HIIT augments muscle carnosine in the absence of dietary beta-alanine intake

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Abstract

Purpose: Cross-sectional studies suggest that training can increase muscle carnosine (MCarn), although longitudinal studies have failed to confirm this. A lack of control for dietary β-alanine intake or muscle fibre type shifting may have hampered their conclusions. The purpose of the present study was to investigate the effects of high-intensity interval training (HIIT) on MCarn. **Methods:** Twenty vegetarian men were randomly assigned to a control (CON; n=10) or HIIT (n=10) group. HIIT was carried out on a cycle ergometer for 12 weeks, with progressive volume (6-12 series) and intensity (140-170% lactate threshold [LT]). MCarn was quantified in whole-muscle and individual fibres; expression of selected genes (*CARNS*, *CNDP2*, *ABAT*, *TauT* and *PAT1*) and muscle buffering capacity *in vitro* ($\beta m_{in\ vitro}$) were also determined. Exercise tests were performed to evaluate total work done (TWD), VO_{2max}, ventilatory thresholds (VT) and LT. **Results:** TWD, VT, LT, VO_{2max} and $\beta m_{in\ vitro}$ were improved in the HIIT group (all $P<0.05$), but not in CON ($p>0.05$). MCarn (in $mmol \cdot kg^{-1}$ dry muscle) increased in the HIIT (15.8±5.7 to 20.6±5.3; $p=0.012$) but not the CON group (14.3±5.3 to 15.0±4.9; $p=0.99$). In type I fibres, MCarn increased in the HIIT (from 14.4±5.9 to 16.8±7.6; p=0.047) but not the CON group (from 14.0±5.5 to 14.9±5.4; p=0.99). In type IIa fibres, MCarn increased in the HIIT group (from 18.8±6.1 to 20.5±6.4; p=0.067) but not the CON group (from 19.7±4.5 to 18.8±4.4; p=0.37). No changes in gene expression were shown. **Conclusion:** In the absence of any dietary intake of β-alanine, HIIT increased MCarn content. The contribution of increased MCarn to the total increase in $\beta m_{in\ vitro}$ appears to be small.

Keywords: Carnosine; β-alanine; Buffering; Training.

Introduction

Hight-intensity interval training (HIIT) is a potent stimulus to improve anaerobic capacity and tolerance to high-intensity efforts (1). These adaptations appear to be, at least in part, through an increase in the physiochemical buffering capacity of the muscle cells (2,3) and by increased H^+ removal capacity (4). Evidence from cross-sectional studies show higher muscle buffering capacity in athletes participating in sports requiring high-intensity efforts in comparison with athletes participating in sports where efforts are of lower intensity, or with non-trained individuals (2,5). This has been confirmed by a longitudinal study that showed improved physiochemical buffering capacity following high-intensity, but not moderate intensity training (3). Skeletal muscle adaptations that account for the increase in physiochemical buffering capacity remain unknown but one possibility is an increase in muscle carnosine (MCarn) content.

Carnosine (β-alanyl-L-Histidine) is a cytoplasmic dipeptide abundantly stored in the skeletal muscle of many vertebrates, including humans. Carnosine synthesis is catalysed by carnosine synthase in a reaction that requires the amino acids L-histidine and β-alanine (6). In human skeletal muscle, β-alanine is the rate-limiting step of carnosine synthesis (7). Increasing dietary intake of β-alanine results in large increases (~60–80%) in MCarn (7-9). A wide range of physiological roles have been attributed to carnosine (10), although pH buffering seems to be particularly relevant within the skeletal muscle under high-intensity exercise, since the pKa of carnosine (*i.e.*, 6.83) (11) lies close to the midpoint of the pH transit-range (between rest and fatigue) in exercised skeletal muscle (12). The H^+ buffering role of carnosine in skeletal muscle is also supported by the fact that glycolytic type II muscle fibres have a higher carnosine content than oxidative type I fibres (13); in humans, carnosine in type II fibres is about 1.5 times higher than in type I fibres (8). Further evidence to support the pH buffering role of carnosine is provided by cross-sectional studies that showed increased MCarn in sprinters compared to non-

sprinters (5), and increased MCarn in professional bodybuilders in comparison with untrained individuals (14).

Although increased MCarn has been hypothesised to be an adaptation induced by long-term high-intensity training (15), longitudinal studies from independent laboratories (16-22) have not shown any significant increase in MCarn following training. Limitations of these studies include lack of dietary control, lack of control for fibre type shifting and training protocols of insufficient intensity and/or duration. Since diet is one of the most influential factors affecting MCarn (7-10,19), any changes elicited by training may have been masked in studies where dietary beta-alanine intake was not controlled. Since type II fibres have ~50% more carnosine than type I fibres, training-induced changes in fibre type distribution and fibre cross-sectional area may have affect measured changes in MCarn. Furthermore, previous studies may have used training protocols of insufficient intensity (16,17) and/or duration (18-22) to induce measurable increases in MCarn. Insufficient training stimuli appears to be critical in light of recent data suggesting that lactate and H^+ may act as triggers for the skeletal muscle to adapt and improve H^+ handling (23). Altogether, these limitations may have rendered previous studies unable to properly test the principle that chronic exercise training increases MCarn. In this proof-of-principle investigation, we used a very specific experimental set-up to test whether HIIT can increase MCarn. To provide sufficient training stimulus, we used a 12-week HIIT protocol, longer than those previously shown to increase muscle buffering capacity (3,24); to control for the potential influence of diet and muscle fibre type shifting, only vegetarians were enrolled and MCarn was determined in individual muscle fibres. We hypothesised that HIIT would induce increases in MCarn, thereby explaining, at least in part, the enhanced muscle buffering capacity shown with this type of training.

Methods

Participants

Twenty young, healthy vegetarian men volunteered to participate in this study. They were randomly assigned to either an untrained control (CON, n=10) or a HIIT (n=10) group, with groups being matched according to baseline maximal oxygen uptake (VO_{2max}). One participant from the HIIT group withdrew from the study due to personal reasons unrelated to the study, meaning that 19 participants completed the study (CON, n=10; HIIT, n=9; (see Table, Supplemental Digital Content 1, which presents the participants' baseline characteristics). Inclusion criteria were: to be physically active (*i.e.*, participation in non-structured exercise and sporting activities 1-3 times per week), and to have been on a vegetarian diet for at least one year prior to the study. Exclusion criteria were: sedentary lifestyle, diagnosed chronic diseases, use of any nutritional supplements 3 months prior to the study, participation in any structured training program 6 months prior to the study, smoking, and continued use of medications. Except for the training protocol for the HIIT group, all participants were requested to maintain similar levels of physical activity throughout the study; compliance with this request was verbally confirmed. They were also asked not to change their food habits, which was confirmed by food diaries. None of the participants consumed any food containing β-alanine across the study. Participants were fully informed about the risks associated with participation before completing a health screen and providing written consent. The study was approved by the Institutional Ethics Committee (Approval Number: 14647713.9.0000.5391) and conformed to the 2013 version of the *Declaration of Helsinki*.

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Experimental Design

126 This was a randomised, controlled, parallel-group trial with participants assessed before
127 (PRE) and after (POST) a 12-week intervention period comprising HIIT or no exercise training
128 (control). Upon first arrival at the laboratory (1 week before the intervention), height, body
129 mass and skinfold thicknesses were recorded, and a resting muscle biopsy was taken from the
130 *m. vastus lateralis*; another muscle biopsy was taken 72-96 h after the intervention period had
131 been completed. Muscle samples were analysed for the following parameters: whole muscle
132 MCarn, isolated muscle fibre MCarn, *in vitro* muscle buffering capacity ($\beta m_{in\ vitro}$), total protein,
133 fibre type distribution and expression of selected genes. Exercise capacity was assessed PRE
134 and POST using a graded exercise test (GXT) and a multiple-bout Wingate Test. Exercise
135 training started within 7 days of the completion of the preliminary tests. Before and after the
136 intervention period, food intake was assessed by three 24-h food diaries.
137
138 *Anthropometry*
139 Body mass was measured PRE and POST to the nearest 100 g on a digital scale (100
140 CH, Welmy, São Paulo, Brasil). Height was measured on a wall-mounted stadiometer and
141 skinfold thickness (Chest, Abdomen, Thigh) was measured in triplicate using a calibrated
142 Harpenden caliper by the same experienced anthropometrist. Body density was calculated using
143 the Jackson & Pollock equation (25) and %fat using the equation of Siri (26).
144
145 *Exercise capacity tests*
146 GXT and multiple-bout Wingate Tests were conducted at least 48-h apart. Participants
147 were asked to abstain from alcohol and exercise 48 h prior to all tests. Testing time was
148 individually standardised. Participants were instructed to arrive at the laboratory 2-4 h post-
149 prandial. On the days preceding the post-intervention tests, participants were instructed to
150 repeat the same meals, portion sizes and timing as per their PRE assessment.

