24 weeks β-alanine supplementation on carnosine content, related genes and exercise

Running title: 24 weeks β-alanine supplementation

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ABSTRACT

Introduction: Skeletal muscle carnosine content can be increased through β-alanine supplementation, but the maximum increase achievable with supplementation is unknown. No study has investigated the effects of prolonged supplementation on carnosine-related genes or exercise capacity. **Purpose:** To investigate the effects of 24-weeks of β-alanine supplementation on muscle carnosine content, gene expression and high-intensity cycling capacity ($CCT_{110\%}$). **Methods:** Twenty-five active males were supplemented with 6.4 $g \cdot day^{-1}$ of sustained release β-alanine (BA) or placebo (PL) over a 24-week period. Every 4 weeks participants provided a muscle biopsy and performed the $CCT_{110\%}$. Biopsies were analysed for muscle carnosine content and gene expression (*CARNS*, *TauT*, *ABAT*, *CNDP2*, *PHT1*, *PEPT2* and *PAT1*). **Results:** Carnosine content was increased from baseline at every time point in BA (all $P<0.0001$; Week 4: +11.37±7.03 $mmol \cdot kg^{-1}dm$, Week 8: +13.88±7.84 $mmol \cdot kg^{-1}dm$, Week 12: +16.95±8.54 $mmol \cdot kg^{-1}dm$, Week 16: +17.63±8.42 $mmol \cdot kg^{-1}dm$, Week 20: +21.20±7.86 $mmol \cdot kg^{-1}dm$, Week 24: +20.15±7.63 $mmol \cdot kg^{-1}dm$), but not PL (all $P=1.00$). Maximal changes were +25.66±7.63 $mmol \cdot kg^{-1}dm$ (range: +17.13 to +41.32 $mmol \cdot kg^{-1}dm$), and absolute maximal content was 48.03±8.97 $mmol \cdot kg^{-1}dm$ (range: 31.79 to 63.92 $mmol \cdot kg^{-1}dm$). There was an effect of supplement ($P=0.002$) on *TauT*; no further differences in gene expression were shown. Exercise capacity was improved in BA ($P=0.05$) with *possible* to *almost certain* improvements across all weeks. **Conclusions:** Twenty-four weeks of β-alanine supplementation increased muscle carnosine content and improved high-intensity cycling capacity. Downregulation of *TauT* suggests it plays an important role in muscle carnosine accumulation with β-alanine supplementation, while the variability in changes in muscle carnosine content between

individuals suggests that other determinants other than the availability of β-alanine may also bear a major influence on muscle carnosine content.

Keywords: Skeletal muscle carnosine, chronic β-alanine supplementation, carnosine-related genes, high-intensity cycling capacity, muscle biopsy

INTRODUCTION

The physiological roles of carnosine (β-alanyl-L-histidine) are pleiotropic and have been associated with effects on muscle buffering capacity, metal-ion chelation and antioxidant scavenging (9). Dietary supply of histidine-containing dipeptides is a major determinant of skeletal muscle carnosine content (18) and increases with β-alanine supplementation have been shown using chromatographic (*i.e.*, HPLC) quantification of muscle biopsy samples (15, 17, 19) and magnetic resonance spectroscopy (3, 12, 13).

Stellingwerff et al. (33) demonstrated that the rate of increase in muscle carnosine over 4 weeks was linearly related to the β-alanine dose given (1.6 and 3.2 $g \cdot day^{-1}$), while the absolute change was dependent on the total amount ingested. An average dose of 5.2 $g \cdot day^{-1}$ for 4 weeks increased carnosine content in the *m. vastus lateralis* from 19.9±1.9 to 30.1±2.3 $mmol \cdot kg^{-1}$dm; a further 6 weeks of supplementation at 6.4 $g \cdot day^{-1}$ increased carnosine content to 34.7±3.7 $mmol \cdot kg^{-1}$dm (19). These data demonstrate that maximum accumulation of carnosine takes more than 4 weeks of β-alanine supplementation at a mean dose of 5.2 $g \cdot day^{-1}$. It is unknown if there is an upper limit to muscle carnosine content and whether this differs between individuals. It is possible that the ergogenic and therapeutic benefits of an increase in muscle carnosine may be maximised when this reaches its peak content. It would be of interest to determine the kinetics of carnosine accumulation in muscle with prolonged β-alanine supplementation.

A number of genes and their resulting proteins regulate the processes affecting muscle carnosine content; the uptake of β-alanine and carnosine into skeletal muscle, the local synthesis of carnosine, the hydrolysis of carnosine, and the transamination of β-alanine. The genes

74 controlling these processes are: *CARNS* (carnosine synthesis), *TauT, PAT1, $ATB^{0,+}$* (β-alanine
75 transport), *CNDP1, CNDP2* (carnosine hydrolysis), *ABAT* (β-alanine transaminase), and *PEPT1,*
76 *PEPT2, PHT1, PHT2* (carnosine/histidine transport). The expression of some of these genes
77 have been examined (14), but the influence of β-alanine supplementation on their expression
78 remains unknown in humans. In particular, transport of β-alanine into muscle (via *TauT*),
79 synthesis of muscle carnosine (via *CARNS*) and deamination of β-alanine (via *ABAT*) have been
80 suggested to play important roles in the regulation of carnosine synthesis (14). The examination
81 of the changes in expression of carnosine-related genes following prolonged β-alanine
82 supplementation could provide important information as to the mechanisms by which increased
83 β-alanine availability increases muscle carnosine content.
84
85 The efficacy of β-alanine supplementation to improve exercise capacity and performance has
86 been demonstrated (20, 31). Improvements during a high-intensity cycling capacity test at 110%
87 of maximum power output ($CCT_{110\%}$) have been verified independently, showing that time to
88 exhaustion (TTE) was improved by 11.9% (19), 12.1% (30) and 14.0% (11). The improved
89 exercise capacity shown by Hill et al. (19) was linear to changes in muscle carnosine, although
90 no studies have examined the association between muscle carnosine and exercise changes over a
91 longer time period with multiple data points.
92
93 We aimed to determine whether: a) a ceiling for carnosine accumulation in skeletal muscle exists
94 following twenty-four weeks of β-alanine supplementation, b) carnosine content influences the
95 expression of genes responsible for regulating carnosine in muscle, and c) the changes in muscle
96 carnosine are related to changes in high-intensity exercise capacity. We hypothesised that: a)

97 long-term β-alanine supplementation would lead to saturation of the muscle carnosine content, b)
98 prolonged supplementation would downregulate genes involved in the control of the carnosine
99 content in muscle, and c) that the increases in muscle carnosine would be paralleled by
100 improvements in exercise capacity.
101

METHODS

Participants

Twenty-five physically active healthy males (age 27 ± 4 y, height 1.75 ± 0.09 m, body mass 78.9 ± 11.7 kg), who participated in exercise (e.g., running, cycling, team sports) 1-3 times per week, volunteered. Participants were requested to maintain similar levels of physical activity and dietary intake for the duration of the study and compliance with this request was verbally confirmed with individuals throughout. Individuals completed a food intake diary during weeks 4-8 and 16-20 on two non-consecutive weekdays and one weekend day. Energy and macronutrient intake was analysed by a nutritionist using specific software (Avanutri, Rio de Janeiro, Brazil). Habitual consumption of β-alanine was calculated based upon specific tables taken from the literature (1, 24). Exclusion criteria included, i) supplementation of creatine or β-alanine in the 6 months prior to the study, ii) ongoing supplementation of any dietary supplement except carbohydrate and whey protein, and iii) vegetarian diet. The study was first approved by the institution's Ethical Advisory Committee. Participants provided written informed consent after completing a health screen.

