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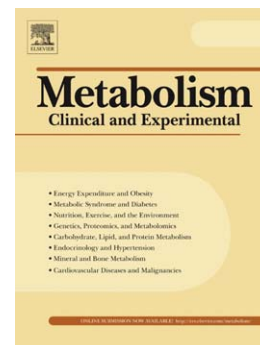
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Metabolic characteristics of keto-adapted ultra-endurance runners

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ABSTRACT

Background: Many successful ultra-endurance athletes have switched from a high-carbohydrate to a low-carbohydrate diet; but they have not previously been studied to determine the extent of metabolic adaptations.

Methods: Twenty elite ultra-marathoners and ironman distance triathletes performed a maximal graded exercise test and a 180 min submaximal run at 64% VO_2max on a treadmill to determine metabolic responses. One group habitually consumed a traditional high-carbohydrate (HC; $n=10$, %carbohydrate:protein:fat = 59:14:25) diet, and the other a low-carbohydrate (LC; $n=10$, 10:19:70) diet for an average of 20 mo (range 9 to 36 mo).

Results: Peak fat oxidation was 2.3-fold higher in the LC group (1.54 ± 0.18 vs 0.67 ± 0.14 g/min; $P=0.000$) and it occurred at a higher percentage of VO_2max (70.3 ± 6.3 vs $54.9 \pm 7.8\%$; $P=0.000$). Mean fat oxidation during submaximal exercise was 59% higher in the LC group (1.21 ± 0.02 vs 0.76 ± 0.11 g/min; $P=0.000$) corresponding to a greater relative contribution of fat (88 ± 2 vs $56 \pm 8\%$; $P=0.000$). Despite these marked differences in fuel use between LC and HC athletes, there were no significant differences in resting muscle glycogen and the level of depletion after 180 min of running (-64% from pre-exercise) and 120 min of recovery (-36% from pre-exercise).

Conclusion: Compared to highly-trained ultra-endurance athletes consuming a HC diet, long-term keto-adaptation results in extraordinarily high rates of fat oxidation, whereas muscle glycogen utilization and repletion patterns during and after a 3 hr run are similar.

Key Words: carbohydrate, fat, metabolism, exercise, glycogen

Abbreviations: VO_{2max} = maximal oxygen consumption, HC = high-carbohydrate, LC = low-carbohydrate, FASTER = Fat Adapted Substrate use in Trained Elite Runners, RPE = ratings of perceived exertion, DXA = dual-energy X-ray absorptiometry, RER = respiratory exchange ratio, USG = urine specific gravity, ELISA = enzyme-linked immunosorbent assay, NEFA = non-esterified fatty acid, HOMA = homeostatic model assessment of insulin resistance, IP – immediately post-exercise, PE-120 (120 min post-exercise)

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INTRODUCTION

The concept that a diet high in carbohydrate is necessary for optimizing exercise performance gained credence in the late 1960s when it was discovered that muscle glycogen depletion was associated with fatigue, and that a high-carbohydrate diet maintained muscle glycogen and performance (1-3). Decades later, a great deal of evidence has accumulated in support of consuming carbohydrate before, during and after exercise (4,5). Textbooks and position statements (6) reinforce the supremacy of the high-carbohydrate fueling paradigm for maximizing performance and recovery.

Less appreciated is the perspective that there is no essential requirement for dietary carbohydrate because humans possess a robust capacity to adapt to low carbohydrate availability. Around the same time the importance of glycogen was recognized in the 1960s, ground-breaking work on the metabolic adaptations to starvation revealed an elegant mechanism by which humans switch to using lipid-based fuels (7). After a few weeks of starvation when glycogen levels significantly decrease, hepatic ketone production increases dramatically to displace glucose as the brain's primary energy source, while fatty acids supply the majority of energy for skeletal muscle. Glucose production from non-carbohydrate sources via gluconeogenesis supplies carbons for the few cells dependent on glycolysis. Similar metabolic adaptations favoring near-exclusive reliance on lipid-based fuels occur after several weeks of a ketogenic diet when carbohydrate is restricted to very low levels, protein is kept moderate, and dietary fat is emphasized (8-10).

A critical mass of experimental data on ketogenic diets and ketone physiology has been generated in the last decade demonstrating the safety and therapeutic efficacy in managing a range of clinical conditions (11-13). Beyond the clinical applications, it was demonstrated more

than three decades ago that consumption of a ketogenic diet with less than 10 g of carbohydrate per day for 4 wk did not compromise endurance performance in elite cyclists (10). These keto-adapted cyclists derived >90% of their fuel from the oxidation of fat while exercising at 64% maximal oxygen consumption, corresponding to an average fat oxidation of 1.5 g/min.

Maximal rates of fat oxidation in humans are generally thought to be much less, even in highly trained endurance athletes with very high aerobic capacity. In the largest study to date that examined peak rates of fat oxidation during exercise, the highest reported value in any single individual was 1.0 g/min (14), 50% lower than the mean value recorded for five keto-adapted cyclists (10). A handful of other studies have examined diets lower in carbohydrate and higher in fat on metabolism during exercise (15-18). Although these studies consistently show greater fat oxidation, values are well below previously reported maximal rates (10). This is likely due to a higher carbohydrate intake and insufficient time to allow full keto-adaptation; thus resulting in less pronounced shifts in fuel use.

There is a distinct lack of research on keto-adapted athletes who have been restricting carbohydrate for several consecutive months despite the fact that many endurance athletes have adopted a low-carbohydrate lifestyle in the past few years. Metabolic studies in this group may yield new insights into human capacities to adapt and thrive under different nutritional inputs. Therefore, we designed the FASTER (Fat Adapted Substrate use in Trained Elite Runners) study to compare metabolic differences between competitive ultra-marathoners and ironman distance triathletes consuming low-carbohydrate (LC) and high-carbohydrate (HC) diets.

METHODS

Experimental Approach

This was a cross-sectional study comparing two groups of elite ultra-endurance athletes habitually consuming either a LC or HC diet. Athletes were carefully matched for age, physical characteristics, primary competition distance, and competition times. With one exception, all athletes lived in the United States and traveled via plane or car to our laboratory for two consecutive days of testing. On Day 1, participants performed a maximal oxygen consumption test during which peak fat oxidation was determined. Day 2 consisted of a treadmill run at 64% of maximal oxygen consumption for 3 hr to determine metabolic responses before, during, and after exercise. Subjects were informed of the purpose and possible risks of the investigation prior to signing an informed consent document approved by the Institutional Review Board.