Exercise capacity and aerobic fitness were determined by GXT before and after the intervention. The test was performed on an electronically-braked cycle ergometer (Excalibur Sport, Lode, Groningen, the Netherlands) and consisted of 4-min stages with a 1-min break between stages. The test intensity started at 50 W and increased by 30 W every 4 min until volitional exhaustion or until the participant could no longer maintain 70 rev·min^{-1}. Strong verbal encouragement was provided to each participant as they approached the end of the test. Time to exhaustion was defined as the time completed during the test until fatigue, while maximum power output was determined as the highest power output achieved during the test.

Breath by breath concentrations of O_2 and CO_2 in expired air and ventilation were determined using a portable gas analysis system (K4b2, Cosmed, Rome, Italy), which was calibrated according to the manufacturer's instructions before and verified after each test. VO_{2max} was determined as the highest 30-s rolling VO_2 average during the GXT. The first ventilatory (anaerobic) threshold was determined as the break point between the increase of carbon dioxide output (VCO_2) and VO_2. The second ventilatory threshold (respiratory compensation point) was determined as the lowest ventilatory equivalent for carbon dioxide (VE/VCO_2 ratio) before a systematic increase. To determine lactate threshold, capillary blood samples were taken from fingertips in heparinized tubes at rest and immediately after each of the 4-min stages during GXT. Samples were immediately transferred to microtubes containing ice-cold 2% NaF and then centrifuged at 2000 g for 5 min at 4°C; plasma was kept at -85°C until analysis, which occurred in a single batch. Plasma lactate was determined spectrophotometrically (Victor 3/1420 Multi-Label Counter, PerkinElmer Inc., Massachusetts, United States of America) in micro-assays using an enzymatic method (Katal, Interteck, São Paulo, Brazil). Lactate threshold was calculated by the modified D-max method (27).

High-intensity intermittent performance was assessed using 3 bouts of the 30-s lower-body Wingate test interspersed by 3-min passive recovery periods. The test was conducted on

a mechanically-braked cycloergometer (Biotec 2100, Cefise, Brazil) with resistance being set at 5% of participant's body mass. One habituation session was carried out at PRE, when the position on the cycle ergometer was individually recorded and replicated in all subsequent sessions. The testing protocol started following a standardised 5-min warm-up against no mechanical resistance. Strong verbal encouragement was given throughout every bout. Total mechanical Work Done (TWD) was calculated for the overall test session. The coefficient of variation (CV) for TWD was 2.8%.

Muscle Biopsies

Muscle samples (~70-150 mg) were obtained under local anaesthesia (3 ml, 1% xylocaine) from the mid-portion of the *m. vastus lateralis* using the percutaneous needle biopsy technique with suction (28), as previously described (29). Samples were obtained PRE and POST from the same leg, as close as possible to one another, and were snap-frozen in liquid nitrogen, where they were stored until analysed. Samples were freeze-dried and dissected free of any visible blood, fat and connective tissue before being powdered.

$\beta m_{in\ vitro}$ *determination*

The non-bicarbonate buffering capacity of skeletal muscle was determined using the homogenate titration method (3,24). An aliquot of ~2-3 mg of freeze-dried muscle was homogenised on ice for 3 min in a 10 mM NaF solution (100 µl for every 3 mg of dry muscle). Homogenates were placed in a 37°C water bath for 5 min before and during pH measurements. Muscle homogenate pH was measured using a glass microelectrode (Microelectrodes Inc. New Hamphsire, USA) attached to a digital pH meter (Fisher Scientific Accumet AB15). Muscle homogenates were initially adjusted to a pH of 7.1 with a 0.02 M NaOH solution and then titrated to a pH of 6.5 by the serial addition of 10 mM HCl. The final result is reported as

millimoles H^+ per kilogram dry muscle required to change a whole pH unit (from 7.1 to 6.1 – $\beta m_{in\ vitro}$). The CV for $\beta m_{in\ vitro}$ determined in our laboratory was 10.46% (in 12 muscle samples taken at rest from Wistar rats from an unrelated study).

Chromatographic determination of total muscle and individual fibre carnosine content

Total MCarn content was quantified by high-performance liquid chromatography (HPLC - Hitachi, Hitachi Ltd., Tokyo, Japan) coupled to a U.V. detector, according to the method described by Mora et al. (30). Deproteinised muscle extracts were obtained from 3-5 mg freeze-dried samples according to the protocol described by Harris et al. (7). The intra-assay CV for total MCarn in our samples was 1.34%.

To quantify carnosine in individual muscle fibres, a more sensitive, fluorescence-based method employing pre-column derivatisation was adapted from Dunnett & Harris (31). Approximately 20–40 single muscle fibres were isolated from each muscle sample. Two 0.5–1.0 mm pieces were cut from each individual fibre; one piece was dissolved and stored overnight at 4ºC in a sealed tube on a protein extraction buffer (15 µl of 0.06M tris-hydroxymethyl-aminomethane pH 6.8, 1% w/v SDS, 0.6% w/v EDTA, 15% w/v glycerol, 5% v/v mercaptoethanol, and bromophenol blue) for myosin heavy chain isoform (MHC) characterisation via SDS-PAGE electrophoresis, as described by Hill et al. (8). The remainder of each fibre was weighed on a quartz-fibre fish-pole balance calibrated to 0.01 µg using DNA strands, the weights of which were determined after dissolving in 1 ml of water by comparison of the absorbance at 260 nm against a standard curve (absorbance *vs.* weight) prepared using the same material (8).

Each isolated fibre (or a pool of fibres of the same type) was extracted by the addition of 200 µL (or 300 µL) of ultrapure water and vortexed for 3 min. After preparing single fibre extracts, carnosine was determined using a Hypersil ODS (3 µm, 150X4.6 mm I.D.) analytical

column (Shandon, Runcom, UK), at 23°C, utilizing a binary gradient formed from solvent A [12.5 mM sodium acetate, pH 7.2 tetrahydrofuran (995:5, v/v)] and solvent B [12.5 mM sodium acetate, pH 7.2 - methanol-acetonitrile (500:350:150, v/v)]. Gradient composition was: 0 to 1.5 min, 0% solvent B; 1.5 to 10 min, 35% B; 10 to 26 min, 60% B; 26 to 30 min, 100% B; 30 to 35 min, 100% B; 35 to 45 min, 0% B. Flow-rate started at 2.0 $ml \cdot min^{-1}$ until 10 min, then gradually decreased to 1.0 $ml \cdot min^{-1}$ at 26 min until finish. Detector excitation and emission wavelengths were 340 nm and 450 nm. The derivatisation reagent was stored in the dark at 2°C and was prepared by mixing 80 µL of an OPA (40mg) plus absolute ethanol (800 µL) solution to 4 µL of β-mercaptoethanol and 1 mL of a 0.4M borate buffer (pH 9.65). For derivatisation, extract and reagent (1:1 v:v) were reacted for 30 s prior to injection. Fresh reagent was used with each new sample batch. Quantification of both chromatographic methods was performed using peak areas and regression equations obtained from standard curves. The intra-assay CV for carnosine determination in individual fibres was 4.97%.

