

1 **24 weeks  $\beta$ -alanine supplementation on carnosine content, related genes and exercise**

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3 Running title: 24 weeks  $\beta$ -alanine supplementation

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23 **ABSTRACT**

24 **Introduction:** Skeletal muscle carnosine content can be increased through  $\beta$ -alanine  
25 supplementation, but the maximum increase achievable with supplementation is unknown. No  
26 study has investigated the effects of prolonged supplementation on carnosine-related genes or  
27 exercise capacity. **Purpose:** To investigate the effects of 24-weeks of  $\beta$ -alanine supplementation  
28 on muscle carnosine content, gene expression and high-intensity cycling capacity (CCT<sub>110%</sub>).  
29 **Methods:** Twenty-five active males were supplemented with 6.4 g·day<sup>-1</sup> of sustained release  $\beta$ -  
30 alanine (BA) or placebo (PL) over a 24-week period. Every 4 weeks participants provided a  
31 muscle biopsy and performed the CCT<sub>110%</sub>. Biopsies were analysed for muscle carnosine content  
32 and gene expression (*CARNS*, *TauT*, *ABAT*, *CNDP2*, *PHT1*, *PEPT2* and *PATI*). **Results:**  
33 Carnosine content was increased from baseline at every time point in BA (all  $P < 0.0001$ ; Week 4:  
34 +11.37±7.03 mmol·kg<sup>-1</sup>dm, Week 8: +13.88±7.84 mmol·kg<sup>-1</sup>dm, Week 12: +16.95±8.54  
35 mmol·kg<sup>-1</sup>dm, Week 16: +17.63±8.42 mmol·kg<sup>-1</sup>dm, Week 20: +21.20±7.86 mmol·kg<sup>-1</sup>dm,  
36 Week 24: +20.15±7.63 mmol·kg<sup>-1</sup>dm), but not PL (all  $P = 1.00$ ). Maximal changes were  
37 +25.66±7.63 mmol·kg<sup>-1</sup>dm (range: +17.13 to +41.32 mmol·kg<sup>-1</sup>dm), and absolute maximal  
38 content was 48.03±8.97 mmol·kg<sup>-1</sup>dm (range: 31.79 to 63.92 mmol·kg<sup>-1</sup>dm). There was an effect  
39 of supplement ( $P = 0.002$ ) on *TauT*; no further differences in gene expression were shown.  
40 Exercise capacity was improved in BA ( $P = 0.05$ ) with *possible to almost certain* improvements  
41 across all weeks. **Conclusions:** Twenty-four weeks of  $\beta$ -alanine supplementation increased  
42 muscle carnosine content and improved high-intensity cycling capacity. Downregulation of *TauT*  
43 suggests it plays an important role in muscle carnosine accumulation with  $\beta$ -alanine  
44 supplementation, while the variability in changes in muscle carnosine content between

45 individuals suggests that other determinants other than the availability of  $\beta$ -alanine may also bear  
46 a major influence on muscle carnosine content.

47

48 **Keywords:** Skeletal muscle carnosine, chronic  $\beta$ -alanine supplementation, carnosine-related  
49 genes, high-intensity cycling capacity, muscle biopsy

50

51 **INTRODUCTION**

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

58

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

70

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

74 controlling these processes are: *CARNS* (carnosine synthesis), *TauT*, *PATI*, *ATB*<sup>0,+</sup> (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<sub>110%</sub>) 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  $\beta$ -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

## 102 **METHODS**

### 103 **Participants**

104 Twenty-five physically active healthy males (age  $27 \pm 4$  y, height  $1.75 \pm 0.09$  m, body mass  $78.9$   
105  $\pm 11.7$  kg), who participated in exercise (e.g., running, cycling, team sports) 1-3 times per week,  
106 volunteered. Participants were requested to maintain similar levels of physical activity and  
107 dietary intake for the duration of the study and compliance with this request was verbally  
108 confirmed with individuals throughout. Individuals completed a food intake diary during weeks  
109 4-8 and 16-20 on two non-consecutive weekdays and one weekend day. Energy and  
110 macronutrient intake was analysed by a nutritionist using specific software (Avanutri, Rio de  
111 Janeiro, Brazil). Habitual consumption of  $\beta$ -alanine was calculated based upon specific tables  
112 taken from the literature (1, 24). Exclusion criteria included, i) supplementation of creatine or  $\beta$ -  
113 alanine in the 6 months prior to the study, ii) ongoing supplementation of any dietary supplement  
114 except carbohydrate and whey protein, and iii) vegetarian diet. The study was first approved by  
115 the institution's Ethical Advisory Committee. Participants provided written informed consent  
116 after completing a health screen.

