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The effect of β-alanine supplementation on high intensity cycling capacity in normoxia and hypoxia

--Manuscript Draft--

Full Title:	The effect of β-alanine supplementation on high intensity cycling capacity in normoxia and hypoxia
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Keywords:	Exercise capacity - Carnosine - Hypoxia - Supplementation - Buffering
Abstract:	The availability of dietary beta-alanine (BA) is the limiting factor in carnosine synthesis within human muscle due to its low intramuscular concentration and substrate affinity. Carnosine can accept hydrogen ions (H+), making it an important intramuscular buffer against exercise-induced acidosis. Metabolite accumulation rate increases when exercising in hypoxic conditions, thus an increased carnosine concentration could attenuate H+ build-up when exercising in hypoxic conditions. This study examined the effects of BA supplementation on high intensity cycling capacity in normoxia and hypoxia. In a double-blind design, nineteen males were matched into a BA group (n=10; 6.4g·d-1) or placebo group (PLA; n=9) and supplemented for 28 days, carrying out two pre- and two post-supplementation cycling capacity trials at 110% of powermax, one in normoxia and one in hypoxia (15.5% O2). Hypoxia led to a 9.1% reduction in exercise capacity, but BA supplementation had no significant effect on exercise capacity in normoxia or hypoxia (P>0.05). Blood lactate accumulation showed a significant trial x time interaction post-supplementation (P=0.016), although this was not significantly different between groups. BA supplementation did not increase high intensity cycling capacity in normoxia, nor did it improve cycling capacity in hypoxia even though exercise capacity was reduced under hypoxic conditions.
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Response to Reviewers:	Our responses have been submitted in the associated files. They are also copied here below. Dear Reviewers Many thanks for taking the time to review our manuscript. Please find below the comments you made along with our responses, which we believe have improved the manuscript. During our revisions we have come across a very recently published article looking for the mechanism of fatigue when cycling in hypoxic conditions. (Mira et al 2020 doi: 10.1249/MSS.0000000000002331). We feel that we should include a comment on this article, and so have added the following text to the discussion: It should, however, be noted that a recent study has suggested the mechanism of fatigue when exercising in a hypoxic environment may not be due to impaired motor function as a result of peripheral or central fatigue, but could be related to the hypoxia affecting areas within the brain (Mira et al. 2020). More research is warranted to determine the mechanism of fatigue in hypoxia, and whether increased muscle carnosine content via BA supplementation can lead to improved exercise capacity in hypoxia… Comments: Reviewer #1: The manuscript entitled "The effect of β-alanine supplementation on high intensity cycling capacity in normoxia vs hypoxia" investigated the influence of most likely improved intramuscular carnosine content after ~28 days of B-alanine supplementation on supramaximal cycling test until exhaustion in two distinct condition: normoxia and

hypoxia. The authors' hypothesis suggests that the supplementation of B-alanine and consequently increment on intramuscular carnosine content would mitigate the acidosis effects induced by high intensity exercise and contribute to the improvement of the cycling performance, especially in the hypoxia condition. Surprisingly, the results showed no difference on cycling performance when compared pre- and post-supplementation, as well as between B-alanine and placebo groups.
The study was conducted in a counter-balanced double-blinded experimental design, which, despite simple, perfectly fit to test the hypothesis, and that must be emphasized. Also, the manuscript is well written, denoting the authors expertise in this particular field of sport science.
The following topics highlighted here, are minor details intending to improve the manuscript quality.

Many thanks for this positive summary.

Title

I would suggest changing "normoxia vs hypoxia" to "normoxia and hypoxia". The term "versus (vs)", in my point of view, might induce to the interpretation that the normoxia condition may influenced the hypoxia condition and vice-versa, however those are two independent conditions in the present experimental design.

Yes, we agree completely and have changed the title to reflect this.

Introduction

As well as the entire manuscript, the introduction section is really well written and easy to follow the "train of thought" since it goes right to the point.