Experimental Design

Participants attended the laboratory on nine occasions. The first two visits were for the determination of maximal cycling power output and a familiarisation with the exercise protocol. The remaining seven visits were for the completion of the main trials, each separated by 4 weeks; one main trial was completed before supplementation (Week 0) followed by one main trial every 4 weeks for 24 weeks (Weeks 4–24) during double-blinded supplementation with β-alanine or placebo (Panel A, Figure 1).

Participants were randomly allocated to receive either β-alanine (BA) or placebo (PL) in a 2:1 ratio (*i.e.*, two participants were allocated in BA for each participant in PL); individuals were matched for maximum cycling power output (W_{max}; BA = 283 ± 42 W, PL = 286 ± 52 W) using a block randomisation method.(2) An unbalanced design was adopted *a priori* in order to minimise the number of individuals being biopsied (12). Individuals were supplemented for 24 weeks with either 6.4 $g \cdot d^{-1}$ β-alanine (CarnoSyn®, NAI, USA) or an equivalent amount of placebo (maltodextrin; NAI, USA); two 800 mg tablets taken four times per day at 3–4 hour intervals. Participants completed a log to verify compliance (BA: 95 ± 6%; PL: 93 ± 6%); one individual, who was in BA, did not adhere to the supplementation protocol and was thus removed from any analyses. Blinding occurred via an outside researcher not involved in direct data collection who provided the researchers with identical white pots containing only participant names.

Experimental Procedures

Preliminary Testing

Height and body mass (BM) were recorded upon arrival at the first laboratory session, and BM was further recorded at Weeks 12 and 24. W_{max} was determined by completing a graded cycling exercise test to exhaustion (Lode Excalibur, Germany). The participants' second visit to the laboratory comprised a familiarisation session of the main exercise protocol (described below).

Main Trials

Participants abstained from alcohol, caffeine and strenuous exercise and completed a food record for the 24 h period prior to the initial trial. They adopted the same dietary intake prior to each trial. Participants arrived at the laboratory at the same time of day a minimum of 2 h following their last consumption of food and 4 h since their last supplement ingestion. A cannula was inserted into the antecubital vein for venous blood collection. The participants then underwent a muscle biopsy of the *m. vastus lateralis* before performing the $CCT_{110\%}$ (Panel B, Figure 1).

Muscle biopsies

Muscle biopsies were taken at rest using a 5 mm biopsy Allandale needle (Northern Hospital Supplies, Edinburgh, UK) by a method adapted from Bergstrom (6), described in detail elsewhere (27). The dominant leg was prepared through an incision along the *m. vastus lateralis* muscle under local anaesthesia (lidocaine 1%, Linisol) of the skin. Two muscle samples (~50 mg for HPLC analysis and ~50 mg for polymerase chain reaction [PCR] analysis) were taken and immediately frozen in liquid nitrogen and stored at -80 ºC. All biopsies followed the same standardised pattern across individuals. The location of each initial biopsy was at a point 25 cm proximal from the tuberositas tibiae and 5 cm lateral from the midline of the femoral course. A second incision was performed adjacent (~1 cm) to the first. Thereafter, the incisions performed in the weeks following were made superior to the previous ones, resulting in three pairs of parallel incisions and one single incision at the most superior point.

Chromatographic determination of carnosine

Total muscle carnosine content was determined by HPLC (Hitachi, Hitachi Ltd., Tokyo, Japan), as per Mora et al. (26). All chromatography was carried out at room temperature. Samples were

analysed in duplicate and injected via an auto sampler using a cut injection method with a total aspirated volume of 70 µL; 30 µL was discarded, 10 µL injected for analysis and the remaining 30 µL also discarded. Prior to all injections, samples were visually inspected for air bubbles, any of which were subsequently removed manually by the experimenter. Standard curves for carnosine were performed prior to each analysis session using concentrations of 0.1, 0.5, 1, 2.5, and 5 mM, showing excellent linearity (R^2=0.996±0.005).

The column used for chromatographic separation was an Atlantis HILIC silica column (4.6×150 mm, 3 µm; (Waters, Massachusetts, USA) attached to an Atlantis Silica column guard (4.6×20 mm, 3 µm). The method used two mobile phases: Mobile phase A: 0.65 mM ammonium acetate, in water/acetonitrile (25:75) (v/v). Mobile phase B: 4.55 mM ammonium acetate, in water/acetonitrile (70:30). The pH of both solutions was adjusted to 5.5 using hydrochloric acid and thereafter filtered under vacuum through a 0.2 µm filter membrane.

The separation condition comprised of a linear gradient from 0 to 100% of solvent B in 13 min at a flow rate of 1.4 $mL \cdot min^{-1}$. Separation was monitored using an ultraviolet detector at a wavelength of 214 nm. The column was equilibrated for 5 min under the initial conditions before each injection. Quantification was performed using peak areas, which were calculated by computer software coupled to the chromatographer and individually inspected for error and consistency by a researcher. Peak area for the standard curve was plotted and a regression equation obtained, from which interpolations were used to calculate the content. Limits of detection for the current method were 0.5125 $mmol \cdot kg^{-1}$dm and the inter-assay coefficient of variation (CV) of carnosine measurement of the same freeze-dried muscle extracted separately

on nine occasions was 0.9±1.2%. The intra-assay CV of carnosine between duplicate injections of all analyses (N=175) was 4.0±4.5%. To determine the reliability of the extraction method, several samples (N=11) were reanalysed following a new extraction phase, showing a variation of 2.5±2.1% from initial content.

Real-time PCR

Real time PCR was used to determine the expression of selected genes related to carnosine metabolism; *CARNS, TauT, ABAT, CNDP1, CNDP2, PAT1, ATB$^{0,+}$, PEPT1, PEPT2, PHT1* and *PHT2*. The reference gene used was *EEF1A1*. Primer synthesis was outsourced (IDT, Iowa, USA) and primer sets are shown in Supplemental Digital Content 1 (Table, Supplemental Digital Content 1, Forward and reverse primer sets). Standardisation of primers revealed good expression at forward and reverse concentrations of 100 mM for *PHT1*, 200 mM for *TauT*, 300 mM for *CNDP2*, *PEPT2* and *PAT1*, and 400 mM for *CARNS* and *ABAT*. There was poor or no expression of *CNDP1*, *PepT2*, *ATB$^{0,+}$* or *PHT2* using concentrations between 100 and 400 mM; therefore, expression of these genes was not performed.

