J Appl Physiol 127: 713–725, 2019.
First published June 27, 2019; doi:10.1152/japplphysiol.00060.2019.

RESEARCH ARTICLE

Twice-a-day training improves mitochondrial efficiency, but not mitochondrial biogenesis, compared with once-daily training

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Submitted 29 January 2019; accepted in final form 24 June 2019

Ghiarone T, Andrade-Souza VA, Learsi SK, Tomazini F, Ataide-Silva T, Sansonio A, Fernandes MP, Saraiva KL, Figueiredo RC, Tourneur Y, Kuang J, Lima-Silva AE, Bishop DJ. Twice-a-day training improves mitochondrial efficiency, but not mitochondrial biogenesis, compared with once-daily training. *J Appl Physiol* 127: 713–725, 2019. First published June 27, 2019; doi: 10.1152/japplphysiol.00060.2019.—Exercise training performed with lowered muscle glycogen stores can amplify adaptations related to oxidative metabolism, but it is not known if this is affected by the "train-low" strategy used (i.e., once-daily versus twice-a-day training). Fifteen healthy men performed 3 wk of an endurance exercise (100-min) followed by a high-intensity interval exercise 2 (twice-a-day group, $n = 8$) or 14 h (once-daily group, $n = 7$) later; therefore, the second training session always started with low muscle glycogen in both groups. Mitochondrial efficiency (state 4 respiration) was improved only for the twice-a-day group (group × training interaction, $P < 0.05$). However, muscle citrate synthase activity, mitochondria, and lipid area in intermyofibrillar and subsarcolemmal regions, and PGC1α, PPARα, and electron transport chain relative protein abundance were not altered with training in either group ($P > 0.05$). Markers of aerobic fitness (e.g., peak oxygen uptake) were increased, and plasma lactate, O_2 cost, and rating of perceived exertion during a 100-min exercise task were reduced in both groups, although the reduction in rating of perceived exertion was larger in the twice-a-day group (group × time × training interaction, $P < 0.05$). These findings suggest similar training adaptations with both training low approaches; however, improvements in mitochondrial efficiency and perceived effort seem to be more pronounced with twice-a-day training.

NEW & NOTEWORTHY We assessed, for the first time, the differences between two "train-low" strategies (once-daily and twice-a-day) in terms of training-induced molecular, functional, and morphological adaptations. We found that both strategies had similar molecular and morphological adaptations; however, only the twice-a-day strategy increased mitochondrial efficiency and had a superior reduction in the rating of perceived exertion during a constant-load exercise compared with once-daily training. Our findings provide novel insights into skeletal muscle adaptations using the "train-low" strategy.

carbohydrate; exercise training; glycogen; mitochondria; transmission electron microscopy

INTRODUCTION

Since the pioneering work of John Holloszy in the 1960s (23), it has been known that exercise is a potent stimulus to promote mitochondrial biogenesis (i.e., the generation of new mitochondrial components leading to increased mitochondrial content and function) (16). Although the exercise stimulus appears to be the most important determinant of these changes in mitochondrial content and function (17, 18, 40), recent studies have suggested that initiating some training sessions with reduced muscle glycogen stores, known as the train-low strategy, can better improve training-induced adaptations in markers of oxidative metabolism when compared with starting all training sessions with normal muscle glycogen stores (for review, see 21, 27). In support of this, it has been reported that 3 wk of continuous cycle exercise [90 to 100 min at ~70% of peak oxygen uptake ($\dot{V}O_{2peak}$)] followed 1 to 2 h later with a high-intensity interval training (HIIT) session, in which the second training session (i.e., HIIT) always starts with reduced muscle glycogen, leads to greater increases in citrate synthase (CS) activity, when compared with traditional training (25, 58). However, although CS activity is commonly used as an indirect marker of mitochondrial content (35), there is no consensus on its validity when compared with the "gold-standard" method of transmission electron microscopy (TEM) (40). Therefore, although increased CS activity following 3 wk of using the "train-low" approach suggests the potential for this

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kind of training to induce greater increases in mitochondrial content, this assumption requires confirmation with the more precise technique of TEM. Furthermore, no study has investigated changes in mitochondrial respiratory function when using a "train-low" approach; therefore, whether the train-low strategy produces greater increases in mitochondrial content and/or respiratory function remains underexplored.

Another issue is that different approaches to reduce pre-exercise muscle glycogen have been used, and it has not been determined which train-low model is better to promote mitochondria biogenesis [for review, see Impey et al., (27]. One train-low model is based on performing a first exercise session to reduce muscle glycogen stores, which is then followed by a second exercise session 1–3 h later: the so-called "twice-a-day" approach (9, 20, 25, 41, 58). Another often-used train-low model is the "sleep-low" or "once-daily" training model (33, 37). In this approach, a first exercise session to reduce muscle glycogen content is performed in the evening, followed by either a low carbohydrate (CHO) meal or an overnight fast and a second training session the next day (~11- to 14-h interval between the two training sessions).

Although both train-low approaches involve starting the second exercise session with reduced muscle glycogen stores, an important difference between the two strategies is the close proximity of the two training sessions with the twice-a-day approach. This could potentiate the exercise-induced increase in genes related to mitochondrial biogenesis in the second session, which suggests that better training adaptations with this strategy might not only be linked to starting the second exercise session with reduced muscle glycogen but could also be influenced by the proximity of the previous exercise session. In support of this assumption, we have recently observed greater exercise-induced increases in the mRNA expression for the isoforms related to mitochondrial biogenesis (e.g., PGC1α, PGC1α1, PGC1α4, PPARα, and PPARβ/δ mRNAs) during a second exercise session with the twice-a-day compared with the once-daily approach even though starting muscle glycogen levels were the same (1). As training-induced adaptations have been proposed to be associated with cumulative, transient increases in mRNA expression (47), it could be hypothesized that performing two training sessions in close proximity may result in greater mitochondrial adaptations than when the same two training sessions are performed on separate days. However, to date, this hypothesis has not been tested. Therefore, the aim of present study was to assess whether the magnitude of the molecular, functional, and morphological adaptations linked to mitochondrial biogenesis differ between these two types of train-low strategies.

MATERIAL AND METHODS

Participants

Fifteen healthy men (26.2 ± 5.3 yr, 179 ± 7 cm, and 75.8 ± 10.6 kg) volunteered to participate in this study, after they had been informed about the possible risks involved in the procedures. None of the participants were tobacco smokers or used dietary supplements. Participants were advised to avoid any other physical activity from 48 h before the initial test and throughout the study. The Ethics Committee of the Federal University of Pernambuco approved all procedures for this study, which were conducted according to the principles expressed in the Declaration of Helsinki. All participants provided written informed consent to participate in the study on their first testing day.

Study Design

An overview of the experimental design is shown in Fig. 1. The study was divided into three phases: *1*) pretraining, *2*) training, and *3*) posttraining.

Phase 1: pretraining. During the first visit, anthropometric measures were performed to determine body mass, height, and percentage body fat. The body fat percentage was calculated using the skinfolds at the chest, abdomen, and thigh (28). A biopsy of the vastus lateralis muscle was then performed, with local anesthesia under the skin and fascia (Lidocaine 2%), using the percutaneous Bergström biopsy needle technique adapted for manual suction (13). The biopsy samples were quickly separated into three portions. The first portion (~4 mg) was immediately immersed in fixative for later structural analysis of mitochondria and lipids by TEM. The second portion (~50 mg) was immediately (within 10 to 15 s) snap-frozen in liquid nitrogen for subsequent analysis of relative protein abundance, CS and β-HAD activity. The third portion (~15 mg) was immediately immersed in ice-cold preservation solution (BIOPS) for mitochondrial respiration analysis. The pre- and posttraining biopsies were performed at the same time of day (± 1 h) and with similar pretest nutrition (see *Nutritional control before tests*). Participants then rested for 10 min and performed a graded exercise test (GXT) until exhaustion on a cycle ergometer (Ergo Fit 167, Pirmansens, Germany) for determination of $\dot{V}O_{2peak}$, peak power output (PPO), and the first (LT1) and second (LT2) lactate threshold. After the GXT, participants rested for 30 min and were familiarized with the 48-kJ cycling time-trial (48-kJ TT) procedures. The familiarization with the TT was performed 3 times, on the same cycle ergometer, with a 20-min interval between each trial. Forty-eight hours later, participants returned to the laboratory and performed a 100-min constant-load exercise at the intensity corresponding to 50% of the difference between LT1 and LT2 (Δ50), followed by a 5-min rest and then a 48-kJ TT.

Phase 2: training. Participants were matched for physical fitness (PPO, $\dot{V}O_{2peak}$, and LT1 and LT2) and allocated to one of two experimental groups. One group ($n = 8$) trained twice-a-day, three times a week, whereas another group ($n = 7$) trained once-daily, six days a week. Both groups trained for 3 wk, based on previous research that has reported this duration is sufficient to observe significant changes in CS activity, cytochrome c oxidase subunit IV content, and exercise performance (25, 58). The training protocol consisted of a combination of two types of training. The first training session was an endurance exercise to reduce muscle glycogen stores. The second training session involved HIIT. The group training twice-a-day performed the two training sessions on the same day, with 2 h between each session. The group training once-daily performed the first training session (endurance exercise) in the evening and the second training session (HIIT) on the morning of the next day (14 h between each session). We have previously observed that both types of training result in a similar reduction in muscle glycogen stores (1). Participants only performed the prescribed training and followed a strict dietary plan throughout the study (see *Endurance HIIT Sessions and Nutritional Control During the Training*).