Participants

We targeted male ultra-endurance runners 21-45 years of age consuming a LC ($n=10$) or a HC ($n=10$) diet who were in the top 10% of finalists competing in sanctioned running events ≥ 50 km and/or triathlons of at least half iron-man distance (113 km). Several athletes had sponsors (55%), course records (30%), national records (10%), international records (10%), and national/international-level appearance for Team USA (25%). Interested athletes completed questionnaires to assess their medical, diet, training, and running competition histories. As a pre-screening technique to determine if participants met dietary inclusion criteria, a registered dietitian had at least one phone call and email communication with each athlete about their habitual diet. Eligible participants were required to measure and weigh all foods consumed and provide a detailed description for 3-days (2 weekdays and 1 weekend day). All subjects were

contacted through phone calls and emails to obtain home recipes, local restaurant menus, and nutritional information as necessary for determining accurate nutrient information. Habitual diet information was entered into commercial nutrient analysis software (Nutritionist Pro™, Axxya Systems, Stafford, TX). Subjects consuming a LC diet, defined as <20% energy from carbohydrate and >60% from fat, consistently for at least 6 months were eligible for the LC group. Subjects consistently consuming >55% energy from carbohydrate were considered for the HC group. Athletes were excluded if they did not consume the appropriate diet for the allotted amount of time or had any health issues, including, but not limited to diabetes, heart disease, kidney, liver, or other metabolic or endocrine dysfunction, current injury, anti-inflammatory medication use, anabolic drug use, or prone to excessive bleeding.

Exercise Protocols

Maximal Aerobic Capacity. Athletes were in racing condition but refrained from any competitions for a minimum of 7 days prior to testing. They were instructed to maintain their habitual diet leading up to testing and to record their diet for the day before and the day of travel (Testing Day 1). Subjects arrived at the laboratory between 1600-1900 hours following a 4 hr fast. Upon arrival, height and body weight were recorded, after which athletes were familiarized with the equipment and procedures prior to completing a maximal oxygen consumption (VO₂max) test on a motorized treadmill (956i Treadmill, Precor, Woodinville, WA) using indirect calorimetry (TrueOne 2400, ParvoMedics, Sandy, UT). Subjects were fitted with a facemask and headgear (7450 Series Silicone V2™ Oro-Nasal Mask, Hans Rudolph, Shawnee, KS) with an adaptor ring to attach the two-way air chamber and gas collection hose to the mask (Adapter 7450 V2™, Hans Rudolph, Shawnee, KS). The protocol involved a 3 min walking

warm-up at 3.5 mph, followed by 2 min stages that incrementally increased speed and incline linearly to elicit ~5% increase in VO_2 at each stage. The test was considered complete when subjects voluntarily stopped the treadmill, which occurred within 18 min. Ratings of perceived exertion (RPE) on a 0-10 scale and heart rate were measured at each stage.

Breath-by-breath gas exchange measurements of VO_2 and VCO_2 were collected and recorded every 30 sec and used to calculate oxygen uptake, minute ventilation, carbohydrate and fat oxidation rates, and respiratory exchange ratio (RER) with the assumption that the urinary nitrogen excretion was negligible. Peak fat oxidation was determined from the highest recorded 30 sec interval. Following testing, subjects consumed a meal (dinner) consistent with the macronutrient percentages of their habitual diet. After dinner, all subjects were instructed to fast overnight, restrict caffeine and abstain from taking over-the-counter medications. To ensure hydration, liberal consumption of water was strongly encouraged for the rest of the evening.

Submaximal Exercise Protocol. Athletes returned to the laboratory for testing the following morning at 0600 after a 10 hr overnight fast (**Fig 1**). Each subject provided a small urine sample to assess specific gravity (Model A300CL, Spartan, Japan) as a measure of hydration (all subjects had a USG ≥ 1.025). Body composition was determined via dual-energy X-ray absorptiometry (DXA) (Prodigy, Lunar Corporation, Madison, WI), height was measured to the nearest 0.1 cm and total body weight was recorded to the nearest 0.1 kg on a digital scale (OHAUS Corp., Fordham Park, NJ). The facemask and headgear were used to determine resting energy expenditure by indirect calorimetry for 10 min after which a seated blood sample was obtained from a forearm vein. Subjects were then moved to a hospital bed for muscle biopsy preparation and acquisition.

After these baseline measures, subjects consumed a shake (5 kcal/kg body mass) with a macronutrient distribution (%carbohydrate:fat:protein) of 5:81:14 in the LC group and 50:36:14 in the HC group. On average, this translated into shakes consisting of 343 kcal, 4.3 g carbohydrate, 31.3 g fat, and 12.6 g protein for the LC group and 332 kcal, 42.7 g carbohydrate, 13.7 g fat, and 12.4 g protein in the HC group. Proportions varied, but both the LC and HC shakes were made from heavy cream, olive oil, whey protein, walnut oil, and strawberries. The HC shake also contained banana and agave syrup. Subjects rested quietly for 90 min after ingestion of the shake. Indirect calorimetry measurements were taken during the last 10 min and a second pre-exercise blood sample was taken.

Subjects then moved to the treadmill and began running at an intensity equivalent to 64% VO_2max for 180 min. Slight adjustments to the pace were made during the first 10 min based on real-time measurements of oxygen uptake. During the endurance exercise test, subjects were allowed to drink water ad libitum but no other nutritional supplementation was consumed. The treadmill was stopped briefly at 60 and 120 min to obtain blood. Heart rate and RPE were recorded every 30 min, and indirect calorimetry measurements were obtained for 10 min at the following intervals: 50-60, 110-120, 140-150, and 170-180 min.

After 180 min of running, subjects moved to a wheel chair and blood was obtained immediately. Subjects were moved to a bed and a second muscle biopsy was performed ~15 min after completing the run. Subjects then consumed a shake identical to the pre-run shake. Indirect calorimetry measurements and blood were taken at 30, 60, and 120 min post-exercise. A final muscle biopsy was taken 120 min post-exercise. The entire day of testing lasted about 8 hr finishing mid-afternoon (see Fig 1), which is the primary reason for including the shakes before and after exercise since no other caloric intake was allowed during testing.

Blood Draws and Muscle Biopsies

An indwelling Teflon cannula or a 21G butterfly needle was inserted into an antecubital vein of the subject. The intravenous line was kept patent with normal saline solution flushes. Prior to each blood draw, 3 mL of blood were extracted and discarded to avoid inadvertent saline dilution of the blood. During each draw, blood was collected into appropriate tubes to allow for whole blood, serum, and plasma analyses. Blood was centrifuged at 1500 x g for 15 min and 4°C, aliquoted into storage tubes, and stored in ultra-low freezers for batch analysis.

