Regular Article

Title: Repeated-sprint training under hypoxic conditions induces lactate metabolic and mitochondrial adaptations in fast-twitch dominant skeletal muscle

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Running title: Fiber-type specific adaptations to hypoxic repeated-sprint training

Abstract

Since the influence of hypoxia on mitochondrial adaptations in different muscle fiber types remains unclear, this study examined the impacts of hypoxic repeated sprint training (RST) on lactate metabolism and mitochondrial-related protein content, with a special focus on potential differences between different fiber type composition. Eight-week-old ICR mice were randomly divided into five groups: normoxic sedentary control (CON), normoxic 2set-RST (2set-NT), normoxic 4set-RST (4set-NT), hypoxic 2set-RST (2set-HT), and hypoxic 4set-RST (4set-HT). Two or four sets of 5×40-s sprints (recovery: 20-s, inter-set = 5-min) were performed for four weeks (three times/week) under either normoxia (FiO_2 = 20.9%) or hypoxia (FiO_2 = 13%). Skeletal muscle samples were analyzed for mitochondrial and metabolism-related protein content.

In the fast-twitch (FT)-dominant plantaris muscle, peroxisome proliferator-activated receptor gamma coactivator-1α (PGC-1α) and monocarboxylate transporter 4 (MCT4) were significantly higher in the 4set-NT and 4set-HT compared to the CON ($p<0.01$). Notably, PGC-1α and MCT4 remained unchanged in the 2set-NT but were significantly elevated in the 2set-HT ($p<0.05$). Pyruvate dehydrogenase (PDH) E1α subunit was higher in the 2set-HT ($p<0.05$) and 4set-HT ($p<0.01$) compared to the CON. Additionally, mitochondrial oxidative phosphorylation (OXPHOS) subunit complex I increased significantly only in the 4set-HT ($p<0.05$). In contrast, no significant changes were observed in the slow-twitch-dominant soleus muscle.

These findings suggest that hypoxic RST selectively increases proteins involved in lactate metabolism and mitochondria, especially in the FT-dominant plantaris. Notably, several proteins (PGC-1α, PDH E1α, and MCT4) were elevated under hypoxia even with lower training loads (2 sets).

Keywords: hypoxia; repeated sprint training; mitochondria; muscle fiber type; training volume

低酸素条件下における反復スプリントトレーニングが速筋線維主体の骨格筋の乳酸代謝およびミトコンドリア適応に及ぼす影響

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要旨

本研究では、低酸素環境下での反復スプリントトレーニング (RST) が乳酸代謝およびミトコンドリア関連タンパク質量にもたらす効果を速筋線維主体の足底筋と遅筋線維主体のヒラメ筋にて検討した。

8 週齢の ICR マウスを無作為常酸素安静対照群 (CON)、常酸素 2 セット-RST 群 (2set-NT)、常酸素 4 セット-RST 群 (4set-NT)、低酸素 2 セット-RST 群 (2set-HT)、低酸素 4 セット-RST 群 (4set-HT) に分けた。常酸素 (FiO_2 = 20.9%) または低酸素 (FiO_2 = 13%) 条件下において、トレッドミル上での 5x40 秒スプリント走行運動 (スプリント間休息：20 秒、セット間休息：5 分) を 2 セットまたは 4 セット行い、週 3 回の頻度で 4 週間実施した。介入終了後に足底筋とヒラメ筋を摘出し、ウェスタンブロット法にてミトコンドリアおよび代謝関連のタンパク質量を定量化した。

足底筋のペルオキシソーム増殖因子活性化受容体 γ 共活性化因子-1α (PGC-1α) およびモノカルボン酸輸送担体 4 (MCT4) が CON 群と比較して 4set-NT 群および 4set-HT 群において有意に高値を示した ($p<0.01$)。CON 群と比較して PGC-1α および MCT4 は 2set-HT 群で有意に高値を示した ($p<0.05$) が、2set-NT 群では有意差は認められなかった。ピルビン酸脱水素酵素 (PDH) E1α サブユニットは、CON 群と比較して 2set-HT 群 ($p<0.05$) および 4set-HT 群 ($p<0.01$) において高値を示した。さらに、ミトコンドリア酸化的リン酸化 (OXPHOS) サブユニット複合体 I は 4set-HT 群においてのみ CON 群より有意に高値を示した ($p<0.05$)。一方、ヒラメ筋においては有意な変化は認められなかった。

以上の結果より、低酸素環境での RST は速筋線維主体の足底筋において乳酸代謝およびミトコンドリアに関与するタンパク質量を選択的に増加させることが示唆された。また、少ないトレーニング負荷 (2 セット) において、

70　複数のタンパク質 (PGC-1α、PDH E1α、MCT4) は低酸素条件下でのみ上昇す
71　ることが示唆された。

1 Introduction

Repeated Sprint Training (RST) has emerged as a highly effective approach for enhancing athletic performance. By implementing brief, “all-out” or near-maximal effort, RST elicits metabolic changes comparable to those caused by traditional endurance training protocols [1]. Incorporating hypoxic conditions further enhances these adaptations, especially benefiting athletes in team sports, combat disciplines, and racquet sports, as well as any sport that involves repeated accelerations [2]. Incorporating hypoxic conditions into RST, as a form of the “living low, training high” model, effectively avoids the adverse effects associated with prolonged hypoxic residence in the traditional “living high, training low” model, such as sleep disturbances, reduced protein synthesis, and appetite suppression while still inducing significant training adaptations [3]. Compared to sprint training in normoxia, RST in hypoxia has consistently been shown to improve exercise performance. A systematic review involving 347 athletes reported that hypoxic RST enhanced all monitored variables, such as repeated sprint ability (RSA) max and VO_2max and was more effective than RST in normoxia [4]. Supportive evidence from elite cross-country skiers showed that six hypoxic RST sessions increased the number of sprints during RSA testing, while no improvement was observed following normoxic RST [5]. Similarly, youth soccer players showed a significant improvement in repeated agility performance after 5 weeks of hypoxic training compared to normoxic training [6]. Additionally, five consecutive days of hypoxic RST were found to enhance peak power output in competitive sprinters [7]. Overall, these findings suggest that hypoxic RST offers potential advantages for performance-related variables, especially those linked to RSA.