Freeze-dried muscle was homogenized and total RNA isolated using Trizol reagent (Invitrogen, Carlsbad, California). Nucleic acid concentration (DNA and RNA) was determined by measuring the optical density at 260 nm with a micro spectrophotometer (NanoDrop ND2000, Thermo Scientific). RNA purity was determined by calculating the absorbance ratio at 260 nm and 280 nm, and RNA integrity checked on a 1% agarose gel stained with ethidium bromide. A 10 μL volume containing a total of 1 μg of RNA completed with ultrapure water was added to 10 μL of a specific cDNA reverse transcription kit solution (2X RT, Applied Biosystems, Thermo

Fisher Scientific, Waltham, USA). The reverse transcription reaction was performed at 25^{o}C for 10 min, followed by 37^{o}C for 120 min and 5 min at 85^{o}C according to the manufacturers' instructions.

Real-time PCR for each gene was performed in duplicate with a 2 μL reaction volume of 5–20 ng cDNA, 11 μL SYBR Green Master Mix (Applied Biosystems, California, USA), 100–400 mM of each primer and completed with water to make 22 μL. Gene expression analyses were carried out using the following cycle parameters: "hold" at 95^{o}C for 20 s; 40 "cycles" of 95^{o}C for 3 s, and 60^{o}C for 30 s; "melt" consisting of a gradual ramp from 65 to 95^{o}C at an increase of 1^{o}C·s^{-1}. The fluorescence intensity was quantified and amplification plots analysed by a sequence detector system (Rotor Gene-Q, Qiagen, Hilden, Germany). The intra-assay CV for the comparative cycle threshold (Ct) between the duplicate injections was between 4.5 and 7.5% for all genes measured. Results were obtained using the comparative Ct method. Delta-Ct (DCt) values were calculated in every sample for each gene of interest as follows: Ct(gene of interest) – Ct(reference gene). Relative changes in the expression level of the genes (DDCt) were calculated by subtraction of the DCt at baseline (Week 0) from the corresponding DCt at the time points of interest (Weeks 4 – 24). Finally, relative quantification (fold change) was calculated using the 2^{-DDCt} equation (34).

Exercise protocols

W_{max} and $CCT_{110\%}$

Each individual performed a W_{max} test with results subsequently used to perform the $CCT_{110\%}$ in all subsequent sessions, as described by Saunders et al. (32). Time-to-exhaustion (TTE, s) was

recorded as the outcome measures for all tests. The $CCT_{110\%}$ has been shown to be a reliable test with a CV of 4.4% for TTE following a solitary familiarisation session (32). The CV between the familiarisation and baseline time trials in the current study was 4.9 ± 3.4 for TTE; this value (4.9%) was used to determine improvements above the variation of the test.

Blood collection and analyses

Finger-prick blood samples were taken pre-, immediately post- and 5-min post-exercise and analysed for lactate concentration (Accutrend Lactate, Roche Diagnostics, Switzerland). Venous blood samples were taken at identical times from the antecubital vein using heparin-coated syringes and analysed for blood pH, bicarbonate and base excess (Rapid Point 350, Siemens, Germany). The pre-, immediately post- and 5-min post-exercise intra-assay CVs for pH, bicarbonate and base excess ranged from 0.07±0.03% to 2.77±2.2%. Samples were taken with the individuals in a supine position except immediately post-exercise, which was taken in a seated upright position while the participant remained on the cycle ergometer.

Statistical Analyses

Data were analysed using the SAS statistical package (SAS 9.2, SAS Institute Inc., USA), and are presented as mean±1SD unless stated. Muscle carnosine, gene expression and exercise data were analysed using mixed model analysis with individuals assumed as a random factor and supplementation (2 levels; BA and PL) and week (7 levels; Week 0-24) assumed as fixed factors. Tukey post-hoc tests were performed whenever a significant F-value was obtained and the significance level was set at $P \leq 0.05$ and a tendency towards an effect was set at $P < 0.1$. Magnitude based inferences (MBIs; (5, 21)) were used to determine the practical significance of

β-alanine on $CCT_{110\%}$; the smallest worthwhile improvement in TTE was 3.56 s (32). The means and SDs for BA and PL were used to calculate effect sizes for muscle carnosine and TTE (22). Blood data were analysed using a mixed model with individuals assumed as a random factor and supplementation (2 levels; BA and PL), week (7 levels; Weeks 0 to Week 24) and time (3 levels; Pre-exercise, Post-exercise, 5-min Post-exercise) assumed as fixed factors. Body mass was analysed using a mixed model with individuals assumed as a random factor and supplementation (2 levels; BA and PL) and week (3 levels; Week 0; Week 12; Week 24) assumed as fixed factors. Food intake was analysed using a mixed model with individuals assumed as a random factor and supplementation (2 levels; BA and PL) and week (2 levels; Weeks 4-8 and Weeks 16-20) assumed as fixed factors. Pearson's correlations were performed to determine any associations between initial muscle carnosine content and absolute changes over time.

RESULTS

Muscle carnosine

There were no significant differences in pre-supplementation (Week 0) carnosine content between BA (22.37±4.46 mmol·kg^{-1}dm) and PL (23.18±5.89 mmol·kg^{-1}dm; P=1.00). There was a main effect of supplementation (P<0.0001) and week (P<0.0001), and a supplementation × week interaction (P<0.0001). Carnosine content increased from Week 0 at every time point in BA (all P<0.0001; Week 4: +11.37±7.03 mmol·kg^{-1}dm, Week 8: +13.88±7.84 mmol·kg^{-1}dm, Week 12: +16.95±8.54 mmol·kg^{-1}dm, Week 16: +17.63±8.42 mmol·kg^{-1}dm, Week 20: +21.20±7.86 mmol·kg^{-1}dm, Week 24: +20.15±7.63 mmol·kg^{-1}dm) with no changes across time in PL (all P=1.00; Figure 2). Effect sizes from Week 0 were all huge in BA (Week 4: 1.96; Week 8: 1.93; Week 12: 2.24; Week 16: 2.25; Week 20: 2.86; Week 24: 2.81) and ranged from negligible to medium effects in PL (0.06 to -0.48).

