately ($r = 0.12$, $p = 0.58$) and 30 min ($r = 0.03$, $p = 0.90$) after exercise were not correlated with exercise-induced relative changes in energy intake. No significant relationship was observed between exercise-induced absolute change in energy intake and AUCs of ghrelin ($r = -0.05$, $p = 0.81$) or PYY_{3-36} ($r = -0.34$, $p = 0.11$).

4. Discussion

The present study was designed to determine the impact of a 20 km run on appetite regulation in well-trained long distance runners. We found that absolute energy intake during a buffet test meal after the exercise period was significantly reduced compared with that after a rest period of identical duration. However, this reduction in energy intake was not associated with exercise-induced acylated ghrelin or PYY_{3-36} responses.

4.1. Exercise-Induced Ghrelin and PYY_{3-36} Responses

Plasma acylated ghrelin concentrations were significantly decreased after exercise, in agreement with previous studies [6,18,19]; however, the magnitude of the reduction in plasma acylated ghrelin concentrations (14% reduction) was smaller compared to previously reported values [14,18,20]. A relatively small reduction in plasma acylated ghrelin could be due to a moderate GH response, as exercise-induced GH elevations suppress ghrelin [21–23]. In the present study, because the magnitude of the exercise-induced GH response was modest (pre-exercise, 1.8 ± 0.5 ng/mL; immediately after exercise, 8.9 ± 1.8 ng/mL), it may have resulted in a relatively small ghrelin response. Furthermore, plasma PYY_{3-36} concentrations were not significantly elevated after the exercise period. There have been inconsistencies in the reported effect of acute exercise on PYY_{3-36} response [12–14,24]. These inconsistencies may be due to whether breakfast was consumed prior to exercising or not. In fact, two studies that demonstrated exercise-induced PYY_{3-36} elevations provided a standard breakfast before the exercise period [12,14], while exercise in the present study was completed following an overnight fast.

4.2. Energy Intake Following 20 km Run

The most important finding in the present study was a significant reduction in energy intake after a 20 km run in well-trained long distance runners. In addition, a detailed analysis of the selected menus during the buffet test meal indicated that lowered energy intake in EX was due to a reduction in calories consumed from staple foods (e.g., rice and bread) and other foods (e.g., fish or meat), and not from fruits or drinks. Previous reports investigating the effect of acute exercise on appetite regulation among well-trained athletes ($\dot{V}O_{2max}$ above 65 mL/kg/min) are quite limited. Moreover, this is the first study, to our knowledge, demonstrating that acute exercise decreased absolute energy intake in well-trained athletes. Exercise-induced decreases in appetite have been generally accepted to be dependent on exercise intensity [25]. However, the average HR (157 ± 3 bpm) during the 20 km run did not reach a maximal level ($78.0\% \pm 1.3\%$ of estimated maximal HR). Moreover, blood lactate concentrations did not change significantly from baseline, suggesting that exercise intensity was moderate. Resting $\dot{V}O_2$, which was determined after the 20 km run, was significantly elevated 20 min after the exercise period, and resting energy expenditure was increased during the post-exercise period. Therefore, reduction of absolute energy intake with a concomitant increase in resting energy expenditure might promote a negative energy balance. However, caution is necessary since energy expenditure during a 20 km run was not evaluated in the present study. Further investigations need to determine energy expenditure during exercise and post-exercise for confirming energy availability. In addition, the influence of low energy availability on post-exercise recovery and training adaptations is required to explore.