Total protein and whole muscle fibre-type distribution

Total protein was assessed in muscle extracts with the Bradford method using a commercially available kit and following manufacturer's instructions (Bio-Rad, CA, USA). MHC was quantified in whole muscle following the same procedures above described for single fibres, where whole muscle samples were diluted in the same protein extraction buffer previously mentioned, and submitted to the same running conditions via SDS-PAGE electrophoresis. MHC I, IIa and IIx expression were individually quantified in arbitrary units with the aid of computer software (Scion Image) and subsequently normalised to the total arbitrary units within each sample.

mRNA isolation, cDNA synthesis and quantitative polymerase-chain reaction (qPCR)

Real time qPCR was used to determine the levels of expression of selected genes related to carnosine metabolism, namely, *CARNS*, *CNDP2*, *ABAT*, *TauT* and *PAT1*. Primer sets (forward and reverse) for the genes of interest were based on a previous study (32) (see Table, Supplemental Digital Content 2, which presents the primers sequences for gene expression analysis). Primer sequences were confirmed using *in-silico* analysis in a public database. Optimal primer concentrations and reaction conditions were determined for each pair of primers. Wet muscle samples (~20 mg) were homogenised using Trizol reagent (Invitrogen, Carlsbad, California), with RNA being isolated using the phenol-chloroform method. RNA concentrations and purity were determined using a micro spectrophotometer (NanoDrop ND2000, Thermo Scientific). RNA integrity was checked on a 1% agarose gel stained with ethidium bromide. The cDNA was synthesised using a specific cDNA reverse transcription kit solution (2X RT, Applied Biosystems, Thermo Fisher Scientific, Waltham, USA).

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Real-time qPCR for each gene was performed in duplicate in a 100-µl microtube containing 5–20 ng cDNA, 11 µl SYBR Green Master Mix (Applied Biosystems), 100–400 mM of each primer and ultra-pure water for a final volume of 22 µl. Gene expression analyses were carried out using the following cycle parameters: 95°C for 20 s; 40 cycles of 95°C for 3 s, and 60°C for 30 s; melting curve consisted of a gradual ramp from 65 to 95°C at an increase of $1°C \cdot s^{-1}$. Fluorescence intensity was quantified and amplification plots analysed by a sequence detector system (Rotor Gene-Q, Qiagen). Results were obtained using the comparative cycle threshold (Ct) method, as described by Bustin et al. (33). Non-template controls were included in all batches.

Food Intake Analysis

Food intake was assessed PRE and POST by three 24-h food diaries undertaken on separate days (two weekdays and one weekend day). Energy and macronutrient intakes were analysed with a computer software containing nutritional information about local food and ingredients (Virtual Nutri™, São Paulo, Brazil). All participants were instructed on how to complete food diaries by a trained nutritionist; they received a booklet containing instructions and real-sized photos of real food to help them record portion sizes.

HIIT program

The HIIT intervention consisted of a progressive program undertaken three times per week (Monday, Wednesday and Friday) for 12 consecutive weeks. All training sessions were conducted on a mechanically-braked cycle ergometer (828E, Monark, Stockholm, Sweden) and were preceded by a 5-minute warm-up at 50 W. Training intensity was set as a percentage of LT and was based on previous intermittent training protocols that were effective in increasing $\beta m_{in\ vitro}$ (3,24). Exercise intensity was set at 140% (weeks 1 to 3), 150% (weeks 4 to 6), 160% (weeks 7 to 9) and 170% (weeks 10 to 12) of individual power at LT measured at PRE. Participants completed a variable number of 2-minute exercise bouts interspersed with 1-min passive recovery periods, as follows: 6 to 9 bouts during weeks 1 to 3, 8 to 10 bouts during weeks 4 to 6, 9 to 12 bouts during weeks 7 to 9, and 6 to 9 bouts during weeks 10 to 12. At the end of each training session, participants received a standardized snack to improve training adherence. Adherence rate to the training sessions was 95±7% (range: 80-100%). All participants were able to complete the entire training protocol within each session.

Statistical Analysis

Participant characteristics at PRE (body composition, food consumption, exercise capacity and intermittent performance, total protein content, $\beta m_{in\ vitro}$, and MCarn) were compared between groups using unpaired *t*-Tests. These same variables, along with whole muscle MHC distribution and MCarn in fibre types, were analysed using Mixed Models with "group" (HIIT and CON) and "time" (PRE and POST) being fixed factors. Because only two pre-post pairs of data were obtained for type IIx fibres, neither within-group comparisons nor delta analysis were conducted for IIx fibres. Participants were random factors in all Mixed Models. Four different structures of covariance matrices were tested and the Bayesian information criterion (lowest BIC) was used to select the model that best fitted to each individual data set. Single degree of freedom contrast analysis was used for specific single-effect comparisons. Unpaired T-tests were used to compare the absolute variation (i.e., post-pre delta) of the above-mentioned variables between groups. Effect sizes (ES) were calculated using Cohen's *d*; Qualitative descriptors for ES interpretation were assigned as follows: <0.2, negligible effect; 0.2 - 0.39, small effect; 0.40 - 0.75, moderate effect; >0.75, large effect. Analyses were conducted using the SAS software v. 9.3. The significance level set was $P \leq 0.05$ and marginally significant effects were considered when $P \leq 0.1$. Data are presented as mean ± standard-deviation, delta change, ES, and 95% confidence interval (CI).

Results

Effects of HIIT on MCarn in whole muscle

No significant differences between groups were observed for MCarn in whole muscle before training (CON: 14.3±5.3 $mmol \cdot kg^{-1}$ *vs.* HIIT: 15.8±5.7 $mmol \cdot kg^{-1}$; p=0.98). MCarn significantly increased by +4.5±3.3 $mmol \cdot kg^{-1}$ (+35.7%) after training in the HIIT group (group-by-time interaction: F=4.72; p=0.049; within-group effect: p=0.012; ES=0.87; 95%CI=-

0.09 to 1.85), but not in the CON group (+0.3±4.4 mmol·kg^{-1}; +6.3%, within-group effect: *p*=0.99; ES=0.14; 95%CI=-0.78 to 1.08) (Figure 1).

- PLEASE INSERT FIGURE 1 HERE -

Effects of HIIT on MCarn in fibre types

No significant differences between groups were shown at PRE for MCarn in type I (*p*=0.99), IIa (*p*=0.97) or IIx (*p*=0.98) muscle fibres. On the other hand, MCarn was significantly lower at PRE in type I fibres compared to type IIa and IIx (both P<0.05), with no significant difference in MCarn between type IIa and IIx (P>0.05). In type I fibres, a +2.7±3.3 mmol·kg^{-1} (+24.2%) increase in MCarn was shown in the HIIT (group-by-time interaction: F=3.78; *p*=0.067; within-group effect: *p*=0.047; ES=0.36; 95%CI=0.07 to 0.65) but not in the CON group (+0.2±2.0 mmol·kg^{-1}; +1.48%, within-group effect: *p*=0.99; ES=0.17; 95%CI=-0.12 to 0.46) (Figure 2). In type IIa fibres, a significant +2.1±2.2 mmol·kg^{-1} (+13.8%) increase in MCarn was shown in the HIIT (group-by-time interaction: F=9.52; *p*=0.006; within-group effect: *p*=0.067; ES=0.28; 95%CI=0.02 to 0.54), but not in the CON group (-1.2±2.1 mmol·kg^{-1}; -5.64%, *p*=0.37; ES=-0.22; 95%CI=-0.49 to 0.05) (Figure 2). Analysis of the absolute change (delta post-pre) showed a significant difference between groups (*p*=0.007) in Type IIa fibres (+2.04 ± 2.24 mmol·kg^{-1} in the HIIT group *vs.* -1.21 ± 2.08 mmol·kg^{-1} in the CON group). In type I fibres, an increase of similar magnitude was shown in the HIIT (+2.70 ± 3.27 mmol·kg^{-1}) but not in the CON group (+0.16 ± 2.02 mmol·kg^{-1} in the CON group), although this only approached significance (*p*=0.084) (figure 2).

- PLEASE INSERT FIGURE 2 HERE -

Effects of HIIT on $\beta m_{in\ vitro}$

Although, a significant between-group difference was shown at PRE (p=0.018), a significant group-by-time interaction was shown for $\beta m_{in\ vitro}$ (F=7.30; p=0.02). A within-group effect was demonstrated for HIIT (p=0.047; ES=1.20; 95%CI=0.09 to 2.21), but not for CON (p=0.413; ES=-0.80; 95%CI=-1.77 to 0.16) (Figure 3, upper panel). Analysis of the absolute change showed a significant difference between groups (p=0.033) in $\beta m_{in\ vitro}$ (+44.1 ± 53.8 mmol $H^+ \cdot kg\ dm^{-1} \cdot pH\ unit^{-1}$) in the HIIT group *vs.* -36.3 ± 40.5 mmol $H^+ \cdot kg\ dm^{-1} \cdot pH\ unit^{-1}$ in the CON group).

