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Twenty-four Weeks of β-Alanine Supplementation on Carnosine Content, Related Genes, and Exercise

BRYAN SAUNDERS[1], VITOR DE SALLES PAINELLI[1], LUANA FARIAS DE OLIVEIRA[1], VINICIUS DA EIRA SILVA[1], RAFAEL PIRES DA SILVA[1], LUIZ RIANI[1], MARIANA FRANCHI[1], LÍVIA DE SOUZA GONÇALVES[1], ROGER CHARLES HARRIS[2], HAMILTON ROSCHEL[1], GUILHERME GIANNINI ARTIOLI[1], CRAIG SALE[3], and BRUNO GUALANO[1]

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ABSTRACT

SAUNDERS, B., V. DE SALLES PAINELLI, L. F. OLIVEIRA, V. DA EIRA SILVA, R. P. DA SILVA, L. RIANI, M. FRANCHI, L. D. GONÇALVES, R. C. HARRIS, H. ROSCHEL, G. G. ARTIOLI, C. SALE, and B. GUALANO. Twenty-four Weeks of β-Alanine Supplementation on Carnosine Content, Related Genes, and Exercise. *Med. Sci. Sports Exerc.*, Vol. 49, No. 5, pp. 896–906, 2017. **Introduction**: Skeletal muscle carnosine content can be increased through β-alanine (BA) 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**: This study aimed to investigate the effects of 24 wk of BA supplementation on muscle carnosine content, gene expression, and high-intensity cycling capacity ($CCT_{110\%}$). **Methods**: Twenty-five active males were supplemented with 6.4 $g \cdot d^{-1}$ of sustained release BA or placebo for a 24 wk period. Every 4 wk participants provided a muscle biopsy and performed the $CCT_{110\%}$. Biopsies were analyzed 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, and week 24 = +20.15 ± 7.63 $mmol \cdot kg^{-1}$ dm) but not placebo (all $P > 0.05$). Maximal increases 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 BA supplementation increased muscle carnosine content and improved high-intensity cycling capacity. The downregulation of *TauT* suggests it plays an important role in muscle carnosine accumulation with BA supplementation, whereas the variability in changes in muscle carnosine content between individuals suggests that other determinants other than the availability of BA may also bear a major influence on muscle carnosine content. **Key Words:** SKELETAL MUSCLE CARNOSINE, CHRONIC β-ALANINE SUPPLEMENTATION, CARNOSINE-RELATED GENES, HIGH-INTENSITY CYCLING CAPACITY, MUSCLE BIOPSY

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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). The dietary supply of histidine-containing dipeptides is a major determinant of skeletal muscle carnosine content (18), and increases with β-alanine (BA) supplementation have been shown using the chromatographic (i.e., high-performance liquid chromatography [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 for 4 wk was linearly related to the BA dose given (1.6 and 3.2 $g \cdot d^{-1}$), whereas the absolute change was dependent on the total amount ingested. An average dose of 5.2 $g \cdot d^{-1}$ for 4 wk increased carnosine

content in the vastus lateralis muscle from 19.9 ± 1.9 to 30.1 ± 2.3 mmol·kg^{-1} dm; a further 6 wk of supplementation at 6.4 g·d^{-1} increased carnosine content to 34.7 ± 3.7 mmol·kg^{-1} dm (19). These data demonstrate that the maximum accumulation of carnosine takes more than 4 wk of BA supplementation at a mean dose of 5.2 g·d^{-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 maximized when this reaches its maximal content. It would be of interest to determine the kinetics of carnosine accumulation in muscle with prolonged BA supplementation.

Several genes and their resulting proteins regulate the processes affecting muscle carnosine content: the uptake of BA and carnosine into skeletal muscle, the local synthesis of carnosine, the hydrolysis of carnosine, and the transamination of BA. The genes controlling these processes are *CARNS* (carnosine synthesis); *TauT*, *PAT1*, and *ATB$^{0,+}$* (BA transport); *CNDP1* and *CNDP2* (carnosine hydrolysis); *ABAT* (BA transaminase); and *PEPT1*, *PEPT2*, *PHT1*, and *PHT2* (carnosine/histidine transport). The expression of some of these genes have been examined (14), but the influence of BA supplementation on their expression remains unknown in humans. In particular, the transport of BA into muscle (via *TauT*), the synthesis of muscle carnosine (via *CARNS*), and the deamination of BA (via *ABAT*) have been suggested to play important roles in the regulation of carnosine synthesis (14). The examination of the changes in the expression of carnosine-related genes after prolonged BA supplementation could provide important information as to the mechanisms by which increased BA availability increases muscle carnosine content.