Phase 3: posttraining. Posttraining measurements were performed in an identical manner as pretraining. To minimize any acute exercise effect on the skeletal muscle responses (12, 39), the posttraining muscle biopsy was performed 48 h after the last training session. As the posttraining muscle biopsy was performed 3 wk after the pretraining muscle biopsy, which provides sufficient time to recover from any disturbances to myocellular signaling pathways provoked by muscle biopsy per se (19, 54, 55), the posttraining muscle biopsy was performed on the same leg of the pretraining muscle biopsy 2 to 3 cm above the first incision. Thus, a potential effect of comparing molecular adaptations from different legs was avoided. Ten minutes after

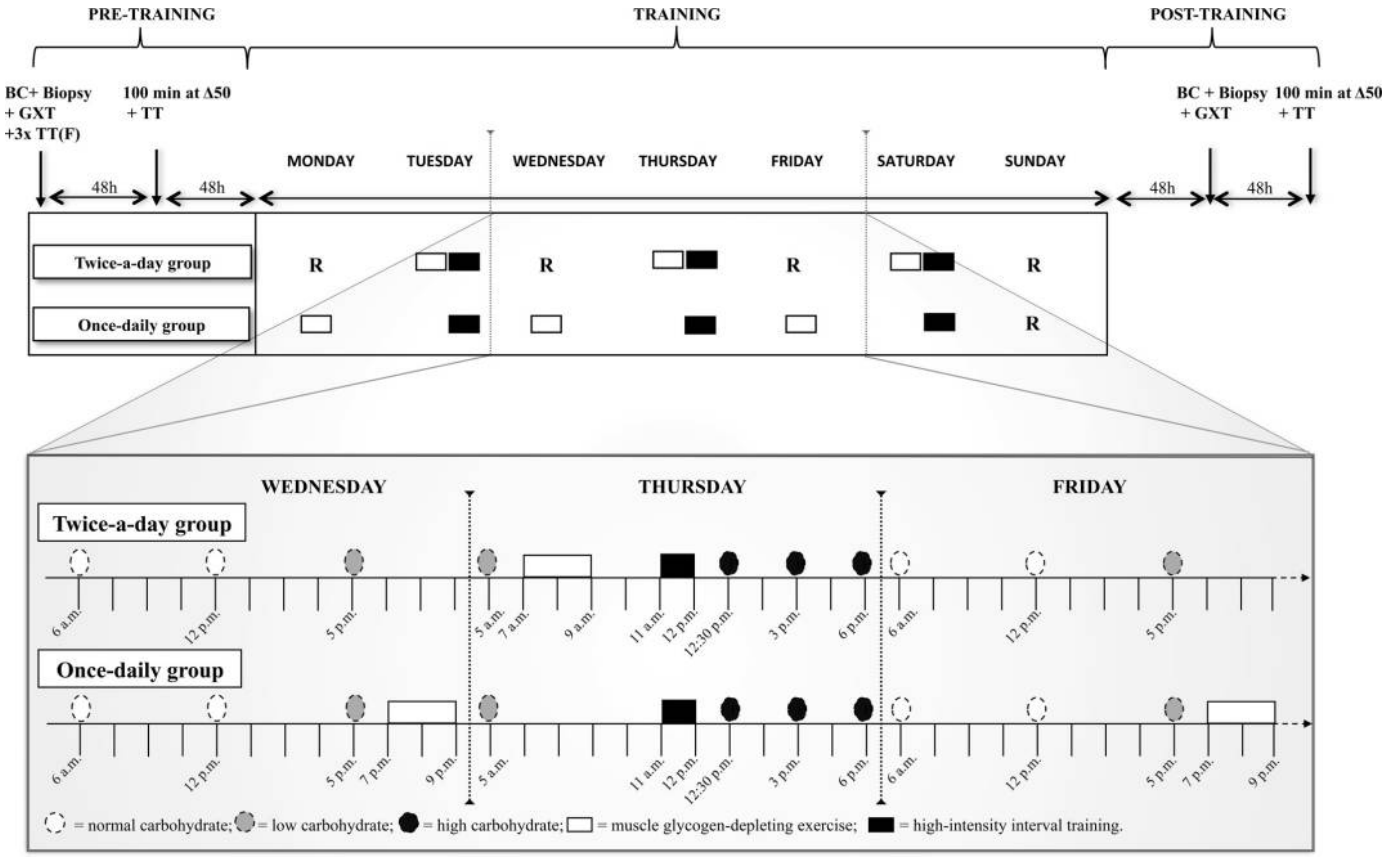

Fig. 1. Overview of the study design. The posttraining Δ50 test was performed at the same absolute pretraining workload. Δ50 = 50% of the difference between the first and second lactate threshold; BC, body composition; F, familiarization; GXT, graded exercise test; HR, heart rate; R, rest; RER, respiratory exchange ratio; RPE, rating of perceived exertion; TT, 48-kJ time-trial; $\dot{V}_E$, ventilation; $\dot{V}_{O_2}$, oxygen uptake.

the biopsy participants performed a GXT, and 48 h later the 100-min constant-load exercise at the same absolute pretraining intensity followed by the 48-kJ TT. All experimental tests and training sessions were performed in a thermoneutral environment (21.3 ± 1.3°C, 40.5 ± 5.1% relative humidity; Fig. 1).

Nutritional Control Before Tests

Participants completed a food record describing time, food preparation, and quantity of each meal consumed during the 24 h before both the first and second pretraining test days. They were instructed to repeat the same food consumption (breakfast, lunch, and dinner) during the 24 h before both posttraining test days. The pre- and posttraining tests and muscle biopsy were performed in the afternoon, ± 3 h after the last meal. Participants were instructed to refrain from alcohol and caffeine consumption for 24 h preceding each test.

GXT

The test began at a work rate of 50 watts (W), followed by 25 W increments every 4 min, with a 1-min recovery between stages. Participants maintained a pedal frequency between 70 and 80 rev/min throughout the test. The test was stopped when participants failed to maintain the pedal frequency within the requested range. Participants wore a facemask, and O_2 uptake ($\dot{V}_{O_2}$), CO_2 production ($\dot{V}_{CO_2}$), and ventilation ($\dot{V}_E$) were recorded breath-by-breath with an automatic gas analyzer (Cortex Metalyzer 3B, Cortex Biophysik, Leipzig, Germany). The O_2 and CO_2 concentrations were analyzed using electrochemical cell and infrared analyzers, respectively, which were calibrated with gases of known concentration before each test. The volume transducer was calibrated using a 3-L syringe. The heart rate (HR) was recorded using an HR monitor (Polar T 31/34, Kempele, Finland). A capillary blood sample (40 μL) was also collected from the ear lobe at the end of each 4-min bout for subsequent lactate determination.

The blood samples were transferred to microtubes containing 10 μL of EDTA and centrifuged at 1,500 *g* for 10-min at 4°C (Hermle Labortechnik GmbH, Germany) for plasma separation from red and white cells. The plasma lactate concentration was analyzed in duplicate in a Spectrophotometer (Biospectro, SP-22, Curitiba, Brazil) using a commercial kit (Labtest, Lagoa Santa, Brazil).

Breath-by-breath $\dot{V}_{O_2}$ values were converted to 30-s averages, and $\dot{V}_{O_{2peak}}$ was determined when two or more of the following criteria were met: an increase in $\dot{V}_{O_2}$ of less than 2.1 $mL \cdot kg^{-1} \cdot min^{-1}$ on two consecutive stages, a respiratory exchange ratio (RER) greater than 1.1, a blood lactate concentration higher than 8.0 mmol/L, and an HR ± 10 beats/min of the maximal age-predicted HR (24). The peak HR was defined as the highest value obtained at the end of the test. The PPO was calculated from the last stage of work completed, added to the fractional time of the last incomplete stage multiplied by the increment (25 W) (32). To determine the LT1, the power output preceding the first increase in lactate concentration above the resting level was visually identified. Two independent investigators determined LT1; in the case of disagreement, a third investigator was consulted. The LT2 was determined by the modified Dmax method. The curve of the lactate-power was adjusted with a third-order polynomial regression curve, and the first and last points (i.e., LT1 and final lactate point) were connected with a straight line. The LT2 was determined by the point on the polynomial regression curve that produced the maximal perpendicular distance to the straight line (3, 4).

100-Min Constant-Load Endurance Test and 48-kJ TT

Participants warmed up at 90% of LT1 for 5 min and then started a 100-min constant-load endurance test at Δ50 (the intensity corresponding to 50% of the difference between LT1 and LT2), maintaining a pedal revolution between 70 and 80 rev/min throughout the test. The rating of perceived exertion (RPE) was assessed at 15, 25, 75, and 100 min of exercise. Gas exchange was measured for 5 min at 15, 25, 75, and 100 min of exercise. The same absolute power was used pre- and posttraining.

After the test, participants rested for 5 min and were then required to complete a 48-kJ TT as quickly as possible. Participants were blinded to elapsed time but were able to see their accumulated work and instantaneous power.

The HR, RPE, $\dot{V}O_2$, $\dot{V}E$, $\dot{V}CO_2$, and RER at rest and during the constant-load exercise (10, 25, 50, 75 and 100 min) were calculated using the mean values of the last 5 min. Oxygen cost was calculated by dividing the mean $\dot{V}O_2$ (mL/min) during the entire test by the work rate measured in watts (W) and expressed as $mL \cdot min^{-1} \cdot W^{-1}$ (57). The fat and CHO oxidation rates (g/min) were calculated using standardized equations (29).

Endurance Training Session

After a standardized, 5-min warm-up at 90% of LT1 (twice-a-day: 79 ± 11 W and once-daily: 86 ± 16 W, $P > 0.05$), participants cycled at Δ50 (twice-a-day: 128 ± 22 W and once-daily: 131 ± 15 W, $P > 0.05$) for 100 min. After a 5-min rest, to reduce the muscle glycogen content of type II muscle fibers further, 6 × 1-min intermittent exercise bouts, interspersed with 1 min of passive rest, were performed at 125% of PPO (twice-a-day: 296 ± 45 W and once-daily: 295 ± 27 W, $P > 0.05$). Participants maintained a pedaling frequency between 70 and 80 rev/min during the entire session. The same power was maintained for every training session. This protocol has been observed to reduce muscle glycogen content by ~45% (1).

HIIT Session

The HIIT consisted of a 5-min warm-up at 90% of LT1, followed by 10 × 2-min at an intensity corresponding to 20% of the difference between LT2 and PPO (twice-a-day: 182 ± 34 W and once-daily: 179 ± 19 W, $P > 0.05$), with a 1-min passive recovery between the bouts (11). Participants maintained a pedaling frequency between 70 and 80 rev/min during each bout. The same power was maintained for every training session.

Nutritional Control During the Training

Participants received a list of all meals to be followed during the training period and were asked to send photographs of key meals to a nutritionist. For the once-daily group, the meals before the endurance exercise session (breakfast, lunch, and snack) were set up respecting each individual's habitual diet (45%–65% CHO, 10%–35% protein, and 20%–35% lipids) (53). Participants consumed a standardized, low-CHO meal (7% CHO, 60% lipids, and 33% protein) 2 h before each endurance exercise session (~5 PM). The endurance training session occurred on Mondays, Wednesdays, and Fridays (starting at 7 PM and ending at 9 PM). After training, participants consumed an amount of water equivalent to 150% of the body mass lost to prevent dehydration (52) and then fasted until the following morning (Tuesday, Thursday, and Saturday mornings). They ate a standardized, low-CHO breakfast (7% CHO, 60% lipids, and 33% proteins) at ~5 AM to avoid muscle (1) and hepatic glycogen resynthesis (45). Participants started the HIIT session (~11 AM) after an additional ~6 h fast (Fig. 1).

The twice-a-day group followed the same dietary pattern as the once-daily group, conserving the same composition and meal schedule in the day before the training (low-CHO meal ~5 PM). On the training day, after a 12-h fast, a standardized breakfast was provided (~5 AM) with a low-CHO content (7% CHO, 60% lipids, and 33% protein); participants fasted for a further ~2 h and then performed the endurance training session (~7 AM). They consumed an amount of water equivalent to 150% of the body mass lost, rested for further 2 h, and then performed the HIIT session (~11 AM). Both the endurance and HIIT training sessions were performed on Tuesdays, Thursdays, and Saturdays.