Along with these functional outcomes, hypoxic RST has been shown to place greater metabolic stress on skeletal muscle, which may facilitate the activation of

transcriptional coactivators involved in mitochondrial biogenesis, such as peroxisome proliferator-activated receptor gamma coactivator-1α (PGC-1α), thereby potentially aiding mitochondrial adaptations [8, 9, 10]. Hypoxic RST may also offer specific molecular benefits, including increased levels of hypoxia-inducible factor-1α (HIF-1α), carbonic anhydrase III, and monocarboxylate transporter 4 (MCT4), along with improved glycolytic capacity and blood perfusion in working muscles [11, 12]. However, most previous studies have primarily focused on performance outcomes, with relatively few investigations examining metabolic adaptations to hypoxic RST [8, 9, 10]. Additionally, evidence for fiber type-specific adaptations remains particularly limited, despite the fact that the inherent metabolic and structural differences between muscle fiber types are expected to cause different adaptive responses to hypoxic RST. Fast-twitch (FT) fibers, which have a glycolytic phenotype, are preferentially recruited during high-intensity sprint, with recruitment increasing in an intensity-dependent manner [13]. This pattern contrasts with low-intensity continuous exercise, which mainly involves oxidative slow-twitch (ST) fibers with minimal recruitment of FT fibers. Furthermore, skeletal muscle HIF-1α expression has been reported to be fiber-type-dependent, with higher expression in FT than in ST [14]. Previous research investigated the muscle type differences on the hypoxia-induced muscle phenotype, but not by the training [15]. Based on Suzuki (2019), it seems that FT- and ST-dominant muscle would respond differently with a combination of hypoxic exposure and normoxic (endurance) exercise [16], however, it remained unknown about the hypoxic exercise especially with RST from the previous literature. To address this gap, the present study employed a rodent model to systematically compare a FT-dominant muscle with an ST-dominant muscle, aiming to identify potential fiber type-specific differences in mitochondrial and metabolic adaptations to hypoxic RST.

This study aims to thoroughly examine the effects of hypoxic RST on skeletal muscle metabolic adaptation, focusing on two main questions: (1) the dose-response relationship between hypoxic RST training volume and metabolic adaptation; (2) fiber type-specific differences in responses to hypoxic RST. By addressing these questions, this study aims to clarify how hypoxic RST impacts skeletal muscle metabolic and mitochondrial adaptations.

2 Methods

2.1 Experimental Animals and Housing Conditions

Six-week-old male and female Institute of Cancer Research (ICR) mice were obtained from CLEA Japan (Tokyo, Japan). The mice were housed in an environment maintained at 23±2℃ under a 12-hour light/dark cycle (dark period: 7:00-19:00). All experiments were carried out during the dark phase when the mice were most active. During the one-week acclimatization period, three to four mice were housed together in standard cages. Afterwards, each mouse was housed individually. The mice had free access to standard laboratory chow (MF, 3.6 kcal/g; 60% kcal from carbohydrate, 13% kcal from fat, 27% kcal from protein; Oriental Yeast, Tokyo, Japan) and tap water. All procedures and protocols involving animals were conducted in accordance with the ethical standards of the Committee on Animal Care and Use, The University of Tokyo (approval no. 2021-1).

2.2 Experimental Procedures

2.2.1 Experimental Adaptation

The mice underwent a 1-week acclimatization to the standard housing environment, followed by treadmill adaptation sessions. The adaptation sessions were conducted at 10 m/min for 10 min per day over 3 consecutive days on a treadmill equipped with an electroshock grid (Model: MK-680, Muromachi Kikai Co., Japan) at

147 the rear of the belt.

148 **2.2.2 Maximal Incremental Exercise Capacity Test**

149 On the 4th day, after completing the acclimatization process, a maximal
150 incremental exercise capacity test was conducted on a treadmill set at a 20° incline.
151 Following a 5-min warm-up at 10 m/min, the speed was increased by 3 m/min every 2
152 min until exhaustion. The maximum running speed (Vmax) was defined as the highest
153 speed at which the mice could no longer continue running despite gentle encouragement.
154 After the test, the mice rested for 1 day before beginning a 4-week training intervention.

155 **2.2.3 Creation of Hypoxic Environment**

156 The hypoxic environment was created in a hypoxic tent (213×213×198 cm) using
157 a hypoxia generator (Everest Summit II, Hypoxico, USA). The generator supplied
158 hypoxic air with an oxygen concentration of 13.0-13.3% at approximately 116 L/min
159 through a plastic tube to the tent, simulating an altitude of 3,800 m. The oxygen
160 concentration inside the tent was monitored using an oxygen analyzer (OXY-1S, JIKCO,
161 Nagoya, Japan), while carbon dioxide (CO_2), temperature, and humidity levels were
162 measured with an NDIR sensor (MonotaRO, Osaka, Japan).

163 **2.2.4 Intervention**

164 Prior to the 4-week sprint training intervention, eight-week-old ICR mice were
165 randomly divided into five groups (n = 8 per group; 4 males and 4 females each): a
166 normoxic sedentary control group (CON), which received no exercise intervention and
167 was kept under normoxia, two normoxic training groups that performed either 2 sets
168 (2set-NT) or 4 sets (4set-NT) of sprints, and two hypoxic training groups that also
169 performed either 2 sets (2set-HT) or 4 sets (4set-HT) of sprints. Normoxia was defined
170 as FiO_2 = 20.9% (CO_2<0.1%; temperature: 22.0-25.0°C; relative humidity: 60.0%-
171 70.0%), and hypoxia as FiO_2 = 13.0-13.3% under identical CO_2, temperature, and

humidity.

The 2set-NT and 2set-HT groups performed 2 sets of sprint training per session at 80% of their Vmax, which was determined through the maximal incremental exercise capacity test conducted prior to the training intervention. Meanwhile, the 4set-NT and 4set-HT groups performed 4 sets at the same intensity. Each set consisted of five 40-second sprints with 20-second recoveries between sprints and 5 minutes between sets. To match hypoxic exposure time, the 2set-HT group remained in the hypoxic environment after training until their total exposure time equaled that of the 4set-HT group. The intervention lasted 4 weeks, with 3 training sessions per week, for a total of 12 sessions.

Body weight was measured on the day of the initial training session (pre-weight) and again on the day of tissue sampling. Weight change was calculated as the difference between these two measurements. Total food intake was determined by the total amount of chow consumed from 8:00 a.m. on the day of the first training session to 8:00 a.m. on the day of sampling.