The efficacy of BA supplementation to improve exercise capacity and performance has been demonstrated (20,31). Improvements during a high-intensity cycling capacity test at 110% of maximum power output ($CCT_{110\%}$) have been verified independently, showing that time to exhaustion (TTE) was improved by 11.9% (19), 12.1% (30), and 14.0% (11). The improved exercise capacity shown by Hill et al. (19) was linear to changes in muscle carnosine, although no studies have examined the association between muscle carnosine and exercise changes for a longer period with multiple data points.

We aimed to determine whether (a) a ceiling for carnosine accumulation in skeletal muscle exists after 24 wk of BA supplementation, (b) the carnosine content influences the expression of genes responsible for regulating carnosine in muscle, and (c) the changes in muscle carnosine are related to changes in high-intensity exercise capacity. We hypothesized that (a) long-term BA supplementation would lead to saturation of the muscle carnosine content, (b) prolonged supplementation would downregulate genes involved in the control of the carnosine content in muscle, and (c) increases in muscle carnosine would be paralleled by improvements in exercise capacity.

METHODS

Participants

Twenty-five physically active healthy males (age = 27 ± 4 yr, height = 1.75 ± 0.09 m, body mass [BM] = 78.9 ± 11.7 kg), who participated in exercise (e.g., running, cycling, and team sports) one to three 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 nonconsecutive weekdays and one weekend day. Energy and macronutrient intake was analyzed by a nutritionist using specific software (Avanutri, Rio de Janeiro, Brazil). Habitual consumption of BA was calculated based on specific tables taken from the literature (1,24). Exclusion criteria included (i) supplementation of creatine or BA in the 6 months before 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 familiarization of the exercise protocol. The remaining seven visits were for the completion of the main trials, each separated by 4 wk; one main trial was completed before supplementation (week 0) followed by one main trial every 4 wk for 24 wk (weeks 4–24) during a double-blinded supplementation with BA or placebo (PL) (Fig. 1A).

Participants were randomly allocated to receive either BA or 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 randomization method. (2) An unbalanced design was adopted *a priori* to minimize the number of individuals being biopsied (12). Individuals were supplemented for 24 wk with either 6.4 g·d^{-1} BA (CarnoSyn®, Natural Alternatives International, Inc., Carlsbad, CA) or an equivalent amount of PL (maltodextrin, Natural Alternatives International, Inc.); two 800-mg tablets taken four times per day at 3- to 4-h 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 BM were recorded on arrival at the first laboratory session, and BM was further