Baseline content (Week 0) ranged from 11.67 to 28.97 mmol·kg^{-1}dm in BA, and 15.14 to 34.89 mmol·kg^{-1}dm in PL. All individuals increased muscle carnosine content above baseline levels. The absolute maximal changes in muscle carnosine was +25.66±7.63 mmol·kg^{-1}dm, ranging from +17.13 to +41.32 mmol·kg^{-1}dm. The absolute maximal content was 48.03±8.97 mmol·kg^{-1}dm, ranging from +31.79 to +63.92 mmol·kg^{-1}dm (Table 1). The time-to maximal content was 17±7 weeks and ranged from 4 to 24 weeks; one individual showed maximal carnosine content at Week 4, four at Week 12, one at Week 16, four at Week 20 and five at Week 24. Initial muscle carnosine content (Week 0) was significantly correlated to the absolute carnosine content at Weeks 8 (r=0.52, P=0.05), 16 (r=0.58, P=0.03) and 20 (r=0.57, P=0.03), but not weeks 4 (r=0.29, P=0.29), 12 (r=0.48, P=0.07) or 24 (r=0.37, P=0.18). There was a significant

297 correlation between muscle carnosine content at Week 0 and the absolute maximal content with
298 BA (r=0.53, *P*=0.04). There were no significant correlations between initial muscle carnosine
299 content and the delta change in carnosine at any week (all *P*>0.05) or the delta maximal change
300 (r=0.04, *P*=0.90)
301
302 **Gene expression**
303 There was no effect of supplement, week or any interaction effects for *CARNS*, *ABAT*, *CNDP2*,
304 *PAT1*, *PEPT2* or *PHT1* (all *P*>0.05). There was a significant effect of supplement (*P*=0.002) for
305 *TauT*, with lower values over time in BA (-36.4%, -39.4%, -27.3%, -56.8%, -46.3% and -35.0%
306 at Weeks 4, 8, 12, 16, 20 and 24; Figure 3), although no effect of week (*P*=0.31) or an
307 interaction (*P*=0.59) was shown. There were no significant correlations between muscle
308 carnosine content and any gene at Week 0 (all *P*>0.05).
309
310 **$CCT_{110\%}$**
311 Exercise capacity was not significantly different between BA and PL at Week 0 (*P*=1.00, Figure
312 2). There was a main-effect of supplement (*P*=0.05), and an interaction effect (supplement ×
313 week, *P*=0.05), although *post-hoc* analyses only revealed Week 20 to be significantly different
314 from Week 0 (*P*=0.02, Figure 2). TTE was improved from Week 0 in BA at all time points but
315 not in PL (Table 2). MBIs showed *possible* to *almost certain* improvements across all weeks in
316 BA compared to Week 0; similarly, ES were greater in BA vs. PL at all time points (Table 2).
317
318 Four individuals in BA improved above the variation of the test (>4.9%) at every time point.A
319 further two individuals improved exercise capacity in all but one week with BA. Six individuals

in BA had an improved exercise capacity at between 2 and 4 time points during supplementation and the remaining three showed no improvements at any time point. The week of supplementation corresponding to each individual's best performance was variable, with two individuals showing best performance times following four weeks of supplementation, and two following eight weeks. One individual's best performance was following twelve weeks, three following sixteen weeks, four after twenty weeks and three at the final time point. No individual showed maximal exercise improvements at their individual maximal muscle carnosine content. Muscle carnosine content was significantly correlated to TTE in BA ($r=0.82$, $r^2=0.68$, $P=0.02$), but not PL ($r=0.32$, $r^2=0.10$, $P=0.49$; Supplemental Digital Content 2, Muscle carnosine content and time-to-exhaustion in BA). Absolute changes in muscle carnosine and TTE were significantly correlated ($r=0.804$, $r^2=0.65$, $P=0.05$; Supplemental Digital Content 2, absolute changes in muscle carnosine content and time-to-exhaustion in BA) for BA. No significant correlation between change in muscle carnosine and exercise capacity were shown in PL (all $P>0.05$).

There was no effect of supplement or week on any blood variable (all $P>0.05$) although there was a significant effect of time on all blood measures (all $P<0.001$); blood lactate was increased and pH, bicarbonate and base excess were decreased following exercise compared to pre-exercise (Table, Supplemental Digital Content 3, Blood pH, bicarbonate, base excess and lactate). There were no interactions shown for blood lactate, pH, bicarbonate and base excess (all $P>0.05$).

Dietary intake

There was a main effect of week on total calorie (P=0.02) and carbohydrate (P=0.02) intake, although no main effect of supplement or a supplement x week interaction (all P>0.05). There were no main effects of supplement, week, or supplement x week interactions for total protein or fat intake (all P>0.05). The intake of β-alanine did not differ between groups (P=0.525), and was unchanged over the supplementation period (P=0.203); similarly, there was no supplement x week interaction (P=0.224; Table, Supplemental Digital Content 4, Food intake in BA and PL during weeks 4-8 and 16-20 of supplementation).

DISCUSSION

This is the first study to systematically examine the effects of longer-term β-alanine supplementation on muscle carnosine content, carnosine-related genes and high-intensity exercise capacity at monthly intervals. The novel findings (Figure 4) are that twenty-four weeks of β-alanine supplementation increased muscle carnosine content from baseline at every time point, although the absolute and the time to the highest recorded content was variable between individuals. *TauT* was down-regulated with chronic β-alanine supplementation. High-intensity cycling capacity was improved, with improvements associated with changes in muscle carnosine.

Muscle carnosine content increased by 55% following 4 weeks, which is lower than the relative increases previously shown using HPLC analysis of muscle biopsy samples (17, 19), despite the lower dose of β-alanine used in those studies (mean 5.2 $g \cdot day^{-1}$; (17, 19)). Absolute changes in muscle carnosine at 4 weeks in the present study were greater than those shown by Harris et al. (17) but identical to those of Hill et al. (19), despite that in the previous studies a slightly lower dose (5.2 versus 6.4$g \cdot day^{-1}$) was given. The greatest absolute change in mean carnosine content occurred following 20 weeks of supplementation, and corresponded to a +98±40% increase. This is lower than the +143±151% increases shown by Chung et al. (10) using ^{1}H-MRS following 4 weeks of β-alanine supplementation, although the absolute changes appear quite similar when both data sets are expressed in the same units. Percentage increases misrepresent carnosine changes in muscle, particularly in those with low initial values (*i.e.*, predominant distribution of type I fibres; low meat eaters or vegetarians). Since the contribution of carnosine to muscle buffering capacity (or indeed any suggested physiological mechanism) is dependent upon its actual content in muscle, any exercise or therapeutic benefits received via this mechanism will

depend on the absolute changes in muscle content. The discrepancy between changes in muscle carnosine content and concentration (*i.e.,* absolute *vs.* percentage change) highlights the necessity in determining absolute changes in muscle carnosine content, particularly in studies in which carnosine accumulation is associated with other physiological outcomes (*e.g.*, gene expression or exercise responses).