Thank you

My concern it's regarding two points that must improve this section. First, the final aim for B-alanine supplementation is improve the sport performance and despite the best results of this ergogenic aid has been verified during maximum exercises lasting 60-240 seconds, a couple of others papers has showed positive effects of B-Alanine supplementation on a wide range of sports/exercises modalities. A small note regarding this topic would strengthen the motivation to choose B-Alanine as ergogenic aid instead any other. Ergogenic Effects of β-Alanine Supplementation on Different Sports Modalities: Strong Evidence or Only Incipient Findings? Brisola GMP, Zagatto AM. J Strength Cond Res. 2019 Jan;33(1):253-282.

Thank you for this suggestion. In response, we have updated our exisiting sentence realting to the two full systematic reviews and meta analyses already cited, which both demonstrate the efficacy of B-alanine. The sentence now reads: The efficacy of BA to improve a range of high-intensity exercise outcomes, particularly those lasting between 1 and 10 minutes, indicates the potential for BA to have a positive effect over a wide range of sports/exercises modalities, and has been demonstrated using meta-analytical data (Hobson et al. 2012; Saunders et al. 2017b). Research on its efficacy in alternate environmental conditions, such as hypoxia, is, however, lacking.

My second, and more important point is about the promising findings linking B-Alanine supplementation and attenuation of neuromuscular fatigue induce by muscle acidosis, which affect the excitation-contraction coupling and cross-bridge interactions. This are in line with the authors hypothesis and I think that must be mentioned. Effect of β-alanine supplementation during high-intensity interval training on repeated sprint ability performance and neuromuscular fatigue. Milioni F, de Poli RAB, Saunders B, Gualano B, da Rocha AL, Sanchez Ramos da Silva A, Muller PTG, Zagatto AM. J Appl Physiol (1985). 2019 Dec 1;127(6):1599-1610.

Thank you for this suggestion, but we are not sure that there is direct evidence to show that B-alanine attenuates neuromuscular fatigue induced by metabolic acidosis through an influence on excitation-contraction coupling or cross-bridge interactions. This is certainly not directly shown in the suggested manuscript, where there was a lack of an effect of increased muscle carnosine content on muscle buffering capacity. In the study

	suggested, participants performed maximal voluntary contractions pre- and post-exercise before and after 4 weeks of HIIT, with and without B-alanine. The B-alanine group showed some improvements from pre- to post-supplementation that were not shown with the placebo, but there is nothing here to show that these improvements in maximum voluntary contraction were in anyway as a result of the mechanisms suggested above. Methods What was the cadence maintained during the powermax and CCT tests? We did not record cadence during the powermax or CCT tests. The exercise bike was set in a linear mode such that the resistance is automatically adjusted in line with the cadence to maintain power. Participants were fully familiarised with this setting. The rules of the tests were simply to maintain a cadence above 60rpm. If cadence fell below this then the test was stopped. Why the powermax test was not carried out at the post-test? The powermax might be different at post supplementation, affecting the results. That should be discussed. We have interpreted this reviewer query in two ways, and have therefore responded to both potential meanings. 1) Potential changes in the fitness level of the particpants per se over the supplementation period: Our participants were recreationally active, and all maintained their activity levels over the duration of the supplementation period. This was verbally checked with the participants at the post supplementation trials, and where this was not the case the participants were excluded, as detailed in the methodology. This decision was taken to avoid excessive testing and laboratory visits as the protocol already involved 7, and therefore improve participant retention. We have added the following to the relevant section in the methods: ‘Participants were asked to maintain their usual physical activity routines during the supplementation period to avoid changes in fitness; this was confirmed at the first post supplementation trial and where this had not been adhered to they were excluded from the trial as detailed above.’ 2) Potential changes in the powermax post-supplemetation due to the supplement: The workload for all the CCT110% tests was set relative to the initial powermax test. If we introduced another powermax test after the supplementation period, this would change our baseline and we would no longer be able to test our hypothesis. If the BA supplementation improved powermax, and we used the improved powermax score to reset the CCT110% workload, this would make the test harder and therefore make it less likely to see any effect of the BA, which was the reason the test would have been made harder. It essential to compare changes to the baseline in order to test our hypothesis and therefore no post-supplemetation powermax was included. What was the equipment/method used to induce hypoxia condition? This must be better described. We have added the following detail to the manuscript to clarify this. ‘the tests were performed in an environmental chamber (WIR52-20HS, Design Environmental Ltd., Gwent, Wales, U.K)’ What was the supplement used as placebo? The beta-alanine group took SR CarnoSyn® beta-alanine tablets, which are patented sustained release tablets containing beta-alanine, cellulose, and excipients. The placebo group used matched placebo tablets also manufactured by the same company, wherein beta-alanine is replaced by celluloses, with all other ingredients being the same. We have clarified this within the supplementation section of the methods.