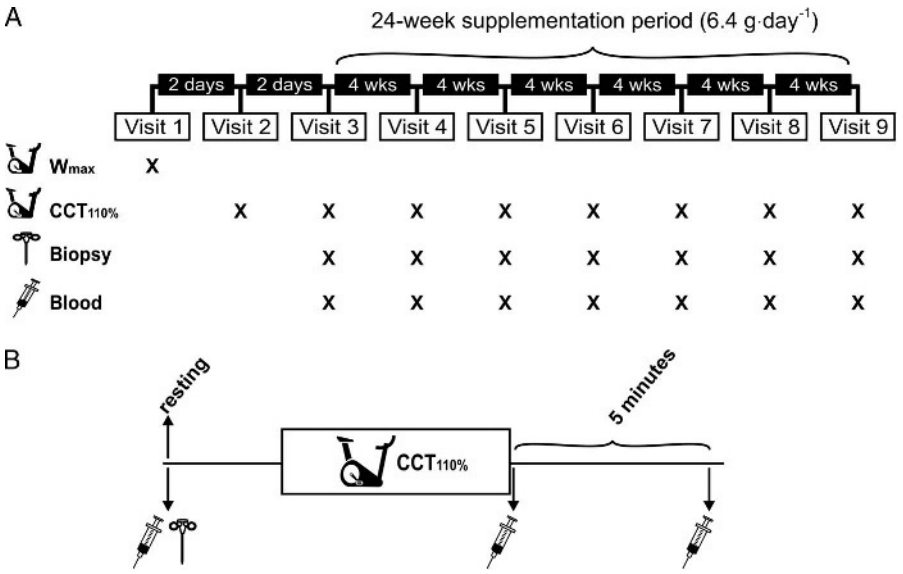

FIGURE 1—A, Experimental design of the study. B, Main trial design. W_{max}, maximum cycling power output test; $CCT_{110\%}$, cycling capacity test at 110% of maximum cycling power output.

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 familiarization session of the main exercise protocol (described in the next section).

Main trials. Participants abstained from alcohol, caffeine, and strenuous exercise and completed a food record for the 24-h period before the initial trial. They adopted the same dietary intake before each trial. Participants arrived at the laboratory at the same time of day a minimum of 2 h after 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 vastus lateralis muscle before performing the $CCT_{110\%}$ (Fig. 1B).

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 vastus lateralis muscle under local anesthesia (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°C. All biopsies followed the same standardized 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 following weeks 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 conducted at room temperature. Samples were analyzed 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 was injected for analysis, and the remaining 30 μL was also discarded. Before 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 before 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, Milford, MA) 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), and mobile phase B, 4.55 mM ammonium acetate, in water/acetonitrile (70:30). The pH level 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 composed of a linear gradient from 0% to 100% of solvent B in 13 min at a flow rate of 1.4 mL·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. The peak area for the standard curve was plotted and a regression equation obtained, from which interpolations were used to calculate the content. The limit of detection for the current method

was 0.5125 mmol·kg^{-1} dm, and the interassay coefficient of variation (CV) of carnosine measurement of the same freeze-dried muscle extracted separately on nine occasions was 0.9% ± 1.2%. The intraassay 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 reanalyzed after 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, Coralville, IA), and primer sets are shown in Supplemental Digital Content 1 (Table, Supplemental Digital Content 1, Forward and reverse primer sets, http://links.lww.com/MSS/A820). The standardization 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, the expression of these genes was not performed.

Freeze-dried muscle was homogenized and total RNA isolated using Trizol reagent (Invitrogen, Carlsbad, CA). Nucleic acid concentration (DNA and RNA) was determined by measuring the optical density at 260 nm with a microspectrophotometer (NanoDrop ND2000; Thermo Fisher Scientific, Waltham, MA). RNA purity was determined by calculating the absorbance ratio at 260 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). The reverse transcription reaction was performed at 25°C for 10 min, followed by 37°C for 120 and 5 min at 85°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, Carlsbad, CA), 100–400 mM of each primer and completed with water to make 22 μL. Gene expression analyses were conducted using the following cycle parameters: "hold" at 95°C for 20 s, 40 "cycles" of 95°C for 3 s, and 60°C for 30 s; "melt" consisting of a gradual ramp from 65°C to 95°C at an increase of 1°C·s^{-1}. The fluorescence intensity was quantified, and amplification plots were analyzed by a sequence detector system (Rotor Gene-Q, Qiagen, Hilden, Germany). The intraassay 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 $2^{-\mathrm{DDCt}}$ 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). 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 after a solitary familiarization session (32). The CV between the familiarization and the 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 before, immediately after, and 5-min after exercise and analyzed 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 analyzed for blood pH, bicarbonate, and base excess (Rapid Point 350; Siemens, Germany). The intra-assay CVs for pH, bicarbonate, and base excess before, immediately after, and 5-min after exercise 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 analyzed using the SAS statistical package (SAS 9.2; SAS Institute Inc., Cary, NC) and are presented as mean ± 1 SD unless otherwise stated. Muscle carnosine, gene expression, and exercise data were analyzed using mixed model analysis with individuals assumed as a random factor and supplementation (two levels; BA and PL) and week (seven levels; weeks 0–24) assumed as fixed factors. Tukey *post hoc* tests were performed whenever a significant F value was obtained. The significance level was set at $P \leq 0.05$, and a tendency toward an effect was set at $P < 0.1$. Magnitude-based inferences (MBI [5,21]) were used to determine the practical significance of BA 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 analyzed using a mixed model with individuals assumed as a random factor and supplementation (two levels; BA and PL), week (seven levels; weeks 0–24), and time (three levels; preexercise, postexercise, and 5-min postexercise) assumed as fixed factors. BM was analyzed using a mixed model with individuals assumed as a random factor, and supplementation (two levels; BA and PL) and week (three levels; week 0, week 12, and week 24) assumed as fixed factors. Food intake was analyzed using a mixed model with individuals assumed as a random factor, and supplementation (two levels; BA and PL) and week (two 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 presupplementation (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, and week 24 = +20.15 ± 7.63 mmol·kg^{-1} dm) with no changes across time in PL (all $P > 0.05$; Fig. 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, and 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 from 15.14 to 34.89 mmol·kg^{-1} dm in PL. All individuals increased muscle carnosine content above baseline levels. The absolute maximal change 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 18 ± 6 wk and ranged from 4 to 24 wk; 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 week 8 ($r = 0.52$, $P = 0.05$), week 16 ($r = 0.58$, $P = 0.03$), and week 20 ($r = 0.57$, $P = 0.03$), but not week 4 ($r = 0.29$, $P = 0.29$), week 12 ($r = 0.48$, $P = 0.07$), or week 24 ($r = 0.37$, $P = 0.18$).

