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Abstract

Purpose: To quantify the effects of a short-term (6-day) low carbohydrate (CHO) high fat (LCHF), and low energy availability (LEA) diet on immune, inflammatory, and iron-regulatory responses to exercise in endurance athletes.

Methods: Twenty-eight elite male race walkers completed two 6-day diet/training phases. During phase 1 (Baseline), all athletes consumed a high CHO/energy availability (CON) diet (65% CHO and $\sim 40 \text{ kcal}\cdot\text{kg}^{-1}$ fat free mass (FFM) $\cdot\text{day}^{-1}$). In phase 2 (Adaptation), athletes were allocated to either a CON (n=10), LCHF (n=8; $<50 \text{ g}\cdot\text{day}^{-1}$ CHO and $\sim 40 \text{ kcal}\cdot\text{kg}^{-1}$ FFM $\cdot\text{day}^{-1}$), or LEA diet (n=10; 60% CHO and $15 \text{ kcal}\cdot\text{kg}^{-1}$ FFM $\cdot\text{day}^{-1}$). At the end of each phase, athletes completed a 25 km race walk protocol at $\sim 75\% \text{ VO}_{2\text{max}}$. On each occasion, venous blood was collected before and after exercise for interleukin-6, hepcidin, cortisol and glucose concentrations, as well as white blood cell counts.

Results: The LCHF athletes displayed a greater IL-6 ($p=0.019$) and hepcidin ($p=0.011$) response to exercise after Adaptation, compared to Baseline. Similarly, post-exercise increases in total white blood cell counts ($p=0.026$) and cortisol levels ($p<0.001$) were larger compared to Baseline following LCHF Adaptation. Decreases in blood glucose concentrations were evident post-exercise during Adaptation in LCHF ($p=0.049$), whereas no change occurred in CON or LEA ($p>0.05$). No differences between CON and LEA were evident for any of the measured biological markers (all $p>0.05$).

Conclusion: Short-term adherence to a LCHF diet elicited small yet unfavorable iron, immune, and stress responses to exercise. In contrast, no substantial alterations to athlete health were observed when athletes restricted energy availability compared to athletes with adequate energy availability. Therefore, short-term restriction of CHO, rather than energy, may have greater negative impacts on athlete health.

Key words: ketogenic, LCHF, low energy availability, hepcidin, REDS, health

Introduction

Energy availability (EA) is defined as the amount of dietary energy remaining for the body's normal physiological function after accounting for the energy cost of exercise, and is expressed relative to fat free mass (FFM) (1). Low energy availability (LEA) in athletes is not uncommon, and can manifest from excessive sustained caloric restriction to alter body composition and/or an inability to recover the energy cost of high or increased training loads underpinned by pathological, deliberate and/or inadvertent origins (2). Relative Energy Deficiency in Sport (RED-S) is a syndrome that describes the long-term health and performance outcomes brought about by extended LEA in both male and female athletes (3). Impaired immune function is one proposed health consequence of RED-S (3), with a review stating that scenarios of LEA are likely to be associated with reductions in catecholamine responses, mucosal immunity, neutrophil function and increased phagocytic activity (4). Alterations in immune function may lead to increased illness susceptibility, with observational data from Olympic-level athletes demonstrating that those who scored highly on an instrument validated to predict a high risk of LEA (5), also showed a positive association with illness, and in particular, symptoms of upper respiratory tract infections, bodily aches, and gastrointestinal disturbances (6). However, other studies have shown no clear association between LEA and immunological function when retrospective analysis of self-report data from 1000 female athletes were considered (7). It is likely that the link between LEA and immunity in athletes is complex, and that many factors potentially mediate this relationship (8).

Of interest, Ackermann et al., (7) reported that female athletes with symptoms of LEA had a 64% greater risk of hematological dysfunction, characterized by a history of anemia, low

hemoglobin, iron and/or ferritin stores. These findings are consistent with the negative hematological consequences proposed by the RED-S model (3), which may be a result of low dietary iron intake as a consequence of reduced caloric intake (9), or alterations to the iron regulatory hormone, hepcidin (10). Regardless, investigation into the health outcomes of RED-S is still in its infancy, and a greater understanding of these underlying health consequences, the time course over which they manifest, and the severity of LEA required to induce alterations is needed.

In parallel to LEA, adherence to a ketogenic low carbohydrate (CHO) high fat (LCHF) diet may also affect both the iron regulatory (11, 12) and immune response (13) to an acute exercise session. This raises the question of whether it is the reduced CHO availability or the EA driving the altered responses. Recent publications from our research group have addressed the effects of 3-weeks adherence to a LCHF diet on immune (13) and iron regulatory responses (11, 12) to exercise in elite endurance athletes. However, our conclusions pertaining to iron metabolism were confounded by differences in changes in iron status that eventuated over time between groups. Accordingly, a short-term ketogenic dietary model, where differences in serum ferritin levels are unlikely to occur, should provide an opportunity to study iron regulatory responses in greater detail. Additionally, our studies of immune function were limited to the investigation of salivary immunoglobulin-A (s-IgA), and therefore, a more detailed understanding of the inflammatory and stress response to exercise in elite athletic cohorts is warranted. Accordingly, the first aim of this study was to assess the impact of a short-term LCHF diet and training intervention on innate immune markers, inflammation, iron regulation and blood lipids in male athletes. Furthermore, studies to date examining the impact of LEA have

primarily been observational in nature and conducted largely in female athletes. Therefore, a second aim of this investigation was to evaluate the health effects of a short-term LEA diet and training intervention in elite male race walkers. Importantly, by integrating both dietary arms within the single study design, we can isolate the effects of LEA from low CHO availability.

Methods

Participants

Twenty-eight elite male race walkers were recruited for this investigation. Athletes participated in one of two training camps: a 2019 training camp (n=20), held in Canberra, Australia (~580 m altitude), or a 2021 training camp (n=8) held in Melbourne, Australia (sea level). Athletes ranged from elite, world championship medalists (n=4) and athletes competing at major international events (n=20), through to national level athletes (n=4), all currently training for either the 20 or 50 km event. No athlete was reported to be anemic (hemoglobin <11.5 g·dL⁻¹ (9)) or consuming oral iron supplements during the study period. Athletes were informed of the risks and requirements of the study before providing written informed consent. Ethics approval was obtained from the Ethics Committee of the Australian Institute of Sport (2019; no. 20181203) and the Australian Catholic University (2021; no. 2020-238HC). Athlete characteristics are presented in Table 1.

This study was implemented during a 4-week training camp comprising of two 6-day dietary phases (Figure 1). During phase 1 (Baseline), all athletes were placed on a high CHO/energy availability control diet (CON), supplying 65% CHO and ~40 kcal·kg⁻¹ FFM·day⁻¹. Athletes were then assigned to one of three parallel group dietary interventions for phase 2

(Adaptation), accounting for each athlete's dietary preference, while matching each group for individual characteristics (age, 20 km personal best time, training status and load). The three dietary interventions were: (i) CON, (ii) LCHF, or (iii) LEA. During both dietary phases, athletes followed a semi-structured training plan, with key sessions completed as a group and all other training recorded in an electronic training diary. Similar training volumes were completed across study phases and between dietary groups, with this data reported in Table 1. On day 6 of each dietary phase (Baseline and Adaptation), athletes completed a 25 km hybrid laboratory-field race walking test, where the iron regulatory, immune, and inflammatory responses to exercise were determined.