Please, improve the description of the pills used. It was used gastro-resistant capsule or tablets?

The tablets used were SR CarnoSyn® beta-alanine tablets, which are patented sustained release tablets containing beta-alanine, cellulose and excipients. We have updated the supplementation section of the methods.

Results

Figure 3 - Since the candle stick graphics are not regularly used in sports science manuscripts, an improvement of the figure legend would benefit the readers' comprehension. Please, describe what means the box and the symbols and horizontal lines inside the box.

We have now changed the second sentence of the figure legend to read as follows: The + denotes the mean, the horizontal line within the box denotes the median, the box shows the second and third quartiles, and the ‘whiskers’ denote the full range.

Discussion

The results are well discussed and make clear the strengths and weakness of the present manuscript; however, one particular topic should be discussed deeper (despite the author already have addressed). As state by the authors the heterogeneity and fitness level of the sample were limiting factors, how beneficial would be extend the supplementation period for more than ~28 days?

We have noted the possibility that an increased supplementation period could have altered our findings. The section now reads as follows: It is unclear why our results are in contrast to these previous studies, given that our participants underwent the same supplementation regime of 6.4g·d-1 of BA as Saunders et al. (2017a) and followed similar exercise protocols to many studies showing an effect, as described above. It could be speculated that supplementation for a more prolonged period of time may have resulted in performance benefits. Indeed, Saunders et al. (2017a) supplemented participants with BA for 24 weeks and carried out performance tests and muscle biopsies every 4 weeks to determine any effects. After the first 4 weeks, muscle carnosine content increased (+11.37 ± 7.03 mmol·kg-1 dm) and exercise capacity improved (BA +5.0% vs PLA +1.8%).

Line 297 - CCT110%

Thank you for spotting the typo, we have now corrected this.

Reviewer #2:

The current manuscript investigates the influence of beta-alanine supplementation on an exercise capacity test conducted in both a normoxic and a hypoxic environment. Exercise capacity was reduced in the hypoxic condition in comparison to normoxia, but BA supplementation did not influence exercise capacity in either condition. The study addresses an interesting question, and uses an appropriate design. The null result is interesting as it contrasts with what was hypothesized based on available data, but the authors do a good job of discussing this, and I believe the publication of null results are essential to advance our estimates of the true effects of various supplementation strategies. I have made some suggestions below on aspects that could be clarified within the manuscript.

Thank you for taking the time to review our manuscript.

Line 57 - 58: While certainly acidosis may impact contractile function as indicated here, it may be interesting to highlight that it may also impact a number of other processes that may be relevant for exercise performance, e.g., reducing the rate of creatine rephosphorylation and impacting oxidative enzyme activity. Perhaps consider expanding this explanation to include these other processes?

We have added these mechanisms, with supporting references, to this section to hopefully provide a more rounded explanation.

Line 62: It would be useful to highlight here whether supplementation similarly improved performance in both conditions, or whether it exerted a greater influence in hypoxia, as may be expected based on the heightened challenge to pH regulation that a hypoxic environment may create.