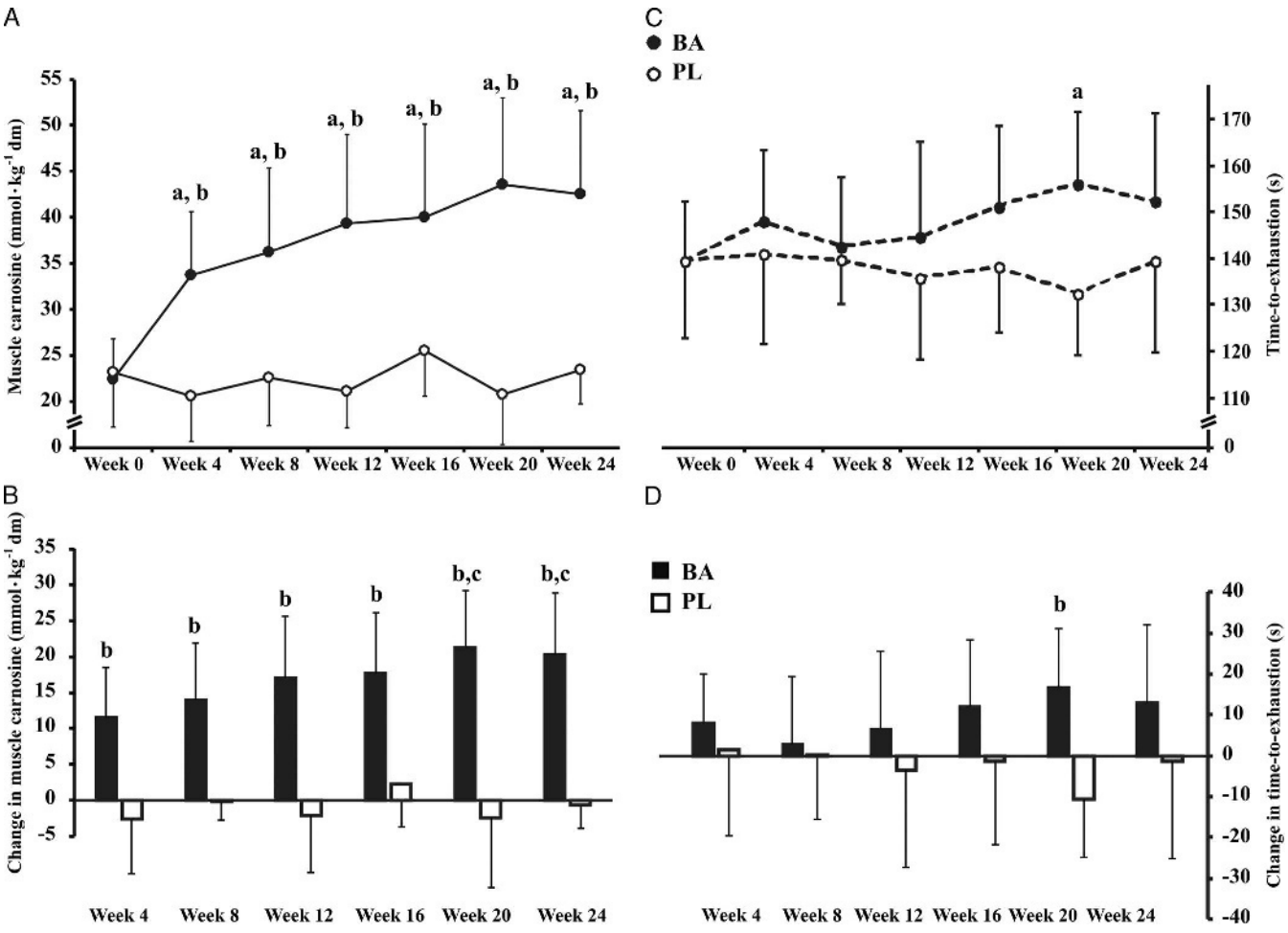

FIGURE 2—A, Muscle carnosine content throughout supplementation in BA (black circles) and PL (white circles). B, Absolute change in muscle carnosine content from week 0 in BA (black bars) and PL (white bars). C, TTE throughout supplementation in BA (black circles) and PL (white circles). D, Absolute change in TTE 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 PL at same time point. [c]$P \leq 0.05$ from weeks 4 and 8. Data are presented as mean ± 1 SD.

TABLE 1. Individual maximal muscle carnosine changes to supplementation in BA.

Participant No.	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 (wk)
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

There was a significant correlation between muscle carnosine content at week 0 and the absolute maximal content with BA ($r = 0.53$, $P = 0.04$). There were no significant correlations between initial muscle carnosine content and the delta change in carnosine at any week (all $P > 0.05$) or the delta maximal change ($r = 0.04$, $P = 0.90$).

Gene expression. There was no effect of supplement, week or any interaction effects for *CARNS*, *ABAT*, *CNDP2*, *PAT1*, *PEPT2* or *PHT1* (all $P > 0.05$). There was a significant effect of supplement ($P = 0.002$) for *TauT*, with lower values over time in BA (−36.4%, −39.4%, −27.3%, −56.8%, −46.3%, and −35.0% at weeks 4, 8, 12, 16, 20, and 24; Fig. 3), although no effect of week ($P = 0.31$) or an interaction ($P = 0.59$) was shown. There were no significant correlations between muscle carnosine content and any gene at week 0 (all $P > 0.05$).