Dietary intervention

All meals were created by an accredited sports dietician and served to athletes in a communal living environment where dietary compliance was monitored. The CON diet aimed to provide 65% CHO, 15% protein and 20% fat, equating to an EA of $40 \text{ kcal}\cdot\text{kg}^{-1} \text{ FFM}\cdot\text{day}^{-1}$. The LEA intervention consisted of 60% of energy from CHO, 25% of energy from protein, 15% of energy from fat with target EA of $15 \text{ kcal}\cdot\text{kg}^{-1} \text{ FFM}\cdot\text{day}^{-1}$. The LCHF diet was ketogenic in nature, providing <50 g of CHO daily and ~80% of energy provided as fat. This diet was isocaloric to the CON intervention, equating to an EA of $40 \text{ kcal}\cdot\text{kg}^{-1} \text{ FFM}\cdot\text{day}^{-1}$.

Target energy requirements were calculated using the following equation: target EI = (target EA * FFM) + EEE (excluding resting metabolic rate (RMR)), where target EA was based on the phase of the study and dietary intervention and FFM determined via dual-energy X-ray absorptiometry. To determine target EI, EEE was prospectively calculated from each athlete's

individualized training plan. For each athlete, EEE was estimated using the Weir equation (14) based on the gas exchange data during a 4-stage submaximal economy test plotted against speed ($3.94 \cdot \text{VO}_2 + 1.11 \cdot \text{VCO}_2$) which gives an estimate of EEE as $\text{kcal} \cdot \text{min}^{-1}$. Thereafter, we calculated estimated energy cost for 1 min of race walking excluding RMR for the same period [i.e. $\text{EEE} (\text{kcal} \cdot \text{min}^{-1}) - (\text{RMR } 24 \text{ h} / 1440)$]. For the purpose of the current study, where planned training was given as distance (km), a simplified method to prospectively estimate EEE was required. Therefore, we calculated EEE per km race walking at each speed of the treadmill test as follows: $\text{EEE} (\text{kcal}/\text{km}) = ((\text{EEE} (\text{kcal}/\text{min}) * 60 \text{ min})) / \text{Speed} (\text{km}/\text{h})$. These values were then averaged over the 4 different speeds and an averaged EEE (kcal/km) value was used in prospective calculations to derive target EI for each athlete and each day. To promote accuracy and compliance, training diaries were checked twice a day (at lunch and dinner). The remaining meals of the day were altered if the athlete's actual training volume differed from what was planned, by an amount resulting in an EA of $>2.4 \text{ kcal} \cdot \text{kg}^{-1}$ FFM (equivalent to ~ 2 km of race walking) to maintain daily EA targets. Dietary analysis was undertaken using FoodWorks computer software (FoodWorks 9; Xyris Software, Australia).

Race walking test protocol

The 25 km hybrid laboratory/field race walk test protocol was performed at the end of each dietary phase (day 6). Athletes arrived at the laboratory at the same time of day for both tests in a rested and fasted state. Initially, an indwelling cannula was inserted into a forearm vein and a venous blood sample collected before athletes were provided with a standardized breakfast. When athletes were adhering to the CON diet (Baseline and Adaptation for CON only), a meal consisting of $2 \text{ g} \cdot \text{kg}^{-1}$ body mass (BM) CHO was consumed. During the Adaptation phase, the

LEA group consumed a meal providing $1 \text{ g}\cdot\text{kg}^{-1}$ BM CHO, whereas the LCHF group were provided with a high-fat meal, consisting of ~80% fat which was isocaloric to that consumed at Baseline and the CON group. Two hours following breakfast, a second venous blood sample was collected, in addition to a fingertip blood sample ($0.6 \mu\text{L}$) that was analyzed for blood glucose concentration.

Athletes then commenced the race walking test protocol, where every 6th kilometer (1, 7, 13, 19, 25 km) was performed on a treadmill in the laboratory at the athlete's ~50 km race walk pace (12 or $13 \text{ km}\cdot\text{h}^{-1}$). This protocol equates to ~75% $\text{VO}_{2\text{max}}$ and has been used previously to investigate the metabolic responses of elite race walkers (15, 16). The remaining distance was completed on an outdoor loop surrounding the laboratory at a consistent speed nominated by the athlete. All athletes completed 4 laps to total 25 km, except for one junior athlete, who completed 3 laps during both phases (19 km total). Upon completing each treadmill bout, athletes were provided with CHO gels equating to $60 \text{ g}\cdot\text{h}^{-1}$ CHO (Baseline and CON). During the Adaptation phase, the LEA group were limited to $30 \text{ g}\cdot\text{h}^{-1}$ CHO, whereas the LCHF group were provided with high fat snacks (cheese and/or high fat cookies) and electrolyte fluids to match for total energy consumed at Baseline ($\sim 240 \text{ kcal}\cdot\text{h}^{-1}$) and by the CON group. Water was provided every ~2 km and consumed *ad libitum*. Immediately post-exercise, venous and fingertip blood samples were collected. Athletes were then provided with a recovery snack 30 min post-exercise ($1.5 \text{ g}\cdot\text{kg}^{-1}$ CHO for all groups during Baseline and CON, an isocaloric low CHO option for LCHF and $0.75 \text{ g}\cdot\text{kg}^{-1}$ CHO for LEA during Adaptation), with an additional venous blood sample collected 1 h post-exercise. Lunch was then provided in accordance with the dietary allocation, and a final venous blood sample collected 3 h post-exercise.

Blood analysis

Five venous blood samples were collected during each 25 km test protocol; on arrival (fasted), immediately pre-exercise, immediately post-exercise, 1 h post-exercise, and 3 h post-exercise. Hematological analysis (including total white cell count, hemoglobin, hematocrit and total neutrophil, lymphocyte monocyte and reticulocyte counts) were performed from a 3 mL EDTA tube (Vacuette, Greiner Bio-One GmbH, Austria) on whole blood via fluorescent flow cytometry on an XN-L 550 analyzer (Sysmex Corporation, Kobe, Japan). Remaining samples were collected into either lithium heparin coated tubes or SST tubes, which were left on the benchtop to clot for 30 min. Bloods were centrifuged at 1500 g at 4 °C for 10 min, aliquoted into 1 mL cryovials and frozen at -80 °C for batch analysis.