We have addressed this comment by rewriting the section and including two further references to clarify the existing data. It now reads: Thus, high-intensity exercise performed in hypoxia will incur even greater metabolic stress and increase the demands on the body's buffering capacity. Nutritional supplementation methods to increase both extracellular (Flinn et al. 2014; Saunders et al. 2014a; Deb et al. 2018b; Haussirth et al. 1995) and intracellular (Saunders et al. 2014a) buffering capacity have been explored as a means to buffer exercise-induced acidosis in normoxia and/or hypoxia. Some studies showed similar performance improvements with these supplements in both hypoxia and normoxia (Deb et al., 2017; Hausswirth et al. 1995), while others showed no improvements in either condition (Flinn et al., 2014). Saunders et al. (2014) did not perform exercise in normoxia but supplementation with beta-alanine and/or sodium bicarbonate was ineffective to improve repeated sprints in hypoxia.

Line 69 - 73: I found this sentence to be quite long and a little difficult to follow and suggest rephrasing to enhance clarity.

We agree, this was very long, and we have now rewritten it for clarity. It now reads: 'Specifically, BA supplementation has been shown to be effective during high-intensity cycling capacity at 110% of previously determined peak power output (CCT110% ; Hill et al. 2007; Sale et al. 2011; Danaher et al. 2014; Saunders et al. 2017a). Such exercise results in high levels of blood lactate and reductions in blood pH (Saunders et al. 2013), likely due to increasing muscle acidosis.'

Line 104: What do you mean by a supplement disclaimer? It would be useful to be more precise on what information was obtained from the participants.

The supplement disclaimer is a document which we ask participants to sign to inform and remind them that, whilst every care is taken to source uncontaminated supplements, the individual is responsible for what supplements they consume. This is, of course, particularly pertinent in those athletes who might need to undertake a doping test, although in the case of the current study on recreational participants this is extremely unlikely to be of direct relevance. As such, we have removed reference to this document from the methods to avoid confusion.

Line 152 - 156: Although it becomes clearer later in the discussion, it is not immediately clear what the purpose of the POMS and PSQI is, and so it may be useful to clarify this within the methods

Yes, we agree an explanation would be beneficial. A sentence has been added to explain their inclusion. It reads: These questionnaires were undertaken to confirm that participants had been having similar sleep quality and were in a similar mood state for each testing session.

Line 163: The justification for undertaking both an independent sample t-test based on changes in performance in the BA and PLA groups, as well as a mixed model ANOVA was not clear to me. What information does the t-test provide that the ANOVA does not?

Thanks for pointing this out. We did all our analysis separately initially to clearly investigate the effect of the hypoxia, the effect of the BA, and then the effect of the BA in the environmental conditions. But as you rightly point out the independent sample t-test comparison was within the Mixed Model ANOVA. Therefore, we have removed reference to the independent sample t-test in the methodology and thank you for highlighting this.

	Line 246: It is not clear to me how you are defining "clearly improving" or "performing worse". How did you distinguish between typical variation and performance improvements/decrements? The identification of "responders" and "non-responders" has been the topic of much discussion recently, and is very difficult to determine in practice. Recent articles have discussed this in more detail and may be useful, e.g., Atkinson et al. 2019 (https://eur03.safelinks.protection.outlook.com/?url=https%3A%2F%2Fdoi.org%2F10.1113%2FEP087712&data=01%7C01%7Cruth.james%40ntu.ac.uk%7Cf258d90a100d476c332708d86d46e04b%7C8acbc2c5c8ed42c78169ba438a0dbe2f%7C0&sdata=LSemIbT2IyN8PtyGhO8QWft5G4m5HqV1jCPRPpRLEvg%3D&reserved=0) or Swinton et al. 2019 (https://eur03.safelinks.protection.outlook.com/?url=https%3A%2F%2Fdoi.org%2F10.3389%2Ffnut.2018.00041&data=01%7C01%7Cruth.james%40ntu.ac.uk%7Cf258d90a100d476c332708d86d46e04b%7C8acbc2c5c8ed42c78169ba438a0dbe2f%7C0&sdata=LdibbVwTQYU44XgmfZrCaJ6jSbJPXGlYdPyxqOCnq9c%3D&reserved=0). Thanks for highlighting this point; as it was not our intention to in anyway denote or define ‘responders’ or ‘non-responders’, or to apply any kind of analysis to this simple interpretation we have removed the leading/misleading terms from the manuscript. The section now reads: Indeed, Figure 2 shows the individual responses in exercise capacity to BA supplementation in our participants, however upon further examination of our data we can find no commonalities between individuals to explain our findings.