117

### 118 **Experimental Design**

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

125  
126 Participants were randomly allocated to receive either  $\beta$ -alanine (BA) or placebo (PL) in a 2:1  
127 ratio (*i.e.*, two participants were allocated in BA for each participant in PL); individuals were  
128 matched for maximum cycling power output ( $W_{\max}$ ; BA =  $283 \pm 42$  W, PL =  $286 \pm 52$  W) using  
129 a block randomisation method.<sup>(2)</sup> An unbalanced design was adopted *a priori* in order to  
130 minimise the number of individuals being biopsied (12). Individuals were supplemented for 24  
131 weeks with either  $6.4 \text{ g}\cdot\text{d}^{-1}$   $\beta$ -alanine (CarnoSyn®, NAI, USA) or an equivalent amount of  
132 placebo (maltodextrin; NAI, USA); two 800 mg tablets taken four times per day at 3–4 hour  
133 intervals. Participants completed a log to verify compliance (BA:  $95 \pm 6\%$ ; PL:  $93 \pm 6\%$ ); one  
134 individual, who was in BA, did not adhere to the supplementation protocol and was thus  
135 removed from any analyses. Blinding occurred via an outside researcher not involved in direct  
136 data collection who provided the researchers with identical white pots containing only participant  
137 names.

138

## 139 **Experimental Procedures**

### 140 Preliminary Testing

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

145

### 146 Main Trials

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

153

#### 154 **Muscle biopsies**

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

166

#### 167 **Chromatographic determination of carnosine**

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

170 analysed in duplicate and injected via an auto sampler using a cut injection method with a total  
171 aspirated volume of 70  $\mu\text{L}$ ; 30  $\mu\text{L}$  was discarded, 10  $\mu\text{L}$  injected for analysis and the remaining  
172 30  $\mu\text{L}$  also discarded. Prior to all injections, samples were visually inspected for air bubbles, any  
173 of which were subsequently removed manually by the experimenter. Standard curves for  
174 carnosine were performed prior to each analysis session using concentrations of 0.1, 0.5, 1, 2.5,  
175 and 5 mM, showing excellent linearity ( $R^2=0.996\pm 0.005$ ).

176

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

183

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

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

197

### 198 **Real-time PCR**

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

208

209 Freeze-dried muscle was homogenized and total RNA isolated using Trizol reagent (Invitrogen,  
210 Carlsbad, California). Nucleic acid concentration (DNA and RNA) was determined by  
211 measuring the optical density at 260 nm with a micro spectrophotometer (NanoDrop ND2000,  
212 Thermo Scientific). RNA purity was determined by calculating the absorbance ratio at 260 nm  
213 and 280 nm, and RNA integrity checked on a 1% agarose gel stained with ethidium bromide. A  
214 10  $\mu$ L volume containing a total of 1  $\mu$ g of RNA completed with ultrapure water was added to 10  
215  $\mu$ L of a specific cDNA reverse transcription kit solution (2X RT, Applied Biosystems, Thermo

216 Fisher Scientific, Waltham, USA). The reverse transcription reaction was performed at 25°C for  
217 10 min, followed by 37°C for 120 min and 5 min at 85°C according to the manufacturers'  
218 instructions.

219  
220 Real-time PCR for each gene was performed in duplicate with a 2 µL reaction volume of 5–20  
221 ng cDNA, 11 µL SYBR Green Master Mix (Applied Biosystems, California, USA), 100–400  
222 mM of each primer and completed with water to make 22 µL. Gene expression analyses were  
223 carried out using the following cycle parameters: “hold” at 95°C for 20 s; 40 “cycles” of 95°C for  
224 3 s, and 60°C for 30 s; “melt” consisting of a gradual ramp from 65 to 95°C at an increase of  
225 1°C·s<sup>-1</sup>. The fluorescence intensity was quantified and amplification plots analysed by a  
226 sequence detector system (Rotor Gene-Q, Qiagen, Hilden, Germany). The intra-assay CV for the  
227 comparative cycle threshold (Ct) between the duplicate injections was between 4.5 and 7.5% for  
228 all genes measured. Results were obtained using the comparative Ct method. Delta-Ct (DCt)  
229 values were calculated in every sample for each gene of interest as follows: Ct(gene of interest) –  
230 Ct(reference gene). Relative changes in the expression level of the genes (DDCt) were calculated  
231 by subtraction of the DCt at baseline (Week 0) from the corresponding DCt at the time points of  
232 interest (Weeks 4 – 24). Finally, relative quantification (fold change) was calculated using the 2<sup>-</sup>  
233 <sup>DDCt</sup> equation (34).