Serum iron, ferritin, transferrin, cholesterol, and C-reactive protein (CRP) were measured on fasting samples with a COBAS Integra 400 automated biochemistry analyzer (Roche Diagnostics, Switzerland). Cortisol (fasted, immediately post- and 1 h post-exercise) and testosterone (fasted only) concentrations, were measured via an Access 2 Immunoassay System (Beckman Coulter, Brea, CA, USA), and were used to calculate testosterone/cortisol (T/C) ratios. Concentrations of IL-6 were analyzed in duplicate using a commercially available ELISA kit pre- and post-exercise (Quantikine HS; R&D Systems, Minneapolis, MN; CV= 3.0%) on a FLUOstar OPTIMA plate reader (BMG Labtech, Offenburg, Germany). Measurement of hepcidin-25 was made on fasting and 3 h post-exercise samples using Intrinsic Hepcidin IDxi ELISA Kit (Intrinsic LifeSciences LLC, CA, USA; CV= 5.9%) according to the manufacturer's instructions. All fingertip capillary blood samples were analyzed immediately for blood glucose concentration (FreeStyle Optium Neo, Abbott Diabetes Care, Victoria, Australia).

Statistical analysis

All statistical analyses were completed using general linear mixed modelling using the R package lme4, with models estimated using Restricted Maximum Likelihood. Visual inspection of residual plots did not reveal any obvious deviations from homoscedasticity or normality for any variable. Models included fixed effects for dietary group*testing phase (resting variables) or dietary group*testing phase*time point (post-exercise responses), with random intercepts for subject identification, camp and environmental temperature included to adjust for baseline levels and inter-individual homogeneity. Given the strong influence that iron status can have on the post-exercise hepcidin response (17), serum ferritin was used as a covariate in the analysis of hepcidin, as undertaken previously (18). Statistical significance of the fixed effects were determined using Type II Wald tests with Kenward–Roger degrees of freedom. Where significant fixed effects were evident, Tukey’s post-hoc comparisons were performed to identify specific condition differences. Significance was set at $p < 0.05$.

Results

Dietary analysis

Actual dietary intake during both dietary phases is reported in Table 2. All athletes achieved their planned dietary targets during each phase. There were no differences in energy, macro- or micronutrient intake between the 3 diets at Baseline (all $p > 0.05$). Consistent with the design of the diets, during the Adaptation phase EI and EA were substantially lower in LEA compared to CON and LCHF ($p < 0.001$). CHO intake was also greatest in CON compared to LEA ($p < 0.001$), with further decreases evident in LCHF ($p < 0.001$). Fat intake was greatest in LCHF and lowest in LEA, with both groups being significantly different to CON ($p < 0.001$).

During the Adaptation phase, dietary iron intake was higher in the CON group compared to both LCHF ($p < 0.001$) and LEA ($p < 0.001$).

Cholesterol and hormone concentrations

Resting cholesterol and hormone levels are reported in Table 3. A significant interaction effect was evident for total cholesterol ($p = 0.047$), which showed a decrease during Adaptation in LEA ($p = 0.030$), whereas no change was evident in CON ($p = 1.000$) or LCHF ($p = 0.066$). Similarly, LDL cholesterol decreased in LEA during Adaptation ($p = 0.008$), with no differences between phases in CON ($p = 0.999$) or LCHF ($p = 0.409$). Significant interaction effects were also observed for triglycerides ($p = 0.004$), as there was a significant decrease in LCHF following Adaptation ($p = 0.041$), whereas CON ($p = 0.518$) and LEA ($p = 0.220$) did not change. Testosterone concentrations declined following Adaptation ($p < 0.001$), however no differences between dietary groups were observed ($p = 0.180$).

Iron status

Variables related to the assessment of iron status are presented in Table 4. No significant differences in serum ferritin levels were evident between phases ($p = 0.085$), however there was a non-significant tendency for LEA to increase (+6%) compared to LCHF (-10%) and CON (-14%), which decreased following Adaptation ($p = 0.058$). While there were no differences in hemoglobin between groups at Baseline ($p > 0.05$), LCHF displayed higher hemoglobin concentrations compared to CON ($p = 0.010$) following Adaptation. This occurred because of a small decrease in CON (-2.8%; $p = 0.095$) and a slight increase in LCHF following Adaptation (+2.0%; $p = 0.420$). Changes to reticulocyte (%) between phases differed among groups

($p=0.006$). A decrease in reticulocytes was seen in LCHF (-20%; $p=0.051$), while there were no changes in CON (+7%; $p=0.658$) or LEA (+5%; $p=0.889$).

Inflammatory and iron regulatory response to exercise

Mean time to complete the 25 km race walk protocol for all athletes was $2:03 \pm 0:06$ (h:min). Completion time was similar between phases for CON (+9 sec; $p=1.000$) and LEA (+1 min 22 sec; $p=0.836$), however LCHF was significantly slower during Adaptation compared to Baseline (+7 min 28 sec; $p<0.001$). A significant 3-way interaction was evident for IL-6 (Figure 2A; $p=0.010$). Specifically, within LCHF, post-exercise IL-6 concentrations were significantly higher after Adaptation compared to Baseline (26.6 vs. 12.4 $\text{pg}\cdot\text{mL}^{-1}$; $p=0.019$), whereas no differences between phases were evident for CON ($p=0.782$) or LEA ($p=0.999$). Furthermore, the magnitude of the post-exercise increase in IL-6 levels were greater in LCHF following Adaptation compared to CON (+13.1 $\text{pg}\cdot\text{mL}^{-1}$; $p=0.010$), but not LEA (+9.6 $\text{pg}\cdot\text{mL}^{-1}$; $p=0.133$). Subsequently, hepcidin concentrations were increased 3 h post-exercise in all groups (Figure 2B; $p<0.001$) and were significantly associated with resting ferritin levels ($p<0.001$). Significant 3-way interactions ($p=0.003$) show that increases in hepcidin levels were largest within LCHF ($p=0.011$) after Adaptation (+301%) compared to Baseline (+126%). No differences between phases were evident for either the CON ($p=0.896$) or LEA groups ($p=1.000$).

The immune and stress response to exercise

White blood cell counts (Figure 3A; $p<0.001$) and neutrophils (Figure 3B; $p<0.001$) increased post-exercise and remained elevated at 1 h post-exercise, with a larger response evident during Adaptation compared to Baseline ($p=0.026$ and $p<0.001$, respectively).

Lymphocyte levels increased slightly post-exercise, before decreasing below pre-exercise levels at 1 h post-exercise (Figure 3C; $p < 0.001$). Monocyte concentrations also increased post-exercise (Figure 4D; $p < 0.001$) returning to fasting levels at 1 h post-exercise ($p = 0.090$). The post-exercise increase in both lymphocyte and monocytes was largest in LCHF at Adaptation when compared to Baseline ($p < 0.001$). At no point were differences between phases detected for CON or LEA ($p > 0.05$).

Cortisol decreased at 1 h post-exercise compared to fasting levels at Baseline in LCHF ($p < 0.001$) and LEA (Figure 4A; $p = 0.017$). In LCHF, no post-exercise changes in cortisol occurred during Adaptation ($p = 0.999$). As a result, cortisol levels in LCHF were higher immediately post-exercise ($+321 \text{ nmol}\cdot\text{L}^{-1}$; $p < 0.001$) and 1 h post-exercise ($+283 \text{ nmol}\cdot\text{L}^{-1}$; $p < 0.001$) during Adaptation compared to Baseline. Similarly, cortisol levels did not change during Adaptation in LEA ($p = 0.793$). However, no differences between phases for LEA were evident at post-exercise ($+142 \text{ nmol}\cdot\text{L}^{-1}$; $p = 0.143$) or 1 h post-exercise ($+75 \text{ nmol}\cdot\text{L}^{-1}$; $p = 0.960$). No changes in blood glucose were evident post-exercise (Figure 4B). The exception occurred in LCHF, with a reduction in glucose post-exercise during Adaptation ($p = 0.05$).