Muscle biopsies were obtained from the superficial portion of the vastus lateralis using the percutaneous needle technique with suction. The biopsy site was prepped and the skin was anesthetized by a local subcutaneous injection of 2% lidocaine hydrochloride. A small incision (~1 cm) was made through the skin and muscle fascia and a 5 mm diameter sterile biopsy needle (Surgical Instruments Engineering Ltd, Midlothain, United Kingdom) was introduced into the muscle to a depth of ~2 cm. To ensure adequate sample sizes a double-chop method combined with suction was used. The muscle sample was removed from the needle and divided into multiple pieces of roughly equal size. The specimens were cleaned of connective tissue and blood, flash frozen in liquid nitrogen, and stored at -80°C for later determination of glycogen. The incision was covered with sterile gauze and compression was applied to prevent bleeding. The incision was then closed with a single suture. In order to avoid possible impairment of glycogen synthesis resulting from microtrauma in the area near the biopsy (19), we performed the immediate post-exercise biopsy on the opposite leg, and the 2 hr post-exercise biopsy 3 cm apart from the first incision site.

Biochemical Analyses

Frozen samples were thawed only once before analysis. Plasma glucose and triglycerides were measured using a Cobas C 111 analyzer (Roche Diagnostics, Indianapolis IN). Serum insulin was analyzed by ELISA (Calbiotech, Spring Valley, CA). Serum glycerol (Cayman Chemical, Ann Arbor, MI), ketones and total non-esterified fatty acids (NEFA) (Wako Diagnostics, Mountain View, CA) were analyzed by enzymatic assays. Insulin, ketones, lactate, NEFA, and glycerol had intra-assay CVs of 4.31, 6.40, 4.65, 4.51 and 5.31% respectively. All samples were run in duplicate except for those measured on Cobas. Glucose and insulin values were used to calculate an index of insulin resistance [HOMA-IR; calculated as $\text{Glucose (mmol/L)} \cdot \text{Insulin } (\mu\text{IU/mL}) / 22.5$] from the baseline resting sample (20). Glycogen was determined from a portion of muscle (10 mg) that was hydrolyzed in 500 μl of 2N HCl by heating at 99°C for 2 hr, with occasional vortexing. Any weight lost to heating was replaced using ddH₂O. The solution was neutralized with 500 μl 2N NaOH and 50 μl of Tris buffer (pH 6.5). 1 ml of glucose hexokinase reagent (ThermoFisher), including 20 μl of supernatant of the hydrolysis product was prepared, and the free glycosyl units were then spectrophotometrically assayed in triplicate at 340 nm on a Nanodrop 2000c spectrophotometer (21). Coefficient of variation was 4.2%.

Statistical Analyses

Independent t-tests were used to examine differences between LC and HC groups for dietary intake and physical characteristics. For biochemical responses to exercise, we used a two-way repeated measures analysis of variance with group (HC and LC) as a between factor and time (i.e., exercise-induced responses) as a within factor. Fisher's least significant

difference post hoc was used to examine pairwise comparisons when significant main or interaction effects were observed. The alpha level for significance was set at $p \leq 0.05$.

RESULTS

Subject Characteristics and Habitual Diet. There were no significant differences between groups in physical characteristics or aerobic capacity (**Table 1**). Two athletes in each group were ironman distance triathletes while the remainder competed primarily in running events ranging from 80 to 161 km (50 to 100 miles). The main difference between groups was their habitual diet (**Table 2**). Average time on a LC diet was 20 mo (range 9 to 36 mo). In the LC group, an overwhelming majority of energy intake was derived from fat (70%), primarily saturated and monounsaturated fatty acids. Only ~10% of their energy intake was from carbohydrate. Conversely, the HC group consumed over half their energy as carbohydrate (59%). Absolute protein was not significantly different between groups, but LC athletes consumed a greater relative amount than HC athletes (19 vs 14% of total energy). Daily nutrient intake determined from food records the 2 days prior to testing showed similar dietary patterns as the habitual diet and were not significantly different (**Supplemental Table 1**).

Peak Fat Oxidation. Peak fat oxidation was on average 2.3-fold higher in the LC group (1.54 ± 0.18 vs 0.67 ± 0.14 g/min; $P=0.000$), with every subject in the LC group (range 1.15 to 1.74 g/min) exceeding the highest value in the HC group (range 0.40 to 0.87 g/min) (**Fig 2A**). The percent of maximal aerobic capacity where peak fat oxidation occurred was also significantly higher in the LC group (70.3 ± 6.3 vs $54.9 \pm 7.8\%$; $P=0.000$) (**Fig 2B**).

Submaximal Substrate Oxidation. All 20 subjects completed 180 min of running. The average percent maximum oxygen consumption during exercise was similar in the LC ($64.7 \pm$

0.0%) and HC ($64.3 \pm 0.0\%$) groups. Ratings of perceived exertion over the 3 hr run were also similar between groups gradually increasing from 3.0 ± 1.3 at the start of exercise to 5.1 ± 1.9 at the end of exercise in the LC group and from 2.9 ± 0.9 to 5.2 ± 2.9 in the HC group. Absolute energy expenditure during the run was not different between the LC (12.4 ± 0.1 kcal/min) and HC (12.2 ± 0.2) groups; however, substrate oxidation patterns at rest and during exercise were significantly different (**Fig 3**). At rest prior to exercise, the RER was significantly ($P=0.000$) lower in the LC (0.72 ± 0.05) than the HC (0.86 ± 0.08) group, indicating a contribution from fat of 95 vs 47%, respectively. During 3 hr of exercise, RER fluctuated between 0.73 and 0.74 translating into relatively stable and higher fat oxidation rates of ~ 1.2 g/min in the LC group, whereas fat oxidation values were significantly lower in the HC group at all time points (**Fig 3A**). The rate of carbohydrate oxidation in the LC group was stable during exercise and significantly ($P=0.000$) lower than the HC group (**Fig 3B**). The average contribution of fat during exercise in the LC and HC groups were 88 and 56%, respectively.

Circulating Metabolites. Circulating markers of lipid metabolism indicated a significantly greater level of ketogenesis (**Fig 4A**) and lipolysis (**Fig 4B**) in the LC athletes. Serum non-esterified fatty acids were higher at the start of exercise in LC athletes, but peak levels at the end of exercise were not significantly different between groups (**Fig 4C**). Plasma triglycerides were not different between groups (**Fig 4D**).