Tail blood was collected immediately after the 1st, 6th (end of week 2), and 12th (end of week 4) training sessions to measure blood lactate concentration using a Lactate Pro2 (Arkray, Japan) portable lactate analyzer. Twenty-four hours after the final training session, under isoflurane inhalation anesthesia, euthanasia was performed by bloodletting via the inferior vena cava. The mice were rapidly dissected, and the bilateral soleus and plantaris muscles were collected and weighed (wet weight, mg). The muscle-to-body weight ratio was calculated by normalizing the combined muscle weight to the body weight (g) measured on the sampling day. The collected samples were immediately frozen in liquid nitrogen and subsequently stored at -80°C until analysis.

To evaluate the independent effect of hypoxic exposure without compromising the primary aim of the main experiment (i.e., comparison of the dose-response relationship between hypoxic RST training volume and metabolic adaptation), we conducted an additional experiment. This experiment employed identical environmental conditions and an exposure schedule to those of the hypoxic training groups in the main experiment (hypoxic condition: FiO_2 = 13%, with the same exposure duration, frequency, and total intervention period). ICR mice were randomly assigned to a normoxic sedentary group (CON, n = 8) or a hypoxic sedentary group (Hse, n = 8) and were exposed to normoxic or hypoxic conditions, respectively. At the end of the intervention period, animals were euthanized at 24 hours after the final exposure, and the plantaris muscle was rapidly excised and snap-frozen in liquid nitrogen. Analytical procedures were identical to those described for the main experiment.

2.3 Analytical Procedures

2.3.1 Tissue Homogenization Process

Protein was isolated from soleus and plantaris muscles using an ice-cold radio-immunoprecipitation assay buffer (RIPA lysis buffer, 50 mol/L Tris-HCl pH 7.4, 150 mol/L NaCl, 0.25% deoxycholic acid, 1% NP-40, and 1 mol/L ethylenediaminetetraacetic acid [EDTA]) supplemented with a protease inhibitor mixture (cOmplete Mini, EDTA-free; Roche Applied Science, Mannheim, Germany) and a phosphatase inhibitor mixture (PhosSTOP; Roche Applied Science). Muscle samples, 5 mm stainless steel beads, and RIPA lysis buffer volume 15-20 times the wet weight of the muscle were placed in 2 mL screw-cap microtubes. The samples were homogenized with a bead mill at 3,600 rpm for 4-6 cycles of 30 seconds each. The tissue was then vortexed on ice for 30 minutes to facilitate solubilization and centrifuged at 4°C at 2,800 rpm for 15 minutes. The supernatant was collected as the

sample solution for further analysis.

2.3.2 Citrate Synthase (CS) Maximal Activity Measurement

Because the maximum activity of citrate synthase (CS) is highly correlated with mitochondrial content in skeletal muscle, it is commonly used as an indicator of mitochondrial content in various studies [17]. The maximum activity of CS was measured using the method by Srere [18]. The sample solution was diluted fivefold with homogenization buffer (100 mM KH_2PO_4, 0.05% bovine serum albumin (BSA), pH 7.3) and used for the CS activity assay. In a 96-well microplate at 37°C, 10 µL of the sample solution was mixed with Tris-HCl, 5,5'-dithiobis (2-nitrobenzoic acid, DTNB), acetyl-CoA, and oxaloacetate. Absorbance changes were measured at 412 nm using a microplate spectrophotometer (SpectraMax ABS Plus, Molecular Devices Japan, Tokyo).

2.3.3 Western Blot Analysis

Protein concentrations in the samples were measured using the Bicinchoninic Acid (BCA) method (BCA Protein Assay Kit, Fujifilm Wako, Osaka, Japan). The samples were diluted with RIPA lysis buffer and sample buffer (0.25 mol/L Tris-HCl, pH 6.8, 8% SDS, 40% glycerol, 0.02% bromophenol blue, 20% 3-mercapto-1,2-propanediol, Fujifilm Wako) to a concentration of 1.0 µg/µL and prepared for western blotting. The samples were divided into two portions; one was boiled at 90°C for 5 minutes, while the other was used for oxidative phosphorylation (OXPHOS) western blotting. The samples were electrophoresed on 7.5-15% SDS-PAGE gels, loading 3-10 µg of protein per lane. Electrophoresis was performed at 150 V for 60 minutes. Afterward, the proteins were transferred to a polyvinylidene fluoride (PVDF) membrane (Merck KGaA, Darmstadt, Germany) using wet transfer at 75-100 V for 75 minutes. After transfer, the membranes were blocked with PVDF blocking reagent (Toyobo, Osaka,

Japan) for 60 min at room temperature. After three washes with 1% Tween 20 Tris-buffered saline (TTBS), the membrane was incubated with primary antibody solution at room temperature for 1 hour, then at 4°C overnight. Following primary antibody incubation, the membrane was washed three times with TTBS and incubated with secondary antibody (Anti IgG (H+L) mouse or rabbit, American Qualex, San Clemente, CA) for 60 minutes. After secondary antibody incubation, the membrane was washed three times with TTBS. Finally, the membrane was reacted with chemiluminescence substrate (Pierce ECL Western Blotting Substrate, Thermo Fisher Scientific, Waltham, MA) and visualized using a ChemiDoc system (Bio-Rad). Densitometric analysis of the captured images were performed using Bio-Rad Quantity One software ver. 4.6.1. The membrane was stained with Ponceau S solution (P7170-1L; Sigma-Aldrich) to verify equal protein loading. Primary antibodies used for Western blot analysis included: PGC-1α (Millipore, #516557, 1:3000); Total OXPHOS Rodent WB Antibody Cocktail (Abcam, #ab110413, 1:5000); Pyruvate dehydrogenase (PDH) E1α subunit (Abcam, #ab168379, 1:4000); Cytochrome c oxidase (COX) IV (Abcam, #ab14744, 1:2000); and antibodies against MCT1 and MCT4, which were raised in rabbits against the C-terminal region of their respective MCTs (Qiagen, Tokyo, Japan) [19].

2.4 Statistical Analysis

All experimental data are presented as mean ± standard deviation (SD). The normality of the data was assessed using the Shapiro-Wilk test, and the homogeneity of variances was tested with Levene's test. Statistical analyses were performed using Prism 9 for macOS (GraphPad Software, San Diego, CA, USA).

Blood lactate concentrations were analyzed using three-way repeated measures analysis of variance (ANOVA) with factors for training time (1st, 6th, 12th), oxygen concentration (normoxia vs. hypoxia), and set number (2 sets vs. 4 sets). When

significant main effects or interactions were detected, post-hoc comparisons were conducted using Tukey's multiple comparisons test identified specific group differences. Sphericity was checked with Mauchly's test, and if violated, degrees of freedom were corrected with the Greenhouse-Geisser adjustment. For other experimental outcomes (e.g., protein concentration), one-way ANOVA was performed to compare differences among the five experimental groups (CON, 2set-NT, 4set-NT, 2set-HT, 4set-HT). Post hoc pairwise comparisons used Tukey's test. For the additional experiment, each variable was compared between the CON and Hse groups using an unpaired t-test. Statistical significance was considered at $p<0.05$.