To accelerate muscle glycogen recovery, participants in both groups drank a beverage containing 1.2 g/kg body mass of CHO + 0.4 g/kg of protein immediately after completing the HIIT (~12 PM) (Fig. 1) (2, 56). They also consumed high CHO meals at 12:30 PM, 3 PM, and 6 PM (75% CHO, 10% lipids, and 15% proteins).

Mitochondria Respiration

Mitochondrial oxygen consumption was measured using a Clark oxygen electrode (Hansatech Instruments, Pentney King's Lynn, UK) (6, 22, 51). The muscle fibers (4.19 ± 0.18 mg wet wt) were immersed in a biopsy preservation solution (BIOPS: 2.77 mmol/L CaK_2EGTA, 7.23 mmol/L K_2EGTA, 5.77 mmol/L Na_2ATP, 6.56 mmol/L $MgCl_2 \cdot 6H_2O$, 20 mmol/L taurine, 50 mol/L 2-(*N*-morpholino)ethanesulfonic acid hydrate (MES), 15 mmol/L Na_2phosphocreatine, 20 mmol/L imidazole, and 0.5 mmol/L dithiothreitol (DTT), pH 7.1) (48) and mechanically dissected using forceps. Saponin (50 μg/mL, Sigma) was used to permeabilize the plasma membrane by gentle shaking for 30 min in BIOPS at 4°C. The saponin was then removed by rinsing the fibers for three 10-min intervals at 4°C in 5 mL MiR05 medium (0.5 mmol/L EGTA, 3 mmol/L $Cl_2.6H_2O$, 60 mmol/L potassium lactobionate, 20 mmol/L taurine, 10 mmol/L KH_2PO_4, 20 mmol/L HEPES, 110 mmol/L sucrose, and 1 g/L bovine serum albumin, pH 7.1) (6, 48). Mitochondrial respiration was measured in duplicate in MiR05 at 37°C based on previously described methods (6). Cytochrome c was added to confirm mitochondrial integrity as a quality control measure. A low oxygen consumption rate (state 2) was left over a 5-min period by using 5 mM pyruvate plus 2 mM malate. Then, 5 mM ADP was injected into the chamber, resulting in an increase in respiration (state 3). After 5 min of state 3 respiration, respiration was inhibited using 70 μM atractyloside (inhibitor of mitochondrial inner membrane ATP/ADP exchange) as an estimate of state 4 respiration. The respiratory control ratio (RCR) was calculated as the ratio between state 3 (with ADP in excess) and state 4 (respiration inhibited). State 4 respiration and RCR were used as indicators of mitochondrial efficiency (7, 30).

TEM

Muscle samples were fixed overnight in a solution containing 2.5% glutaraldehyde and 4% paraformaldehyde in 0.1 mol/L cacodylate buffer. After fixation, the samples were washed twice in the same buffer, postfixed in a solution containing 1% OsO_4, 5 mmol/L $CaCl_2$ and 0.8% $(K_4Fe(CN)_6$ in 0.1 mol/L cacodylate buffer, pH 7.2, dehydrated in acetone, and embedded in Embed 812 (Electron Microscopy Science, Washington, PA). Polymerization was carried out at 60°C for 3 days (50). Ultrathin sections were collected on 300 mesh nickel grids, counterstained with 5% uranyl acetate and lead citrate and examined with a FEI Tecnai G2 Spirit Biotwin (North America 361 NanoPort, Hillsboro, OR) TEM. All the longitudinally oriented fibers were photographed by an independent observer who was blind to training status and group at ×20,000 magnification in a randomized systematic order, including 12 images from the subsarcolemmal region and 12 images from the myofibrillar region [6 central intermyofibrillar (IMF) and 6 superficial IMF]. This blinded investigator also analyzed the lipid droplets and mitochondrial fragments using ImageJ software to calculate total mitochondria and lipid droplet area. The mitochondria and lipid area from IMF and subsarcolemmal (SS) region were expressed relative to the myofibrillar area and the surface area of the fibers (43, 44). The reference for SS space quantification was the cytoplasmic space between the sarcolemma and the first layer of myofibrils (43, 44).

Table 1. *Nutritional analysis for the main meals during the training period for both the twice-a-day and once-daily groups*

	Energy, Kcal	CHO, %	Protein, %	Lipid, %
Twice-a-day				
Normal CHO meals	1,998.6 ± 289.3	54.1 ± 3.1	19.8 ± 3.1	26.1 ± 3.0
Low CHO meals	1,666.9 ± 255.6	7.0 ± 0.0	33.0 ± 0.0	60.0 ± 0.0
High CHO meals	3,068.1 ± 340.9	75.0 ± 0.0	16.7 ± 0.2	8.4 ± 0.2
Once-daily				
Normal CHO meals	1,697.7 ± 503.9	60.4 ± 6.2	16.0 ± 3.7	23.6 ± 6.6
Low CHO meals	1,582.9 ± 60.5	7.0 ± 0.0	33.0 ± 0.0	60.0 ± 0.0
High CHO meals	2,992.0 ± 565.8	75.0 ± 0.0	16.6 ± 0.3	8.4 ± 0.3

Values are means ± SD. Macronutrient composition is presented as % of total calorie intake. Normal CHO meals (breakfast + lunch of *day 1*); low-CHO meals (dinner of day 1 + breakfast of day 2); high CHO meals (drink + lunch + dinner of *day 2*). There were no significant differences between groups ($P > 0.05$). CHO, carbohydrate.

CS Activity

CS activity was determined according to a previous report (17). CS activity was determined spectrophotometrically at 30°C by measuring the appearance of the CoA-SH [acetyl-CoA + oxaloacetate + H_2O ↔ citrate + CoA-SH + H^+ (side reaction: CoA-SH + 5,5′-dithiobis-(2-nitrobenzoic acid) → mercaptide ion)]. The diluted muscle homogenates were used for the assay. Homogenates for CS activity were prepared (40 μL of 3 mM acetyl-CoA, and 25 μL of 1 mM 5,59-dithiobis (2-nitrobenzoic acid) and added to 165 μL 100 mM Tris buffer (pH 8.3) kept at 30°C. CS activity was determined in triplicate on a microtiter plate by adding 5 μL of 8 mg/mL muscle homogenate. After addition of 15 μL of 10 mM oxaloacetic acid, the plate was immediately placed in an xMark-Microplate Spectrophotometer (Bio-Rad Laboratories, Hercules, CA) at 30°C, and after 30 s of linear agitation, absorbance at 412 nm was recorded every 15 s for 3 min. CS activity were expressed as moles per hour per kilogram protein.

β-Hydroxyacyl-CoA Dehydrogenase Activity

β-Hydroxyacyl-CoA dehydrogenase (β-HAD) activity was determined spectrophotometrically at 30°C by measuring the disappearance of NADH (AcAcCoA + NADH→NAD^+ + alcohol β-HAD). Homogenates for β-HAD activity were prepared in a buffer [HEPES 5 mM (pH 8.7), EGTA 1 mM, DTT 1 mM, and 0.1% Triton X-100 (pH 8.3)] kept at 30°C. β-HAD activity was determined in triplicate on a microtiter plate by adding 25 μL of muscle homogenate. After addition of 5 μL AcAcCoA, the plate was immediately placed in an xMark-Microplate Spectrophotometer (Bio-Rad) at 30°C, and after 30 s of linear agitation, absorbance at 340 nm was recorded every 15 s for 3 min. β-HAD activity was expressed as mmoles per hour per kilogram protein.

Quantitative Western Blot Analysis

Approximately 20 mg of frozen muscle tissue was homogenized using a TissueLyser II (Qiagen, Valencia, CA) in a 1:20 dilution of ice-cold radioimmunoprecipitation assay (RIPA) buffer (pH 7.4) containing: 0.15 mol/L NaCl, 1% Triton-X100, 0.5% sodium deoxycholate, 0.05 mol/L Tris, 0.1% SDS, 0.1 mol/L EDTA, 1% protease inhibitor cocktail (cat. no. P-8340, Sigma-Aldrich, St. Louis, MI), and phosphatase inhibitor cocktail (cat. no. P-5726, Sigma-Aldrich). Homogenates were rotated end-over-end for 60-min at 4°C, and the supernatant (i.e., crude cytosolic) was then collected and the pellet discarded. Protein content of muscle homogenates was measured in triplicate using a Bradford assay (Bio-Rad protein assay dye reagent concentrate, Bio-Rad) against bovine serum albumin standards (cat. no. A-9647, Sigma-Aldrich). The coefficient of variation for the protein assay was lower than 5%.

RIPA-buffered homogenate was diluted in 4X Laemmli buffer (0.25 mol/L Tris, 8% SDS, 40% glycerol, 0.04% bromophenol blue, 20% 2-mercaptoethanol) and equal amounts of total protein (10–20 μg) were loaded on Criterion 4%–20% TGX Stain-Free Precast Gels or 12% TGX Stain-Free gels casted using a FastCast Acrylamide kit (Bio-Rad). All samples for a participant were loaded in adjacent lanes on the same gel. Four to six different dilutions of a mixed-homogenate internal standard were also loaded on each gel and a calibration curve plotted of density against protein content. From the subsequent linear regression equation protein abundance was calculated from the measured band intensity for each sample on the gel (42). Gel electrophoresis ran for 90 min at 80 to 150 V. Proteins were turbo-transferred to a 0.2-μm PVDF membrane at 25 V for 10 min. Membranes were blocked for 60 min at room temperature in 5% nonfat dry milk diluted in Tris-buffered saline with 0.1% Tween-20 (TBST). Membranes were then washed in TBST and incubated overnight at 4°C with the appropriate primary antibody: monoclonal anti-PGC-1 (Cell Signaling Technology catalog no. 2178), rabbit polyclonal anti-PPARα (cat. no. ab-24509), antibody cocktail anti-OXPHOS Complex I-V (ab110411) diluted (1:1,000) in 5% BSA, and 0.02% sodium azide in TBST. Following TBST washes the membranes were incubated in the relevant secondary antibody, goat anti-rabbit IgG (Perkin Elmer/NEF812001EA), diluted (1:10,000) in 5% nonfat dry milk in TBST for 60 min at room temperature. After further washes, membranes were incubated in chemiluminescent solution (1.25 mmol/L 294 luminol, 0.2 mmol/L ρ-coumaric acid, 100 mmol/L Tris pH 8.5, 0.009% H_2O_2) for 2 min, and images were taken with a ChemiDoc Imaging System fitted (Bio-Rad). Densitometry was performed with Image Laboratory 5.0 software (Bio-Rad). See Supplemental Fig. S1 for representative Western blots (https://doi.org/10.6084/m9.figshare.8251343.v1).