- PLEASE INSERT FIGURE 3 HERE -

Effects of HIIT on total protein content

No significant differences between groups for total protein were shown at PRE (CON: 1.79 ± 1.45 $\mu g.\mu L^{-1}$ *vs.* HIIT: 1.24 ± 0.68 $mg.\mu L^{-1}$ - p=0.99), and no significant group-by-time interaction effect was shown for total protein content (F=0.37; p=0.55; Figure 2, bottom panel). Analysis of the absolute change showed no significant difference between groups (p=0.670) for total protein content (-0.21 ± 1.57 $mg.\mu L^{-1}$ in the HIIT group *vs.* -0.55 ± 1.82 $mg.\mu L^{-1}$ in the CON group).

Effects of HIIT on MHC shifting

No significant differences between groups were shown for MHC expression at PRE (all p>0.05). Type I MHC expression significantly decreased -5.5±7.2% in the HIIT group (group-by-time interaction: F=4.94; p=0.043; within-group effect: p=0.08; ES=-0.83; 95%CI=-1.86 to 0.19), but not the in the CON group (+2.1±4.9%; within-group effect: p=0.449; ES=0.08; 95%CI=-0.9 to 1.07). This was paralleled by a trend towards a significant within-group effect

374 in type IIa MHC expression in the HIIT group (+4.5±7.7%; group-by-time interaction: F=1.48;
375 p=0.241; within-group effect: p=0.09; ES=0.48; 95%CI=-0.51 to 1.48), but not in the CON
376 group (+0.5±3.9%; within-group effect: p=0.97; ES=0.03; 95%CI=-0.95 to 1.02). Type IIx
377 MHC expression did not change in any of the groups (CON group: -2.6±4.6%; ES=-0.16;
378 95%CI=-1.18 to 0.86; HIIT group: +1.3±12.9%; ES=0.16; 95%CI=-0.86 to 1.18) (Table 1).
379
380 - PLEASE INSERT TABLE 1 HERE -
381
382 *Effects of HIIT on the expression of selected genes in the skeletal muscle*
383 No significant changes were shown for the expression of the genes *CNDP-2*, *ABAT*,
384 *TauT*, *PAT1* and *CARNS* (group-by-time interaction effects: all p>0.05) (Figure 4).
385
386 - PLEASE INSERT FIGURE 4 HERE -
387
388 *Effects of HIIT on exercise capacity*
389 There was a significant +10.1% (+3907 J) improvement in TWD in the HIIT group
390 (group-by-time interaction effect: F=22.96, p=0.0003; within-group effect: p<0.0001; ES=0.99;
391 95%CI=0.01 to 1.98) but not in the CON group (-2.17%, -1010 J, p=0.20; ES=-0.09; 95%CI=-
392 1.02 to 0.83). The absolute change in TWD was significantly different between groups
393 (p=0.0003; Figure 5). There was a significant +23.7% (+401 s) increase in time-to-exhaustion
394 in the HIIT group (group-by-time interaction: F=5.40; p=0.027; within-group effect: p=0.004;
395 ES=1.32; 95%CI=0.30 to 2.34) but not in the CON group (+0.6%; +9 s, p=0.78; ES=-0.17;
396 95%CI=-1.10 to 0.75). HIIT significantly increased maximum power output by 21.1% (+41.25
397 W; group-by-time interaction: F=6.67; p=0.015; within-group effect: p=0.003; ES=1.29;
398 95%CI=0.27 to 2.31), whilst no changes were shown for the CON group (-2.2%; -5 W, within

399 group effect: p=0.58; ES=-0.16; 95%CI=-1.34 to 0.53). The absolute change in time-to-
400 exhaustion (p=0.0002) and maximum power output (p=0.0004) were significantly different
401 between groups (Figure 5).
402
403 - PLEASE INSERT FIGURE 5 HERE -
404
405 *Effects of HIIT on VO_{2max} and ventilatory thresholds*
406 No group-by-time interaction was shown for VO_{2max} (F=0.98; p=0.32). However, a
407 significant difference between groups (p=0.045) was found for delta VO_{2max}, with the HIIT
408 group showing a +3.25 $ml \cdot kg^{-1} \cdot min^{-1}$ increase (+8.11%; ES=0.52; 95%CI=-0.42 to 1.46)
409 following training whereas the CON group showed a +0.65 $ml \cdot kg^{-1} \cdot min^{-1}$ increase (+1.58%;
410 ES=0.09; 95%CI=-1.03 to 0.83; Figure 5).
411 Time to reach the 1st and the 2nd VT were +43.8% and +19.4% longer after the
412 intervention in the HIIT group (group-by-time interaction: F=6.04, p=0.020 and F=5.63,
413 p=0.024, for the 1st and 2nd VT respectively; within-group effect: p=0.012; ES=1.14;
414 95%CI=0.14 to 2.14 and p=0.014; ES=1.08; 95%CI=0.09 to 2.08, for the 1st and 2nd VT
415 respectively), but not in the CON group (within-group effect: -9.57%, p=0.39; ES=-0.36;
416 95%CI=-1.49 to 0.40 and -5.13%, p=0.42; ES=-0.35; 95%CI=-1.29 to 0.58 for the 1st and 2nd
417 VT, respectively). Significant differences were shown between groups for the delta changes in
418 the time to reach the 1st and 2nd VT (both p=0.009; both in Figure 5).
419
420 *Effects of HIIT on body weight and body fat percentage*
421 There was no group-by-time interaction for body weight (F=0.85; p=0.37) or body fat
422 percentage (F = 0.23; p=0.63). Similarly, there were no significant differences between groups
423 in the absolute changes in body weight (p=0.40) or body fat percentage (p=0.64).

Food intake

No main effects or interaction effects were shown for calories and macronutrient intake between groups across the study (see Table, Supplemental Digital Content 3, which presents the food consumption data).

- PLEASE INSERT SUPPLEMENTAL DIGITAL CONTENT 3 HERE -

Discussion

Using robust methods and a tightly controlled experimental design, we were able to prove, for the first time, the principle that chronic high-intensity exercise training increases MCarn, which occurs independently of dietary supply of β-alanine. Increased MCarn, therefore, seems to be part of the milieu of muscle adaptive responses to HIIT. Since increased MCarn was independent of dietary or supplemental intake of β-alanine, it must have resulted from intrinsic changes in the skeletal muscle, which is supported by the significant increase shown in both type I and II individual fibres. These findings are particularly relevant, since the adaptive response of MCarn to high-intensity training has remained doubtful, with evidence for increased MCarn being limited to cross-sectional studies and not confirmed by interventional studies.

Several longitudinal studies did not show increased MCarn with exercise training. These studies have limitations, however, including a lack of control for dietary intake of β-alanine (the most influential factor on MCarn) (7-9) and the use of training protocols of insufficient volume (18-22) and/or intensity (16,17) unlikely to result in significant changes in MCarn. This argument is supported by cross-sectional studies suggesting increased MCarn could be an adaptive response to long-term high-intensity, but not endurance, training (5). Only one of these

studies (18) measured MCarn in isolated muscle fibre types, meaning that most studies did not account for changes in fibre distribution induced by training. Nonetheless, the only study that measured carnosine in fibre types did not control dietary intake of β-alanine. Thus, previous studies may have lacked sufficient sensitivity and control to determine changes in MCarn content with training.