3 Results

3.1 Body Weight Changes, Food Intake, Muscle Wet Weight

After 4 weeks of intervention, significant differences in body weight changes were observed among the groups ($p<0.05$). However, no significant differences were found in total food intake between the groups. The muscle-to-body weight ratio (mg/g) in both the plantaris and soleus muscles did not differ significantly among the groups (Table 1).

[Insert Table 1 here]

3.2 Changes in Blood Lactate Concentrations During the Training Intervention

Blood lactate concentration was measured as an outcome variable to assess if hypoxic training caused changes in the blood lactate response. A three-way repeated measures ANOVA was used to examine the effects of training time, oxygen concentration, and set number on blood lactate concentrations (Figure 1). Results in Table 2 showed significant main effects of training time [$F (1.728, 48.38) = 13.37$, $p<0.001$] and oxygen concentration [$F (1, 28) = 122.1$, $p<0.001$], but no effect of set number [$F (1, 28) = 2.042$, $p = 0.1641$]. There were also significant two-way interactions between training time and oxygen concentration [$F (2, 56) = 3.424$, $p =$

297 0.040], and between training time and set number [F (2, 56) = 6.294, p = 0.003]. A
298 significant three-way interaction was found [F (2, 56) = 4.098, p = 0.022], showing that
299 the response patterns changed depending on the combination of all three factors. Post-
300 hoc analysis with Tukey's multiple comparisons test indicated that blood lactate
301 concentrations were consistently higher in hypoxic training groups compared to
302 normoxic groups at all time points.

303 [Insert Figure 1 here]

304 [Insert Table 2 here]

305 After the first training session, lactate levels were significantly higher in the 2set-
306 HT group (7.1±1.9 mM) than in the 2set-NT group (3.5±0.6 mM, $p<0.05$), and higher
307 in the 4set-HT group (11.1±3.9 mM) than in the 4set-NT group (3.9±1.2 mM, $p<0.05$).
308 At the 6th training session (end of week 2), lactate levels remained elevated in the 2set-
309 HT group (7.0±1.4 mM) compared with the 2set-NT group (3.6±0.7 mM, $p<0.05$), and
310 in the 4set-HT group (7.3±1.8 mM) compared with the 4set-NT group (3.1±1.0 mM,
311 $p<0.01$). By the 12th training session (end of week 4), 2set-HT (6.5±1.4 mM) remained
312 significantly higher than 2set-NT (2.5±0.8 mM, $p<0.01$), and 4set-HT (5.6±1.8 mM)
313 was also significantly higher than 4set-NT (2.7±0.8 mM, $p<0.05$). No significant
314 differences were observed between the 2 sets and 4 sets protocols within the same
315 oxygen condition, nor between hypoxic training groups across time points.

316 **3.3 Mitochondrial Enzyme Activities and Respiratory Chain Complexes**

317 Plantaris Muscle: PGC-1α, a master regulator of mitochondrial biogenesis, was
318 significantly increased in the 2set-HT, 4set-HT, and 4set-NT groups compared to the
319 CON group ($p<0.05$), while the 2set-NT group showed no significant difference (Figure
320 2A). CS maximal activity, a marker of mitochondrial content, showed no significant
321 differences among the groups (Figure 2B). Similarly, the protein level of COX IV, a

critical component of mitochondrial OXPHOS complex IV involved in the electron transport chain step, did not differ significantly among the groups (Figure 2C). However, the protein content of OXPHOS complex I (Figure 2D) was significantly upregulated only in the 4set-HT group compared to the CON group ($p<0.05$). The protein contents of OXPHOS complex II-V (Figure 2E-H) showed no significant differences among the groups.

[Insert Figure 2 here]

Soleus Muscle: PGC-1α protein content (Figure 3A), CS maximal activity (Figure 3B), COX IV protein content (Figure 3C), and OXPHOS complex I-V protein contents (Figure 3D-H) showed no significant differences among the groups.

[Insert Figure 3 here]

3.4 Proteins Contents of Lactate Transporters and Pyruvate Dehydrogenase

Plantaris Muscle: Protein content of PDH E1α (Figure 4A) was significantly increased in the 2set-HT ($p<0.05$) and 4set-HT ($p<0.01$) groups compared to the CON group, while neither 2set-NT nor 4set-NT showed significant differences from CON. MCT4 (Figure 4C) protein content was significantly increased in the 4set-NT, 2set-HT, and 4set-HT groups compared to the CON group ($p<0.01$), with no significant difference between the 2set-NT and CON groups. MCT1 (Figure 4B) showed no significant differences among the groups.

Soleus Muscle: In the soleus muscle, there were no significant differences among groups in the protein content of PDH E1α (Figure 4E), MCT1 (Figure 4F), or MCT4 (Figure 4G).

[Insert Figure 4 here]

3.5 Effects of hypoxic exposure without exercise on protein contents related to energy metabolism

Figure 5 shows data from the additional experiment to evaluate the independent effects of hypoxic exposure on representative indices that showed significant changes in the main experiment. In the plantaris muscle, there were no significant differences between CON and Hse in the protein content of OXPHOS complex I (Figure 5A), PGC-1α (Figure 5B), PDH E1α (Figure 5C), and MCT4 (Figure 5D).

[Insert Figure 5 here]

4 Discussion

The primary goal of this study was to examine the dose-response relationship of hypoxic RST. Therefore, we compared each training group with its respective normoxia sedentary control to assess whether hypoxic RST at different set numbers could induce significant metabolic adaptations. The main findings of this study show that under hypoxic training conditions, adaptive responses did not differ significantly between the 2set-HT and 4set-HT, except for mitochondrial OXPHOS complex I. Conversely, when RST was performed in 2 sets, certain adaptations, such as PGC-1α, PDH E1α, and MCT4, did not appear in normoxia, but emerged under hypoxia. Notably, in FT fiber-predominant muscles, training in hypoxia led to increases in specific mitochondrial constituent proteins and lactate metabolism-related proteins, whereas these adaptations were not observed in ST fiber-predominant muscles. Despite the upregulation of PGC-1α, there were no significant changes in CS maximal activity or COX IV protein content, both recognized as markers of mitochondrial content.