Table 2. *Body composition and aerobic fitness parameters before and after the three weeks exercise training program in both experimental groups*

	Twice-a-day		Once-daily	
	Pretraining	Posttraining	Pretraining	Posttraining
Body mass, kg*	78.8 ± 12.8	77.8 ± 12.9	72.5 ± 7.0	71.7 ± 8.0
Body fat, %*	14 ± 4	12 ± 3	13 ± 5	12 ± 3
Fat mass, kg*	11.1 ± 5.1	9.3 ± 3.2	9.6 ± 3.6	9.0 ± 4.5
Fat-free mass, kg	67.6 ± 8.6	67.3 ± 10.0	62.9 ± 7.2	62.7 ± 6.8
PPO, W*	237 ± 36	270 ± 42	235 ± 22	265 ± 24
$\dot{V}O_{2peak}$, mL·kg^{-1}·min^{-1}*	36.1 ± 4.3	39.2 ± 5.5	37.9 ± 7.8	40.2 ± 6.5
$\dot{V}O_{2peak}$, L/min	2.8 ± 0.4	3.0 ± 0.5	2.7 ± 0.6	2.9 ± 0.6
[Lac]peak, mmol/L*	9.5 ± 2.4	11.3 ± 3.0	10.5 ± 1.3	11.0 ± 1.5
LT1, W*	88 ± 13	138 ± 33	96 ± 17	132 ± 12
LT1, L/min*	1.4 ± 0.2	1.8 ± 0.4	1.4 ± 0.3	1.6 ± 0.3
LT2, W*	167 ± 34	201 ± 41	164 ± 19	201 ± 18
LT1, L/min*	2.1 ± 0.4	2.5 ± 0.5	2.0 ± 0.5	2.4 ± 0.5

Values are means ± SD. [Lac]peak, peak of plasma lactate concentration; LT1, first lactate threshold; LT2, xecond lactate threshold; PPO, peak power output; $\dot{V}O_{2peak}$, peak oxygen uptake. *Main effect of training, $P < 0.05$.

Statistical Analysis

Statistical analysis was performed using the GraphPad Prism software version 7.0a. All data were checked for normality by the Kolmogorov-Smirnov test. To investigate the effect of training and group on all dependent variables, a two-way mixed-model ANOVA was used [factors: training (pre- and posttraining) and group (twice-a-day and once-daily)]. Changes in parameters measured during the 100-min constant-load endurance test were assessed using three-way mixed-model ANOVA [factors: time (rest, 15, 25, 50, 75, 100 min), training (pre- and posttraining), and group (twice-a-day and once-

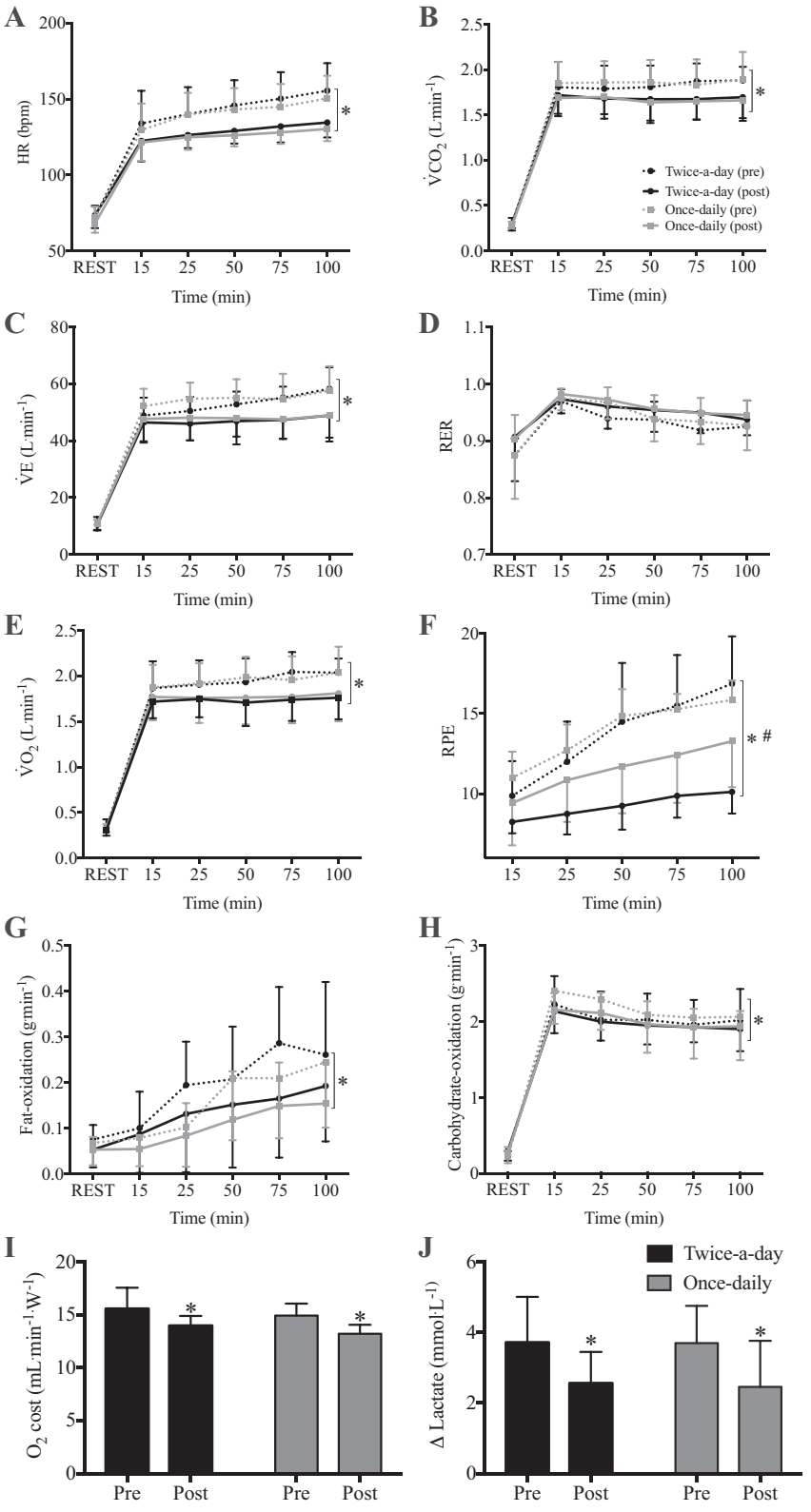

Fig. 2. Physiological responses during the 100-min endurance test pre- and posttraining for the twice-a-day and the once-daily groups. HR (*A*), $\dot{V}CO_2$ (B), $\dot{V}E$ (*C*), RER (*D*), $\dot{V}O_2$ (*E*), RPE (*F*), fat oxidation (*G*), CHO oxidation (*H*), O_2 cost (*I*), ΔLactate (*J*). Values are mean ± SD *Significant main effect of training ($P < 0.05$) for $\dot{V}E$, $\dot{V}O_2$, $\dot{V}CO_2$, HR, RPE, fat and CHO oxidation rates, O_2 cost, and ΔLactate. #Significant group × training × time interaction ($P < 0.05$) for RPE. ΔLactate, lactate accumulated above rest; CHO, carbohydrate; HR, heart rate; RPE, rating of perceived exertion; $\dot{V}CO_2$, Carbon dioxide production; $\dot{V}E$, ventilation; $\dot{V}O_2$, oxygen uptake.

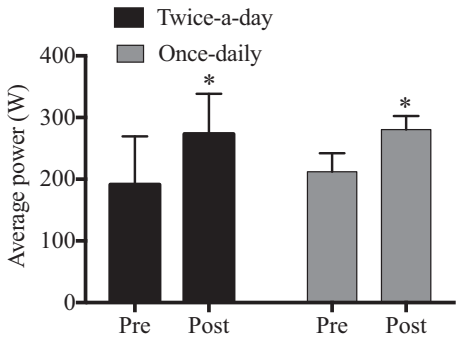

Fig. 3. Average power during the 48-kJ time-trial for both the twice-a-day and once-daily training groups. Values are mean ± SD *Significant main effect of training ($P < 0.05$).

daily)]. The post hoc analysis was performed using Bonferroni test. Significance was set at $P \leq 0.05$. Values are expressed as mean ± SD.

RESULTS

Dietary Control

Participants adhered to the prescribed dietary protocols, and there was a similar mean energy intake and macronutrient distribution (all $P > 0.05$) between the groups (Table 1).

Body Composition

The baseline parameters of body composition were similar between the groups at pretraining (all $P > 0.05$, Table 2). There was a significant but similar reduction in body mass and fat mass in both groups posttraining (main effect of training, $P = 0.04$ and $P = 0.05$, respectively). No significant alteration in fat-free mass was observed with training in either group (main effect of training, $P = 0.37$).

GXT

The baseline parameters obtained in the GXT test were similar between groups at pretraining (all $P > 0.05$, Table 2). There was a main effect of training for PPO ($P = 0.01$), $\dot{V}O_{2peak}$ ($P = 0.05$), peak blood lactate concentration ($P = 0.01$), and power at LT1 ($P = 0.01$) and LT2 ($P = 0.01$) but without an effect of group (all $P > 0.05$) or a training × group interaction (all $P > 0.05$).

100-Min Constant-Load Endurance Test and Time Trial

Pretraining $\dot{V}_E$, $\dot{V}O_2$, $\dot{V}CO_2$, HR, fat and CHO oxidation rates, oxygen cost, and plasma lactate were similar between groups (all $P > 0.05$, Fig. 2), and all were reduced with training (main effect of training, all $P < 0.05$), without an effect of group (all