234

### 235 **Exercise protocols**

236  $W_{\max}$  and CCT<sub>110%</sub>

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

239 recorded as the outcome measures for all tests. The CCT<sub>110%</sub> has been shown to be a reliable test  
240 with a CV of 4.4% for TTE following a solitary familiarisation session (32). The CV between the  
241 familiarisation and baseline time trials in the current study was  $4.9 \pm 3.4$  for TTE; this value  
242 (4.9%) was used to determine improvements above the variation of the test.

243

#### 244 **Blood collection and analyses**

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

253

#### 254 **Statistical Analyses**

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

262  $\beta$ -alanine on CCT<sub>110%</sub>; the smallest worthwhile improvement in TTE was 3.56 s (32). The means  
263 and SDs for BA and PL were used to calculate effect sizes for muscle carnosine and TTE (22).  
264 Blood data were analysed using a mixed model with individuals assumed as a random factor and  
265 supplementation (2 levels; BA and PL), week (7 levels; Weeks 0 to Week 24) and time (3 levels;  
266 Pre-exercise, Post-exercise, 5-min Post-exercise) assumed as fixed factors. Body mass was  
267 analysed using a mixed model with individuals assumed as a random factor and supplementation  
268 (2 levels; BA and PL) and week (3 levels; Week 0; Week 12; Week 24) assumed as fixed factors.  
269 Food intake was analysed using a mixed model with individuals assumed as a random factor and  
270 supplementation (2 levels; BA and PL) and week (2 levels; Weeks 4-8 and Weeks 16-20)  
271 assumed as fixed factors. Pearson's correlations were performed to determine any associations  
272 between initial muscle carnosine content and absolute changes over time.

273

274 **RESULTS**

275 **Muscle carnosine**

276 There were no significant differences in pre-supplementation (Week 0) carnosine content  
277 between BA ( $22.37 \pm 4.46$  mmol·kg<sup>-1</sup>dm) and PL ( $23.18 \pm 5.89$  mmol·kg<sup>-1</sup>dm;  $P=1.00$ ). There was  
278 a main effect of supplementation ( $P<0.0001$ ) and week ( $P<0.0001$ ), and a supplementation ×  
279 week interaction ( $P<0.0001$ ). Carnosine content increased from Week 0 at every time point in  
280 BA (all  $P<0.0001$ ; Week 4:  $+11.37 \pm 7.03$  mmol·kg<sup>-1</sup>dm, Week 8:  $+13.88 \pm 7.84$  mmol·kg<sup>-1</sup>dm,  
281 Week 12:  $+16.95 \pm 8.54$  mmol·kg<sup>-1</sup>dm, Week 16:  $+17.63 \pm 8.42$  mmol·kg<sup>-1</sup>dm, Week 20:  
282  $+21.20 \pm 7.86$  mmol·kg<sup>-1</sup>dm, Week 24:  $+20.15 \pm 7.63$  mmol·kg<sup>-1</sup>dm) with no changes across time  
283 in PL (all  $P=1.00$ ; Figure 2). Effect sizes from Week 0 were all huge in BA (Week 4: 1.96;  
284 Week 8: 1.93; Week 12: 2.24; Week 16: 2.25; Week 20: 2.86; Week 24: 2.81) and ranged from  
285 negligible to medium effects in PL (0.06 to -0.48).

286

287 Baseline content (Week 0) ranged from 11.67 to 28.97 mmol·kg<sup>-1</sup>dm in BA, and 15.14 to 34.89  
288 mmol·kg<sup>-1</sup>dm in PL. All individuals increased muscle carnosine content above baseline levels.  
289 The absolute maximal changes in muscle carnosine was  $+25.66 \pm 7.63$  mmol·kg<sup>-1</sup>dm, ranging  
290 from +17.13 to +41.32 mmol·kg<sup>-1</sup>dm. The absolute maximal content was  $48.03 \pm 8.97$  mmol·kg<sup>-1</sup>  
291 dm, ranging from +31.79 to +63.92 mmol·kg<sup>-1</sup>dm (Table 1). The time-to maximal content was  
292  $17 \pm 7$  weeks and ranged from 4 to 24 weeks; one individual showed maximal carnosine content  
293 at Week 4, four at Week 12, one at Week 16, four at Week 20 and five at Week 24. Initial  
294 muscle carnosine content (Week 0) was significantly correlated to the absolute carnosine content  
295 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  
296 ( $r=0.29$ ,  $P=0.29$ ), 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<sub>110%</sub>**