In the present study, our training protocol increased TWD, time-to-exhaustion, maximum power output, VO_{2max}, and the 1st and 2nd VT, thereby confirming the efficacy of our intervention. HIIT also increased whole muscle MCarn by 35%, with this increase not accounted for by diet, since we exclusively recruited vegetarian participants who consumed virtually no β-alanine/carnosine. The inclusion of vegetarians only, along with the individual fibre type analysis, were intentionally chosen in our experimental set-up, as it would allow us to more conclusively test the principle that chronic high-intensity exercise training increases MCarn. Although similar increases in MCarn may not necessarily occur in omnivores, or may not be detectable if these variables are not controlled, our results clearly show that the principle holds true that training can increase MCarn content in the absence of β-alanine ingestion. Baseline MCarn content was lower than those we have previously shown in omnivores (~15 vs. 23 $mmol \cdot kg^{-1}dm$ (9). The mean increases shown here (4.61 ± 3.05 $mmol \cdot kg^{-1}dm$) are approximately one third of those shown following 4 weeks of β-alanine supplementation at high doses (7-9). Furthermore, there was a ~5% reduction in type I and a ~5% increase in type II fibres in response to HIIT, which is line with other studies showing a reduction in type I MHC and an increase in type IIa MHC after sprint training (34). This could have been a confounding factor in the interpretation of the role of training on carnosine synthesis. Our results confirm previous studies (8, 13, 18), showing that type II fibres have ~1.5 times more carnosine than type I fibres, meaning that fibre shifting accounted for an ~10% increase in whole muscle MCarn in this study. The remaining increase in MCarn (~25%) can be almost entirely attributed

474 to the increased MCarn content in individual muscle fibres. Interestingly, our data demonstrate
475 that absolute increases in MCarn with HIIT is similar between different fibre types which is in
476 line with results following β-alanine supplementation (8). This strengthens the notion that fibre
477 types, despite having different baseline carnosine contents, seem to respond similarly to stimuli
478 that lead to increased carnosine. The cellular and molecular mechanisms responsible for such
479 differences in baseline carnosine content between fibre types, and for the similarities in
480 carnosine responses to different stimuli (e.g., supplementation and training), remain unknown.
481 To explore mechanisms that could account for increased MCarn content within the
482 skeletal muscle, we quantified the expression of genes involved in carnosine homeostasis.
483 None of the investigated genes showed altered expression in response to training. It must be
484 noted that gene expression does not necessarily represent alterations in protein content and,
485 most importantly, the biological activity of the protein. However, we did not have enough
486 sample to carry out the analyses of protein content and enzymatic activity, which we
487 acknowledge as a limitation in this study. Furthermore, gene expression was determined at one
488 time point 72-96 hours following the last training session, which does not exclude the
489 possibility that changes in gene expression could have occurred at different time points.
490 Increased MCarn can only be a consequence of increased synthesis, decreased degradation, or
491 both. Considering our vegetarian sample had virtually zero dietary intake of β-alanine,
492 increased synthesis could only be possible if the endogenous production of β-alanine increased
493 with training, or that its degradation within muscle decreased with training. Assuming that the
494 conversion of β-ureidopropionate to β-alanine in the liver is 10 $\mu mol \cdot h^{-1}$ (35), and that 1 kg of
495 dry muscle is equivalent to 4.3 kg of wet muscle, a ~5 $mmol \cdot kg^{-1}$ dry muscle increase in
496 carnosine (~50 mmol increase in a 70-kg individual with 40 kg of wet muscle) would require
497 the β-alanine synthesis rate to increase ~2.5 times above baseline in order to provide substrate
498 for the synthesis of 50 mmol of carnosine (assuming no losses of β-alanine). Other possible

pathways for β-alanine synthesis is the decarboxylation of aspartate to produce β-alanine in kidneys or muscle via GADL1 activity (36), the transamination of malonate semialdehyde to produce β-alanine via beta-alanine aminotransferase, or the transamination of L-alanine to form β-alanine via β-alanine-pyruvate transaminase. Although not determined herein, the activities of these enzymes could have been affected by training. In addition to increased β-alanine synthesis, another and perhaps more plausible explanation is a reduction in carnosine degradation in muscle in response to HIIT. Other mechanisms might involve increased β-alanine transport into skeletal muscle cells, perhaps triggered by increased blood flow. These are only speculative, however, and future studies should be specifically designed to explore the underpinning effects of exercise on muscle carnosine turnover.

Previous studies have shown increased muscle buffering capacity following high-intensity training (3,24,37,38). Our results corroborate and further confirm these findings; this is particularly important in our study since it serves as a positive control to ensure that our HIIT protocol elicited an adaptation that could be explained by changes in MCarn. We employed the same HIIT protocol as Edge et al. (3,24), who showed a 25% and 31% increase in $\beta m_{in\ vitro}$ after 5 and 8 weeks; extending this to 12 weeks, our study resulted in a ~40% increase in $\beta m_{in\ vitro}$ (see Figure, Supplemental Digital Content 4, which shows an association between absolute changes in $\beta m_{in\ vitro}$ and the duration of exercise training). The maximum change that an increase of ~4 $mmol \cdot kg^{-1}$ in carnosine would incur for $\beta m_{in\ vitro}$ is ~1.3 $mmolH^{+} \cdot kg^{-1}$; this accounts for ~3% of the total increase in $\beta m_{in\ vitro}$ (~44 $mmolH^{+} \cdot kg^{-1}$ induced by HIIT), suggesting that increased carnosine accounts for a minor part of this response. However, the titration method does not provide an accurate measure of true physiochemical buffering capacity. Homogenisation of muscle results in PCr and ATP hydrolysis leading to increases in inorganic and hexose monophosphates (pKas 6.1-6.8), which contribute to an overestimation of muscle buffering capacity (39). Thus, any attempt to calculate the contribution of carnosine to the

measured $\beta m_{in\ vitro}$ would fundamentally underestimate the true contribution of carnosine, meaning caution should be exercised when interpreting these data.

Although a precise quantification of the contribution of increased MCarn to the increased $\beta m_{in\ vitro}$ is not possible, it is very unlikely that increased MCarn is accountable for the entire increase in $\beta m_{in\ vitro}$; therefore, other intrinsic changes in skeletal muscle induced by HIIT must have occurred to account for the total increase in $\beta m_{in\ vitro}$. We measured total protein in muscle extracts, since they exert some buffering action, although our results indicate that increased protein is not part of this response. Other possibilities include increased phosphates, and proteins rich in histidine residues (the only amino acid capable of contributing to $\beta m_{in\ vitro}$ over this pH range), such as myoglobin. These have not been measured in our study and future studies should explore what exact mechanisms beyond increased carnosine that fully account for the increase in $\beta m_{in\ vitro}$. Metabolomic studies could help to identify ionisable compounds with pKa close to the physiological transit-range that are responsive to HIIT-type interventions.

- PLEASE INSERT SUPPLEMENTAL DIGITAL CONTENT 4 HERE -

In conclusion, this study demonstrated that HIIT increases MCarn in whole muscle and in isolated muscle fibre types, independent of diet. Whether HIIT can elicit similar effects in omnivores, this still merits further investigation. These results challenge the current belief that exercise training cannot increase MCarn content and demonstrate that this can occur in the absence of β-alanine/carnosine ingestion (7-9). These novel data demonstrate that the increase in MCarn accounts for a small part of the total increase in muscle buffering capacity with HIIT. The increase in MCarn induced by training corresponds, in absolute values, to one-third of the typical increase brought about by β-alanine supplementation; it is driven not only by fibre type shift, but also by an intrinsic increase in MCarn that occurs in both type I and type II fibres and

549 does not depend on the dietary supply of β-alanine. Further research should determine whether
550 this exercise training protocol elicits similar or greater increases in muscle carnosine content in
551 individuals who ingest beta-alanine in their diet. In addition, further research should examine
552 whether these responses are affected by training status and which types of exercise training (e.g.
553 different intensities and volumes) elicit the greatest accumulation of MCarn.
554
555
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563
564
565 **Competing Interests**
566 The authors declare that they have no competing interests. No funding was received for
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569 American College of Sports Medicine. The results of the study are presented clearly, honestly,
570 and without fabrication, falsification, or inappropriate data manipulation.
571
572
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Data analysis and interpretation – Vitor de Salles Painelli, Kleiner Marcio Nemezio, Isabel Andrade, Ana Jessica Pinto, Mariana Franchi, Luiz Riani

Statistical expertise – Vitor de Salles Painelli, Guilherme Giannini Artioli

738 **Figures captions**

739

740 **Figure 1.** Effects of high-intensity interval training (HIIT) on muscle carnosine content
741 measured in whole muscle.

742

743 **Figure 2.** Effects of high-intensity interval training (HIIT) on carnosine content measured in
744 individual fibres grouped by type. No delta analyses were carried-out for type IIx fibres due to
745 the low number of pre-post pairs of data.