Plasma glucose and serum insulin were not significantly different between groups at rest and during exercise but increased during the last hour of recovery in the HC athletes, likely due to the greater amount of carbohydrate in the shake (**Fig 5A and 5B**). There was no significant difference between groups in insulin resistance as determined by HOMA. Serum lactate

responses were variable, but were significantly higher in LC athletes during the last hour of exercise (**Fig 5C**).

Muscle Glycogen. Compared to baseline, muscle glycogen was significantly decreased by 62% immediately post-exercise and 38% at 2 hr post-exercise in the HC group. The LC group exhibited a similar pattern; muscle glycogen was decreased by 66% immediately post-exercise and 34% at 2 hr post-exercise (**Fig 6A**). There were no significant differences in pre-exercise or post-exercise glycogen concentrations between groups. There was a high degree of variability in muscle glycogen concentrations pre-exercise in both groups. In contrast, the depletion and resynthesis patterns showed a more uniform response, especially the amount of glycogen synthesized during the 2 hr recovery period in LC athletes (44.8 ± 7.5 ; 95% CI 40.2–49.4 $\mu\text{mol/g w.w.}$), which was one-third less variable than HC athletes (34.6 ± 23.9 ; 95% CI 19.8–49.4 $\mu\text{mol/g w.w.}$) (**Fig 6B**). Interestingly, in all ten LC athletes the total amount of carbohydrate oxidized during the 3 hr run as calculated from indirect calorimetry (mean \pm SD; 64 ± 25 g) was lower than the total amount of glycogen disappearance (mean \pm SD; 168 ± 65 g), assuming 10 kg of active tissue.

DISCUSSION

We studied two groups of highly-trained competitive ultra-endurance athletes who were well matched in regards to training status and physical characteristics. The main difference was that the LC athletes consumed 6-times less dietary carbohydrate than LC athletes (82 vs 684 g/day) for an average of 20 mo. The most notable findings were that compared to HC athletes, the LC keto-adapted runners showed: 1) two-fold higher rates of peak fat oxidation during graded exercise, 2) greater capacity to oxidize fat at higher exercise intensities, 3) two-fold

higher rates of fat oxidation during sustained submaximal running, and 4) no differences in pre-exercise muscle glycogen concentrations, the rate of glycogen utilization during exercise, and the rate of glycogen synthesis during recovery. Thus, we show for the first time that chronic keto-adaptation in elite ultra-endurance athletes is associated with a robust capacity to increase fat oxidation during exercise while maintaining normal skeletal muscle glycogen concentrations.

The whole body maximal fat oxidation rates in the LC athletes were similar to those observed in elite keto-adapted cyclists (10), which are noteworthy considering they are ~50% higher than previously reported maximal rates of fat oxidation in the literature (14). During submaximal exercise, fat contributed to 88 vs 56% of energy expended in LC and HC athletes, respectively. Several studies have consistently reported that short-term consumption of a high-fat diet in highly trained endurance athletes increases fat oxidation during submaximal exercise (reviewed in 22). However, no studies have showed the same degree of shift towards fat oxidation as observed in LC athletes reported here. In the LC ultra-endurance runners studied here, a combination of greater degree of carbohydrate restriction and longer period of adaptation likely resulted in a more robust capacity for fat oxidation during exercise.

Serum glycerol and non-esterified fatty acid concentrations increased rapidly during exercise and decreased during recovery. Whereas fatty acids were similar between groups, glycerol concentrations were ~2-fold higher in LC athletes. Phinney et al (10) also reported similar circulating fatty acids concentrations before and after keto-adaptation. Serum glycerol is a better indicator of adipose tissue lipolysis compared to fatty acids since adipose tissue and skeletal muscles have lower glycerol kinase activity and thus are not able to use glycerol as effectively. Still some reports suggest muscle can use circulating glycerol for intramuscular triglyceride synthesis (23). Previous studies have indicated a greater capacity to transport

circulating fatty acids into muscle after a short-term low-carbohydrate/high-fat diet (24,25). Thus, these data indicate a higher rate of adipose tissue lipolysis in LC athletes resulting in a greater release of both glycerol and fatty acids into the circulation, with greater overall uptake of fatty acids into skeletal muscle.

Carbohydrate oxidation was significantly lower in the LC ultra-endurance runners, but unexpectedly muscle glycogen concentrations were not different between groups. It was previously reported that a 4 wk ketogenic diet in elite cyclists decreased resting muscle glycogen by half and the rate of glycogen use during exercise by 4-fold (10). Other studies have shown that a low-carbohydrate/high-fat diet decreases resting glycogen and the rate of glycogen use during submaximal exercise (25,26). The duration of the LC diet was shorter (4 wk) in the work by Phinney (10), suggesting that complete adaptations in glycogen homeostasis and kinetics may take several months. The different glycogen responses could also be due to lower carbohydrate intake, which was <10 g/day in cyclists (10) versus 86 g/day in the LC runners. A short-term glycogen loading effect is unlikely since food logs were recorded for the two days leading up to testing and indicated the average carbohydrate intake was 64 g/day in LC athletes (**Supplemental Table 1**). The small relative contribution of carbohydrate to energy expenditure in LC athletes, but similar use of glycogen as HC athletes, indicates a decreased reliance on circulating glucose in the keto-adapted athlete.

The muscle glycogen responses in the LC athletes are provocative in light of data previously reported in highly-trained Alaskan sled dogs (27,28). Sled dogs have an innately high endurance capacity and often perform several hours of running at submaximal intensity while consuming a high-fat/low-carbohydrate diet. Dogs running 160 km/day for 5 days showed no cumulative muscle glycogen depletion despite eating a diet consisting of only 15% carbohydrate

(27). In a subsequent study (28), dogs ran 140 km/day for 4 days and showed a 66% reduction in muscle glycogen after the first 140 km run (similar to the 64% reduction in muscle glycogen in the LC athletes) and a progressive increase in muscle glycogen over subsequent days of running. A more recent study reported that trained Alaskan sled dogs eating a 16% carb diet showed an unexpected high rate of carbohydrate oxidation during exercise that was associated with a significant increase in gluconeogenesis from glycerol and increased lactate oxidation (29). Thus, highly-trained high-fat adapted sled dogs show very different fuel utilization patterns that predicted based on studies done in trained humans eating a high-carbohydrate diet.