We hypothesised that changes in muscle carnosine content would be mirrored by changes in the expression of carnosine-related genes. *TauT* was downregulated with supplementation, although no other changes in gene expression were shown. Since *TauT* is the primary transporter of β-alanine into muscle (4), our data support the suggestion that increases in muscle carnosine may be more dependent upon the transport of β-alanine into the muscle than the activity of carnosine synthase (*CARNS; (14))*, since this will directly influence the availability of β-alanine for muscle carnosine synthesis. Decreasing the activity of *TauT* during prolonged increases in circulating β-alanine through oral supplementation may be the body's mechanism to best maintain intramuscular homeostasis of muscle carnosine by limiting the uptake of β-alanine into muscle. Blancquaert et al. (8) suggested that the homeostasis of muscle carnosine is tightly regulated by the transamination of circulating levels of β-alanine via GABA-T and AGXT2; the current data suggest that the downregulation of *TauT* can also play a role in the regulation of muscle carnosine content, perhaps contributing to increased transamination of circulating levels due to decreased uptake into muscle, although this was not measured here. The lack of any other changes in gene expression in this study is in contrast to the increased expression of *CARNS*, *TauT* and *ABAT* shown following β-alanine supplementation in mice (14). However, the dose of β-alanine that these mice received is equivalent to a supra-physiological dose in humans and it is

unclear when the mice received their final dose in relation to the timing of analysis. In the current study, participants were requested to arrive at the laboratory four hours following the ingestion of a dose of β-alanine. These results are understandable given circulating levels of β-alanine return to normal 4 hours following an equivalent dose (17). A limitation of our study is that only gene expression was analysed; post-transcriptional events may result in disparate kinetics between gene and protein expression, influencing inferences (25). Further research should ascertain whether expression of these genes and proteins is modified in the hours following acute β-alanine ingestion and whether these change over time with prolonged supplementation.

The highest carnosine contents ranged from 31.79 to 63.92 $mmol \cdot kg^{-1}dm$, and were dependent on the initial content in muscle. Interestingly, five individuals showed their highest values at 24 weeks, with four of those still showing increases in excess of 6 $mmol \cdot kg^{-1}dm$ from the previous time point. For these participants it is possible that further increases in carnosine would have occurred with additional supplementation. The variability in the kinetics of carnosine accumulation shown here is unlike that of creatine in muscle, since 5-7 days of creatine supplementation at a dose of 20 to 30 $g \cdot day^{-1}$ is sufficient to reach maximal content which falls within a narrow physiological range across individuals (140-160 $mmol \cdot kg^{-1}dm$; (16, 29)). Lower initial doses of creatine supplementation lead to a longer time-to-peak content in individuals (23). Although one individual attained maximal content within four weeks of supplementation, the remaining participants showed maximal content during the final twelve weeks of supplementation. It cannot be dismissed that the current supplementation protocol may have been suboptimal in attaining peak carnosine content in muscle. The effects of higher or lower

doses may result in a different expression profiles in the genes or enzymes associated with carnosine synthesis (*i.e.*, lower downregulation of *TauT*) and further investigation is warranted to determine whether maximal content can be attained sooner.

Trained individuals have greater increases in muscle carnosine concentration with supplementation (7), possibly as a result of better delivery of β-alanine to the muscle due to increased blood flow (28), while it could also be due to a contraction-induced stimulation of *TauT* (7). Thus, increased expression of the β-alanine transporter (or an attenuation of its down regulation) may lead to an increased carnosine accumulation with supplementation. It remains to be determined whether muscle contraction *per se* increases the activity of β-alanine transporters, and greater increases with supplementation in highly trained individuals cannot be ruled out.

All individuals increased muscle carnosine from initial content with supplementation, which suggests that all individuals can show some degree of carnosine accumulation following β-alanine supplementation. Mean muscle carnosine contents increased most in the first 4 weeks, although this quickly dropped off as evidenced by a difference from the previous time point only at week 4. Nonetheless, an increased content in the final weeks of supplementation from the first eight suggest that total content continued to increase. Stellingwerff et al. (33) showed a linear response with supplementation with a high dependence on initial concentrations and the total amount of β-alanine consumed, which explained ~80% of the variance in carnosine concentration in their study. Although the initial carnosine content in the present study was related to the content at several time points and the maximal content attained, individual analysis revealed that not all individuals increased carnosine content linearly. These differences may be

443 related to the two lower doses used in the aforementioned study (1.6 and 3.2 $g \cdot d^{-1}$), which
444 resulted in far lower increases in muscle carnosine concentration. Thus, it appears that the uptake
445 kinetics of muscle carnosine content may be dependent upon the dose ingested.
446
447 These are the first data to show that muscle carnosine may not increase continuously until
448 maximal content in all individuals, given that carnosine content decreased at certain time points
449 across the 24-week period. Interestingly, these decreases occurred despite on-going
450 supplementation with β-alanine. The physiological mechanisms underpinning this response can
451 only be speculated upon but may include a down regulation of the transport of β-alanine into the
452 muscle cell, a reduction in the activity of the carnosine synthase enzyme or an increased
453 degradation of carnosine by carnosinases. These possibilities seem unlikely to explain the results
454 of the current study, given that we only showed an effect of β-alanine on *TauT*, although we
455 determined the relative expression of the genes that encode their associated protein(s), which can
456 be dependent on sampling time. Other possible explanations include the potential for
457 experimental or analytical error, although we feel this is unlikely given the control measures that
458 were undertaken to ensure the quality of muscle sampling, the extraction procedure and the
459 HPLC analysis. One other clear possibility is that the location of the muscle biopsy contributed
460 to the changes in muscle carnosine content across the study due to sample to sample differences
461 in the amount of type I and II muscle fibres collected in the biopsy sample. Since muscle
462 carnosine is not homogeneously distributed across muscle fibres in the *m. vastus lateralis* (19),
463 this may have resulted in variation between biopsies. It is, however, unlikely that these
464 differences within the same mixed muscle sample would have accounted for the magnitude of
465 the changes observed in muscle carnosine content. In addition, muscle carnosine content varied

by ~17% within the placebo group across twenty-four weeks, which is similar to those shown in the *m. gastrocnemius* over 9 weeks.(3) These interesting and novel findings pose several important questions worthy of further investigation, including a) why some individuals show decrements in muscle carnosine with β-alanine supplementation and others do not, b) what physiological mechanisms contribute to this process, and c) what is the biochemical fate of the carnosine that is eliminated from the skeletal muscle.

Supplementation with β-alanine improved exercise capacity and MBIs showed *possible* to *almost certain* improvements across all weeks with β-alanine with effect sizes suggesting moderate to very large effects. Similar exercise improvements have been shown using the $CCT_{110\%}$ on three independent occasions following 4 weeks of β-alanine supplementation (12-14%; (11, 19, 30)), with further improvements following 10 weeks of supplementation (~16%; (19)). Thus, it was hypothesised that greater exercise improvements would be shown in the current study when supplementation was extended past 10 weeks, although this was not the case. The smaller improvements shown here may have been due to large variability in exercise responses, perhaps due to differences in the buffering contribution of carnosine between individuals. The buffering contribution of carnosine has been estimated to be ~8%, although it is likely to be higher (19). Since its relative contribution to muscle buffering is dependent on total buffering capacity, it could be postulated that some individuals may be less responsive to changes in muscle carnosine content than others. However, this could not explain why no individual's peak performance coincided with their peak muscle carnosine content; it cannot currently be ruled out that changes in muscle buffering are offset by changes in other compounds. Nonetheless, exercise capacity in the current study was associated with muscle carnosine content and data suggests that 24 weeks

of β-alanine supplementation improves high-intensity exercise capacity, although variability exists with several less or non-responsive individuals. Future studies should evaluate exercise capacity with β-alanine supplementation on multiple occasions to account for variability in exercise responses.