Discussion

This study demonstrates that adherence to a short-term ketogenic LCHF diet results in acute perturbations to both the immune and iron regulatory response to exercise. Conversely, 6 days of LEA did not cause any alterations to markers of immune function or iron metabolism when compared to athletes adhering to a high CHO/energy control diet. By including both a LEA and LCHF diet in our investigation, we were able to isolate the effects of low energy from low

CHO availability, and demonstrate that the short-term restriction of CHO, rather than EA, appears to have a greater detrimental impact on markers of athlete health.

Low carbohydrate high fat diet

To address the first aim of this investigation, we assessed the influence of the ketogenic LCHF diet on a range of health indices, with a particular interest in furthering our understanding of how iron metabolism may be affected. Increases in the iron regulatory hormone, hepcidin, have been reported 3-6 h after exercise (19, 20), resulting in the inhibition of the iron export transporter ferroportin, therefore potentially limiting iron absorption during the post-exercise period. Post-exercise hepcidin levels can be up-regulated via an augmented IL-6 response to exercise when training under conditions of low CHO availability (21). Badenhorst and colleagues (21) reported a trend towards increased inflammatory-induced hepcidin release after 24 h of low CHO intake in trained athletes. However, in their study the low CHO condition still provided a moderate CHO intake of $3 \text{ g}\cdot\text{kg}^{-1} \text{ BM CHO}$, compared to the current study, where CHO intake in LCHF was restricted to $\sim 0.5 \text{ g}\cdot\text{kg}^{-1} \text{ BM}$. Therefore, it stands to reason that the ketogenic version of a LCHF diet could elicit larger perturbations to the iron regulatory response to exercise. Indeed, a previous investigation by our group examined the impact of 3 weeks adherence to a LCHF diet on hepcidin levels in elite athletes (11). Here, an increased IL-6 response was clearly evident in keto-adapted athletes, with a tendency for increased hepcidin concentrations also apparent. However, it is likely that the hepcidin response was confounded by the differences in serum ferritin that occurred over the 3-week period, as ferritin levels were lower in the high CHO, compared to LCHF group. Differences in serum ferritin are known to have a strong influence on hepcidin concentrations (17), and as a result, it is difficult to

determine whether it was the LCHF intervention, or ferritin, that was affecting hepcidin concentrations. Therefore, in the current study we utilized a 6-day intervention to induce the desired physiological training adaptations relevant to the LCHF diet (shifts in fuel utilization and ketone concentrations (22, 23)) to assess the impact of the diet on iron regulation without confounding changes in serum ferritin manifesting. For the first time, we have clearly demonstrated that six days of exposure to the LCHF diet, combined with a low CHO pre-exercise meal, amplified the post-exercise hepcidin response compared to Baseline. When considering the reduced dietary iron content provided by a LCHF diet (~ 25% less than CON), and the potential impairment to iron absorption resulting from an increased post-exercise hepcidin response, athletes intending to adopt LCHF diets over a prolonged period should be vigilant in monitoring their iron status to ensure adequate levels are maintained.

Despite the reported impairment to iron regulation in LCHF athletes, no reduction to red blood cell production was apparent here, but rather, small increases in hemoglobin and hematocrit were detected. In previous investigations of the LCHF diet, the same outcome of similar magnitude was observed, which were attributed to an arbitrary shift in plasma volume (11). However, the consistency of this response may instead point towards the occurrence of a dietary-induced plasma volume contraction (-2.4% in LCHF, compared to +4.4% in CON (24)). This outcome may reflect the increased urinary sodium and water loss that occurs with adherence to the LCHF, in association with changes in insulin concentrations (25). Furthermore, this modest shift in plasma volume may need to be considered in the interpretation of concentration-based biomarkers. Notably, a marked decline in reticulocyte counts, a marker of erythropoiesis stimulation, was evident in keto-adapted athletes, which occurs independent of plasma volume

shifts. The importance of this finding is currently unclear; however, a greater focus on hematological adaptations which could influence training adaptation is warranted. Finally, the described alterations to reticulocyte counts, hemoglobin concentrations and plasma volume shifts could impact the athlete biological passport (26); an outcome that requires further investigation.

It has consistently been shown that acute CHO restriction can also impair the immune and inflammatory response to exercise (27, 28), implying that strategies to achieve high CHO availability around exercise can be a partially effective counter-measure against exercise-induced immune impairment (29). In the present study, we showed acute, modest alterations to white blood cell counts and the stress response to exercise in athletes adhering to a LCHF diet. Previously, our group reported a decrease in self-reported physical readiness and general health in athletes during the initial (1 week) adaptation to a LCHF diet (13), which coincides with the immune alterations observed in the present study. With that in mind, self-reported wellness returned to pre-study levels after 3 weeks of adherence to a LCHF diet, which aligned with s-IgA secretion rates similar to levels measured in athletes consuming CHO-rich diets (13). Therefore, while we showed that immune resistance was lowered during the initial adaptation to a LCHF diet, the long-term impact is less concerning once an athlete has adapted to the nutritional perturbation. Indeed, other work has shown a ~70% increase in s-IgA secretion rate after 31 days adherence to a LCHF diet, and markers of immune function were not compromised (30). In the current study, although white blood cell counts and their subsets were elevated above Baseline by ~50-100% in LCHF, they were only slightly above the clinical range of normal (31). Therefore, the clinical relevance of the current findings, and their impact on illness susceptibility requires further examination. Given the uncertainty around the significance of these changes,

more clinically meaningful markers of immune functioning, such as vaccination or experimental infection models and multi-omics, should be explored to identify whether these dietary approaches increase an athlete's risk of illness, particularly during the early adaptation phase to a LCHF diet.

Low energy availability

The second aim of this investigation was to determine the health implications of short-term energy restriction, to ultimately distinguish the effects of low CHO availability, from energy availability. To our knowledge, this investigation is the first to induce LEA in elite male endurance athletes, using a novel personalized approach for calculating EEE in the field, which allows athletes to complete their periodized and fluctuating daily training volumes and energy expenditure supported by appropriately fluctuating changes in energy and nutrient intake. To date, the majority of studies assessing EA have either employed cross-sectional observations of highly trained athletes (7, 32), or interventional studies in moderately trained individuals in which daily exercise and energy intake were clamped across the intervention (33, 34). While these studies contribute important information, concerns have been raised regarding the 'one-off' assessment of EA (35), which can fail to truly represent the chronic state. Furthermore, the reliance on self-reported food intake and training diaries in free-living scenarios can be problematic (36), as they can lead to either an under- or over-estimation of EA (37). The current intervention manipulated EA in a real-world manner, in which elite male athletes completed a periodized training program (daily programs varying according to mode, frequency, intensity and duration), and had their energy intake adjusted to achieve the target for high and low EA. This outcome was achieved by: 1) provision and supervision of a rigorously controlled dietary plan,

with 2) individualization of EEE calculations based on the athlete's prospective training plan, and 3) real-time manipulation of EI over the day according to actual training completed.