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  $\times$   
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

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

334

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

341

342 **Dietary intake**

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

350

351 **DISCUSSION**

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

359  
360 Muscle carnosine content increased by 55% following 4 weeks, which is lower than the relative  
361 increases previously shown using HPLC analysis of muscle biopsy samples (17, 19), despite the  
362 lower dose of  $\beta$ -alanine used in those studies (mean 5.2 g·day<sup>-1</sup>; (17, 19)). Absolute changes in  
363 muscle carnosine at 4 weeks in the present study were greater than those shown by Harris et al.  
364 (17) but identical to those of Hill et al. (19), despite that in the previous studies a slightly lower  
365 dose (5.2 versus 6.4g·day<sup>-1</sup>) was given. The greatest absolute change in mean carnosine content  
366 occurred following 20 weeks of supplementation, and corresponded to a +98±40% increase. This  
367 is lower than the +143±151% increases shown by Chung et al. (10) using <sup>1</sup>H-MRS following 4  
368 weeks of  $\beta$ -alanine supplementation, although the absolute changes appear quite similar when  
369 both data sets are expressed in the same units. Percentage increases misrepresent carnosine  
370 changes in muscle, particularly in those with low initial values (*i.e.*, predominant distribution of  
371 type I fibres; low meat eaters or vegetarians). Since the contribution of carnosine to muscle  
372 buffering capacity (or indeed any suggested physiological mechanism) is dependent upon its  
373 actual content in muscle, any exercise or therapeutic benefits received via this mechanism will

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

379  
380 We hypothesised that changes in muscle carnosine content would be mirrored by changes in the  
381 expression of carnosine-related genes. *TauT* was downregulated with supplementation, although  
382 no other changes in gene expression were shown. Since *TauT* is the primary transporter of  $\beta$ -  
383 alanine into muscle (4), our data support the suggestion that increases in muscle carnosine may  
384 be more dependent upon the transport of  $\beta$ -alanine into the muscle than the activity of carnosine  
385 synthase (*CARNS*; (14)), since this will directly influence the availability of  $\beta$ -alanine for muscle  
386 carnosine synthesis. Decreasing the activity of *TauT* during prolonged increases in circulating  $\beta$ -  
387 alanine through oral supplementation may be the body's mechanism to best maintain  
388 intramuscular homeostasis of muscle carnosine by limiting the uptake of  $\beta$ -alanine into muscle.  
389 Blancquaert et al. (8) suggested that the homeostasis of muscle carnosine is tightly regulated by  
390 the transamination of circulating levels of  $\beta$ -alanine via GABA-T and AGXT2; the current data  
391 suggest that the downregulation of *TauT* can also play a role in the regulation of muscle  
392 carnosine content, perhaps contributing to increased transamination of circulating levels due to  
393 decreased uptake into muscle, although this was not measured here. The lack of any other  
394 changes in gene expression in this study is in contrast to the increased expression of *CARNS*,  
395 *TauT* and *ABAT* shown following  $\beta$ -alanine supplementation in mice (14). However, the dose of  
396  $\beta$ -alanine that these mice received is equivalent to a supra-physiological dose in humans and it is

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

406

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

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

423

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

431

432 All individuals increased muscle carnosine from initial content with supplementation, which  
433 suggests that all individuals can show some degree of carnosine accumulation following  $\beta$ -  
434 alanine supplementation. Mean muscle carnosine contents increased most in the first 4 weeks,  
435 although this quickly dropped off as evidenced by a difference from the previous time point only  
436 at week 4. Nonetheless, an increased content in the final weeks of supplementation from the first  
437 eight suggest that total content continued to increase. Stellingwerff et al. (33) showed a linear  
438 response with supplementation with a high dependence on initial concentrations and the total  
439 amount of  $\beta$ -alanine consumed, which explained ~80% of the variance in carnosine  
440 concentration in their study. Although the initial carnosine content in the present study was  
441 related to the content at several time points and the maximal content attained, individual analysis  
442 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·d<sup>-1</sup>), 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