MCT1 is predominantly expressed in ST muscle fibers, facilitating lactate uptake and oxidation, whereas MCT4 is mainly expressed in FT muscle fibers and is upregulated by HIF-1α under hypoxia, mediating the efflux of glycolytically produced

lactate [20, 21]. In the present study, post-exercise blood lactate concentration was higher in the hypoxic training group, consistent with a previous finding [22], likely due to increased reliance on glycolysis and MCT4-mediated lactate export under hypoxia. However, blood lactate concentration depends on the balance between lactate appearance (Ra) and disappearance (Rd) [23]. Despite the upregulation of MCT4, blood lactate gradually decreased during training in the 4set-HT group, suggesting that Rd increased more than Ra. The fixed running speed used in this experiment may have led to a gradual decline in relative exercise intensity as training progressed, thereby reducing Ra. In contrast, Rd is likely associated with enhanced oxidative capacity. Previous studies have shown that exercise training increases mitochondrial content in skeletal muscle and enhances respiratory function [24], while hypoxic conditions can also improve mitochondrial respiratory function [25], and the resulting increase in oxidative capacity further facilitates lactate clearance. Hypoxia is also known to activate vascular endothelial growth factor and Nitric oxide-related signaling, leading to angiogenesis and vasodilation, which facilitate lactate removal [26, 27]. Furthermore, elevated PDH abundance and activation promote pyruvate entry into the TCA cycle, enhancing aerobic metabolism of lactate-derived substrates [28]. Although we could not fully investigate, combined mitochondrial and microvascular adaptations reported in previous studies could be a plausible explanation for the enhanced lactate clearance observed after hypoxic training.

In this study, training under hypoxia elicited adaptations that were not present under normoxia at the same training volume, especially when RST was performed in 2 sets. These adaptations were likely driven by enhanced metabolic stress under hypoxic conditions [29, 30]. In this study, hypoxic training induced some metabolic and mitochondrial adaptations that were not observed under normoxia, particularly in the

2set-HT group. The upregulation of PGC-1α, along with the selective increases in MCT4 and PDH E1α, indicates that a smaller absolute workload under hypoxic conditions was sufficient to elicit greater metabolic stress and activate signaling pathways related to mitochondrial biogenesis [31, 32, 33, 34], thereby promoting mitochondrial and metabolic adaptations in the plantaris muscle [35]. Second, these adaptations may also occur through lactate signaling. Lactate functions as a potent circulating metabolic signal [36] capable of modulating various cellular signaling pathways [37]. Mouse studies have shown that exogenous lactate upregulates the mRNA expression of PGC-1α [38, 39, 40], MCT4, and PDH E1α in skeletal muscle [41, 42]. Our preliminary experiments demonstrated that hypoxic exposure alone (without exercise) significantly increased blood lactate concentrations (normoxic: 3.0±0.6 mM vs. hypoxic: 4.9±0.9 mM, $p<0.01$), confirming that hypoxia contributes an additional effect to elevated blood lactate. When combined with exercise at matched absolute work intensities, hypoxic conditions consistently maintained higher post-exercise blood lactate levels relative to normoxic conditions [43, 44]. Based on these results, we propose that repeated sprint training under hypoxia promotes lactate accumulation and activates lactate-dependent signaling cascades, thus enhancing skeletal muscle metabolic adaptations.

Comparisons between 2set-HT and 4set-HT revealed similar changes in most mitochondrial OXPHOS proteins, except for complex I, which was upregulated only during higher-volume hypoxic training. This difference likely reflects distinct regulatory mechanisms: PGC-1α shows a threshold-like response, plateauing at relatively low training volumes [45, 46], whereas complex I upregulation may be more sensitive to cumulative hypoxic exposure [47] and training volume [48]. In the present study, complex I increased while complex II did not, which seems inconsistent with

previous reports indicating that the maximal mitochondrial respiration induced by complex I is nearly equal to that induced by complex II [49]. However, changes in OXPHOS complexes protein levels might not necessarily match changes in mitochondrial respiration. A previous study also showed this decoupling, revealing that after high-intensity interval exercise under normoxic conditions, complex I protein increased while complex II remained unchanged, yet the respiratory capacity of both complexes were enhanced [48]. Because only OXPHOS protein levels were measured in the present study, mitochondrial respiratory function could not be directly assessed. Future research should explore the effects of hypoxic training on mitochondrial respiratory capacity adaptations.

Meanwhile, these adaptations were fiber type-specific: they were evident in the FT-dominant plantaris but not in the ST-dominant soleus. In the soleus, none of these protein levels or CS maximal activity showed significant alterations. A plausible explanation is that glycolytic muscles exhibit higher HIF-1α expression than oxidative muscles [14], as demonstrated in rodent studies, indicating that FT fibers are particularly sensitive to HIF-1-mediated regulation under hypoxia. These adaptations may be driven by transcription factors that regulate oxygen signaling and transport and may contribute to the improved contractile performance of FT fibers [50]. Considering the very short half-life of HIF-1α under normoxia (<5 min) [51, 52] compared to our 24-hour interval between the last intervention and tissue sampling, we did not measure HIF-1α since transient changes would likely be undetectable. As previous study showed that repeated-sprint exercise at a similar hypoxic level (FiO_2 = 14.6%) significantly increases skeletal muscle HIF-1α mRNA [11], it is possible that HIF-1α expression was elevated in this study as well. Both plantaris (FT-dominant) and soleus (ST-dominant) muscles are recruited during high-intensity treadmill running at steep inclines [53]. In

our study, with the treadmill set at 20° incline and 80% Vmax, both muscle types were expected to be recruited. Therefore, the lack of significant mitochondrial and metabolic adaptation in soleus likely reflects differential adaptive responses of distinct muscle fiber types to high-intensity exercise in hypoxic conditions, rather than insufficient muscle recruitment.

In the present study, PGC-1α protein content in the plantaris muscle was significantly elevated in the 2set-HT, 4set-NT, and 4set-HT groups, consistent with previous reports [45, 54]. PGC-1α is considered the master regulator of mitochondrial biogenesis. However, CS maximal activity, a conventional marker of mitochondrial content, did not increase proportionally. In other words, the elevation in PGC-1α was not accompanied by corresponding changes in CS activity, indicating a disconnect between molecular signaling and enzymatic markers. Previous studies have also shown that sprint or other high-intensity exercise, whether under normoxic or hypoxic conditions, does not necessarily lead to an increase in CS activity [55, 56]. Within high-intensity interval training (HIIT), brief but intense stimuli are sufficient to upregulate PGC-1α; however, maximal activity and mitochondrial structural proteins, such as OXPHOS, do not always increase in parallel, a pattern supported by earlier findings [57, 58]. Another possible explanation is that the activity of PGC-1α might be disrupted under hypoxic conditions. A previous study indicated that hypoxic stimulation increases the monomethylation of PGC-1α at K224 [59]. This modification was shown to reduce PGC-1α's activity, leading to a decrease in mitochondrial biogenesis.