A provocative finding was that LC athletes appeared to break down substantially more glycogen (>100 g) than the total amount of carbohydrate oxidized during the 3 hr run. This was the case in all ten LC athletes. Why would athletes with high rates of acetyl CoA generation from fatty acids bother breaking down muscle glycogen if those carbons are not terminally oxidized? Although speculative, we believe the reason may be to provide a source of glucose for the pentose phosphate pathway (PPP) and a source of pyruvate to form oxaloacetate. The PPP generates important 5 carbon sugars and reducing power (i.e., NADPH) for biosynthetic reactions such as ribonucleic acid synthesis and maintaining glutathione levels, respectively. Glyceraldehyde-3-phosphate and fructose 1,6 bisphosphate generated from the PPP can also enter glycolysis after the energy input stage, thereby generating ATP through substrate level phosphorylation and increasing formation of pyruvate. Pyruvate may be necessary in a keto-adapted athlete for two reasons. First, pyruvate can be used as an anaplerotic substrate by pyruvate carboxylase to generate oxaloacetate. At the onset of exercise several TCA cycle intermediates increase in concentration including fumarate, citrate and malate, however oxaloacetate remains low. Thus, in the keto-adapted athlete glycogen breakdown may be

necessary to ensure a constant source of oxaloacetate for optimal TCA functioning. Second, pyruvate can be converted to lactate or alanine in muscle, which may then serve as gluconeogenic substrate for the liver.

Rates of muscle glycogen synthesis in humans are highest when large amounts of carbohydrate are consumed immediately post-exercise (30), yet the LC athletes had similar rates of glycogen repletion compared to the HC athletes, despite receiving a negligible amount of carbohydrate after exercise (4 vs 43 g) and more fat (31 vs 14 g). When no carbohydrate or energy is provided after prolonged exercise, a small amount of muscle glycogen synthesis occurs (31) presumably due to hepatic gluconeogenesis providing a source of glucose for glycogen. Horses supplemented with fat after exercise showed impaired glycogen synthesis, but 3 wk of a high-fat diet resulted in similar glycogen repletion as horses fed a high-carbohydrate diet (32). An obvious question that arises is what is the carbon source for glycogen synthesis in the absence of carbohydrate intake post-exercise? Although speculative, lactate and/or glycerol, which were two-fold higher at the end of exercise in LC athletes and then sharply decreased during recovery, may have provided a source of carbons for glycogen synthesis during recovery (33). Lactate conversion to glycogen could occur directly (lactate glyconeogenesis) or indirectly via the Cori cycle. Interestingly, lactate and ketones are both transported across cell membranes by monocarboxylic acid transporters, which are upregulated after a ketogenic diet (34). Lactate was shown to account for up to 18% of skeletal muscle glycogen synthesized after high-intensity exercise (35). It could be that lactate rapidly replenished liver glycogen and an ability to maintain hepatic glucose output in the face of limited exogenous carbohydrate intake. Lactate Regardless of the mechanism, these results suggest that long-term consumption of a low-carbohydrate/high-fat diet in highly-trained ultra-marathoners results in adaptations in the homeostatic regulation of

muscle glycogen that act to preserve levels similar to those observed when exogenous carbohydrate availability is high.

LC athletes had ~3-fold higher levels of circulating beta-hydroxybutyrate and total ketones at rest, during exercise, and recovery. As expected, ketone levels increased progressively during exercise and peaked 30 min into recovery consistent with the previously reported post-exercise ketosis, also referred to as the Courtyce-Douglas effect (36). When ketone levels exceed 0.5 mmol/L, they become a preferred fuel for the brain (37). Since ketones are derived from fatty acids, the keto-adapted athlete has access to and an ability to utilize a stable, abundant source of fuel in the form of beta-hydroxybutyrate. Beyond its role as a metabolite, beta-hydroxybutyrate in the range of concentrations observed in the LC athletes has recently been shown to be a potent inhibitor of histone deacetylases, which in turn upregulates expression of antioxidant genes (38). It has also been observed that beta-hydroxybutyrate is associated with a reduction in the generation of reactive oxygen species by mitochondria (39). It remains to be demonstrated whether LC athletes derive benefit in terms of cognitive function or recovery from these unique metabolic and signaling effects of beta-hydroxybutyrate.

Since this was a cross-sectional study it is possible that LC athletes had naturally higher rates of fat oxidation or were somehow better suited to respond to a low-carbohydrate diet. Long-term prospective diet intervention studies would be helpful to better understand the time course and variability in adaptations to a low-carbohydrate diet. While we showed distinct differences in metabolic responses between LC and HC athletes, we did not measure performance. It will be important for future work to address a range of performance outcomes from endurance to anaerobic capacity to strength/power, as well as sport and/or military-specific tasks. All the LC athletes were initially high-carbohydrate athletes. Thus, they made the choice to switch to a very

low-carbohydrate diet and had enough self-perceived benefit to continue this lifestyle. From a practical standpoint, these observations broaden the dietary options available for endurance athletes to include a very low level of carbohydrate.

In summary, these results provide the first documentation of the metabolic adaptations associated with long-term consumption of a very low-carbohydrate/high-fat diet in highly trained keto-adapted ultra-endurance athletes. The enhanced ability to oxidize fat during exercise across a range of intensities is striking, as is the ability to maintain 'normal' glycogen concentrations in the context of limited carbohydrate intake. Keto-adaptation provides an alternative to the supremacy of the high-carbohydrate paradigm for endurance athletes.

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FIGURE LEGENDS

Figure 1. Experimental protocol to determine metabolic responses to submaximal exercise.

Figure 2. Individual peak fat oxidation rates (**A**) and the exercise intensity eliciting peak oxidation (**B**) during a maximal graded treadmill test. Mean responses between groups were significantly different (Independent t-test, $P=0.000$). Circles indicate mean and 95% CI. LC = low-carbohydrate diet group; HC = high-carbohydrate diet group.

Figure 3. Fat (**A**) and Carbohydrate (**B**) oxidation rate during 180 min of running at 65% VO_{2max} and 120 min of recovery. All time points were significantly different between groups. LC = low-carbohydrate diet group; HC = high-carbohydrate diet group.

Figure 4. Circulating concentrations of ketones (**A**), glycerol (**B**), non-esterified fatty acids (**C**), and triglycerides (**D**). LC = low-carbohydrate diet group; HC = high-carbohydrate diet group. BOHB = beta-hydroxybutyrate. All variables showed significant main time and interaction (group x time) effects. H and L = Indicates significant ($P\leq 0.05$) difference from the corresponding baseline (BL) value for the HC and LC diet group, respectively. *Indicates significant ($P=0.000$) difference between HC and LC values at that time point.