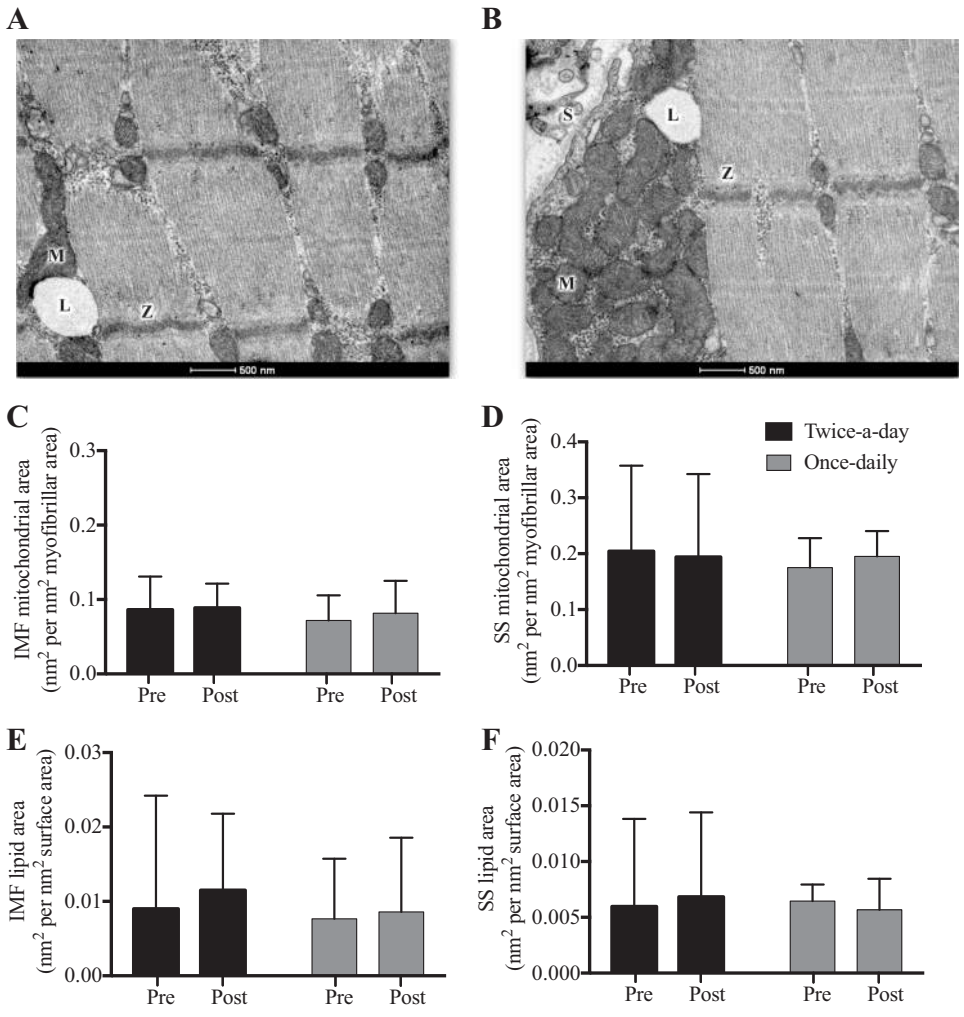

Fig. 4. Representative transmission electron microscopy images of the intermyofibrillar (IMF) region (*A*) and subsarcolemmal (SS) region (*B*) of a skeletal muscle fiber. Relative mitochondrial area and lipid droplet area of IMF (*C* and *D*, respectively). Relative mitochondrial area and lipid droplet area of SS region (*E* and *F*, respectively). Values are mean ± SD. There was no main effect of group or training ($P > 0.05$). L, lipid droplet; M, mitochondria; S, sarcolemma; Z, Z-line.

$P > 0.05$) or a training × group interaction (all $P > 0.05$). There was no training ($P = 0.09$), group ($P = 0.58$), or a training × group interaction effect ($P = 0.56$) for RER (Fig. 2). However, there was a group × time × training interaction for RPE ($P = 0.01$); the RPE responses over time were more attenuated with training in the twice-a-day than in the once-daily group (Fig. 2).

Average power during the pretraining 48-kJ TT was similar between groups (212.3 ± 29.8 W vs. 191.5 ± 78.1 W, $P > 0.05$, Fig. 3) and increased similarly with training in both the once-daily (280.7 ± 21.9 W, 34 ± 17%) and the twice-a-day (273.6 ± 64.9 W, 51 ± 30%) groups (main effect of training, $P = 0.01$), without an effect of group ($P = 0.61$) or a training × group interaction ($P = 0.43$).

TEM, CS Activity and β-HAD Activity

An example of TEM images illustrating differences between the IMF and SS regions is provided in Fig. 4, *A* and *B*. There was no difference between groups at pretraining for the mean total area of mitochondria and lipid droplets in both the SS and IMF regions (all $P > 0.05$, Fig. 4, *C–F*), and 3 wk of exercise training did not change any of these measures (main effect of training, all $P > 0.05$). Consistent with the TEM findings, there were no pretraining differences between the groups for CS activity ($P > 0.05$, Fig. 5*A*) and no significant changes with training (main effect of training, $P = 0.61$). There was no main effect of training ($P = 0.22$) or a training × group interaction ($P = 0.87$) for β-HAD activity; however, there was a main effect of group ($P = 0.01$) with higher values for once-daily compared with twice-a-day (Fig. 5*B*).

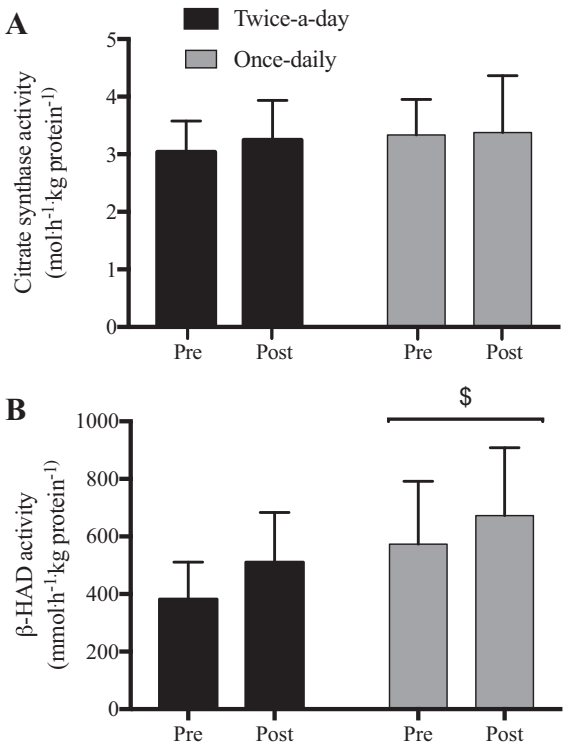

Fig. 5. Citrate synthase activity in the twice-a-day and once-daily training groups. There was no main effect of group or training ($P > 0.05$) (*A*). β-HAD activity in the twice-a-day and once-daily training groups. $There is a main effect of group ($P = 0.001$), with higher values for the once-daily group (*B*). Values are mean ± SD. β-HAD, β-hydroxyacyl-CoA dehydrogenase.

PGC1α, PPARα, and Electron Transport Chain Proteins

There were no pretraining differences between the groups for PGC1α, PPARα, and electron transport chain protein content (all $P > 0.05$, Fig. 6). Three weeks of training was not sufficient to increase PGC1α or PPARα relative protein abundance (main effect of training, $P = 0.52$ and $P = 0.20$, respectively). Similarly, there was no training, group, or training × group interaction (all $P > 0.05$) for subunits of the electron transport chain proteins (complex I to IV).

Mitochondrial Respiration

There were no pretraining differences between the groups for parameters of mitochondrial respiration (all $P > 0.05$, Fig. 7). Training had no effect on state 2 (Malate/Pyruvate) or state 3 (ADP) respiration in both groups ($P = 0.59$ and $P = 0.74$, respectively). However, state 4 respiration decreased in the twice-a-day group (pre: 4.5 ± 0.8 $nmolO_2 \cdot mL^{-1} \cdot min^{-1} \cdot mg$ wet wt^{-1} and post: 2.6 ± 0.9 $nmolO_2 \cdot mL^{-1} \cdot min^{-1} \cdot mg$ wet wt^{-1}), with no effect in the once-daily group (pre: 3.4 ± 1.3 $nmolO_2 \cdot mL^{-1} \cdot min^{-1} \cdot mg$ wet wt^{-1} and post: 3.9 ± 1.0 $nmolO_2 \cdot mL^{-1} \cdot min^{-1} \cdot mg$ wet wt^{-1}) (group × training interaction, $P = 0.01$). A similar pattern was observed for the RCR = state 3/state 4), in which the RCR increased with twice-a-day training (pre: 5.1 ± 1.1 and post: 10.8 ± 3.7), but there was no effect with once-daily training (pre: 7.7 ± 2.4 and post: 5.8 ± 1.7) (group × training interaction, $P = 0.01$). When the respiration was expressed relative to CS activity, a marker of mitochondrial content (35), state 4 respiration was significantly reduced in the twice-a-day group (pre: 1.5 ± 0.4 $nmolO_2 \cdot mL^{-1} \cdot min^{-1} \cdot mg$ wet wt^{-1} and post: 0.8 ± 0.3 $nmolO_2 \cdot mL^{-1} \cdot min^{-1} \cdot mg$ wet wt^{-1}), with no effect in the once-daily group (pre: 1.0 ± 0.3 $nmolO_2 \cdot mL^{-1} \cdot min^{-1} \cdot mg$ wet wt^{-1} and post: 1.3 ± 0.6 $nmolO_2 \cdot mL^{-1} \cdot min^{-1} \cdot mg$ wet wt^{-1}) (group × training interaction, $P = 0.01$). There were no differences for state 2 or state 3 respiration normalized to CS activity ($P = 0.68$ and $P = 0.90$, respectively).

DISCUSSION

For the first time, to our knowledge, we have investigated the effect of two different train-low models on training-induced changes in mitochondria content, using the gold-standard TEM technique, and on mitochondria respiration. In addition, we also explored whether body composition, parameters of aerobic fitness, exercise-induced physiological responses, exercise performance, and relative protein abundance changes with training were dependent of the train-low model used. Three weeks of twice-a-day or once-daily training led to *1*) similar reductions in body mass and body fat, and similar increases in markers of aerobic fitness; *2*) similar attenuation of physiological responses during a submaximal, constant-load exercise and similar increases in subsequent time trial performance; and *3*) no changes in mitochondrial and lipid droplet content, CS activity, or relative protein abundance of PGC1α, PPARα, and the electron transport chain components (complex I-V). However, a reduction in state 4 respiration, which is indicative of improved mitochondrial efficiency (7, 30, 34), and a consequent increase in the RCR, was only found in the twice-a-day