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

472

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

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

493

494 In conclusion, twenty-four weeks of  $\beta$ -alanine supplementation increased muscle carnosine  
495 content up to  $\sim 64$   $\text{mmol}\cdot\text{kg}^{-1}\text{dm}$ , although maximal absolute changes were variable (*i.e.*, +17 to  
496 +41  $\text{mmol}\cdot\text{kg}^{-1}\text{dm}$ ), as was the time-to-maximal content. The transporter *TauT* was  
497 downregulated with  $\beta$ -alanine supplementation, suggesting it plays an important role in the  
498 accumulation of muscle carnosine content during prolonged  $\beta$ -alanine supplementation. Exercise  
499 capacity was improved with supplementation, mirroring changes in muscle carnosine, although a  
500 certain amount of variation was shown. Collectively, these results highlight the variability in  
501 changes in muscle carnosine content between individuals and that a maximal accumulation of  
502 muscle carnosine may not occur within twenty-four weeks at a high dose for all individuals,  
503 suggesting that determinants other than the availability of  $\beta$ -alanine may have a major influence  
504 on muscle carnosine content.

505

506 **ACKNOWLEDGEMENTS**

507 The authors would like to thank National Alternatives International, San Marcos, California for  
508 providing the  $\beta$ -alanine (Carnosyn™) and maltodextrin supplements. We also wish to thank the  
509 Laboratório de Determinantes Energéticos de Desempenho Esportivo (LADESP) for access to  
510 the cycle ergometer used in this study. Finally, our thanks are extended to the participants who  
511 took part in the study for their time and dedication.

512

513 **FUNDING AND CONFLICT OF INTEREST**

514 Bryan Saunders, Vitor de Salles Painelli, Rafael Pires da Silva, Mariana Franchi, Guilherme  
515 Giannini Artioli and Bruno Gualano have been financially supported by Fundação de Amparo à  
516 Pesquisa do Estado de Sao Paulo (FAPESP grant numbers: 2011/19513-2, 2013/04806-0,  
517 2012/13026-5, 2015/22686-7, 2014/11948-8, 2013/14746-4). Bryan Saunders has received a  
518 scholarship from National Alternatives International, San Marcos, California. Luana Farias de  
519 Oliveira and Livia de Souza Gonçalves have been financially supported by Coordenação de  
520 Aperfeiçoamento de Pessoal de Nível Superior (CAPES).

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

524

525 **AUTHOR CONTRIBUTION**

526 Significant manuscript writer – Bryan Saunders, Bruno Gualano, Craig Sale

527 Significant manuscript reviewer/reviser – Hamilton Roschel, Guilherme Giannini Artioli, Roger

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535 Statistical expertise – Bryan Saunders  
536

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628

629

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). <sup>a</sup> $P \leq 0.0001$  from Week 0. <sup>b</sup> $P \leq 0.0001$  from  
640 PL at same time point. <sup>c</sup> $P \leq 0.05$  from Weeks 4 and 8. Data are mean $\pm$ 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  $\beta$ -alanine into muscle; the other  $\beta$ -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  $\beta$ -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