In conclusion, twenty-four weeks of β-alanine supplementation increased muscle carnosine content up to ~64 $mmol \cdot kg^{-1}dm$, although maximal absolute changes were variable (*i.e.*, +17 to +41 $mmol \cdot kg^{-1}dm$), as was the time-to-maximal content. The transporter *TauT* was downregulated with β-alanine supplementation, suggesting it plays an important role in the accumulation of muscle carnosine content during prolonged β-alanine supplementation. Exercise capacity was improved with supplementation, mirroring changes in muscle carnosine, although a certain amount of variation was shown. Collectively, these results highlight the variability in changes in muscle carnosine content between individuals and that a maximal accumulation of muscle carnosine may not occur within twenty-four weeks at a high dose for all individuals, suggesting that determinants other than the availability of β-alanine may have a major influence on muscle carnosine content.

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The results of the present study do not constitute endorsement by ACSM. We declare that the results of the study are presented clearly, honestly, and without fabrication, falsification, or inappropriate data manipulation.

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630 **FIGURES**

631 Figure 1. Panel A: Experimental design of the study. Panel B: Main trial design. W_{max} =
632 maximum cycling power output test; $CCT_{110\%}$ = Cycling capacity test at 110% of maximum
633 cycling power output.

634

635 Figure 2. Panel A: Muscle carnosine content throughout supplementation in BA (black circles)
636 and PL (white circles). Panel B: Absolute change in muscle carnosine content from Week 0 in
637 BA (black bars) and PL (white bars). Panel C: Time-to-exhaustion throughout supplementation
638 in BA (black circles) and PL (white circles). Panel D: Absolute change in time-to-exhaustion
639 from Week 0 in BA (black bars) and PL (white bars). $^{a}P \leq 0.0001$ from Week 0. $^{b}P \leq 0.0001$ from
640 PL at same time point. $^{c}P \leq 0.05$ from Weeks 4 and 8. Data are mean±1SD.

641

642 Figure 3. Fold change across the 24 weeks for *CARNS*, *TauT*, *ABAT*, *CNDP2*, *PAT1*, *PHT1* and
643 *PEPT2*. $^{*}P=0.002$ Main effect of BA.

644

645 Figure 4. Overview of the analyses and results of the current study. There was a downregulation
646 in the *TauT* transporter which transports β-alanine into muscle; the other β-alanine transporter,
647 *PAT1*, was unaffected. Similarly, no changes were shown in the histidine/carnosine transporters
648 *PHT1* and *PEPT2*, which intramuscular expression of *CARNS* and *CNDP2*, which code
649 carnosine synthase (Carn. Synth.) and carnosinase (CN2) was also unchanged. There was no
650 change in the expression of *ABAT*, which encodes the protein responsible for intracellular
651 transamination of β-alanine. There was an increase in muscle carnosine content over the 24 week
652 period, which resulted in an improved high-intensity cycling capacity.

653

SUPPLEMENTAL DIGITAL CONTENT

Supplemental Digital Content 1.doc Forward and reverse primer sets for all genes analysed during standardisation.

Supplemental Digital Content 2.tiff Panel A: Muscle carnosine content and time-to-exhaustion across the supplementation period in BA ($r=0.82$, $r^2=0.68$, $P=0.02$). Panel B: Absolute change (Δ) in muscle carnosine content and absolute change (Δ) in time-to-exhaustion across the supplementation period in BA ($r=0.804$, $r^2=0.65$, $P=0.05$).

Supplemental Digital Content 3.doc Blood pH, bicarbonate, base excess and lactate (mean ± 1SD) at pre-exercise, post-exercise and 5-min post-exercise at every week in BA and PL. *$P<0.001$ from Pre-exercise.

Supplemental Digital Content 4.doc Food intake (mean ± 1SD) in BA and PL during weeks 4-8 and 16-20 of supplementation. *$P=0.02$ Main effect of Week.