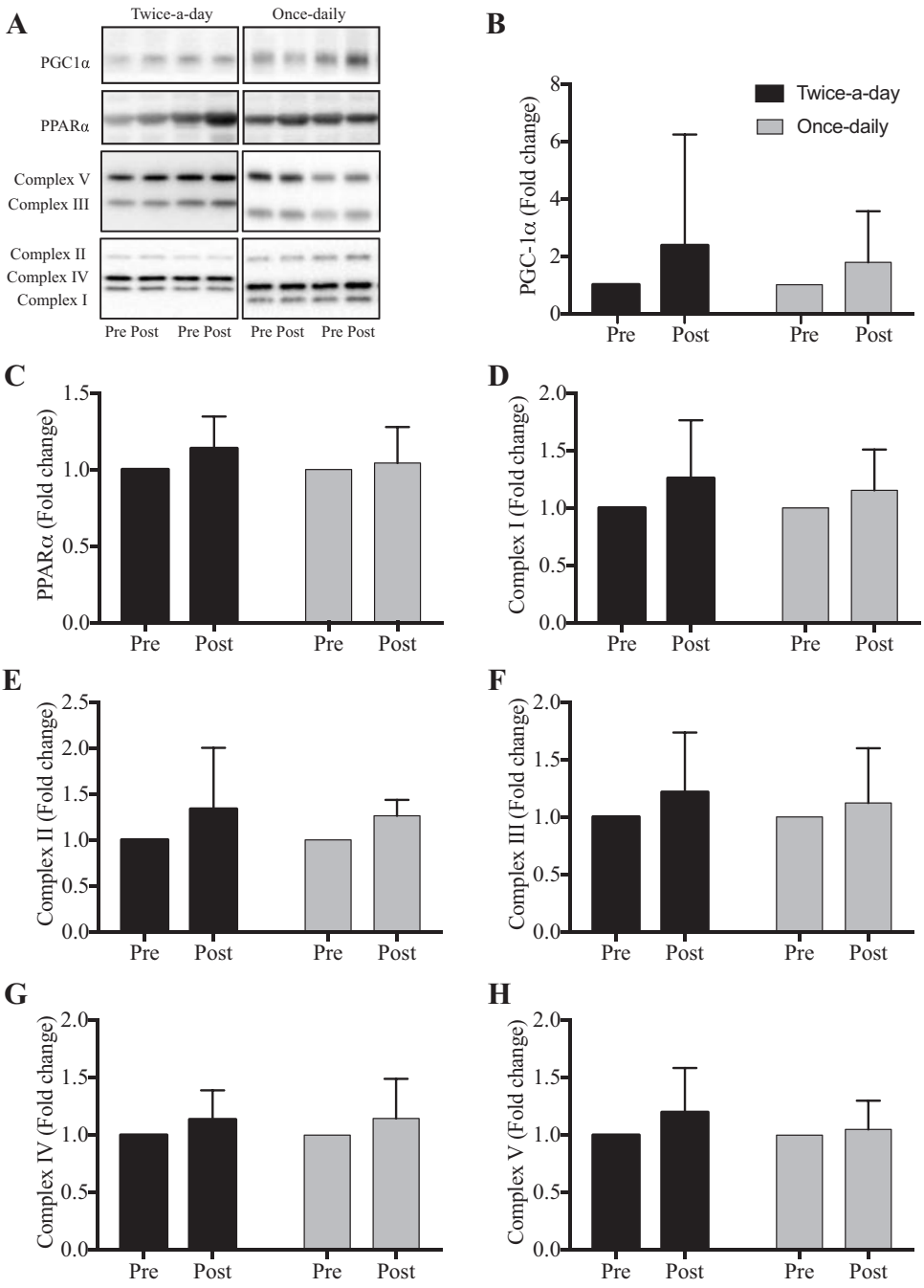

Fig. 6. Representative Western blot bands (*A*). Protein abundance (relative to pretraining values) for PGC1α (B), PPARα (*C*), and complexes I to V (*D–H*) pre- and posttraining for the twice-a-day and once-daily groups. Values are mean ± SD. There was no main effect of group or training ($P > 0.05$).

group. In addition, the RPE during a submaximal, constant-load exercise task was reduced to a greater extent in the twice-a-day group.

In the present study, both groups performed the same training volume and had a similar energy intake and macronutrient distribution during the training period (Table 1). Furthermore, our previous findings with an identical diet and exercise regimen indicate that the first endurance training session was successful to reduce muscle glycogen and that the second training session starts with similar reduced levels of muscle glycogen in both train-low models (~260 mmol/kg wet mass (1). It has recently been proposed that for the train-low approach to be effective and to induce greater molecular adaptations, the second training session should start with muscle glycogen values lower than 300 mmol/kg wet mass (26). Therefore, our approach allowed us to isolate the effects of reduced muscle glycogen levels from the proximity of the two exercise sessions on training-induced adaptations.

Our results indicate that regardless of the training model, a 3-wk train-low program is sufficient to reduce body mass and body fat and to increase markers of aerobic fitness ($\dot{V}O_{2peak}$ and lactate thresholds) (Table 2). The improvement in these endurance-related parameters was accompanied by a significant attenuation in cardiorespiratory responses ($\dot{V}_E$, $\dot{V}O_2$, $\dot{V}CO_2$, HR, and O_2 cost) to a submaximal, constant-load exercise (Fig. 2), with a subsequent improvement in time trial performance (Fig.

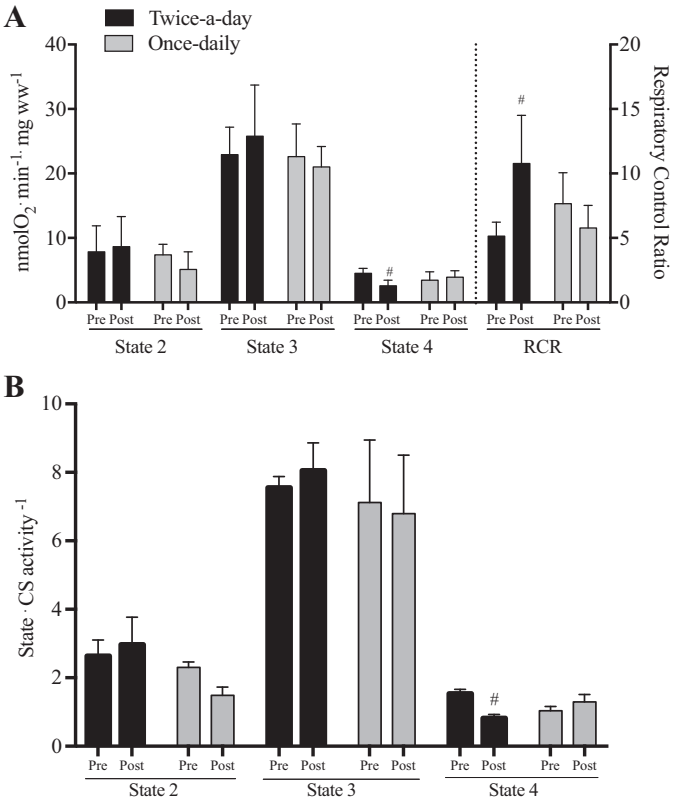

Fig. 7. Skeletal muscle mitochondrial respiration expressed in micromoles O_2 per minute per gram of fiber wet-weight (*A*) and relative to CS activity (*B*). State 2 corresponds to malate/pyruvate, state 3 was the ADP-stimulated respiration, state 4 was the control state of respiration in the presence of actractyloside, and the respiratory control ratio (RCR) was calculated as state 3/state 4 respiration. Values are mean ± SD. #There was a significant group × training interaction with training-induced alterations occurring only in the twice-a-day group ($P < 0.05$).

3). These results indicate that anthropometric, cardiorespiratory, metabolic, and performance improvements were similar between the twice-a-day and once-daily training models. Although it is not possible to compare these findings with previous studies directly, as no other study has directly compared different train-low models, it has been reported that body fat is reduced after either twice-a-day (15) or once-daily (37) training. Additionally, time trial performance has been observed to increase following either twice-a-day (9) or once-daily (37) training. In response to a submaximal exercise task, both HR and blood lactate have been reported to decrease after twice-a-day training (15); although no decrease in HR or blood lactate was reported after once-daily training, there was a reduction in O_2 cost (37). Therefore, our results indicate that both twice-a-day and once-daily approaches are similarly effective to promote anthropometric, cardiorespiratory, and metabolic adaptations, with a subsequent similar improvement in exercise performance.

An interesting and unexpected result was that the twice-a-day training resulted in a greater decrease in the RPE during the constant-load exercise, when compared with once-daily training (Fig. 2). Based on our data, it is difficult to explain the reason for this greater reduction in the perception of exertion for the twice-a-day group. However, one difference between the two approaches is that the once-daily group had 14 h to recover between the endurance exercise (first training session) and the HIIT (second training session) during the training period, whereas the twice-a-day group had only 2 h for recovery between these two training sessions. It is likely, therefore, that the twice-a-day group performed every second training session (HIIT) with greater central and/or peripheral fatigue and ultimately with increased perceived effort (49). This greater "overall stress" for the participants of the twice-a-day group may have improved their ability to tolerate discomfort, leading to a greater reduction in perceived exertion for the same absolute load after training. Reduced central fatigue development and perceived effort during exercise seems to be an important adaptation to a 6-wk HIIT program and might contribute to the improved posttraining endurance performance (46).

To our knowledge, this is the first study using TEM, considered the gold standard to measure mitochondrial content, to address the effects of different train-low approaches on mitochondrial and lipid content and localization. As 2–3 wk of the train-low strategy has been associated with increased CS activity (20, 58), considered an indirect marker of mitochondrial biogenesis (35), we hypothesized that there would be an increase in mitochondrial content as measured by TEM in our study. Contrary to our hypothesis, 3 wk of train-low training did not alter mitochondria or lipid content (Fig. 4). There were also no significant changes in CS activity (Fig. 5). It is unclear why we did not observe an increase in mitochondrial content or CS activity. However, previous studies reporting significant increases in CS activity following similar train-low training programs have used much greater volumes of HIIT (20, 58), and it has previously been reported that changes in CS activity are correlated with training volume (5, 16). Thus, it may be that our HIIT volume was not sufficient to promote a significant increase in mitochondrial content.

Consistent with the TEM and CS activity data, we observed no effect of training on PGC1α, complex I-V, β-HAD activity, or PPARα protein content (Fig. 6). Previous studies investigating the effect of different train-low strategies on PGC1α protein content have produced contradictory observations, with studies showing no effect (58) or increased content (41). Similarly, the effect of different train-low strategies on complex I-V protein content is controversial, with studies reporting no effect (25) or an increase (41, 58). Two studies, however, observed that the train-low strategy increases β-HAD activity (20, 58). This inconsistency may be explained, at least in part, by training duration because an increase in protein content has been reported when training is longer than 3 wk (41). As we observed an improved aerobic fitness and exercise performance, despite no increase in mitochondrial area or the content of PGC1α and complexes I-V, our data are in accordance with the hypothesis that functional alterations may precede molecular changes (5); our data also indicate that this occurs independent of the train-low regime adopted.

Although PPARα activates the transcription of genes involved in fatty acid uptake and oxidation (14), no study has examined the effect of different train-low strategies on PPARα expression. In the present study, PPARα expression was unaltered from pre- to posttraining; however, we found an unexpected reduction in fat oxidation rate for both groups. The effect of train-low strategies on fat oxidation during submaximal exercise is controversial, with one study showing an

increase after 3 wk of training (25), whereas another reported no effect after 4 wk of training (15). The reasons for a reduction in fat oxidation rate after training are unclear but may be related to the training-induced reduction of O_2 cost; therefore, improved exercise efficiency after training may reduce oxidation of both CHO and fat substrates. It should be highlighted, however, that we measured postprandial fat and CHO oxidation rates (~3 h after the last meal). Although meals were replicated from pre- to posttraining tests, it is possible this influenced the observed changes in substrate metabolism (8).