Our data show that 6 days of LEA was insufficient to elicit meaningful alterations in the immune and stress response to exercise in male athletes. Accordingly, either a longer period of adaptation or more severe restriction of energy (along with a lower overall CHO availability of the diet) may be required to observe a negative impact on immunity. This is an important finding for athletes who frequently implement targeted and purposeful periods of acute energy restriction to manipulate body composition to prepare for a key event. This is especially the case for athletes who compete in weight division sports, or events where a high power-to-weight ratio or low levels of fat mass provide a competitive advantage (2). During scenarios of acute energy restriction, it may be that the immune system is prioritized over other body systems, and optimal functioning is preserved even under conditions of very low EA. However further evidence is required to support this assertion. Sex-specific differences between males and females should also be explored, since it may be that females show greater health and physiological perturbations to LEA than their male counterparts (3).

The amount of protein ingested by athletes in LEA states is also suggested to be particularly important for athletes to minimize immune disturbances (8). In the current study, athletes adhering to the LEA intervention exceeded the current recommendations for protein ingestion ($2.1 \pm 0.1 \text{ g}\cdot\text{kg}^{-1} \text{ BM}$) (38, 39), consuming a similar amount to both CON ($2.3 \pm 0.2 \text{ g}\cdot\text{kg}^{-1} \text{ BM}$) and LCHF ($2.2 \pm 0.0 \text{ g}\cdot\text{kg}^{-1} \text{ BM}$). This pattern of protein intake appears to be generally representative of endurance athletes in a state of LEA, with a study of free living, elite,

middle- and long-distance runners and race walkers with symptoms of LEA reporting similar intakes (2.2-2.4 g·kg⁻¹ BM) (32). Evidence for the beneficial effects of protein intake on immune function comes from studies of anorexia nervosa patients, an illness characterized by very low energy intakes, which has generally shown to be protective against infection up until a very low body mass index of <15 kg/m² is reached (40). Conversely, starvation defined as protein-energy-malnutrition, has a strong negative influence on immune functioning (41). Differences in immunity between starvation and anorexia nervosa patients may be attributed to the presence/absence of protein, with several investigations demonstrating similar protein intakes in anorexia nervosa patients and healthy controls, despite the large differences in overall energy intake (42). Therefore, it seems that if athletes are free from malnutrition (including micronutrient deficiencies) and are consuming sufficient protein, illness susceptibility may not be increased by LEA.

Impaired hematological function, inclusive of iron deficiency, has also been linked with LEA in athletes, and has been proposed as a health consequence of RED-S (3). However, in the current study, no differences in iron regulatory markers, including resting and post-exercise hepcidin concentrations, were evident between the LEA and CON groups. This result contrasts earlier reports in both highly-trained endurance athletes (43), and military personnel (44), where resting hepcidin and ferritin levels increased after 3 days of LEA, compared to a diet of adequate EA. Hepcidin can act as a nutrient sensor during times of starvation (45), sequestering iron within the storage protein, ferritin, to ensure sufficient supply for essential physiological functions to be maintained throughout an energy deficit. This outcome has been shown in anorexia nervosa patients, where increased hepcidin concentrations elicited an increase to ferritin

levels (46, 47). In the current study, a non-significant trend towards differences in ferritin between dietary groups was evident after 6 days ($p=0.058$), with slight increases in LEA and small decreases in LCHF and CON. Taken together, these data bring into question the time course of changes in iron metabolism that occur with LEA, particularly with the knowledge that depleted iron stores are a common outcome of chronic LEA, or RED-S (3, 7). It could be hypothesized that hepcidin and ferritin acutely increase with short-term (1-4 day) LEA exposure (43, 44), and return to near normal levels by ~6 days (as seen in the current study). After this time, the secondary effects of LEA, such as the effect of lowered sex hormone concentrations on hepcidin expression, reduced dietary iron intake, and/or increased exercise-induced iron losses (7) may contribute to the eventual depletion of iron stores. However, this time course is highly speculative, and longitudinal studies are required to better understand these dietary-induced changes to iron metabolism.

In contrast to the influence of CHO restriction, the LEA dietary intervention did not affect post-exercise IL-6 and hepcidin concentrations. We speculated that the decreased CHO intake in LEA compared to CON (~5 vs 10 $\text{g}\cdot\text{kg}^{-1}$ BM CHO) may have yielded a larger IL-6 response to exercise, however this was not the case. This result is in contrast to the work of others showing 3 days of LEA reduced muscle glycogen content, and increased the IL-6 response to exercise by ~2-fold (43). This is somewhat surprising given CHO intake, energy intake and EA were similar between studies, however peak IL-6 concentrations were on ~80% higher across all dietary groups in the current investigation compared to previous work (43). Differences between exercise protocols and caliber of athletes may explain these discrepancies. Finally, while we have not seen any effect of the LEA diet on iron regulation, the reduced dietary

iron content diet should be considered. Our results support previous suggestions that the reduced overall caloric intake can lead to reduced dietary iron intake (9), which if sustained long-term, may have negative implications for iron balance.

A final feature of this investigation was assessment of alterations to blood lipid profiles, with the finding of minimal changes associated with the CON or LCHF interventions. This result is in contrast with longer term studies of keto-adaptation in athletes (48), which have reported an increase in total cholesterol, LDL and HDL concentrations compared to athletes who had adhered to a high CHO diet. Increased dietary fat consumption and increased demand for lipid metabolism were postulated as underpinning mechanisms. Similarly, a recent meta-analysis of LCHF diets in athletic populations reported an increase in total cholesterol over a ~3 week period (range 1-24 weeks (49)). When placing our results in context of the current literature, it is likely that our 6-day dietary intervention period was not long enough to elicit substantial changes to blood lipid profiles, similar to those reported previously. Decreases in LDL and total cholesterol, and increases in HDL cholesterol occurred in LEA, indicative of an acute change in lipid metabolism. However, no athletes in this study had cholesterol values outside of the healthy reference range (50), and therefore, these changes are unlikely to be of clinical significance.

Conclusion

Short-term restriction of CHO elicited small yet unfavorable perturbations to the iron, immune, and stress response to exercise in male endurance athletes. In light of suggestions that adherence to a LCHF diet should occur for 'several months' to attain complete keto-adaptation, it is important to consider whether our acute observations of changes to iron regulation and

immune function manifest into long-term concerns for iron status or susceptibility to illness. If necessary, counter-measure strategies that attempt to offset these health conditions (e.g. oral iron or vitamin C supplementation) should be examined in the context of ketogenic LCHF diets. Importantly, no substantial alterations to markers of athlete health were observed when male athletes were exposed to a short period of significantly decreased EA ($15 \text{ kcal}\cdot\text{kg}^{-1} \text{ FFM}\cdot\text{day}^{-1}$). Pending further evaluation of other body systems, short intense periods of LEA may be well-tolerated by male athletes, and could form a useful strategy to acutely manipulate body composition without detectable impairments to indices of athlete health. Future investigations of the long-term implications of LEA should focus on variations in the duration and severity of EA thresholds as well as the effects of periodic rather than chronic exposure. However, our ability to study real-life observations of LEA will likely be limited by the ethical considerations of undertaking deliberate interventions in healthy individuals. In the meantime, athletes implementing short periods of LEA should ensure CHO and protein intake are adequate to maintain immune support.