654 **SUPPLEMENTAL DIGITAL CONTENT**

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

657

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

662

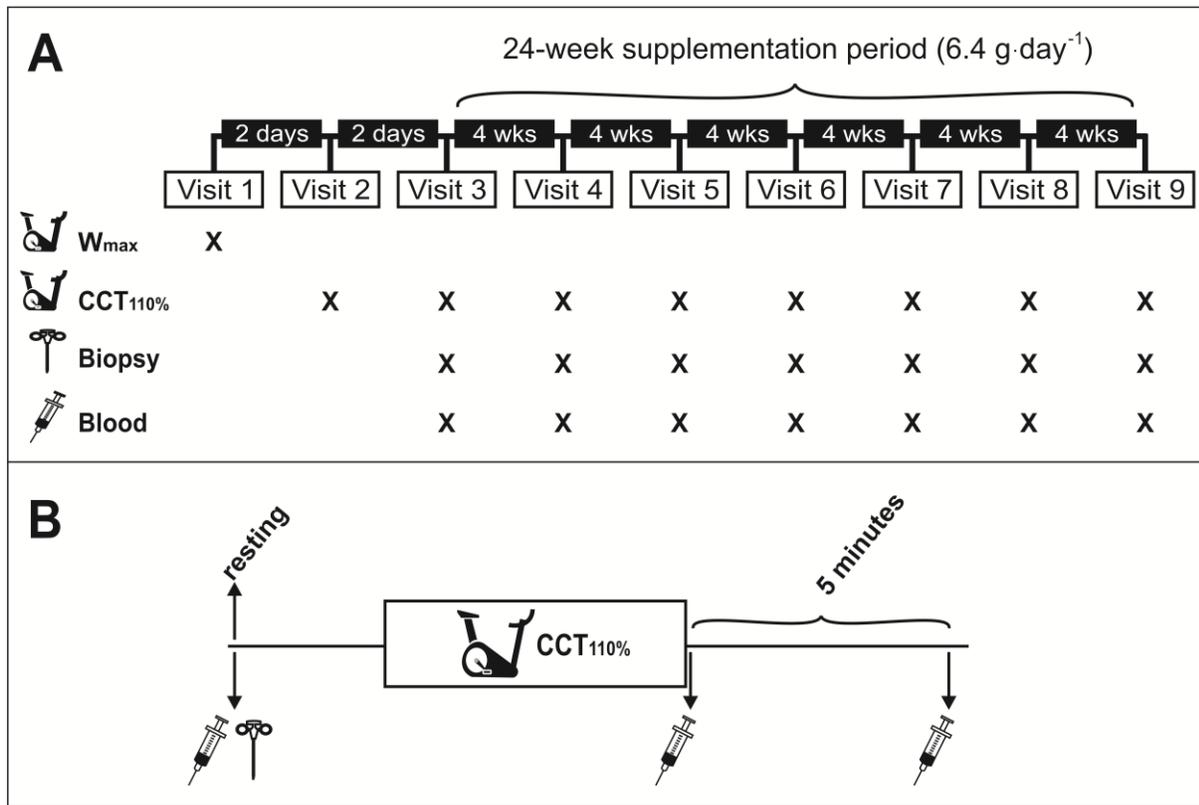
663 Supplemental Digital Content 3.doc Blood pH, bicarbonate, base excess and lactate (mean  $\pm$   
664 1SD) at pre-exercise, post-exercise and 5-min post-exercise at every week in BA and PL.

665 \* $P<0.001$  from Pre-exercise.

666

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

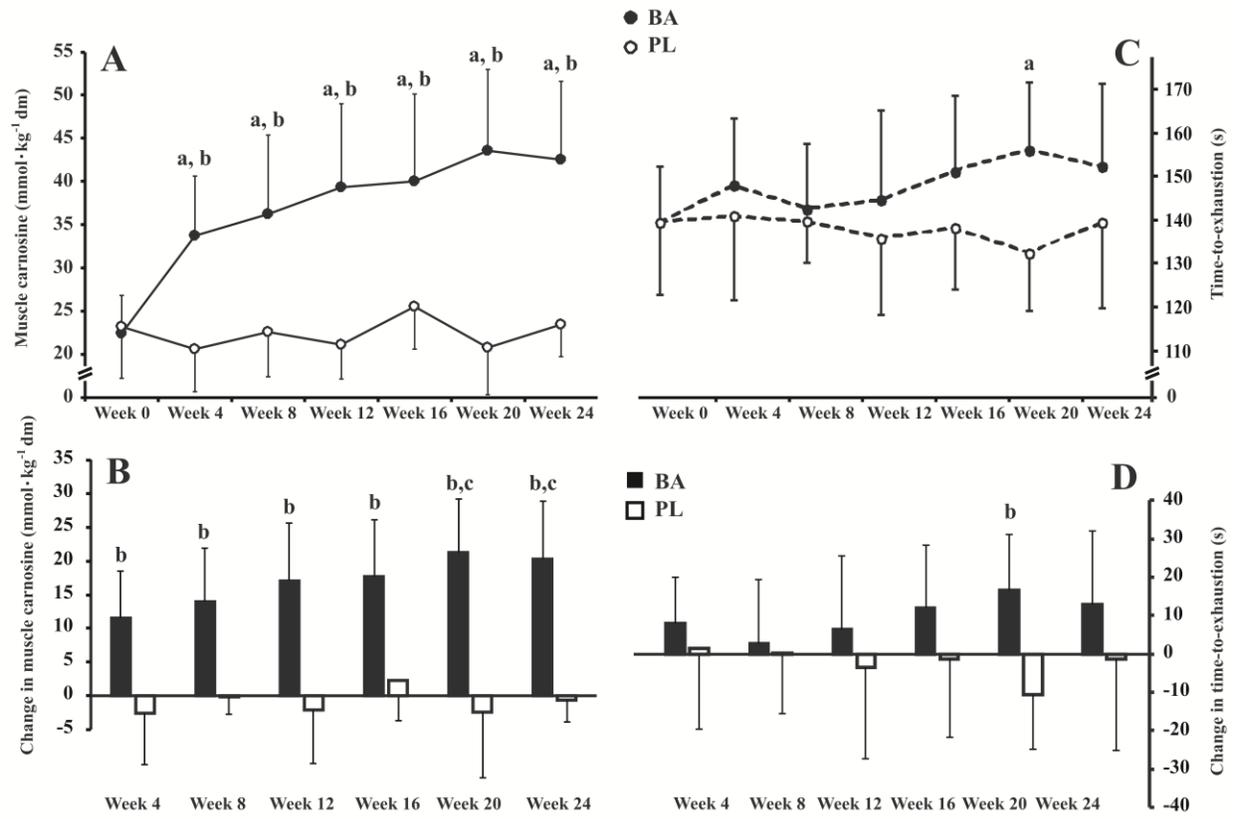
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670