746

747 **Figure 3.** Effects of high-intensity interval training (HIIT) on muscle buffering capacity
748 measured *in vitro* ($\beta m_{in\ vitro}$) (upper panel) and on total protein content (bottom panel).

749

750 **Figure 4:** Gene expression of carnosinase-2 (CNDP-2), beta-alanine transaminase (ABAT),
751 taurine transporter (TauT), proton/amino acid transporter-1 (PAT1) and carnosine-synthase
752 (CARNS). Data are expressed as means and standard deviation of fold change at POST with
753 levels at PRE for both groups arbitrarily set to 1.

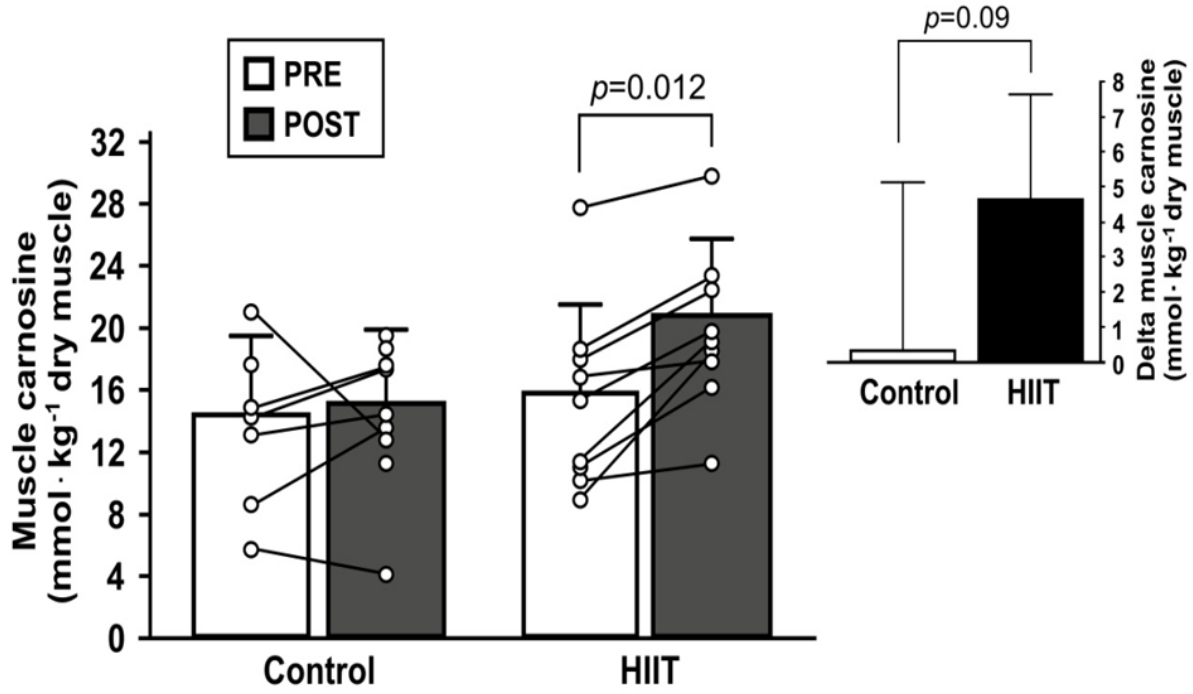
754

755 **Figure 5:** Absolute change in total work done (TWD), time-to-exhaustion, mean power output
756 (MPO), maximal oxygen consumption (VO_{2max}), and time to reach the 1st and 2nd ventilatory
757 thresholds (VT) in the Control (white bars) and Trained (black bars) groups.