Article Title: The effect of β-alanine supplementation on high intensity cycling capacity in normoxia and hypoxia

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Conflict of interest
No funding was received directly for this study. CS has received funding to support a PhD studentship relating to the effects of carnosine on cardiac function from NAI. NAI has also provided CS with supplements for other studies free of charge and has contributed to the payment of open access publication charges for some manuscripts on beta-alanine supplementation. No other authors have a conflict of interest to declare.

Abstract
The availability of dietary beta-alanine (BA) is the limiting factor in carnosine synthesis within human muscle due to its low intramuscular concentration and substrate affinity. Carnosine can accept hydrogen ions (H^+), making it an important intramuscular buffer against exercise-induced acidosis. Metabolite accumulation rate increases when exercising in hypoxic conditions, thus an increased carnosine concentration could attenuate H^+ build-up when exercising in hypoxic conditions. This study examined the effects of BA supplementation on high intensity cycling capacity in normoxia and hypoxia. In a double-blind design, nineteen males were matched into a BA group (n=10; 6.4g·d^{-1}) or a placebo group (PLA; n=9) and supplemented for 28 days, carrying out two pre- and two post-supplementation cycling capacity trials at 110% of powermax, one in normoxia and one in hypoxia (15.5% O_2). Hypoxia led to a 9.1% reduction in exercise capacity, but BA supplementation had no significant effect on exercise capacity in normoxia or hypoxia (P>0.05). Blood lactate accumulation showed a significant trial x time interaction post-supplementation (P=0.016), although this was not significantly different between groups. BA supplementation did not increase high intensity cycling capacity in normoxia, nor did it improve cycling capacity in hypoxia even though exercise capacity was reduced under hypoxic conditions.

Keywords: Exercise capacity – Carnosine – Hypoxia – Supplementation – Buffering

Introduction

Training at high altitude has been used in many sports as it provides an ergogenic benefit to physical performance upon return to sea level (Millet et al. 2010). However, the physiological adaptations and performance benefits from this strategy require many weeks of acclimation and training (Saunders et al. 2019). Acute hypoxic conditions increase metabolic demand and trigger the anticipation of fatigue; there is an increase in heart rate and respiratory rate at rest (Burtscher et al. 2006) and an overload of the body's acid-base systems, since pCO_2 is lower than at sea level (Kayser et al. 1993). Consequently, performing exercise in these conditions poses a substantial challenge, especially for high-intensity exercise characterised by large accumulation of metabolites such as hydrogen ions (H^+), ADP and Pi in skeletal muscles. These cause a reduction in muscle pH, as well as a reduction in arterial and venous blood pH, thereby stressing the body's buffering systems. At rest, the standard intramuscular pH is around 7.0 and arterial and venous blood pH is around 7.4, however, during high-intensity exercise, muscle pH can drop to as low as 6.0 and blood pH can decline to ~7.0 (Pan et al. 1991). Reductions in pH levels during exercise can limit performance by reducing the rate of creatine rephosphorylation (Hogan et al. 1999), by negatively impacting oxidative enzyme activity (Jubrias et al. 2003) and by negatively impacting the excitation-contraction coupling mechanism and cross-bridge interactions (Knuth et al. 2006; Debold et al. 2008). Thus, high-intensity exercise performed in hypoxia will incur even greater metabolic stress and increase the demands on the body's buffering capacity. Nutritional supplementation methods to increase both extracellular (Flinn et al. 2014; Saunders et al. 2014a; Deb et al. 2018b; Haussirth et al. 1995) and intracellular (Saunders et al. 2014a) buffering capacity have been explored as a means to buffer exercise-induced acidosis in normoxia and/or hypoxia. Some studies showed similar performance improvements with these supplements in both hypoxia and normoxia (Deb et al., 2017; Hausswirth et al. 1995), while others showed no improvements in either condition (Flinn et al., 2014). Saunders et al. (2014) did not perform exercise in normoxia but supplementation with beta-alanine and/or sodium bicarbonate was ineffective to improve repeated sprints in hypoxia.