671 **Figure 1**

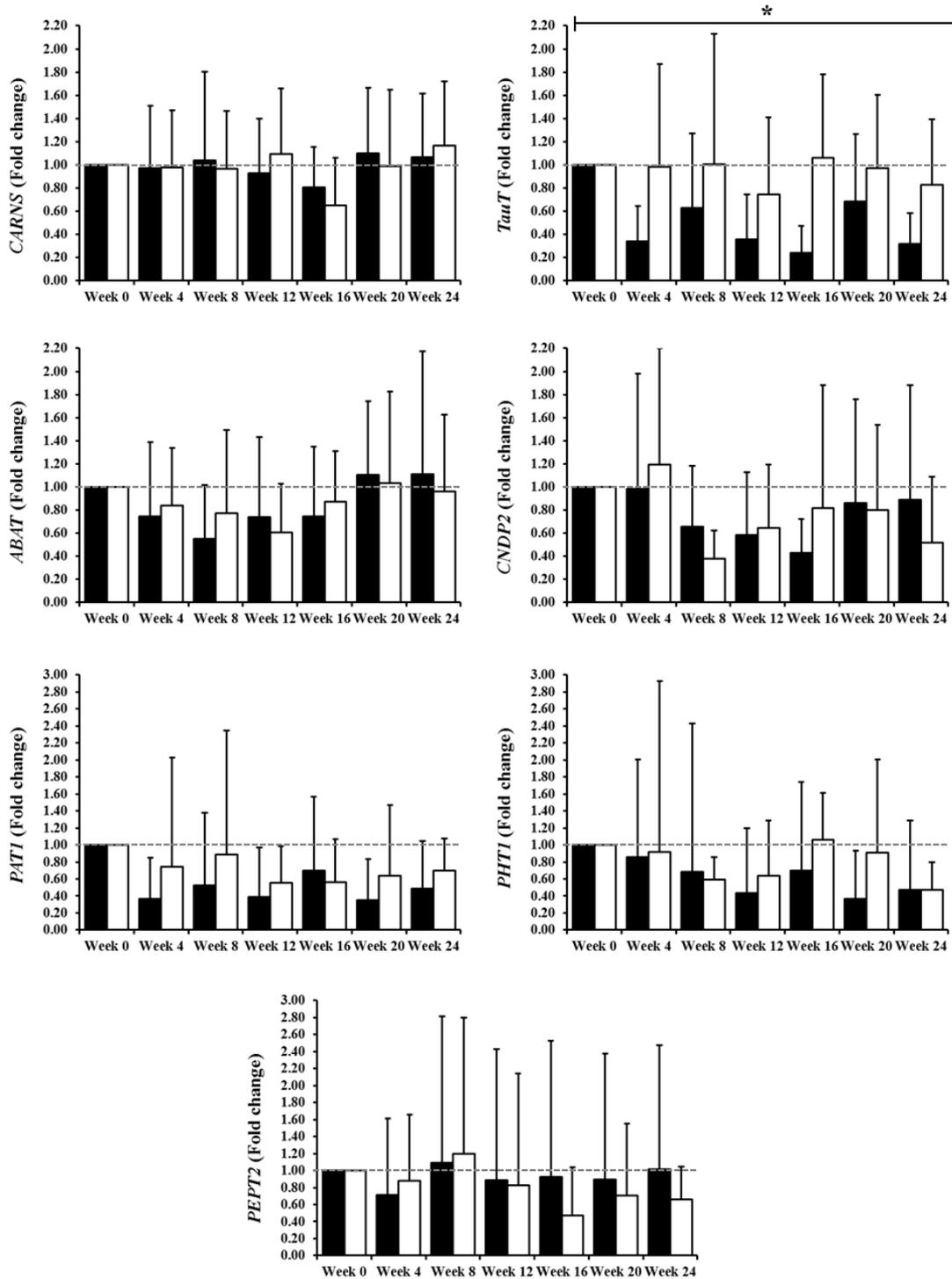
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674 **Figure 2**