There was no alteration in the mitochondrial area or the expression of several proteins; however, we found that the state 4 of mitochondrial respiration was increased in the twice-a-day but not the once-daily group (Fig. 7). Although not significant, state 3 respiration increased by 13% in the twice-a-day group and was reduced 7% in the once-daily group. To our knowledge, this is the first study evaluating mitochondrial respiration after any train-low strategy. The oxidation of substrates provides electrons to the electron transport chain and the movement of these electrons promotes the extrusion of protons toward the intermembrane space, generating an electrochemical gradient (36). The energy provided by reentry of protons through complex V is then used to regenerate ATP (36). However, oxidation is not 100% coupled to the production of ATP, and the mitochondrial membrane potential can be partially dissipated (36). This "leakage/slippage" can be measured by state 4 mitochondrial respiration, when the ADP to ATP conversion is blunted (36). The reduction in our estimate of state 4 mitochondrial respiration, therefore, suggests an improvement in mitochondrial efficiency, perhaps via a reduction in the leakage/slippage of protons through the inner mitochondrial membrane. This is line with earlier studies comparing sedentary and trained individuals that indicate increased coupling between the electron transport chain and phosphorylation (improvement of mitochondrial efficiency) in trained individuals (10, 59). This reduction in state 4 mitochondrial respiration after twice-a-day training remained when respiration was expressed relative to CS activity (Fig. 7), suggesting that the improvement in mitochondrial efficiency was related to functional changes in the mitochondria and not to changes in mitochondrial content.

Another important issue is that adequate training recovery has a potential impact on training adaptations, in particular to prevent overtraining (38). Although training volume and exercise intensity were the same in both groups, the recovery between the second training session (HIIT) and the next training session (endurance training) was 31 h for the once-daily group and 43 h for the twice-a-day group, which means the twice-a-day group had a 28% longer recovery period after each HIIT session. It is plausible that this longer recovery for the twice-a-day group may have influenced some of the training-induced adaptations. It is important to note, however, that the recovery for the once-daily group does not seem to be insufficient as there was no sign of overtraining, such as elevated resting HR, irritability, or lack of appetite (31). In addition, following the classical recommendation to accelerate muscle repair and glycogen recovery, participants ingested a CHO-protein beverage immediately after completing the second training session, which may have attenuated the potential for different recovery times between experimental groups to affect training-induced adaptations.

In conclusion, this is the first study to compare two different train-low models (twice-a-day vs. once-daily approaches) and to investigate their effects on mitochondrial biogenesis using TEM and respirometry. Despite significant improvements in aerobic fitness and endurance performance, our results indicate little influence of the train-low strategy used. However, there was evidence of higher mitochondrial efficiency when using the twice-a-day strategy. In addition, twice-a-day training induced a larger reduction in perceived exertion, which indicates a potential advantage of this strategy. As observed previously (25, 41, 58), these differences between groups did not translate to greater improvements in exercise performance.

ACKNOWLEDGMENTS

Thaysa Ghiarone is grateful to Fundação de Amparo à Ciência e Tecnologia do Estado de Pernambuco (FACEPE) for a Doctorate's degree scholarship.

GRANTS

This study was financed in part by the Coordenação de Aperfeiçoamento de Pessoal de Nível Superior–Brasil (CAPES): Finance Code 001 and Conselho Nacional de Desenvolvimento Científico e Tecnológico (CNPq): Special Visiting Researcher no. 9296127485573053.

DISCLOSURES

No conflicts of interest, financial or otherwise, are declared by the authors.

AUTHOR CONTRIBUTIONS

T.G., V.A.A.-S., T.A.-S., A.E.L.-S., and D.J.B. conceived and designed research; T.G., S.K.L., F.T., A.S., M.P.F., K.L.S., and A.E.L.-S. performed experiments; T.G., Y.T., and A.E.L.-S. analyzed data; T.G., A.E.L.-S., and D.J.B. interpreted results of experiments; T.G. prepared figures; T.G. drafted manuscript; T.G., V.A.A.-S., S.K.L., F.T., T.A.-S., M.P.F., K.L.S., A.E.L.-S., and D.J.B. edited and revised manuscript; T.G., V.A.A.-S., S.K.L., F.T., T.A.-S., A.S., M.P.F., K.L.S., R.C.B.Q.F., Y.T., J.K., A.E.L.-S., and D.J.B. approved final version of manuscript.

REFERENCES

1. **Andrade-Souza VA, Ghiarone T, Sansonio A, Silva KA, Tomazini F, Arcoverde L, Fyffe J, Saner N, Kuang J, Bertuzzi R, Bishop DJ, Lima-Silva AE.** Exercise twice-a-day potentiates skeletal muscle signalling responses associated with mitochondrial biogenesis in humans, which are independent of lowered muscle glycogen content (preprint) *bioRxiv* 547489, 2019. doi:10.1101/547489.
2. **Berardi JM, Price TB, Noreen EE, Lemon PW.** Postexercise muscle glycogen recovery enhanced with a carbohydrate-protein supplement. *Med Sci Sports Exerc* 38: 1106–1113, 2006. doi:10.1249/01.mss.0000222826.49358.f3.
3. **Bishop D, Jenkins DG, Mackinnon LT.** The relationship between plasma lactate parameters, Wpeak and 1-h cycling performance in women. *Med Sci Sports Exerc* 30: 1270–1275, 1998. doi:10.1097/00005768-199808000-00014.
4. **Bishop D, Jenkins DG, McEniery M, Carey MF.** Relationship between plasma lactate parameters and muscle characteristics in female cyclists. *Med Sci Sports Exerc* 32: 1088–1093, 2000. doi:10.1097/00005768-200006000-00008.
5. **Bishop DJ, Granata C, Eynon N.** Can we optimise the exercise training prescription to maximise improvements in mitochondria function and content? *Biochim Biophys Acta* 1840: 1266–1275, 2014. doi:10.1016/j.bbagen.2013.10.012.
6. **Bishop DJ, Thomas C, Moore-Morris T, Tonkonogi M, Sahlin K, Mercier J.** Sodium bicarbonate ingestion prior to training improves mitochondrial adaptations in rats. *Am J Physiol Endocrinol Metab* 299: E225–E233, 2010. doi:10.1152/ajpendo.00738.2009.
7. **Chance B, Williams GR.** The respiratory chain and oxidative phosphorylation. *Adv Enzymol Relat Subj Biochem* 17: 65–134, 1956.
8. **Chryssanthopoulos C, Williams C, Nowitz A, Kotsiopoulou C, Vleck V.** The effect of a high carbohydrate meal on endurance running capacity. *Int J Sport Nutr Exerc Metab* 12: 157–171, 2002. doi:10.1123/ijsnem.12.2.157.