$CCT_{110\%}$. Exercise capacity was not significantly different between BA and PL at week 0 ($P = 1.00$, Fig. 2). There was a main effect of supplement ($P = 0.05$) and an interaction effect (supplement × week, $P = 0.05$), although *post hoc* analyses only revealed week 20 to be significantly different from week 0 ($P = 0.02$, Fig. 2). TTE was increased from week 0 in BA at all time points but not in PL (Table 2). MBI showed *possible* to *almost certain* improvements across all weeks in BA compared with week 0; similarly, ES values were greater in BA versus PL at all time points (Table 2).

Four individuals in BA improved above the variation of the test (>4.9%) at every time point. A further two individuals improved exercise capacity in all but 1 wk 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 after 4 wk of supplementation and two after 8 wk. One individual's best performance was after 12 wk, three after 16 wk, four after 20 wk, 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, http://links.lww.com/MSS/A821). 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, http://links.lww.com/MSS/A821) for BA. No significant correlations 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 after exercise compared with preexercise (Table, Supplemental Digital Content 3, Blood pH, bicarbonate, base excess and lactate, http://links.lww.com/MSS/A822). 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–week interaction (all $P > 0.05$). There were no main effects of supplement, week, or supplement–week interactions for total protein or fat intake (all $P > 0.05$). The intake of BA did not differ between groups ($P = 0.525$) and was unchanged over the supplementation period ($P = 0.203$); similarly, there was no supplement–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, http://links.lww.com/MSS/A823).