Figure 5. Circulating concentrations of glucose (**A**), insulin (**B**), and lactate (**C**). LC = low-carbohydrate diet group; HC = high-carbohydrate diet group. All variables showed significant main time and interaction (group x time) effects. H and L = Indicates significant ($P\leq 0.05$) difference from the corresponding baseline (BL) value for the HC and LC diet group, respectively. *Indicates significant ($P=0.000$) difference between HC and LC values at that time point.

Figure 6. Mean (**A**) and individual (**B**) muscle glycogen concentrations at baseline (pre-exercise), immediate post-exercise (IP), and 120 post-exercise (PE-120). Subjects ran on a

treadmill at 65% VO_2max for 180 min. *Indicates significant ($P=0.000$) difference from baseline. †Indicates significant ($P=0.000$) difference from IP. No significant differences between groups. LC = low-carbohydrate diet group; HC = high-carbohydrate diet group.

ACCEPTED MANUSCRIPT

Table 1. Subject characteristics.

	High-Carbohydrate Diet <i>n=10</i>		Low-Carbohydrate Diet <i>n=10</i>		T-Test
	Mean \pm SD	Range	Mean \pm SD	Range	P Value
Age, yr	32.9 \pm 6.0	22.0–40.0	34.1 \pm 7.1	21.0–45.0	0.689
Height, cm	173.9 \pm 5.3	167.1–182.0	175.7 \pm 7.8	165.1–189.4	0.555
Body mass, kg	66.5 \pm 6.8	57.9–79.9	68.8 \pm 8.2	55.5–81.6	0.491
Body fat, %	9.6 \pm 4.3	4.7–15.5	7.8 \pm 2.4	4.5–12.3	0.266
Lean mass, kg	57.3 \pm 5.0	49.4–64.2	60.9 \pm 7.1	50.2–71.7	0.387
Fat mass, kg	6.5 \pm 3.3	2.8–12.1	5.5 \pm 1.9	3.0–8.8	0.207
VO ₂ max, L/min	4.25 \pm 0.46	3.34–4.86	4.46 \pm 0.39	3.78–4.95	0.299
VO ₂ max, mL/kg/min	64.3 \pm 6.2	54.8–76.0	64.7 \pm 3.7	59.6–71.1	0.850
Competitive Running Experience (yr)	9 \pm 6	4–22	11 \pm 8	1–25	0.583

Table 2. Habitual daily nutrient intake.

	High-Carbohydrate Diet <i>n=10</i>	Low-Carbohydrate Diet <i>n=10</i>	T-Test
	Mean \pm SD	Mean \pm SD	P Value
Energy, kcal	3174 \pm 611	2884 \pm 814	0.380
Protein, g	118 \pm 38	139 \pm 32	0.186
Protein, %en	14.4 \pm 3.5	19.4 \pm 2.4	0.001
Protein, g/kg	1.7 \pm 0.4	2.1 \pm 0.6	0.192
Carbohydrate, g	486 \pm 128	82 \pm 62	0.000
Carbohydrate, %en	59.1 \pm 10.2	10.4 \pm 4.9	0.000
Fat, g	91 \pm 31	226 \pm 66	0.000
Fat, %en	25.0 \pm 7.4	69.5 \pm 6.0	0.000
Saturated fat, g	21 \pm 10	86 \pm 22	0.000
Monounsaturated fat, g	29 \pm 14	82 \pm 42	0.001
Polyunsaturated fat, g	18 \pm 9	28 \pm 17	0.106
Alcohol, %en	1.6 \pm 2.4	0.7 \pm 1.4	0.310
Cholesterol, mg	251 \pm 249	844 \pm 351	0.000
Fiber, g	57 \pm 27	23 \pm 17	0.000

Determined from 3 day dietary food records.