Declarations

The study was funded by a Program Grant from the Australian Catholic University Research Fund awarded to L.M.B. The authors declare no conflicts of interest. The results of this study are presented clearly, honestly and without fabrication, falsification, or inappropriate data manipulation and do not constitute endorsement by the American College of Sports Medicine.

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Figure Captions

Figure 1. Schematic representation of the study protocol involving race walkers on three different dietary interventions (CON = high energy/carbohydrate availability diet; LCHF = low carbohydrate, high fat diet; LEA = low energy availability diet).

Figure 2. A) Interleukin-6 (IL-6) pre- and post-exercise, and B) hepcidin-25 concentrations fasted and at 3 h post-exercise during Baseline and Adaptation phases for athletes in the high energy/carbohydrate availability diet (CON), low carbohydrate high fat diet (LCHF) and low energy availability diet (LEA). Data presented as mean + SD. * Indicates a significant within-group difference to Baseline
\$ Indicates a significant difference compared to CON.

Figure 3. (A) Total white blood cell, (B) neutrophil, (C) lymphocyte and (D) monocyte counts fasted, post- and 1 h post-exercise during Baseline and Adaptation phases for athletes on the high energy/carbohydrate availability diet (CON), low carbohydrate high fat diet (LCHF) and low energy availability diet (LEA). Data presented as mean \pm SD. Grey area represents the general population reference range (31). * Indicates a significant within-group difference to Baseline. \$ Indicates a significantly larger response than both the CON and LEA groups.

Figure 4. (A) Cortisol levels and (B) blood glucose concentrations during the Baseline and Adaptation phases for athletes in the high energy/carbohydrate availability diet (CON), low carbohydrate high fat diet (LCHF) and low energy availability diet (LEA). Data presented as

mean \pm SD. * Indicates a significant decrease from fasting levels. # Indicates a significant within-group difference to Baseline. \$ Indicates a significant decrease from pre-exercise levels.