DISCUSSION

This is the first study to systematically examine the effects of longer-term BA supplementation on muscle carnosine

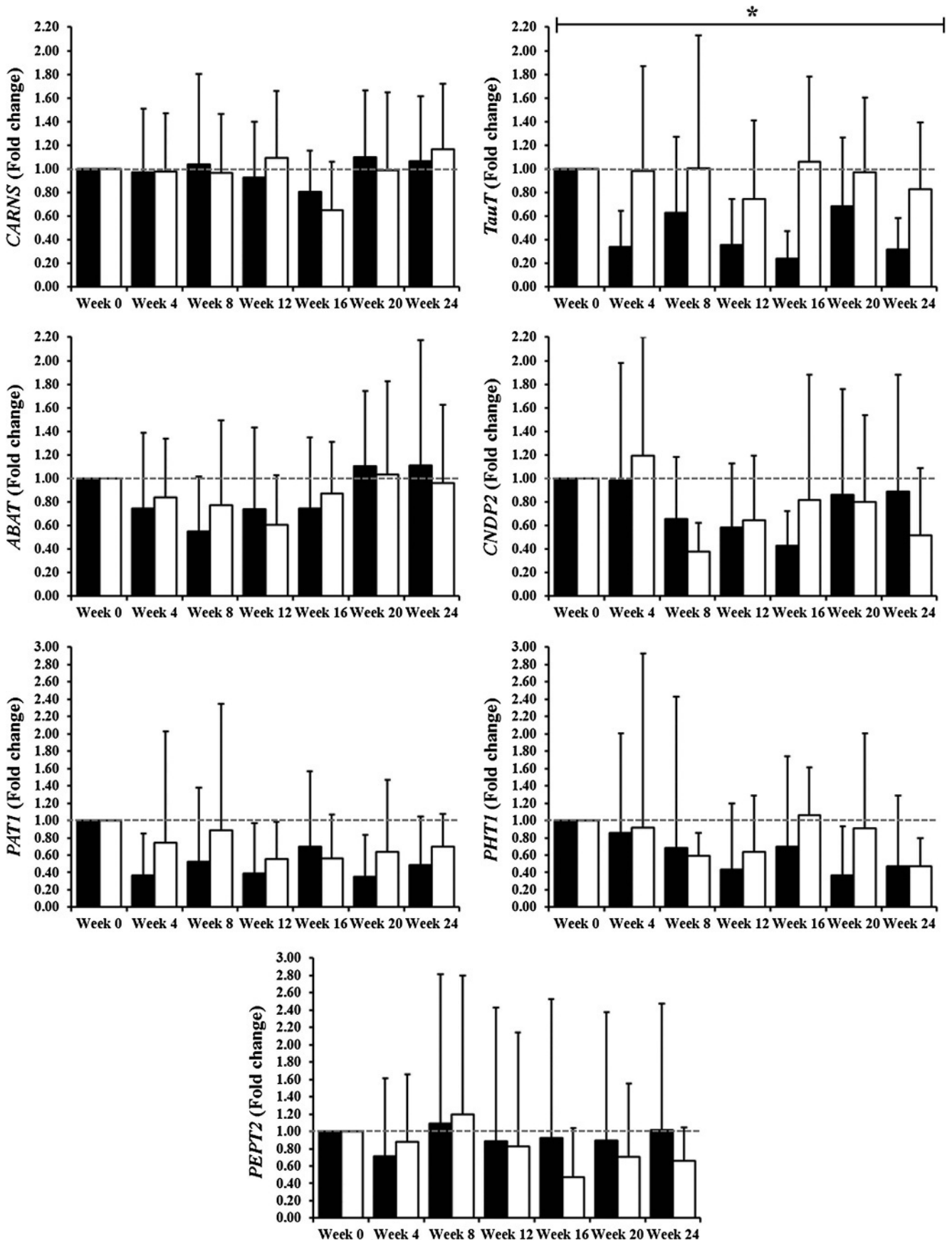

FIGURE 3—Fold change across the 24 wk for *CARNS*, *TauT*, *ABAT*, *CNDP2*, *PAT1*, *PHT1*, and *PEPT2*. *P = 0.002 Main effect of BA.