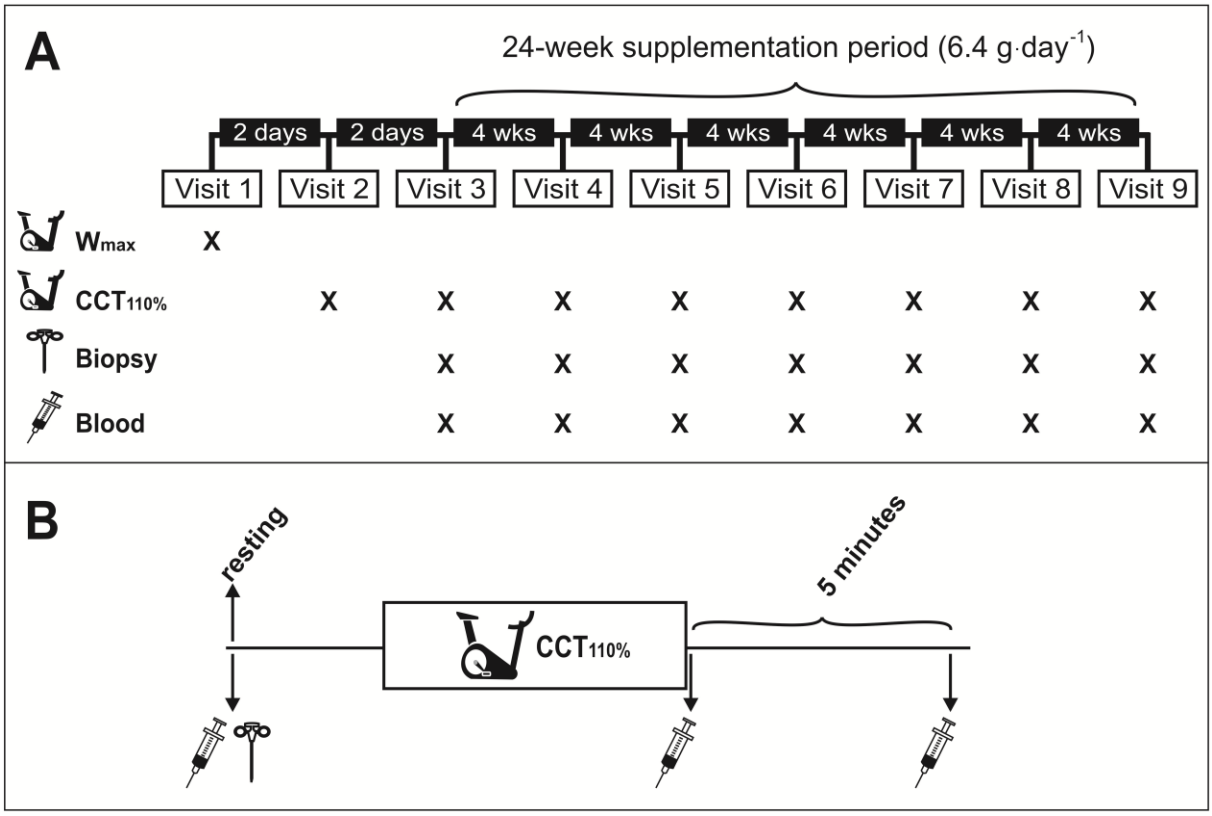
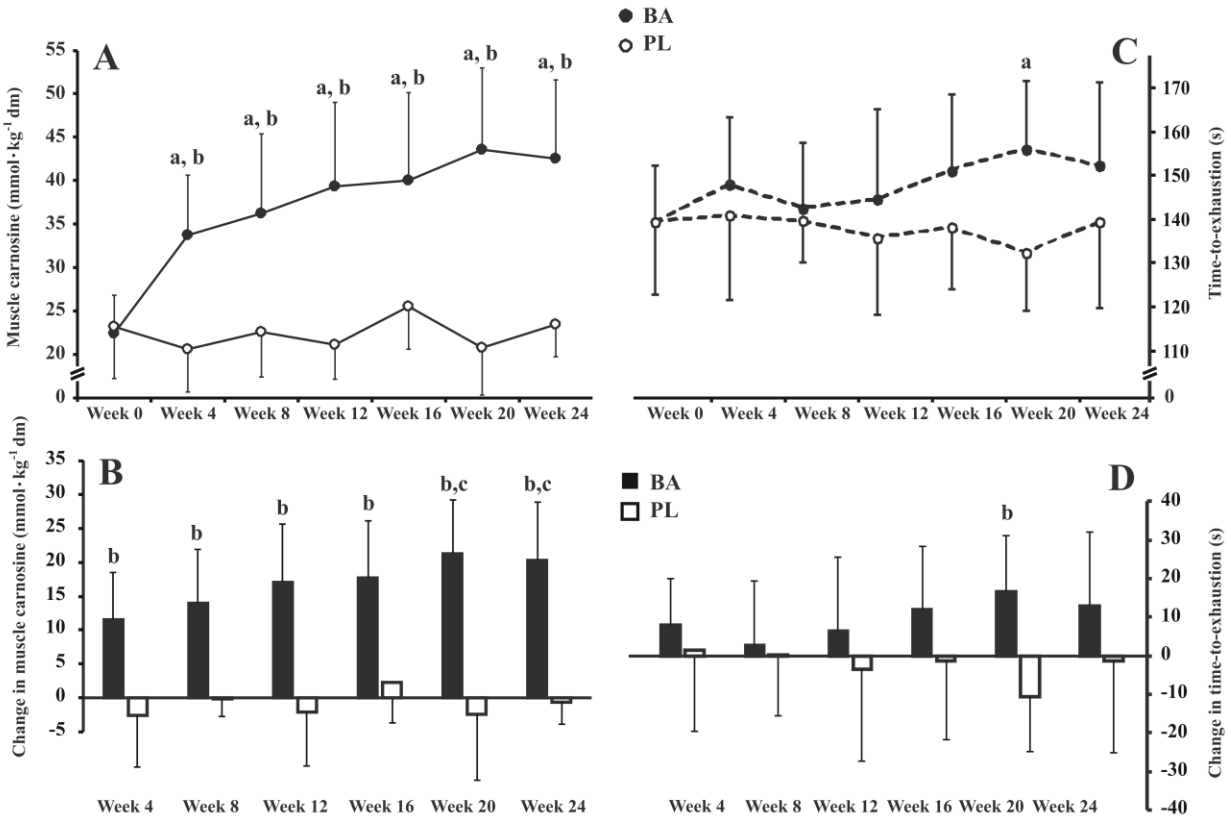

A
24-week supplementation period (6.4 g·day-1)
2 days
2 days
4 wks
4 wks
4 wks
4 wks
4 wks
4 wks
Visit 1
Visit 2
Visit 3
Visit 4
Visit 5
Visit 6
Visit 7
Visit 8
Visit 9
Wmax
CCT110%
Biopsy
Blood
B
resting
CCT110%
5 minutes