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Figure 1

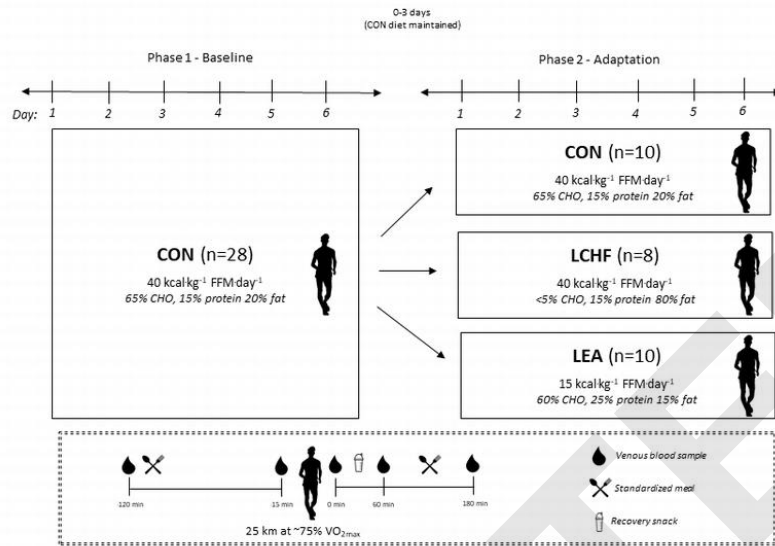


Figure 2

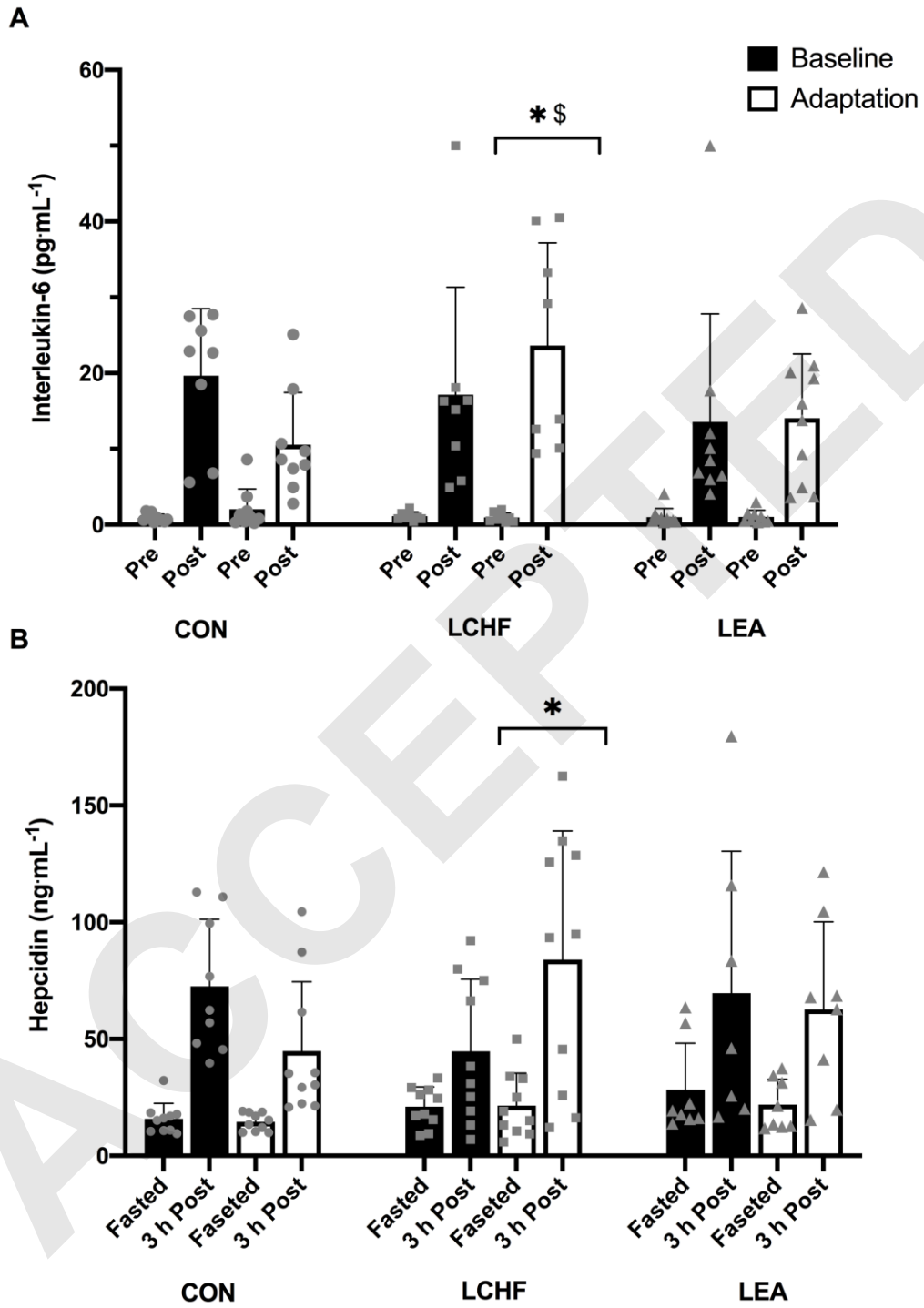


Figure 3

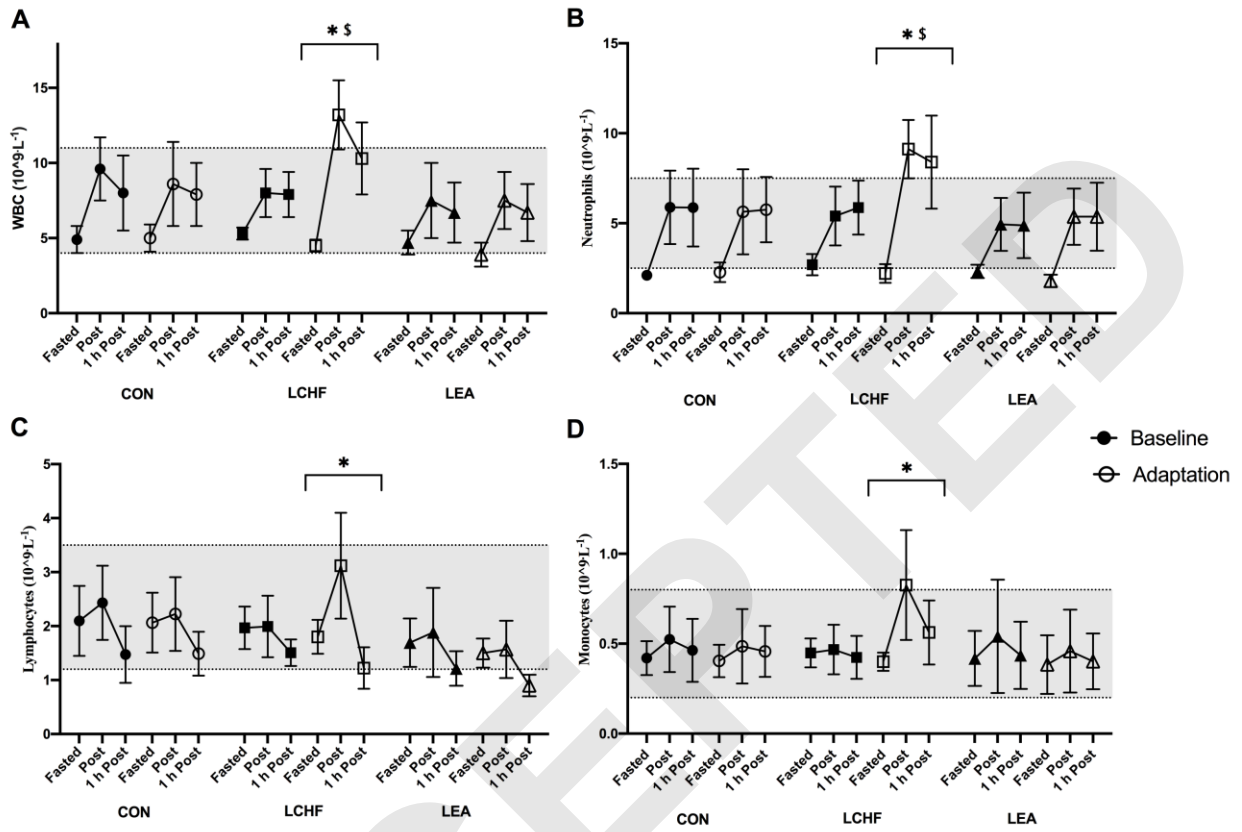


Figure 4

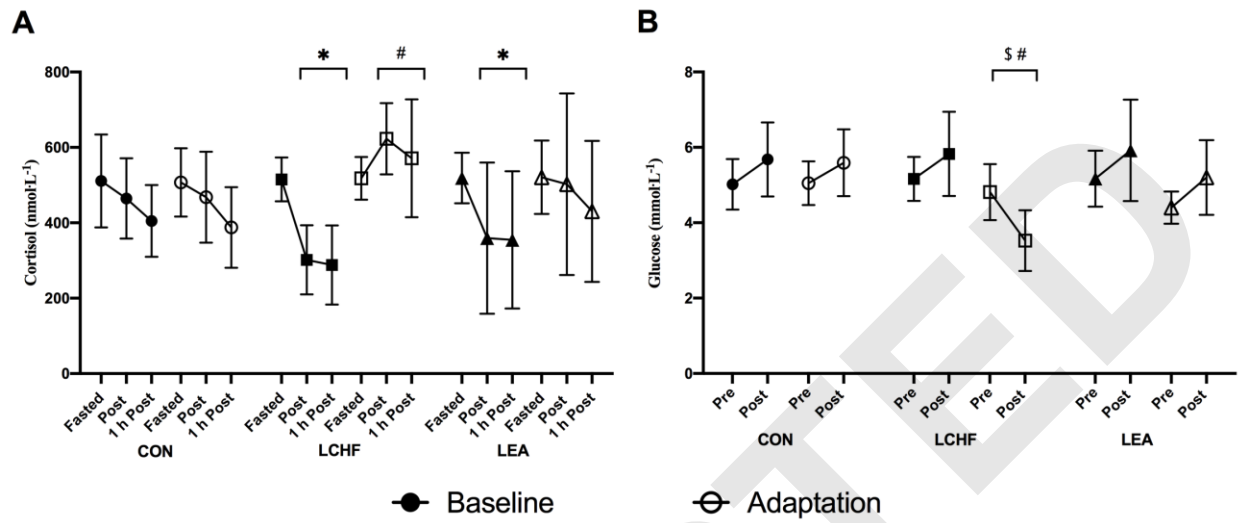


Table 1. Characteristics of athletes allocated to the high energy/carbohydrate availability diet (CON), ketogenic low carbohydrate high fat diet (LCHF) and the low energy availability diet (LEA). Average daily training load was calculated across each 6-day phase. Data presented as mean (SD). No significant differences between groups were evident for any of these variables.