758

759

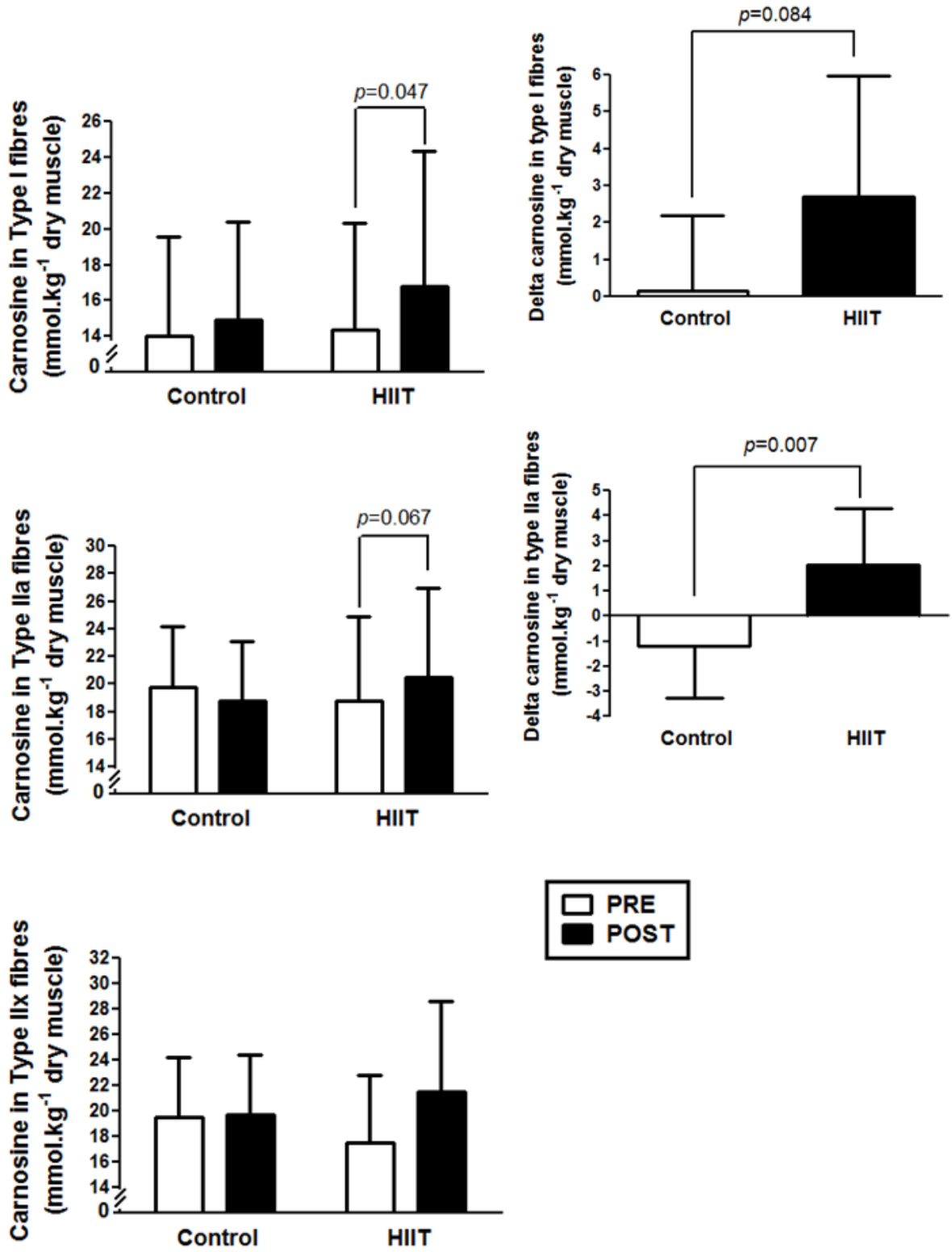
760 **FIGURE 1**



761

762

763 **FIGURE 2**



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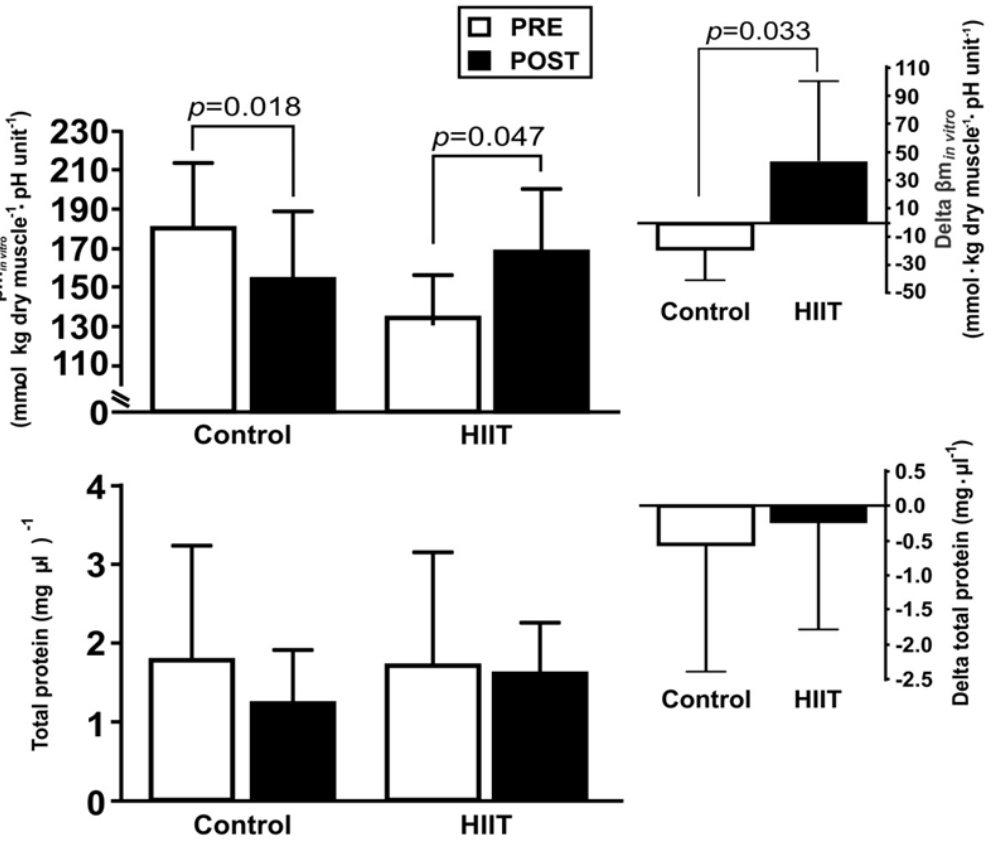
766 **FIGURE 3**



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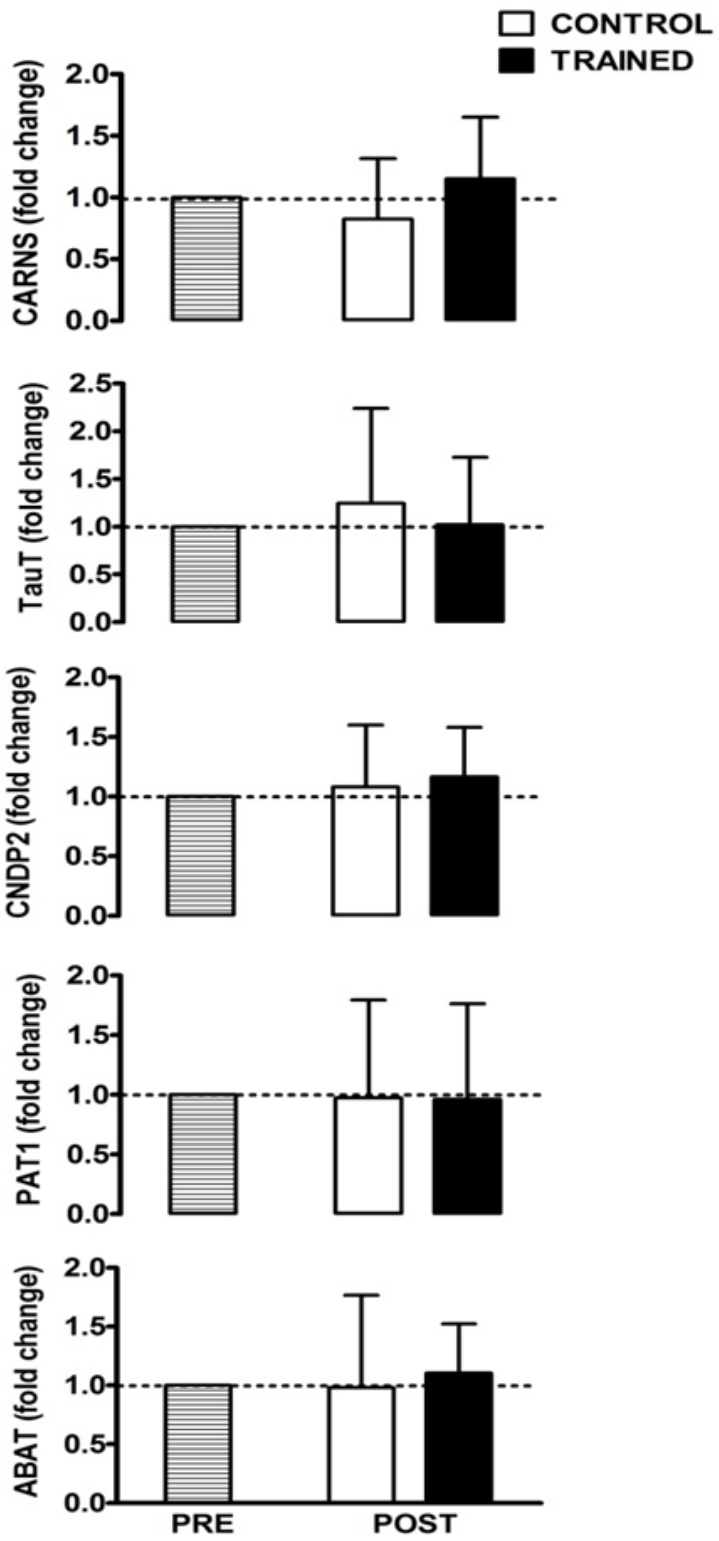
769 **FIGURE 4**



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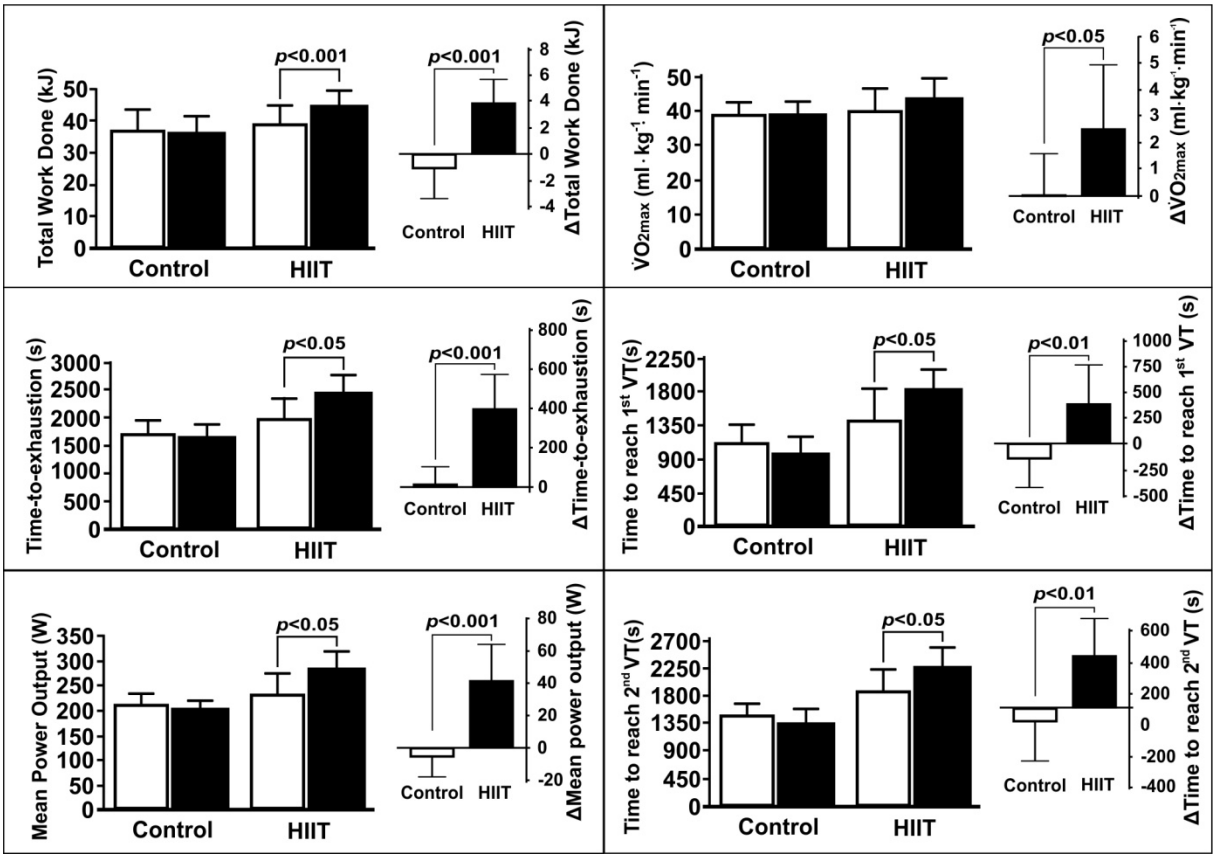
771

772 **FIGURE 5**



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List of Supplemental Digital Content

Supplemental Digital Content 1, Table.doc Participant's baseline characteristics.