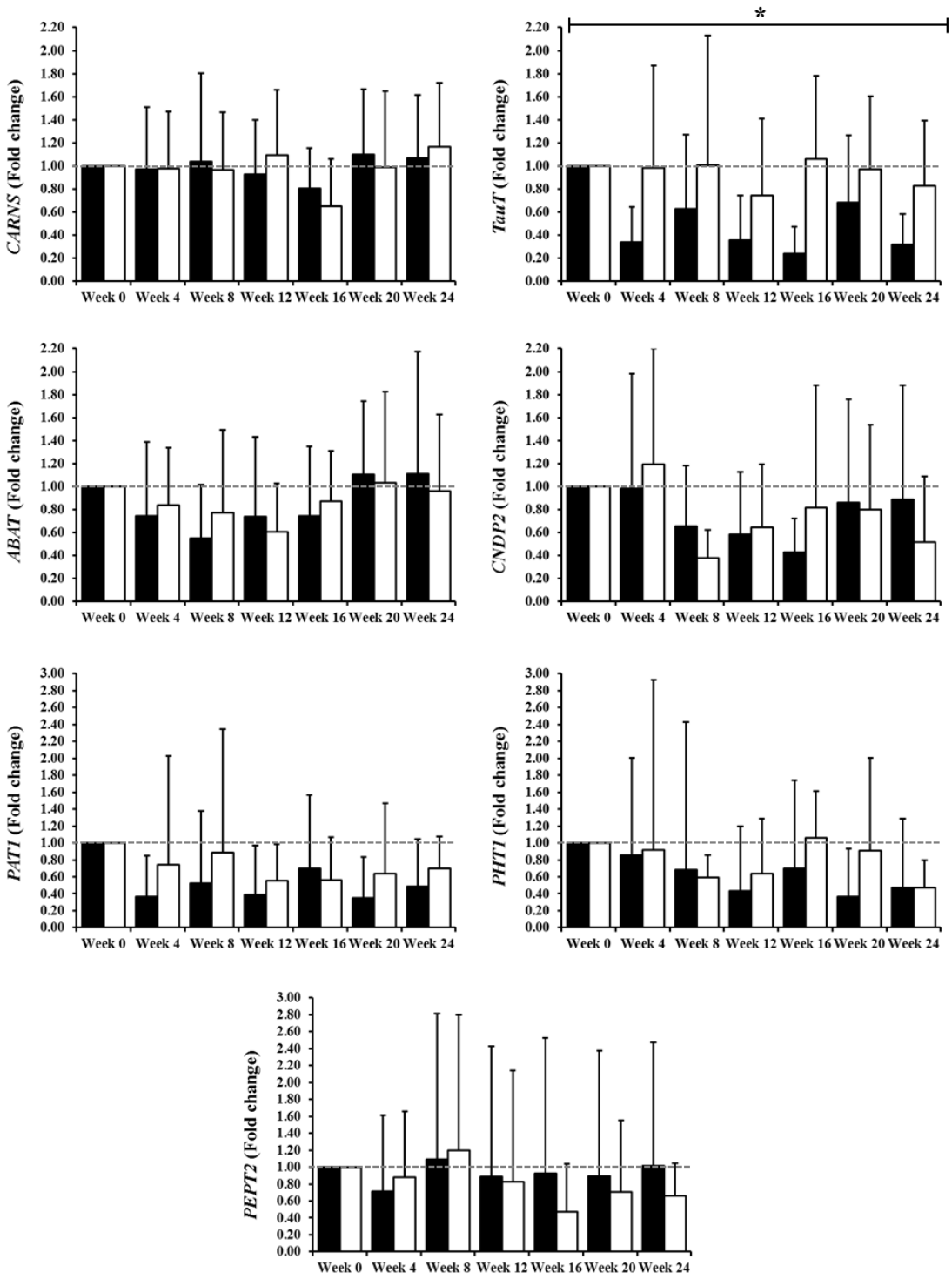
Figure 1



673

674 **Figure 2**

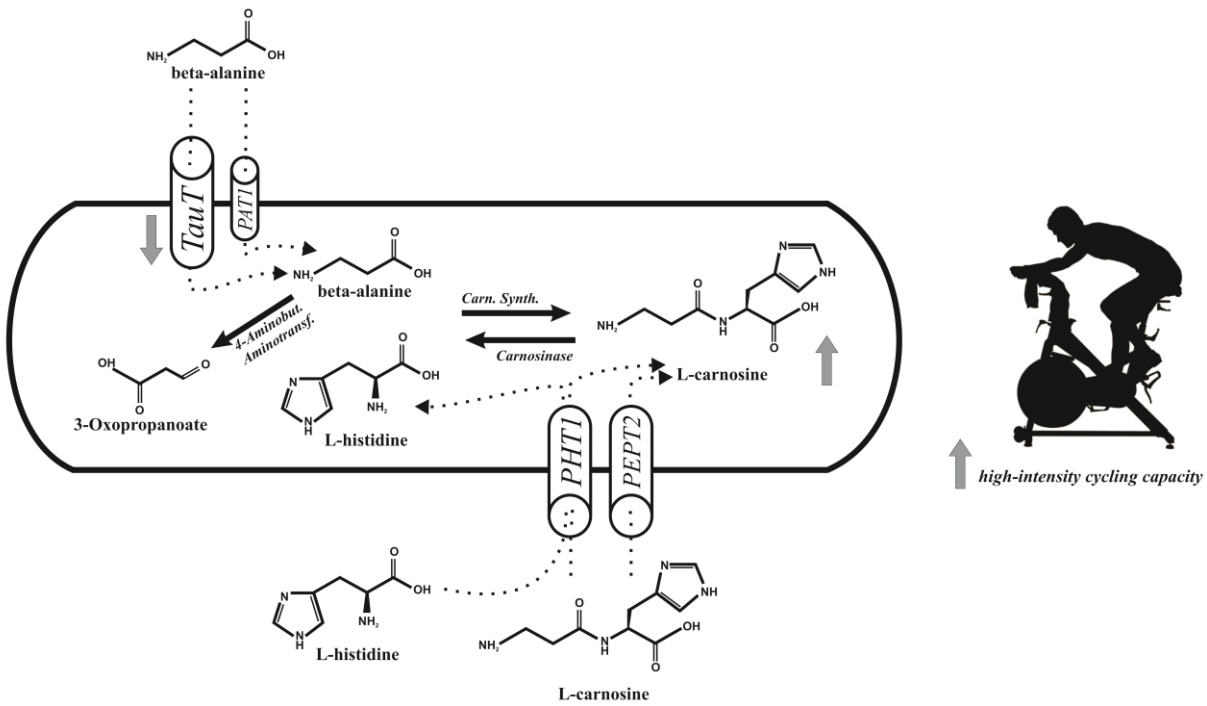
675



676

677 **Figure 3**

678



679

680 **Figure 4**

681

682 **Table 1.**[1]

Participant number	Week 0 (mmol·kg^{-1}dm)	Maximal content (mmol·kg^{-1}dm)	Absolute maximal change (mmol·kg^{-1}dm)	Percentage maximal change (%)	Time-to-maximum (weeks)
1	11.67	31.79	20.13	172.5	20
2	26.97	45.42	18.45	68.4	16
3	19.84	41.94	22.10	111.4	4
4	22.60	63.92	41.32	182.8	12
5	19.19	39.89	20.71	107.9	24
8	19.86	39.29	19.44	97.9	20
10	28.49	59.80	31.31	109.9	24
13	22.99	45.18	22.19	96.5	24
14	22.26	47.42	25.16	113.1	24
16	18.34	54.94	36.60	199.6	24
17	21.71	41.57	19.86	91.5	12
18	28.97	46.10	17.13	59.1	12
22	26.96	54.76	27.80	103.1	20
23	22.50	59.81	37.31	165.8	20
25	23.29	48.62	25.34	108.8	12
Mean	22.37	48.03	25.66	119.2	18
SD	4.46	8.97	7.63	41.5	6
Min	11.67	31.79	17.13	59.1	4
Max	28.97	63.92	41.32	199.6	24

683

684

[1] Table 1. Individual maximal muscle carnosine changes to supplementation in BA.

685 **Table 2.**[2]
686

(vs. Week 0)	TTE					
	BA			PL		
	%change	MBI	ES	%change	MBI	ES
Week 4	+5.7±8.7	89%; *likely*	0.62	+1.8±14.4	31%; *possible*	0.08
Week 8	+2.4±12.6	41%; *possible*	0.25	+1.2±11.9	21%; *unlikely*	0.01
Week 12	+4.8±13.8	70%; *possible*	0.31	-1.3±18.3	15%; *unlikely*	-0.21
Week 16	+8.8±12.0	96%; *very likely*	0.80	+0.1±15.6	19%; *unlikely*	-0.09
Week 20	+12.3±10.4	100%; *almost certainly*	1.21	-7.1±9.9	1%; *almost certainly not*	-0.47
Week 24	+9.7±13.5	96%; *very likely*	0.83	+0.3±17.5	25%; *unlikely*	-0.01

687

[2] Table 2. Likelihood of a positive improvement in TTE (%; qualitative) as determined by MBI and ES at every week versus Week 0.