4.3. Inter-Individual Variability of Exercise-Induced Reduction of Energy Intake

The relatively larger sample size of this study (n = 23) enabled us to perform additional statistical analyses. Although energy intake in EX was significantly lower than in CON, a relatively large degree of individual variability was observed (−40.2% to 12.8%); a similar trend was also reported in previous studies [24,26–28]. To clarify the association between appetite-related hormonal responses and exercise-induced reductions in energy intake, we divided all subjects into two groups (a group with greater reductions in energy intake and a group with smaller reductions in energy intake) based on the average value of the exercise-induced relative change in energy intake (−12.9% ± 2.8%). There were no significant differences between the two groups for acylated ghrelin (p = 0.78) or PYY_{3-36} (p = 0.53) concentrations 30 min after the exercise period (immediately before the buffet test meal), suggesting that reduced energy intake after a 20 km run was not associated with exercise-induced acylated ghrelin or PYY_{3-36} responses. Thus, a plausible factor contributing to the reduction in energy intake after the 20 km run may be an exercise-induced elevation of GLP-1, as GLP-1 has anorexigenic effects. Ellingsgaard et al. [29] suggested that GLP-1 secretion was stimulated by exercise-induced interleukin-6 (IL-6) production in skeletal muscle in rats. IL-6 is an inflammatory cytokine, and long distance running markedly increases IL-6 production in skeletal muscle, as well as in the blood [30]. Ueda et al. [12] also observed a significant inverse correlation between the decrease in energy intake after exercise and an incremental GLP-1 response. Therefore, the impact of GLP-1 concentration mediated by IL-6 elevation after a 20 km run on reduced energy intake should be considered. Furthermore, we found that exercise-induced elevations of Mb concentrations were significantly correlated with exercise-induced relative changes in energy intake. Currently, we are unsure whether increased Mb concentrations directly affected exercise-induced reductions in energy intake. However, future investigations to study the influence of muscle damage and the inflammatory response on appetite regulation would be informative. Another possible factor contributing to the reduction of energy intake might be an exercise-induced elevation of core temperature, since this has been demonstrated to lower energy intake after exercise [31]. However, it is unlikely that an elevation of core temperature had a strong impact in our study, since the 20 km run was completed in a cold environment during winter (ambient temperature: 12.1 °C).

4.4. Macronutrient Intake Following a 20 km Run

The influence of exercise on macronutrient intake distribution remains unclear [10,32,33]. Blundell et al. [34] suggested that acute exercise may alter food preference, which is associated with replenishment of short-term energy stores. In the present study, carbohydrate intake during the buffet test meal was significantly higher in EX than in CON. Considering that fat oxidation (evaluated by RER) was significantly enhanced during the post-exercise period in EX, replenishment of muscle glycogen appeared to be augmented after the 20 km run. Therefore, the increased proportion of carbohydrate is reasonable. Dehydration and thirst may have also contributed to altered macronutrient intake [33]. However, it is unlikely that dehydration occurred since the run was performed in a cold outdoor environment, and a total of 400 mL of water was provided to each participant. Furthermore, body weight did not change significantly after the exercise period (pre: 56.3 ± 1.0 kg, post: 55.8 ± 1.0 kg, $p > 0.05$).

4.5. Limitations

Several limitations should be considered in the present study. First, there were slight, but significant differences in baseline acylated ghrelin and PYY_{3-36} concentrations between the two trials. The reason for this difference is unclear as training volume, dinner before the testing day, and accommodations the night prior to the trial were controlled and matched between the two trials. However, there was no significant correlation between energy intake and acylated ghrelin or PYY_{3-36} concentrations at baseline (acylated ghrelin, EX: $r = -0.16$, p = 0.47, CON: $r = -0.16$, p = 0.47;

PYY_{3-36}, EX: $r = -0.35$, $p = 0.10$, CON: $r = -0.17$, $p = 0.45$). In addition, in the EX trial, the lower acylated ghrelin concentration (anorexigenic effect) might be offset by the lower PYY_{3-36} concentration (orexigenic effect). Therefore, the influence of different acylated ghrelin and PYY_{3-36} concentrations at baseline would have been negligible; Second, the present study was conducted without using randomized counter-balanced design to match environmental condition during 20 km outdoor run, and all subjects completed firstly EX. Unick et al. [35] revealed that energy intake during the meal was similar in spite of the order of exercise trial and rest trial (exercise trial followed by rest trial or rest trial followed by exercise trial). Therefore, it is assumed that the present experimental design without crossover design had little influence on energy intake during buffet test meal; Third, exercise intensity during the 20 km run may have been lower than during a competition. Therefore, the reduction of energy intake after exercise may have been underestimated. Finally, information on energy intake during the rest of the trial day, and on following day, was not recorded. Previous studies reported that a compensatory increase in energy intake after an ad libitum meal was not observed over such a time period [6,13,36]. However, caution is necessary because subjects of above studies [6,13,36] were not competitive endurance athletes. Further investigations are needed to confirm whether compensatory increase in energy intake will happen.

5. Conclusions

A 20 km run significantly decreased subjective hunger, plasma acylated ghrelin concentrations, and absolute energy intake in well-trained long distance runners. However, the exercise-induced reduction of energy intake was not associated with acylated ghrelin or PYY_{3-36} responses.

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Abbreviations

PYY	peptide YY
GLP-1	glucagon-like peptide-1
$\dot{V}O_{2max}$	maximal oxygen uptake
HIIT	high-intensity interval training
VAS	visual analog scale
GH	growth hormone
FFA	free fatty acid
Mb	myoglobin
CK	creatine kinase
CV	coefficients of variation
IL-6	interleukin-6

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