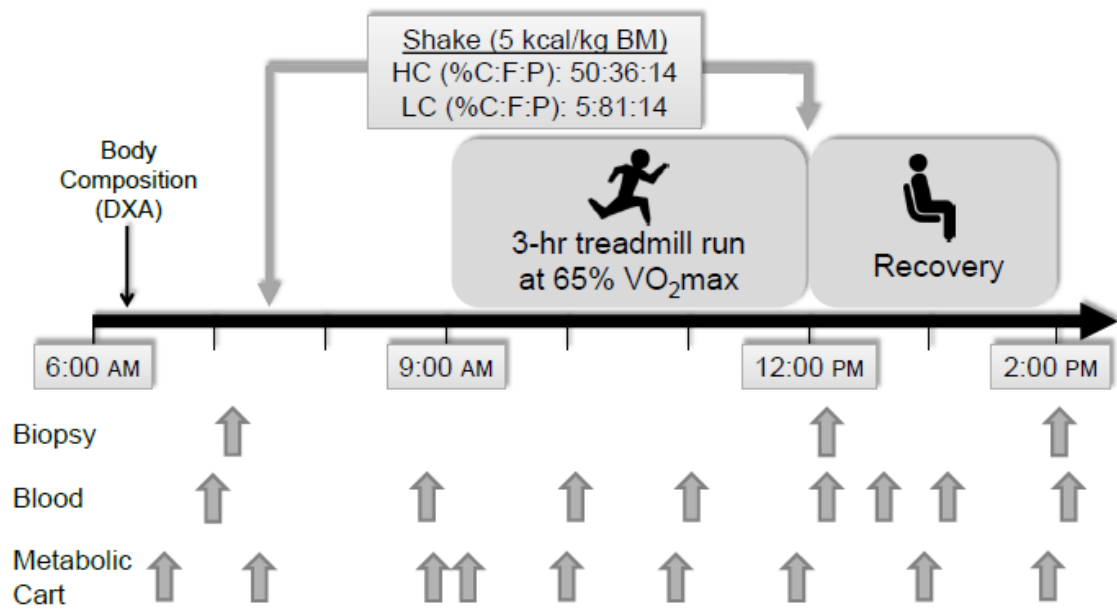


Figure 1

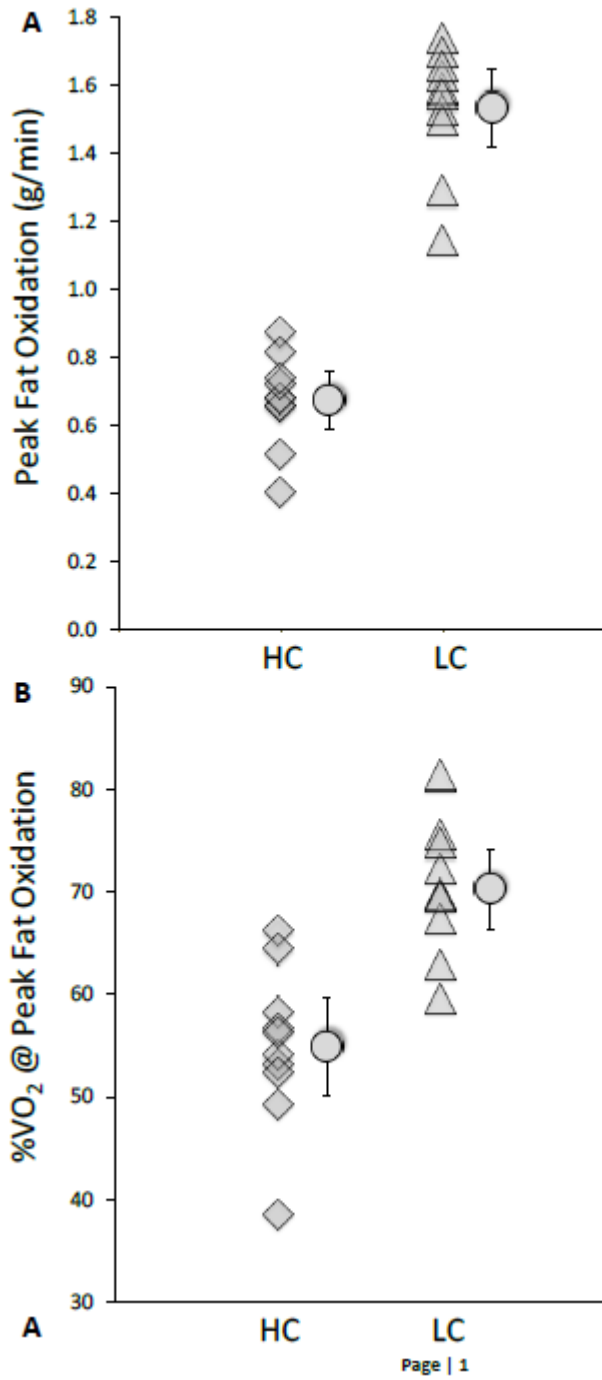
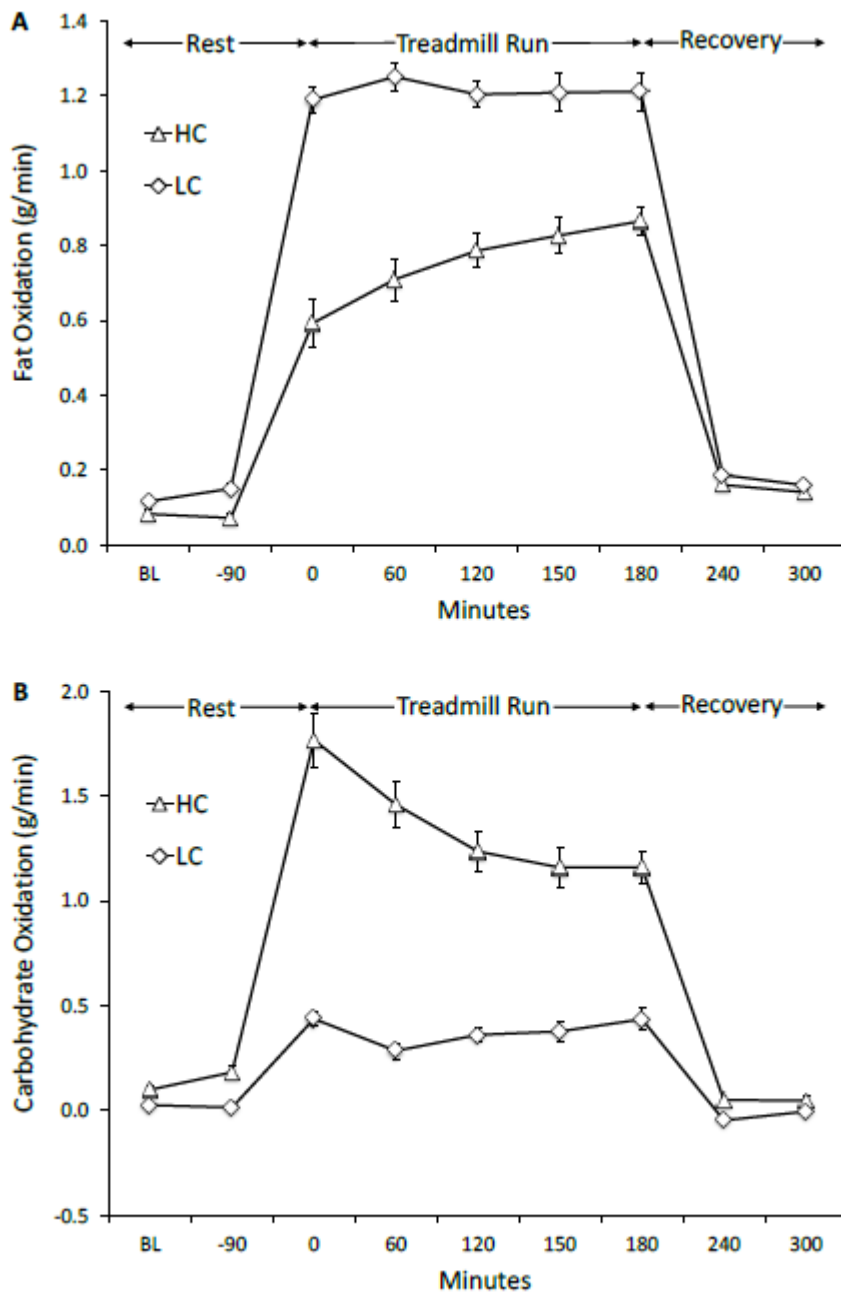