Carnosine is a naturally occurring cytoplasmic dipeptide found in many human tissues and in high concentrations within the skeletal muscle (Harris et al. 2006; Hill et al. 2007; Saunders et al. 2017a; Carvalho et al. 2018) and has the ability to accept H^+ (Smith 1938; Tanokura et al. 1976). Previous research has shown that BA supplementation can augment intramuscular carnosine content after four weeks of supplementation (Harris et al. 2006; Derave et al. 2007; Hill et al. 2007; Saunders et al. 2017a) which can improve high-intensity exercise capacity and performance (Saunders et al. 2017a). Specifically, BA supplementation has been shown to be effective during high-intensity cycling capacity at 110% of previously determined peak power output ($CCT_{110\%}$; Hill et al. 2007; Sale et al. 2011; Danaher et al. 2014; Saunders et al. 2017a). Such exercise results in high levels of blood lactate and reductions in blood pH (Saunders et al. 2013), likely due to increasing muscle acidosis. The efficacy of BA to improve a range of high-intensity exercise outcomes, particularly those lasting between 1 and 10 minutes, indicates the potential for BA to have a positive effect over a wide range of sports/exercises modalities, and has been demonstrated using meta-analytical data (Hobson et al. 2012; Saunders et al. 2017b). Research on its efficacy in alternate environmental conditions, such as hypoxia, is, however, lacking.

The literature suggests that BA supplementation has ergogenic potential to improve exercise performance under acute hypoxic conditions. Exercise in acute hypoxia leads to a reduction in oxygen at the mitochondrial level and a consequent increase in the participation of the glycolytic pathway, thereby increasing the rate of metabolite accumulation (Levine et al. 2008). It causes an earlier onset of muscle acidosis for the same exercise load compared with sea level, and the effects of acute hypoxia reduce performance by 10% of cycling peak power output and 18% of VO_{2max} (Deb et al. 2018b). The increase in H^+ when exercising in hypoxia strain the buffering systems of the body in order to maintain exercise performance. Only two studies have investigated the effects of BA on exercise in hypoxia. Saunders et al. (2014a) used a sprint based, football specific intermittent treadmill task and showed no significant effect of supplementation on 5 x 6s repeated sprint performance at simulated 2500 m (15.5% O_2). Wang et al. (2019) showed that BA supplementation maintained anaerobic working capacity in normobaric hypoxia, but it did not attenuate the onset of fatigue nor enhance repeated sprint performance. However, as indicated by meta-analyses on the topic (Hobson et al. 2012; Saunders et al. 2017b), BA supplementation does not influence performance in exercise tasks lasting <60s. The $CCT_{110\%}$ has been used

extensively in previous BA studies (Hill et al. 2007; Sale et al. 2011; Danaher et al. 2014; Saunders et al. 2017a) and been shown to be malleable to improvements from increased buffering capacity. Furthermore, the $CCT_{110\%}$ is an exercise test of the type and duration which appears to be influenced by acute exposure to hypoxia (Deb et al. 2018a).

Therefore, the aim of the current study was to examine the effects of BA supplementation on high intensity cycling capacity, using the $CCT_{110\%}$, in normoxia and hypoxia. It was hypothesised that BA supplementation would improve high intensity cycling capacity in normoxia and hypoxia, with greater improvements in hypoxia.

Methods

Participants

Twenty-two recreationally active males volunteered to take part in this study (age: 21 ± 2 y, height: 1.78 ± 0.07 m, body mass: 74.40 ± 11.47 kg, Mean ± SD). Three participants were excluded due to injury, illness and difficulties in data collection. Ethical approval for the study was granted by the Nottingham Trent University Human Invasive Ethics Committee (application number: 342). Prior to testing, participants completed a health screen and provided written informed consent. Participants were excluded from the trial if they had surgical history or pain in the lower limbs, were vegan/vegetarian, or had consumed BA supplements within the last three months. Participants were asked to refrain from vigorous exercise and alcohol consumption for 24 hours prior to each trial, which were separated by a minimum of 24 hours and took place at the same time of day. Participants were asked to complete a food diary 24 hours prior to their first trial and to replicate their food and fluid intake prior to subsequent trials. A standardised breakfast, consisting of a cereal bar and a sports drink (total energy content 270 kcal), was also provided for participants to consume two hours prior to attending a trial. Participants were asked to maintain their usual physical activity routines during the supplementation period to avoid changes in fitness; this was confirmed at the first post supplementation trial and where this had not been adhered to, they were excluded from the trial as detailed above.