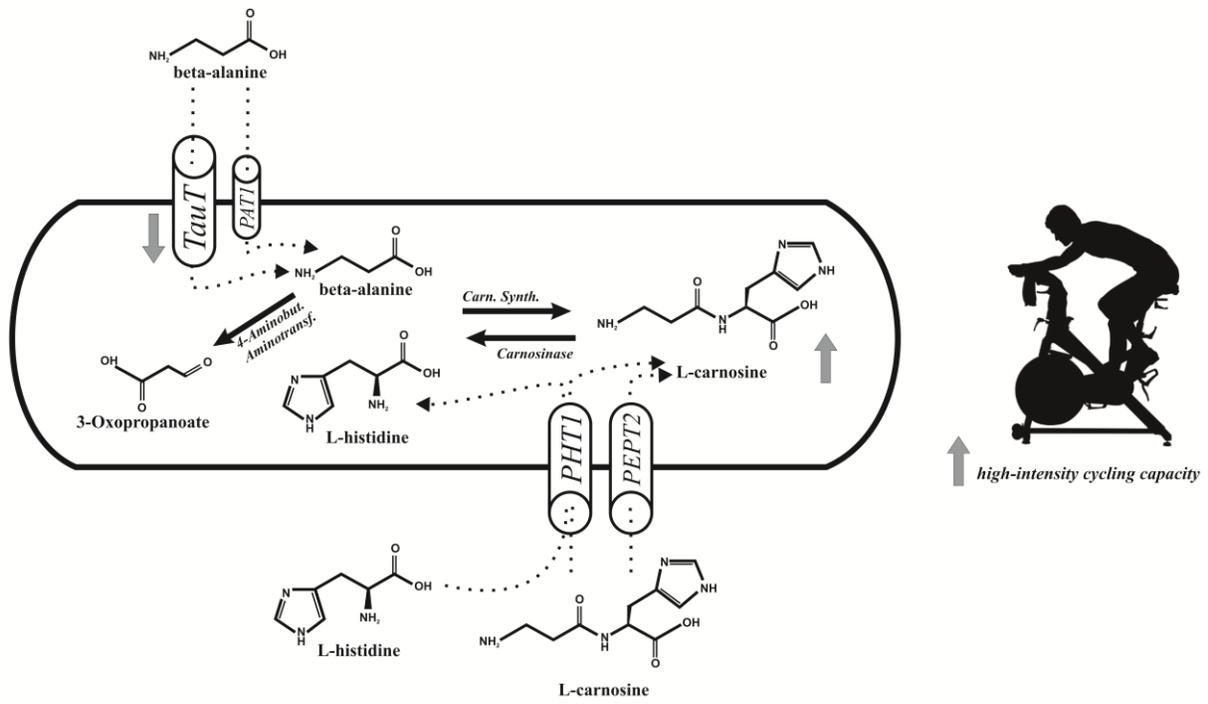
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676

677 **Figure 3**

678



679

680 **Figure 4**

681

682 **Table 1.**<sup>1</sup>

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

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<sup>1</sup> Table 1. Individual maximal muscle carnosine changes to supplementation in BA.

685 **Table 2.**<sup>2</sup>  
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	<b>TTE</b>					
	<b>BA</b>			<b>PL</b>		
	<b>%change</b>	<b>MBI</b>	<b>ES</b>	<b>%change</b>	<b>MBI</b>	<b>ES</b>
<b>Week 4</b>	+5.7±8.7	89%; <i>likely</i>	0.62	+1.8±14.4	31%; <i>possible</i>	0.08
<b>Week 8</b>	+2.4±12.6	41%; <i>possible</i>	0.25	+1.2±11.9	21%; <i>unlikely</i>	0.01
<b>Week 12</b>	+4.8±13.8	70%; <i>possible</i>	0.31	-1.3±18.3	15%; <i>unlikely</i>	-0.21
<b>Week 16</b>	+8.8±12.0	96%; <i>very likely</i>	0.80	+0.1±15.6	19%; <i>unlikely</i>	-0.09
<b>Week 20</b>	+12.3±10.4	100%; <i>almost certainly</i>	1.21	-7.1±9.9	1%; <i>almost certainly not</i>	-0.47
<b>Week 24</b>	+9.7±13.5	96%; <i>very likely</i>	0.83	+0.3±17.5	25%; <i>unlikely</i>	-0.01

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