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

	TTE					
	BA			PL		
(vs Week 0)	%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

content, carnosine-related genes, and high-intensity exercise capacity at monthly intervals. The novel findings (Fig. 4) are that 24 wk of BA 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 downregulated with chronic BA supplementation. High-intensity cycling capacity was improved, with improvements associated with changes in muscle carnosine.

Muscle carnosine content increased by 55% after 4 wk, which is lower than the relative increases previously shown using HPLC analysis of muscle biopsy samples (17,19), despite the lower dose of BA used in those studies (mean = 5.2 $g \cdot d^{-1}$ [17,19]). Absolute changes in muscle carnosine at 4 wk in the present study were greater than those shown by Harris et al. (17) but identical with those of Hill et al. (19), despite that in the previous studies a slightly lower dose (5.2 vs 6.4 $g \cdot d^{-1}$) was given. The greatest absolute change in mean carnosine content occurred after 20 wk 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 after 4 wk of BA 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 fibers; low meat eaters or vegetarians). Because the contribution of carnosine to muscle buffering capacity (or indeed any suggested physiological mechanism) is dependent on 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 hypothesized that changes in muscle carnosine content would be mirrored by changes in the expression of carnosine-related genes. *TauT* was downregulated with supplementation,

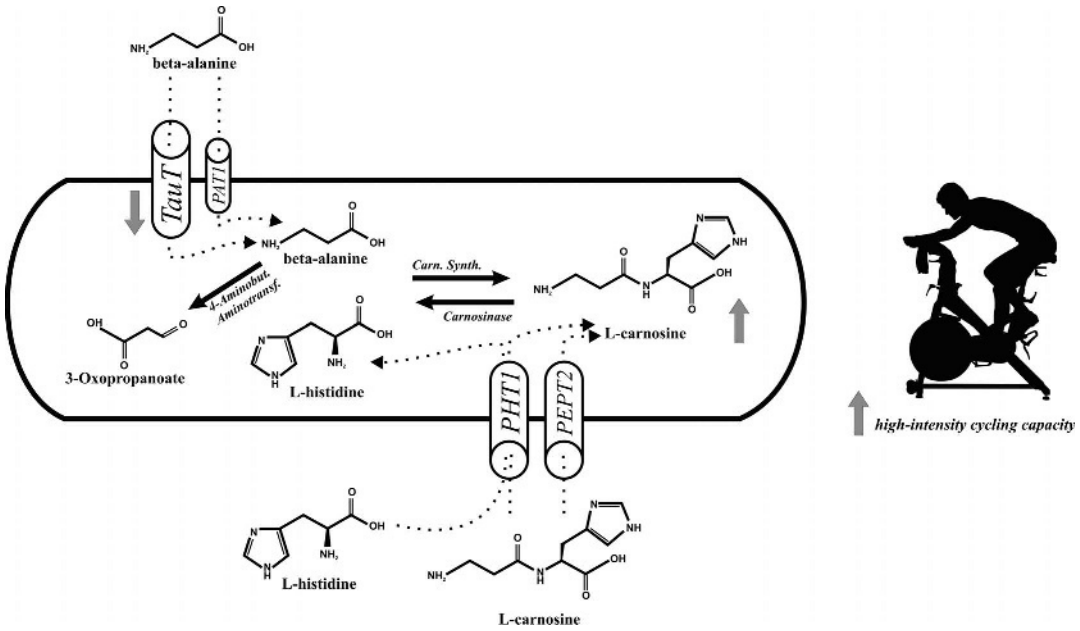

FIGURE 4—Overview of the analyses and results of the current study. There was a downregulation in the *TauT* transporter, which transports BA into muscle; the other BA transporter, *PAT1*, was unaffected. Similarly, no changes were shown in the histidine/carnosine transporters *PHT1* and *PEPT2*, in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