It should be noted that our analysis compared each training group with its corresponding normoxic resting control. Based on this design, we cannot conclude that hypoxic training is superior to normoxic training. Although hypoxic training in the present study induced upregulation of lactate metabolic and mitochondrial adaptations,

whether these adaptations are superior to those induced by normoxic training requires direct inter-group comparison studies to determine. Regarding this issue, previous studies have reported that hypoxic training does not necessarily outperform normoxic training [60, 61]. Moreover, to assess the independent effect of hypoxic exposure, we conducted the additional experiment examining whether hypoxia alone under sedentary conditions would influence the protein variables that showed changes in the main experiment (OXPHOS complex I, PGC-1α, PDH E1α, and MCT4). The results indicated that the levels of these proteins did not significantly differ between CON and Hse. These findings suggest that, under the conditions of main experiment, the observed adaptations are primarily due to the training stimulus rather than to hypoxic exposure itself. However, the absence of a hypoxic sedentary control group in the main experiment limits a full evaluation of hypoxia's independent effect, which remains a limitation of the current study. Furthermore, in this study, exercise capacity was not reassessed, nor was training intensity adjusted during the intervention period. Because hypoxic training can enhance metabolic adaptations in skeletal muscle, the mice's actual exercise capacity likely improved gradually, leading to a progressive decline in relative training intensity in the hypoxic group over time. This process could have resulted in an underestimation of the effects of hypoxic training. Future studies should further investigate the influence of changes in relative training intensity on metabolic adaptations during hypoxic training. Moreover, including a hypoxic sedentary control group would allow clearer distinction between the independent effects of hypoxia and exercise, thereby elucidating the specific mechanisms underlying hypoxia-induced adaptations.

5. Conclusion

Under the present protocol, both 2 sets and 4sets of hypoxic RST induced

selective adaptive changes in lactate metabolism and mitochondrial-related proteins in FT fiber-predominant muscle compared to normoxic sedentary conditions. In contrast, ST fiber-predominant muscle exhibited no detectable molecular-level alterations. These findings indicate that hypoxic RST serves as a muscle fiber type-specific training strategy capable of selectively activating and remodeling the metabolic characteristics of FT fibers, while exerting limited effects on ST fibers. Notably, several proteins (PGC-1α, PDH E1α, and MCT4) were significantly elevated in the 2set-HT condition but not in the 2set-NT condition, demonstrating an additional hypoxic effect even at lower training volumes.

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Conflicts of Interest:

The authors declare that there are no conflicts of interest.

Author Contributions:

W Wang and H Hatta, Y Takahashi, N Takei were responsible for research design and experimental protocols. W Wang and Y Takahashi, T Matsumoto, T Inaba were responsible for data collection. W Wang conducted the data analysis. W Wang and H Hatta, Y Takahashi, N Takei were responsible for data interpretation and manuscript writing and revision. All authors approved the final manuscript following careful review.

Figure 1.

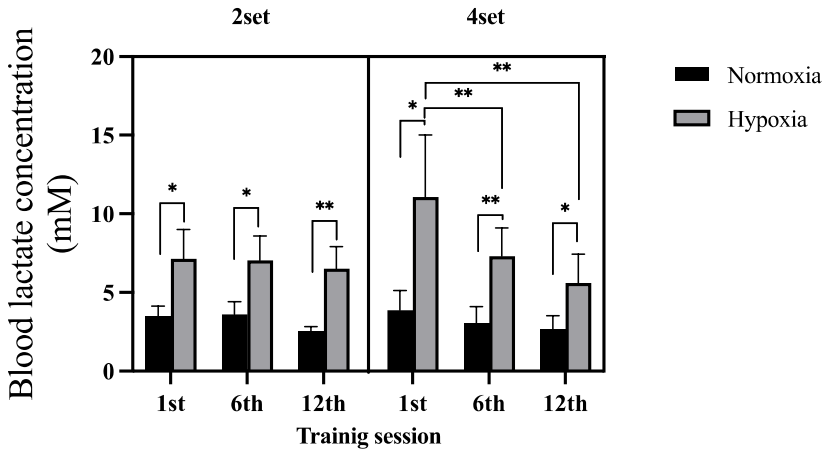

Figure 1. Blood lactate concentrations under different training volumes and oxygen conditions. Data are shown for normoxia (black bars) and hypoxia (gray bars), with 2 sets of RST (left panel) and 4 sets of RST (right panel). Measurements were taken after the 1st (week 0), 6th (end of week 2), and the 12th training session (end of week 4). Values are expressed as mean±SD (n = 8 per group). **$p<0.01$, *$p<0.05$ between the groups. RST: repeated-sprint training.

Figure 2.