	CON (n=10)	LCHF (n=8)	LEA (n=10)
Age (y)	26.7 (7.0)	26.2 (3.9)	30.0 (4.0)
$\dot{V}O_{2max}$ (mL·kg⁻¹·min⁻¹)	63.2 (3.6)	67.5 (5.7)	62.1 (6.1)
10 km Personal Best (min:sec)	41:56 (1:52)	41:41 (2:10)	40:22 (1:15)
Baseline training volume (km·day⁻¹)	20.3 (3.2)	18.3 (3.2)	19.6 (2.7)
Adaptation training volume (km·day⁻¹)	19.3 (3.1)	17.9 (3.1)	19.2 (2.3)

Table 2. Actual dietary intake for athletes adhering to a high energy/carbohydrate availability diet (CON), low carbohydrate high fat diet (LCHF) and low energy availability diet (LEA) during the Baseline and Adaptation phases of the study. Data presented as mean (SD). * Indicates a significant difference to Baseline. # Indicates a significant difference to CON. \$ Indicates a significant difference to LCHF

		Baseline			Adaptation		
		CON	LCHF	LEA	CON	LCHF	LEA
Body Mass	kg	66.6 (6.2)	66.2 (7.7)	67.8 (5.4)	66.1 (6.3)	64.2* (7.5)	65.8* (5.3)
Energy Intake	kcal·day ⁻¹	3824 (623)	3843 (462)	3727 (335)	3970 (537)	3730 (411)	2335 *# ^{\$} (238)
	kcal·kg ⁻¹	58 (6)	59 (4)	55 (2)	61 (5)	57 (3)	35 *# ^{\$} (3)
Energy Availability	kcal·kg ⁻¹	40 (3)	41 (1)	41 (3)	41 (4)	41 (2)	15 *# ^{\$} (2)
	FFM·d ⁻¹						
CHO	g·day ⁻¹	613 (102)	616 (76)	599 (53)	639 (87)	36 *# (6)	338 *# ^{\$} (33)
	g·kg ⁻¹ ·day ⁻¹	9.4 (1.0)	9.5 (0.6)	8.9 (0.4)	9.8 (0.8)	0.5 *# (0.1)	5.0 *# ^{\$} (0.4)
Protein	g·day ⁻¹	144 (22)	144 (17)	141 (14)	148 (21)	145 (16)	141 (14)
	g·kg ⁻¹ ·day ⁻¹	2.2 (0.2)	2.2 (0.1)	2.1 (0.1)	2.3 (0.2)	2.2 (0.0)	2.1 (0.1)
Fat	g·day ⁻¹	83 (15)	84 (11)	79 (9)	84 (13)	330 *# (39)	40 *# ^{\$} (7)
	g·kg ⁻¹ ·day ⁻¹	1.3 (0.2)	1.3 (0.1)	1.2 (0.1)	1.3 (0.2)	5.1 *# (0.4)	0.6 *# ^{\$} (0.1)
Iron	mg·day ⁻¹	21.1 (2.8)	20.2 (2.0)	21.2 (2.7)	23.9 (4.4)	16.0 *# (2.1)	16.2 *# (2.0)
Cholesterol	mg·day ⁻¹	388 (110)	459 (71)	429 (79)	382 (81)	639 *# (152)	302 *# ^{\$} (30)

Table 3. Fasting cholesterol, hormones and C-reactive protein (CRP) levels for athletes adhering to a high energy/carbohydrate availability diet (CON), low carbohydrate high fat diet (LCHF) and low energy availability diet (LEA) during the Baseline and Adaptation phases of the study. Data presented as mean (SD). *Indicates a significant difference to Baseline (p<0.05).

	CON		LCHF		LEA	
	Baseline	Adaptation	Baseline	Adaptation	Baseline	Adaptation
CRP (mg·L ⁻¹)	0.92 (0.59)	0.98 (0.56)	0.79 (0.35)	1.16 (0.96)	0.67 (0.45)	0.76 (0.61)
Testosterone (nmol·L ⁻¹)	19.2 (4.2)	17.9* (4.2)	19.5 (4.3)	16.2* (5.1)	16.1 (5.8)	13.4* (6.4)
Cortisol (nmol·L ⁻¹)	511 (123)	507 (91)	515 (58)	518 (57)	519 (67)	521 (97)
T/C Ratio (au)	0.040 (0.016)	0.036* (0.009)	0.039 (0.011)	0.032* (0.012)	0.031 (0.011)	0.027* (0.013)
Total Cholesterol (nmol·L ⁻¹)	3.22 (0.41)	3.22 (0.41)	3.58 (0.64)	3.05 (0.63)	3.73 (0.39)	3.25* (0.26)
HDL (nmol·L ⁻¹)	1.40 (0.20)	1.49* (0.24)	1.42 (0.26)	1.55* (0.32)	1.56 (0.24)	1.69* (0.31)
LDL (nmol·L ⁻¹)	2.06 (0.43)	2.15 (0.65)	2.48 (0.65)	1.97 (0.74)	2.82 (0.85)	1.99* (0.61)
Triglycerides (nmol·L ⁻¹)	0.63 (0.23)	0.74 (0.17)	0.74 (0.15)	0.50* (0.25)	0.75 (0.24)	0.61 (0.18)
TC/HDL Ratio (au)	2.33 (0.42)	2.20* (0.39)	2.56 (0.43)	2.04* (0.68)	2.46 (0.62)	2.01* (0.52)

Table 4. Iron profile variables for athletes adhering to high energy/carbohydrate availability diet (CON), low carbohydrate high fat diet (LCHF) and low energy availability diet (LEA) during the Baseline and Adaptation phases of the study. Data presented as mean (SD). *Indicates a significant difference to Baseline. #Significant differences compared to CON. \$Significant differences compared to LEA.

	CON		LCHF		LEA	
	Baseline	Adaptation	Baseline	Adaptation	Baseline	Adaptation
Ferritin ($\mu\text{g}\cdot\text{L}^{-1}$)	90 (31)	77 (28)	115 (44)	104 (44)	89 (49)	94 (63)
Iron ($\mu\text{mol}\cdot\text{L}^{-1}$)	16.4 (4.3)	13.3 (3.4)	10.8 (4.3)	12.7 (3.0)	10.5 [#] (4.0)	13.7 (3.4)
Transferrin ($\text{g}\cdot\text{L}^{-1}$)	2.55 (0.32)	2.55 (0.33)	2.68 (0.32)	2.61 (0.34)	2.78 (0.41)	2.69 (0.37)
Transferrin Saturation (%)	25.0 (6.4)	20.4 (5.9)	15.6 (6.0)	19.0 (5.4)	15.2 [#] (7.7)	19.9 (6.8)
Hepcidin ($\text{ng}\cdot\text{mL}^{-1}$)	15.9 (6.6)	14.5 (3.8)	23.0 (8.2)	25.0 (13.4)	25.2 (18.8)	19.0 (11.4)
Hemoglobin ($\text{g}\cdot\text{dL}^{-1}$)	14.2 (0.7)	13.8 (0.7)	15.3 (0.8)	15.6 [#] (1.0)	14.6 (0.8)	14.5 (1.1)
Hematocrit (%)	41.5 (2.3)	40.5 (3.1)	43.8 [#] (2.7)	44.1 [#] (2.9)	42.8 (2.3)	42.3 (2.8)
Reticulocytes ($10^9\cdot\text{L}^{-1}$)	51.8 (9.1)	55.5 (17.4)	48.1 (20.5)	38.7 ^{\$} (13.1)	55.1 (12.4)	57.5 (12.8)
Reticulocytes (%)	1.10 (0.18)	1.18 (0.37)	0.95 (0.39)	0.76 (0.25)	1.13 (0.25)	1.19 (0.23)
Red Blood Cells ($12^9\cdot\text{L}^{-1}$)	4.72 (0.28)	4.59 (0.34)	5.03 (0.32)	5.13 [#] (0.41)	4.90 (0.23)	4.82 (0.28)