although no other changes in gene expression were shown. Because *TauT* is the primary transporter of BA into muscle (4), our data support the suggestion that increases in muscle carnosine may be more dependent on the transport of BA into the muscle than the activity of carnosine synthase (*CARNS* [14]) because this will directly influence the availability of BA for muscle carnosine synthesis. Decreasing the activity of *TauT* during prolonged increases in circulating BA through oral supplementation may be the body's mechanism to best maintain intramuscular homeostasis of muscle carnosine by limiting the uptake of BA into muscle. Blancquaert et al. (8) suggested that the homeostasis of muscle carnosine is tightly regulated by the transamination of circulating levels of BA 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 after BA supplementation in mice (14). However, the dose of BA that these mice received is equivalent to a supraphysiological 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 4 h after the ingestion of a dose of BA. These results are understandable given circulating levels of BA return to normal 4 h after an equivalent dose (17). A limitation of our study is that only gene expression was analyzed; posttranscriptional events may result in disparate kinetics between gene and protein expression, influencing inferences (25). Further research should ascertain whether the expression of these genes and proteins is modified in the hours after acute BA 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 wk, 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 because 5–7 d of creatine supplementation at a dose of 20 to 30 $g \cdot d^{-1}$ 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 4 wk of supplementation, the remaining participants showed maximal content during the final 12 wk 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 BA to the muscle due to increased blood flow (28), although it could also be due to a contraction-induced stimulation of *TauT* (7). Thus, an increased expression of the BA transporter (or an attenuation of its downregulation) may lead to an increased carnosine accumulation with supplementation. It remains to be determined whether muscle contraction *per se* increases the activity of BA 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 after BA supplementation. Mean muscle carnosine contents increased most in the first 4 wk, 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 suggests 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 BA 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 related to the two lower doses used in the aforementioned study (1.6 and 3.2 $g \cdot d^{-1}$), which resulted in far lower increases in muscle carnosine concentration. Thus, it appears that the uptake kinetics of muscle carnosine content may be dependent on the dose ingested.

These are the first data to show that muscle carnosine may not increase continuously until maximal content in all individuals, given that carnosine content decreased at certain time points across the 24-wk period. Interestingly, these decreases occurred despite ongoing supplementation with BA. The physiological mechanisms underpinning this response can only be speculated on but may include a downregulation of the transport of BA into the muscle cell, a reduction in the activity of the carnosine synthase enzyme, or an increased degradation of carnosine by carnosinases. These possibilities seem unlikely to explain the results of the current study, given that we only showed an effect of BA on *TauT*, although we determined the relative expression of the genes that encode their associated protein(s), which can be dependent on sampling time. Other possible explanations include the potential for experimental or analytical error, although we feel this is unlikely given the control measures that were undertaken to ensure the quality of muscle sampling, the extraction procedure, and the HPLC analysis. One other clear possibility is that the location of the muscle biopsy

contributed to the changes in muscle carnosine content across the study because of sample-to-sample differences in the amount of type I and II muscle fibers collected in the biopsy sample. Because muscle carnosine is not homogeneously distributed across muscle fibers in the vastus lateralis muscle (19), this may have resulted in variation between biopsies. It is, however, unlikely that these differences within the same mixed muscle sample would have accounted for the magnitude of the changes observed in muscle carnosine content. In addition, muscle carnosine content varied by ~17% within the PL group across 24 wk, which is similar to those shown in the gastrocnemius muscle for 9 wk (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 BA 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 BA improved exercise capacity, and MBI showed *possible* to *almost certain* improvements across all weeks with BA, with effect sizes suggesting moderate to very large effects. Similar exercise improvements have been shown using the $CCT_{110\%}$ on three independent occasions after 4 wk of BA supplementation (12–14% [11,19,30]), with further improvements after 10 wk of supplementation (~16% [19]). Thus, it was hypothesized that greater exercise improvements would be shown in the current study when supplementation was extended past 10 wk, although this was not the case. The smaller improvements shown here may have been due to large variability in exercise responses, perhaps because of 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). Because 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 suggest that 24 wk of BA supplementation improves high-intensity exercise capacity, although variability exists with several less or nonresponsive individuals. Future studies should evaluate exercise capacity with BA supplementation on multiple occasions to account for variability in exercise responses.

In conclusion, 24 wk of BA 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 BA supplementation, suggesting it plays an important role in the accumulation of muscle carnosine content during prolonged BA 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 24 wk at a high dose for all individuals, suggesting that determinants other than the availability of BA may have a major influence on muscle carnosine content.

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The results of the present study do not constitute endorsement by the American College of Sports Medicine. The authors declare that the results of the study are presented clearly, honestly, and without fabrication, falsification, or inappropriate data manipulation.

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