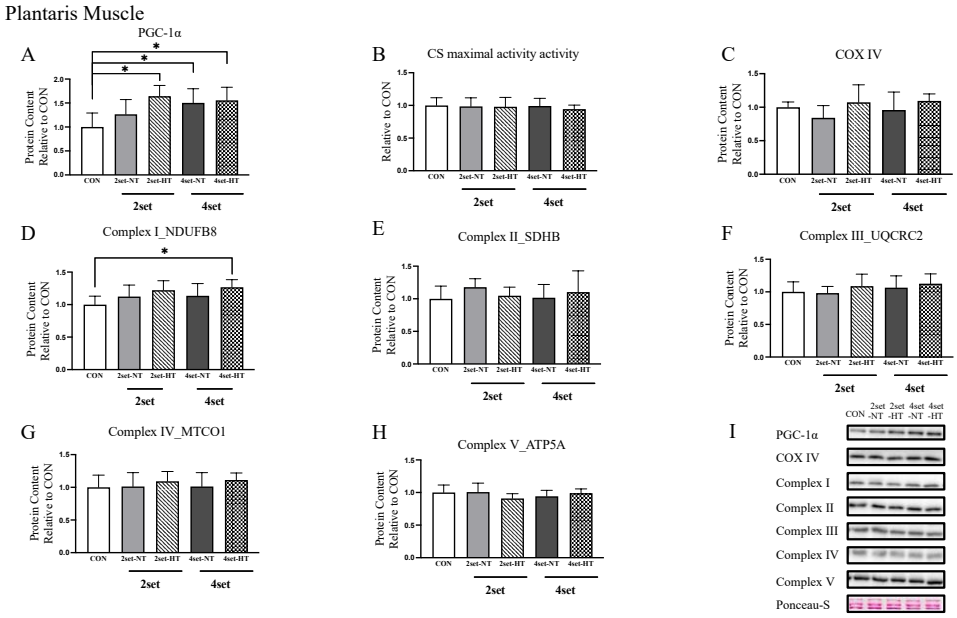

Figure 2. The mitochondrial characteristics and protein content in the plantaris muscle of each experimental group. Panels display (A) PGC-1α protein content, (B) CS maximal activity, (C) COX IV protein content, (D-H) OXPHOS complexes I-V protein contents and (I) Representative western blotting results. The experimental groups include normoxic sedentary control (CON, n=8), 2 sets of RST under normoxia (2set-NT, n=8), 4 sets of RST under normoxia (4set-NT, n=8), 2 sets of RST under hypoxia (2set-HT, n=8), 4 sets of RST under hypoxia (4set-HT, n=8). Values are mean±SD. All data are shown as relative values compared to the control group. *$p<0.05$ between the groups. RST: repeated-sprint training; PGC-1α: peroxisome proliferator-activated receptor gamma coactivator-1α; CS: citrate synthase; OXPHOS: oxidative phosphorylation; NDUFB8: NADH: ubiquinone oxidoreductase subunit B8; SDHB: succinate dehydrogenase complex iron sulfur subunit B; UQCRC2: ubiquinol-cytochrome c reductase core protein 2; MTCO1: mitochondrially encoded cytochrome C oxidase 1; ATP5A: ATP synthase, H^+ transporting, mitochondrial F1 complex, α subunit; COX IV: cytochrome c oxidase IV.

Figure 3.

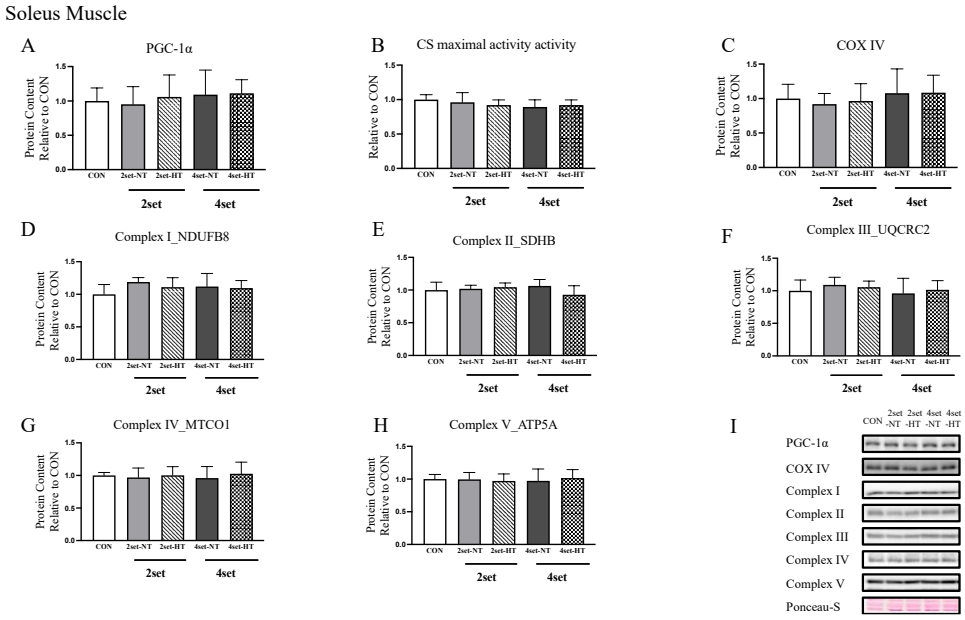

Figure 3. The mitochondrial characteristics and protein content in the soleus muscle of each experimental group. Panels show (A) PGC-1α protein content, (B) CS maximal activity, (C) COX IV protein content, (D-H) OXPHOS complexes I-V protein contents and (I) Representative western blotting results. The experimental groups include normoxic sedentary control (CON, n=8), 2 sets of RST under normoxia (2set-NT, n=8), 4 sets of RST under normoxia (4set-NT, n=8), 2 sets of RST under hypoxia (2set-HT, n=8), 4 sets of RST under hypoxia (4set-HT, n=8). Values are mean ± SD. All data are shown as relative values against the control group. RST: repeated-sprint training; PGC-1α: peroxisome proliferator-activated receptor gamma coactivator-1α; CS: citrate synthase; OXPHOS: oxidative phosphorylation; NDUFB8: NADH: ubiquinone oxidoreductase subunit B8; SDHB: succinate dehydrogenase complex iron sulfur subunit B; UQCRC2: ubiquinol-cytochrome c reductase core protein 2; MTCO1: mitochondrially encoded cytochrome C oxidase 1; ATP5A: ATP synthase, H^+ transporting, mitochondrial F1 complex, α subunit; COX IV: cytochrome c oxidase IV.

Figure 4.