Table 1. Participant characteristics. Mean (SD).

	BA (n = 10)	PLA (n = 9)	*P*
Age (y)	21 (3)	20 (1)	0.114
Height (m)	1.80 (0.08)	1.75 (0.04)	0.355
Body Mass (kg)	73.77 (11.02)	75.09 (12.57)	0.779
Powermax (W)	279 (66)	267 (40)	0.279
Supplement Compliance (%)	99 (2)	95 (6)	0.001
Total supplement consumed (g)	189.7 (4.0)	182.9 (12.0)	0.001

Experimental design

Participants carried out seven separate trials: 1 powermax test, 2 familiarisation $CCT_{110\%}$ trials (normoxia followed by hypoxia), and 4 main trials; 2 pre-supplementation $CCT_{110\%}$ and 2 post-supplementation $CCT_{110\%}$ trials. During the four main $CCT_{110\%}$ trials, chamber conditions were randomised and carried out in a single blind, crossover and counterbalanced design. For the allocation to BA or PLA supplementation groups, subjects were pair-matched for body mass and Powermax, where the first participant of each pair was randomly allocated to one supplement group, with their pair-match being subsequently allocated to the other group (see Table 1). This process was done by only the lead investigator to maintain the double-blind nature of the study.

Experimental protocol

Powermax test. Using a Lode cycle ergometer (Lode B.V., Groningen, The Netherlands) participants were asked to cycle to exhaustion with exercise intensity increasing by 6W every 15s. Exhaustion was defined as when the participant could no longer continue to cycle above 60 rpm despite verbal encouragement from the experimenter. This test was used to determine the relative exercise intensity for participants in the $CCT_{110\%}$ trials.

$CCT_{110\%}$. The reliability of a $CCT_{110\%}$ was assessed by Saunders et al. (2013) who showed the test to be a reliable measurement of performance in recreationally active males (CV of 4.43% for TTE) and suited to a nutritional intervention study. In the current study, the tests were performed in an environmental chamber (WIR52-20HS, Design Environmental Ltd., Gwent, Wales, U.K), in both normoxic (20.9% O_2, 50% humidity and 18 °C) and hypoxic conditions (15.5% O_2, 50% humidity and 18 °C). Participants first rested in the chamber for 10 min before carrying out a 5 min warm-up working at 60% of age predicted max heart rate. The $CCT_{110\%}$ began by first starting at 80% for 15s, followed by 95% for 15s and finally reaching 110%, which was then maintained until exhaustion, again defined as when a participant could no longer cycle above 60rpm. Heart rate (HR; Polar heart rate monitor, Kempele, Finland) and rating of perceived exertion (RPE; Borg 1982) were recorded every minute of the test and immediately upon completion. Finger prick capillary blood samples (70µL) were taken at rest (before the warm-up), immediately after and 5 min after the $CCT_{110\%}$ and analysed for blood pH, blood lactate concentration, base excess and bicarbonate (HCO_3^-), using a blood gas analyser (ABL FLEX 90, Radiometer, Ireland).

Supplementation. Supplementation with either daily BA (patented sustained release tablets (Carnosyn^{SR}, Natural Alternatives International, USA) 6.4 $g \cdot d^{-1}$) or PLA (matched with the CarnoSyn^{SR} tablets, with the BA replaced by celluloses) commenced once a participant completed their last pre- $CCT_{110\%}$ trial. Supplementation was provided in the form of pills and the dosage required participants to take two tablets, four times per day. Supplementation lasted for 29 ± 1 day before the first post-trial commenced. Participants recorded their intake on a supplementation log and were contacted on a weekly basis to try to optimise compliance. Participants continued to supplement up to the last post-trial and returned any left-over pills, along with their supplementation log so that supplement compliance could be calculated (see Table 1).