Supplemental Digital Content 2, Table.doc Primers sequences for gene expression analysis.

Supplemental Digital Content 3, Table.doc Energy and macronutrient intake.

Supplemental Digital Content 4, Figure.tif Illustration of absolute changes in muscle buffering capacity in vitro ($\beta m_{in\ vitro}$) during weeks of exercise training (data adapted from: Edge et al. 2006b; Edge et al. 2006c). A positive and high correlation was observed between the parameters (R^2=0.89). To estimate $\beta m_{in\ vitro}$ (which is the estimated βm over 1 full pH change, data from the present study has been adjusted by dividing by 0.6, in accordance with the procedure used in the two studies by Edge et al.).

SDC 1

	HIIT (n=9)	CONTROL (n=10)	*p*
Age (y)	27±6	29±6	0.56
Body weight (kg)	73.4±9.7	70.3±9.9	0.47
Height (m)	1.78±0.06	1.77±0.06	0.88
BMI (kg/m^2)	23.5±3.0	22.1±2.1	0.26
Time on vegeterian diet (y)	3.5±3.0	5.1±4.2	0.31
W_{max} (W)	233.3±40.9	209.0±24.7	0.14
Time to exhaustion (s)	1970±371	1682±259	0.09
Time to 1st VT (s)	1413±429	1107±261	0.08
Time to 2nd VT (s)	1873±355	1473±212	0.01
VO_{2max} ($ml.kg^{-1}.min^{-1}$)	40.05±6.21	39.14±3.28	0.69
HR_{max} ($beats \cdot min^{-1}$)	193±6	191±6	0.50
TWD - Wingate (J)	38827±5957	36448±6832	0.41
Body fat (%)	15.6±6.7	12.6±3.5	0.26

Data are expressed as mean ± standard-deviation. BMI: body mass index; W_{max}: maximum power output; 2nd VT: second ventilatory threshold; VO_{2max}: maximum oxygen consumption; FC_{max}: maximum heart rate; TWD: total work done; $\beta m_{in\ vitro}$: muscle buffering capacity *in vitro*.

SDC 2

Gene	FWD primer sequence	REV primer sequence
CARNS	5'-GGCGTCAGCAAGAAGTTCGT-3'	5'-CCGGTGCTCTGTCATGTCAA-3'
CNDP2	5'-TTGCTGATGGGCTCTTTGGT-3'	5'-TCGATGTCGTCGTACAGCTTGT-3'
ABAT	5'-CGCACTCTAAAGCCATTCAC-3'	5'-AGATCCTCCACCTCTTCCA-3'
TauT	5'-CGTACCCCTGACCTACAACAAA-3'	5'-CAGAGGCGGATGACGATGAC-3'
PAT1	5'-CATAACCCTCAACCTGCCCAAC-3'	5'-GGGACGTAGAACTGGAGTGC-3'
EEF1A1 (reference gene)	5'-CTGGCAAGGTCACCAAGTCT-3'	5'-CCGTTCTTCCACCACTGAT-3'

CARNS - carnosine sintase; *CNDP2* - carnosinase isoform 2; *ABAT* - beta-alanine transaminase; *TauT* - taurine transporter; *PAT1* - proton-dependent amino acid transporter; *EEF1A1* - Eukaryotic Translation Elongation Factor 1 Alpha 1.

SDC 3

	HIIT		CON		*p**
	PRE	**POST**	**PRE**	**POST**	
Energy (kcal)	2717 ± 1066	2588 ± 899	2041 ± 430	1961 ± 438	0.81
Protein (g)	60.3 ± 32.4	80.7 ± 28.5	84.2 ± 57.9	73.2 ± 28.2	0.23
Protein (%)	11.4 ± 4.5	13.1 ± 4.3	13.1 ± 3.6	14.0 ± 4.4	0.82
CHO (g)	461.3 ± 272.0	406.0 ± 235.9	331.8 ± 102.7	275.0 ± 58.4	0.82
CHO (%)	62.5 ± 12.2	60.2 ± 15.8	56.1 ± 5.9	55.8 ± 6.5	0.65
Fat (g)	76.1 ± 28.4	71.3 ± 44.4	85.3 ± 44.6	67.8 ± 25.9	0.47
Fat (%)	30.9 ± 8.2	26.6 ± 12.7	30.8 ± 5.5	26.1 ± 10.2	0.49
Protein/kg	0.99 ± 0.43	1.10 ± 0.50	0.94 ± 0.27	1.07 ± 0.41	0.91

Data are expressed as mean ± standard-deviation. No significant differences were observed.

* *p* values represent the Group x Time interaction. CHO=carbohydrate.

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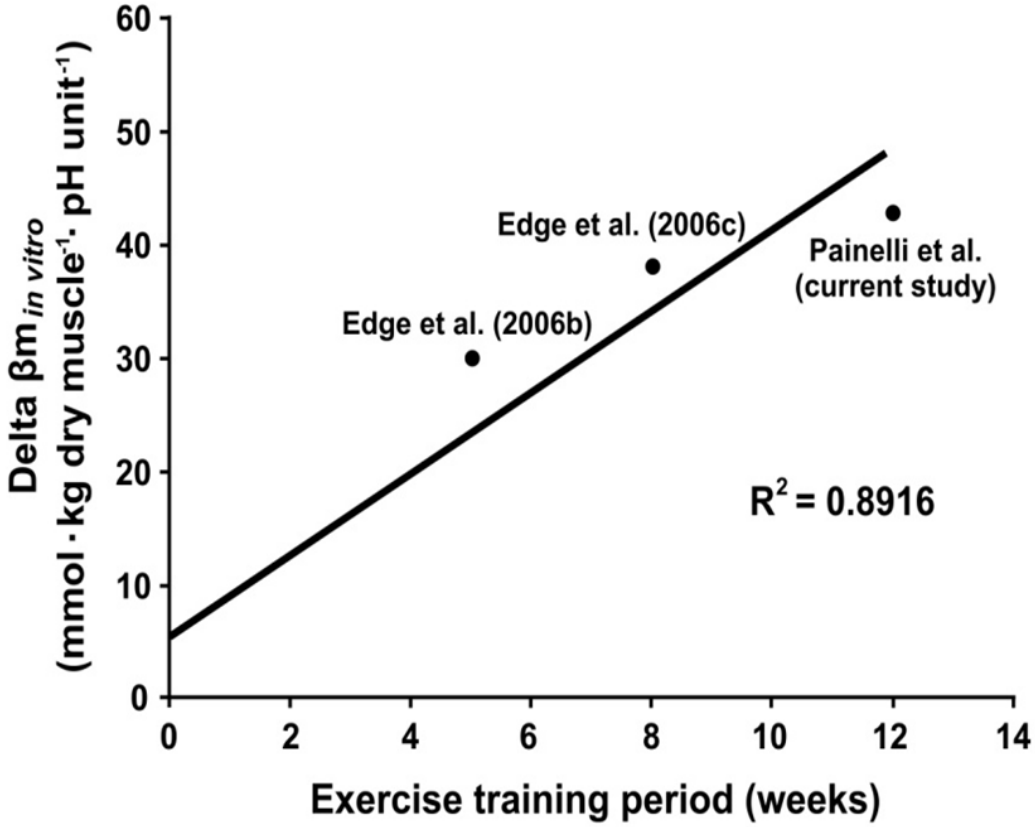

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