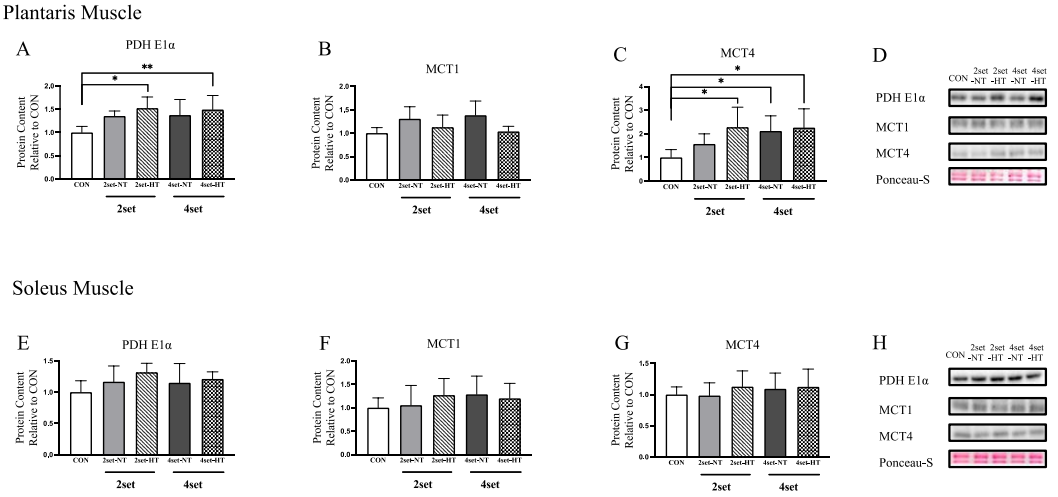

Figure 4. Protein content of lactate transporters and pyruvate dehydrogenase in plantaris and soleus muscles. Panels show (A, E) PDH E1α, (B, F) MCT1, (C, G) MCT4 protein contents, and (D, H) Representative western blotting results. The experimental groups include normoxic sedentary control (CON, n=8), 2 sets of RST under normoxia (2set-NT, n=8), 4 sets of RST under normoxia (4set-NT, n=8), 2 sets of RST under hypoxia (2set-HT, n=8), 4 sets of RST under hypoxia (4set-HT, n=8). Values are mean ± SD. All data are shown as relative values against the control group. **p<0.01, *p<0.05 between groups. RST: repeated-sprint training; PDH E1α: pyruvate dehydrogenase E1α; MCT1: monocarboxylate transporter 1; MCT4: monocarboxylate transporter 4.

Figure 5.

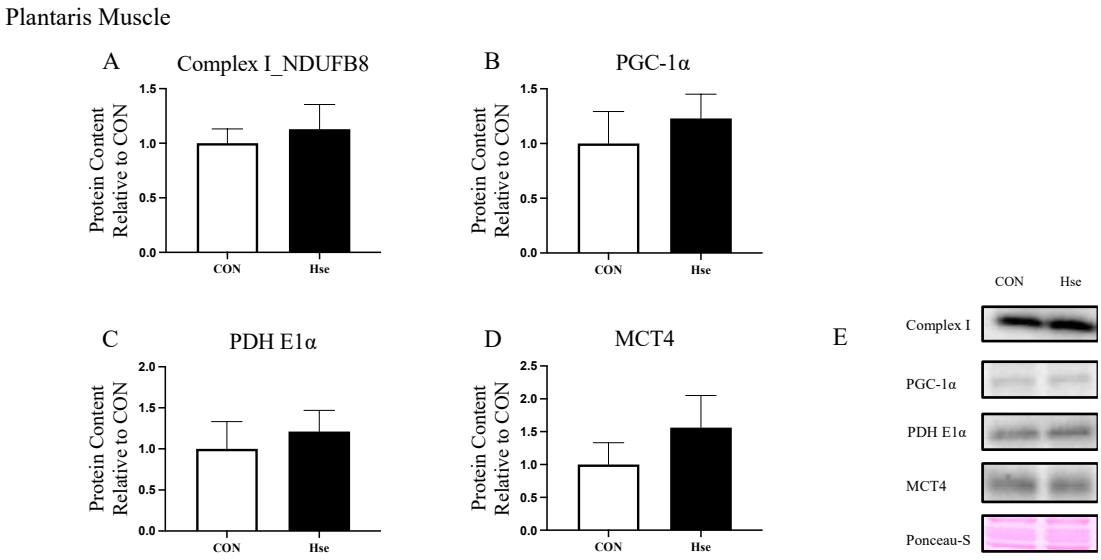

Figure 5. Protein contents of (A) OXPHOS complex I, (B) PGC-1α, (C) PDH E1α, and (D) MCT4 in the plantaris muscle of normoxic sedentary control (CON, n = 8) and hypoxic sedentary control (Hse, n = 8) groups, and (E) representative western blotting results. Values are mean ± SD. All data are shown as relative values against the CON group. Statistical comparisons between CON and Hse were performed using unpaired t-test. PGC-1α: peroxisome proliferator-activated receptor gamma coactivator-1α; NDUFB8: NADH: ubiquinone oxidoreductase subunit B8; PDH E1α: pyruvate dehydrogenase E1α; MCT4: monocarboxylate transporter 4.

Table 1. Physiological parameters of the five experimental groups

Group	CON	2set-NT	4set-NT	2set-HT	4set-HT
Pre-weight (g)	33.6±4.2	34.9±6.0	34.1±5.0	33.3±5.9	35.9±5.6
Weight change (g)	6.7±1.1	5.4±2.1	3.9±1.7*	3.3±1.1**	3.2±1.5**
Total food intake (g)	160.87±13.01	158.49±20.45	150.80±3.86	147.88±12.40	153.89±10.03
Plantaris muscle-to-body weight ratios (mg/g)	0.96±0.08	0.98±0.12	0.96±0.18	1.04±0.12	0.89±0.15
Soleus muscle-to-body weight ratios (mg/g)	0.43±0.03	0.45±0.09	0.43±0.07	0.42±0.04	0.43±0.09

Table 1. Physiological parameters of the five experimental groups (n = 8 each): CON, 2set-NT, 4set-NT, 2set-HT, and 4set-HT. The table includes body weight (g) before the intervention, weight changes (g) after 4 weeks of intervention, total food intake (g), and muscle-to-body weight ratios of the soleus and plantaris muscles (mg/g). Significant differences in body weight changes after 4 weeks were observed between the CON group and the other three groups (4set-NT, 2set-HT, and 4set-HT), with significance levels indicated as *: $p<0.05$, **: $p<0.01$ (compared to CON). The data are expressed as ± SD, and statistical comparisons were performed using one-way ANOVA.

Table 2. Three-way ANOVA Table with Interaction

Source of Variation	SS	DF	MS	F (DFn, DFd)	P value
T	68.29	2	34.14	F (1.72, 48.38) = 13.37	**P<.001**
O	434.4	1	434.4	F (1, 28) = 122.1	**P<.001**
S	7.26	1	7.26	F (1, 28) = 2.042	P = 0.164
T x O	17.49	2	8.744	F (2, 56) = 3.424	**P = 0.040**
T x S	32.15	2	16.07	F (2, 56) = 6.294	**P = 0.003**
O x S	7.26	1	7.26	F (1, 28) = 2.042	P = 0.164
T x O x S	20.93	2	10.46	F (2, 56) = 4.098	**P = 0.022**

Table 2. Three-way ANOVA results for blood lactate concentration post-exercise. T: Training time; O: Oxygen concentration; S: Set number; T × O: Training time × Oxygen concentration interaction; T × S: Training time × Set number interaction; O × S: Oxygen concentration × Set number interaction; T × O × S: Training time × Oxygen concentration × Set number interaction.