9. **Cochran AJ, Myslik F, MacInnis MJ, Percival ME, Bishop D, Tarnopolsky MA, Gibala MJ.** Manipulating carbohydrate availability between twice-daily sessions of high-intensity interval training over 2 weeks improves time-trial performance. *Int J Sport Nutr Exerc Metab* 25: 463–470, 2015. doi:10.1123/ijsnem.2014-0263.
10. **Daussin FN, Zoll J, Ponsot E, Dufour SP, Doutreleau S, Lonsdorfer E, Ventura-Clapier R, Mettauer B, Piquard F, Geny B, Richard R.** Training at high exercise intensity promotes qualitative adaptations of mitochondrial function in human skeletal muscle. *J Appl Physiol (1985)* 104: 1436–1441, 2008. doi:10.1152/japplphysiol.01135.2007.
11. **Edge J, Bishop D, Goodman C.** Effects of chronic $NaHCO_3$ ingestion during interval training on changes to muscle buffer capacity, metabolism, and short-term endurance performance. *J Appl Physiol (1985)* 101: 918–925, 2006. doi:10.1152/japplphysiol.01534.2005.
12. **Egan B, Zierath JR.** Exercise metabolism and the molecular regulation of skeletal muscle adaptation. *Cell Metab* 17: 162–184, 2013. doi:10.1016/j.cmet.2012.12.012.
13. **Evans WJ, Phinney SD, Young VR.** Suction applied to a muscle biopsy maximizes sample size. *Med Sci Sports Exerc* 14: 101–102, 1982.
14. **Gan Z, Burkart-Hartman EM, Han DH, Finck B, Leone TC, Smith EY, Ayala JE, Holloszy J, Kelly DP.** The nuclear receptor PPARβ/δ programs muscle glucose metabolism in cooperation with AMPK and MEF2. *Genes Dev* 25: 2619–2630, 2011. doi:10.1101/gad.178434.111.
15. **Gejl KD, Thams LB, Hansen M, Rokkedal-Lausch T, Plomgaard P, Nybo L, Larsen FJ, Cardinale DA, Jensen K, Holmberg HC, Vissing K, Ørtenblad N.** No superior adaptations to carbohydrate periodization in elite endurance athletes. *Med Sci Sports Exerc* 49: 2486–2497, 2017. doi:10.1249/MSS.0000000000001377.
16. **Granata C, Jamnick NA, Bishop DJ.** Training-induced changes in mitochondrial content and respiratory function in human skeletal muscle. *Sports Med* 48: 1809–1828, 2018. doi:10.1007/s40279-018-0936-y.
17. **Granata C, Oliveira RS, Little JP, Renner K, Bishop DJ.** Mitochondrial adaptations to high-volume exercise training are rapidly reversed after a reduction in training volume in human skeletal muscle. *FASEB J* 30: 3413–3423, 2016. doi:10.1096/fj.201500100R.
18. **Granata C, Oliveira RS, Little JP, Renner K, Bishop DJ.** Training intensity modulates changes in PGC-1α and p53 protein content and mitochondrial respiration, but not markers of mitochondrial content in human skeletal muscle. *FASEB J* 30: 959–970, 2016. doi:10.1096/fj.15-276907.
19. **Guerra B, Gómez-Cabrera MC, Ponce-González JG, Martinez-Bello VE, Guadalupe-Grau A, Santana A, Sebastia V, Viña J, Calbet JA.** Repeated muscle biopsies through a single skin incision do not elicit muscle signaling, but IL-6 mRNA and STAT3 phosphorylation increase in injured muscle. *J Appl Physiol (1985)* 110: 1708–1715, 2011. doi:10.1152/japplphysiol.00091.2011.
20. **Hansen AK, Fischer CP, Plomgaard P, Andersen JL, Saltin B, Pedersen BK.** Skeletal muscle adaptation: training twice every second day vs. training once daily. *J Appl Physiol (1985)* 98: 93–99, 2005. doi:10.1152/japplphysiol.00163.2004.
21. **Hearris MA, Hammond KM, Fell JM, Morton JP.** Regulation of muscle glycogen metabolism during exercise: implications for endurance performance and training adaptations. *Nutrients* 10: 298, 2018. doi:10.3390/nu10030298.
22. **Hinz W, Grüninger S, De Pover A, Chiesi M.** Properties of the human long and short isoforms of the uncoupling protein-3 expressed in yeast cells. *FEBS Lett* 462: 411–415, 1999. doi:10.1016/S0014-5793(99)01568-9.
23. **Holloszy JO.** Biochemical adaptations in muscle. Effects of exercise on mitochondrial oxygen uptake and respiratory enzyme activity in skeletal muscle. *J Biol Chem* 242: 2278–2282, 1967.
24. **Howley ET, Bassett DR JR, Welch HG.** Criteria for maximal oxygen uptake: review and commentary. *Med Sci Sports Exerc* 27: 1292–1301, 1995. doi:10.1249/00005768-199509000-00009.
25. **Hulston CJ, Venables MC, Mann CH, Martin C, Philp A, Baar K, Jeukendrup AE.** Training with low muscle glycogen enhances fat metabolism in well-trained cyclists. *Med Sci Sports Exerc* 42: 2046–2055, 2010. doi:10.1249/MSS.0b013e3181dd5070.
26. **Impey SG, Hammond KM, Shepherd SO, Sharples AP, Stewart C, Limb M, Smith K, Philp A, Jeromson S, Hamilton DL, Close GL, Morton JP.** Fuel for the work required: a practical approach to amalgamating train-low paradigms for endurance athletes. *Physiol Rep* 4: e12803, 2016. doi:10.14814/phy2.12803.
27. **Impey SG, Hearris MA, Hammond KM, Bartlett JD, Louis J, Close GL, Morton JP.** Fuel for the work required: a theoretical framework for carbohydrate periodization and the glycogen threshold hypothesis. *Sports Med* 48: 1031–1048, 2018. doi:10.1007/s40279-018-0867-7.
28. **Jackson AS, Pollock ML.** Generalized equations for predicting body density of men. *Br J Nutr* 40: 497–504, 1978. doi:10.1079/BJN19780152.
29. **Jeukendrup AE, Wallis GA.** Measurement of substrate oxidation during exercise by means of gas exchange measurements. *Int J Sports Med* 26, *Suppl* 1: S28–S37, 2005. doi:10.1055/s-2004-830512.
30. **Johannsen DL, Galgani JE, Johannsen NM, Zhang Z, Covington JD, Ravussin E.** Effect of short-term thyroxine administration on energy metabolism and mitochondrial efficiency in humans. *PLoS One* 7: e40837, 2012. doi:10.1371/journal.pone.0040837.
31. **Johnson MB, Thiese SM.** A review of overtraining syndrome-recognizing the signs and symptoms. *J Athl Train* 27: 352–354, 1992.
32. **Kuipers H, Verstappen FT, Keizer HA, Geurten P, van Kranenburg G.** Variability of aerobic performance in the laboratory and its physiologic correlates. *Int J Sports Med* 6: 197–201, 1985. doi:10.1055/s-2008-1025839.
33. **Lane SC, Camera DM, Lassiter DG, Areta JL, Bird SR, Yeo WK, Jeacocke NA, Krook A, Zierath JR, Burke LM, Hawley JA.** Effects of sleeping with reduced carbohydrate availability on acute training responses. *J Appl Physiol (1985)* 119: 643–655, 2015. doi:10.1152/japplphysiol.00857.2014.
34. **Larsen FJ, Schiffer TA, Borniquel S, Sahlin K, Ekblom B, Lundberg JO, Weitzberg E.** Dietary inorganic nitrate improves mitochondrial efficiency in humans. *Cell Metab* 13: 149–159, 2011. doi:10.1016/j.cmet.2011.01.004.
35. **Larsen S, Nielsen J, Hansen CN, Nielsen LB, Wibrand F, Stride N, Schroder HD, Boushel R, Helge JW, Dela F, Hey-Mogensen M.** Biomarkers of mitochondrial content in skeletal muscle of healthy young human subjects. *J Physiol* 590: 3349–3360, 2012. doi:10.1113/jphysiol.2012.230185.
36. **Liesa M, Shirihai OS.** Mitochondrial dynamics in the regulation of nutrient utilization and energy expenditure. *Cell Metab* 17: 491–506, 2013. doi:10.1016/j.cmet.2013.03.002.
37. **Marquet LA, Brisswalter J, Louis J, Tiollier E, Burke LM, Hawley JA, Hausswirth C.** Enhanced endurance performance by periodization of carbohydrate intake: "sleep low" strategy. *Med Sci Sports Exerc* 48: 663–672, 2016. doi:10.1249/MSS.0000000000000823.
38. **Matos N, Winsley RJ.** Trainability of young athletes and overtraining. *J Sports Sci Med* 6: 353–367, 2007.
39. **McGinley C, Bishop DJ.** Distinct protein and mRNA kinetics of skeletal muscle proton transporters following exercise can influence interpretation of adaptations to training. *Exp Physiol* 101: 1565–1580, 2016. doi:10.1113/EP085921.
40. **Meinild Lundby AK, Jacobs RA, Gehrig S, de Leur J, Hauser M, Bonne TC, Flück D, Dandanell S, Kirk N, Kaech A, Ziegler U, Larsen S, Lundby C.** Exercise training increases skeletal muscle mitochondrial volume density by enlargement of existing mitochondria and not de novo biogenesis. *Acta Physiol (Oxf)* 222: e12905, 2018. doi:10.1111/apha.12905.
41. **Morton JP, Croft L, Bartlett JD, Maclaren DP, Reilly T, Evans L, McArdle A, Drust B.** Reduced carbohydrate availability does not modulate training-induced heat shock protein adaptations but does upregulate oxidative enzyme activity in human skeletal muscle. *J Appl Physiol (1985)* 106: 1513–1521, 2009. doi:10.1152/japplphysiol.00003.2009.
42. **Murphy RM, Lamb GD.** Important considerations for protein analyses using antibody based techniques: down-sizing Western blotting up-sizes outcomes. *J Physiol* 591: 5823–5831, 2013. doi:10.1113/jphysiol.2013.263251.
43. **Nielsen J, Gejl KD, Hey-Mogensen M, Holmberg HC, Suetta C, Krustrup P, Elemans CPH, Ørtenblad N.** Plasticity in mitochondrial cristae density allows metabolic capacity modulation in human skeletal muscle. *J Physiol* 595: 2839–2847, 2017. doi:10.1113/JP273040.
44. **Nielsen J, Mogensen M, Vind BF, Sahlin K, Højlund K, Schrøder HD, Ortenblad N.** Increased subsarcolemmal lipids in type 2 diabetes: effect of training on localization of lipids, mitochondria, and glycogen in sedentary human skeletal muscle. *Am J Physiol Endocrinol Metab* 298: E706–E713, 2010. doi:10.1152/ajpendo.00692.2009.
45. **Nilsson LH, Hultman E.** Liver glycogen in man–the effect of total starvation or a carbohydrate-poor diet followed by carbohydrate refeeding. *Scand J Clin Lab Invest* 32: 325–330, 1973. doi:10.3109/00365517309084355.

46. **O'Leary TJ, Collett J, Howells K, Morris MG.** Endurance capacity and neuromuscular fatigue following high- vs moderate-intensity endurance training: A randomized trial. *Scand J Med Sci Sports* 27: 1648–1661, 2017. doi:10.1111/sms.12854.
47. **Perry CG, Lally J, Holloway GP, Heigenhauser GJ, Bonen A, Spriet LL.** Repeated transient mRNA bursts precede increases in transcriptional and mitochondrial proteins during training in human skeletal muscle. *J Physiol* 588: 4795–4810, 2010. doi:10.1113/jphysiol.2010.199448.
48. **Pesta D, Gnaiger E.** High-resolution respirometry: OXPHOS protocols for human cells and permeabilized fibers from small biopsies of human muscle. *Methods Mol Biol* 810: 25–58, 2012. doi:10.1007/978-1-61779-382-0_3.
49. **Ross EZ, Middleton N, Shave R, George K, Nowicky A.** Corticomotor excitability contributes to neuromuscular fatigue following marathon running in man. *Exp Physiol* 92: 417–426, 2007. doi:10.1113/expphysiol.2006.035972.
50. **Saraiva KL, Silva VA, Dias ES, Peixoto CA.** Morphological changes in the testis induced by diethylcarbamazine. *Reprod Toxicol* 22: 754–759, 2006. doi:10.1016/j.reprotox.2006.07.008.
51. **Sferruzzi-Perri AN, Higgins JS, Vaughan OR, Murray AJ, Fowden AL.** Placental mitochondria adapt developmentally and in response to hypoxia to support fetal growth. *Proc Natl Acad Sci USA* 116: 1621–1626, 2019. doi:10.1073/pnas.1816056116.
52. **Shirreffs SM, Taylor AJ, Leiper JB, Maughan RJ.** Post-exercise rehydration in man: effects of volume consumed and drink sodium content. *Med Sci Sports Exerc* 28: 1260–1271, 1996. doi:10.1097/00005768-199610000-00009.
53. **Trumbo P, Schlicker S, Yates AA, Poos M; Food and Nutrition Board of the Institute of Medicine, The National Academies.** Dietary reference intakes for energy, carbohydrate, fiber, fat, fatty acids, cholesterol, protein and amino acids. *J Am Diet Assoc* 102: 1621–1630, 2002. doi:10.1016/S0002-8223(02)90346-9.
54. **Van Thienen R, D'Hulst G, Deldicque L, Hespel P.** Biochemical artifacts in experiments involving repeated biopsies in the same muscle. *Physiol Rep* 2: e00286, 2014. doi:10.14814/phy2.286.
55. **Volpi E, Chinkes DL, Rasmussen BB.** Sequential muscle biopsies during a 6-h tracer infusion do not affect human mixed muscle protein synthesis and muscle phenylalanine kinetics. *Am J Physiol Endocrinol Metab* 295: E959–E963, 2008. doi:10.1152/ajpendo.00671.2007.
56. **Wallis GA, Hulston CJ, Mann CH, Roper HP, Tipton KD, Jeukendrup AE.** Postexercise muscle glycogen synthesis with combined glucose and fructose ingestion. *Med Sci Sports Exerc* 40: 1789–1794, 2008. doi:10.1249/MSS.0b013e31817e0f7e.
57. **Wilkerson DP, Koppo K, Barstow TJ, Jones AM.** Effect of work rate on the functional 'gain' of Phase II pulmonary O_2 uptake response to exercise. *Respir Physiol Neurobiol* 142: 211–223, 2004. doi:10.1016/j.resp.2004.06.001.
58. **Yeo WK, Paton CD, Garnham AP, Burke LM, Carey AL, Hawley JA.** Skeletal muscle adaptation and performance responses to once a day versus twice every second day endurance training regimens. *J Appl Physiol (1985)* 105: 1462–1470, 2008. doi:10.1152/japplphysiol.90882.2008.
59. **Zoll J, Sanchez H, N'Guessan B, Ribera F, Lampert E, Bigard X, Serrurier B, Fortin D, Geny B, Veksler V, Ventura-Clapier R, Mettauer B.** Physical activity changes the regulation of mitochondrial respiration in human skeletal muscle. *J Physiol* 543: 191–200, 2002. doi:10.1113/jphysiol.2002.019661.