Figure 2



Page | 1

Figure 3

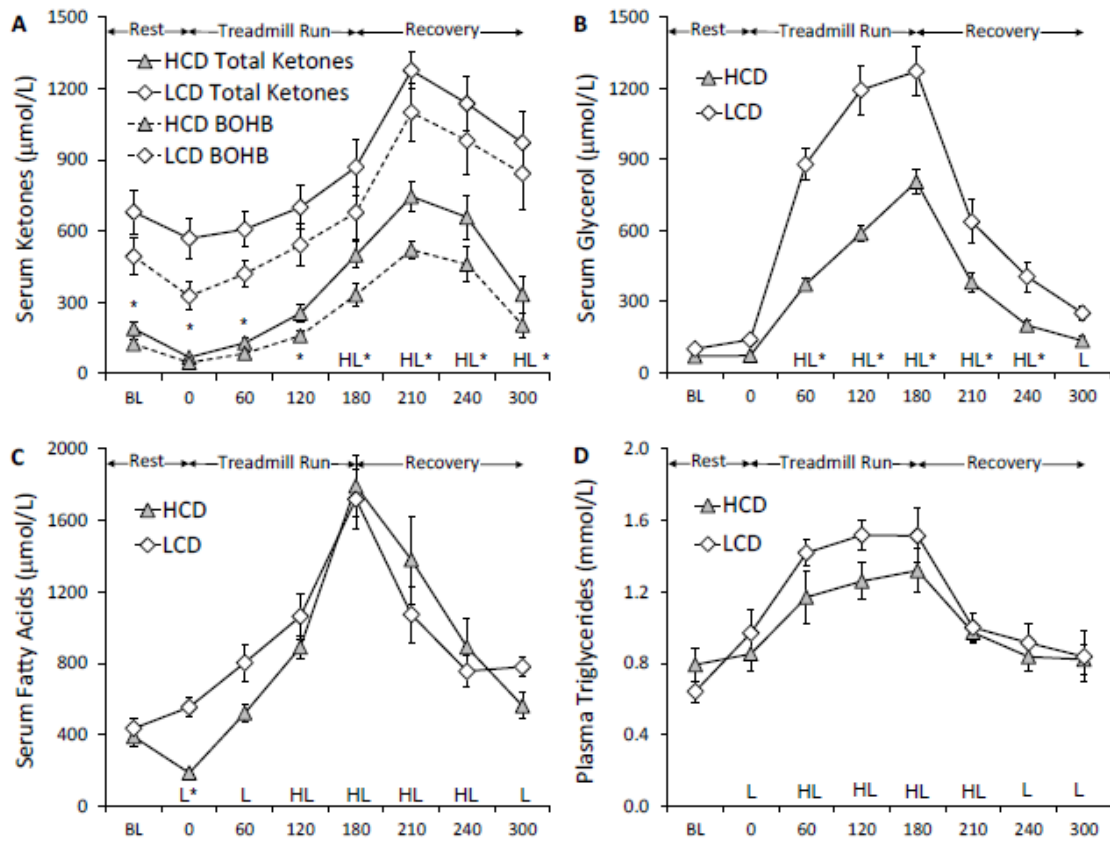
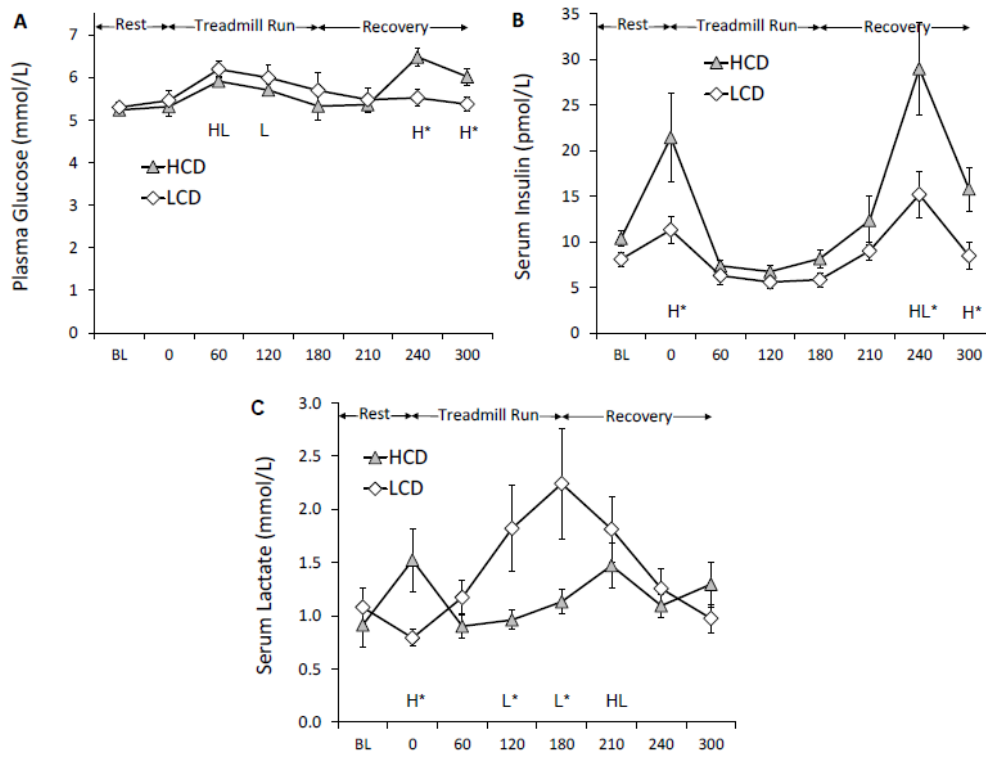


Figure 4



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Figure 5

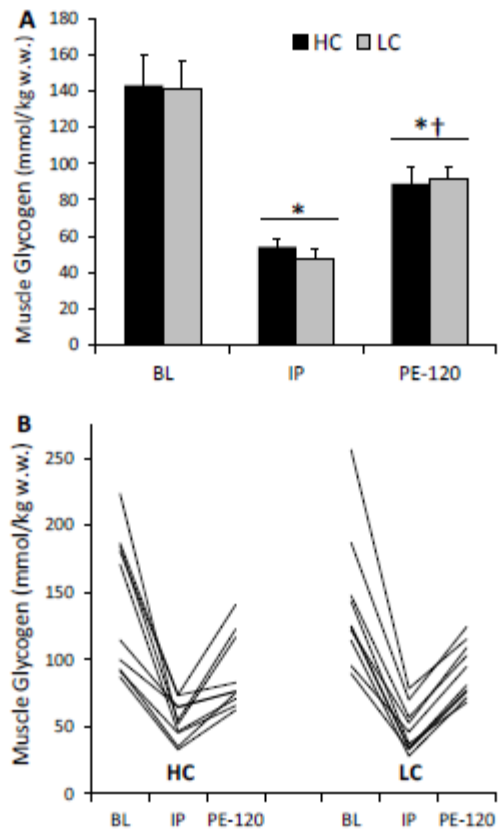


Figure 6