Questionnaires. The Profile of Moods State questionnaire (POMS; McNair et al. 1971) was used to evaluate the mood of the volunteers prior to every trial. It is composed of 65 clauses rated as "Not at All", "A Little", "Moderately", "Quite a Lot" or "Extremely", which is then summed to give the outcome of Total Mood Disturbance. The Pittsburgh sleep quality index (PSQI; Buysse et al. 1989) was used to assess the quality of sleep by global PSQI score. These questionnaires were undertaken to confirm that participants had been having similar sleep quality and were in a similar mood state for each testing session.

Statistical analysis

The Statistical Package for Social Sciences (IBM SPSS Statistics 24) was used to analyse the data collected. Data were first checked for normality of distribution using the Shapiro-Wilk test and all data were shown to be normal. A paired-samples t-test was executed to examine any differences between pre-normoxic and pre-hypoxic $CCT_{110\%}$ trials, to determine the effect of the environmental conditions on participants exercise capacity. Mixed model ANOVA's were conducted to determine whether or not there were differences in exercise capacity, relevant blood markers and questionnaires (POMS and PSQI) pre- to post-supplementation, between groups, and across environmental conditions. Statistical significance was accepted at the $P \leq 0.05$ level. Effect size was calculated using Hedges g for small sample sizes, and effect sizes of about 0.20, 0.5, and 0.8 are considered small, medium, and large, respectively. All data are presented as mean ± 1SD.

Results

Performance analysis

There was a significant reduction in exercise capacity in hypoxia compared to normoxia pre-supplementation ($P < 0.001$; Effect Size -0.85; **Figure 1**). There was no significant difference in ΔTTE between BA and PLA, *i.e.* the effect of BA supplementation ($P = 0.387$; Effect Size 0.05; **Figure 2**). The ANOVA showed no significant effect of BA on TTE in hypoxic or normoxic conditions(Time, $P = 0.578$; Group x Condition x Time, $P = 0.747$; Group x Condition, $P = 0.566$; Condition x Time, $P = 0.506$; Effect Sizes BA hypoxia -0.02, BA normoxia -0.12, PLA hypoxia 0.05, PLA normoxia -0.24; **Figure 3**).

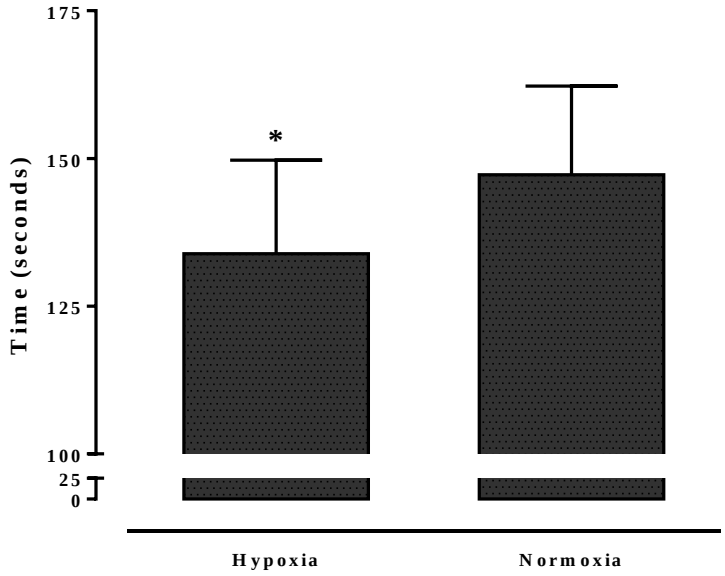

Fig. 1. Comparison of TTE between pre-supplementation hypoxic and normoxic $CCT_{110\%}$ trials. Mean ± SD are shown. * denotes a significant difference between pre-supplementation trials at $P < 0.001$.

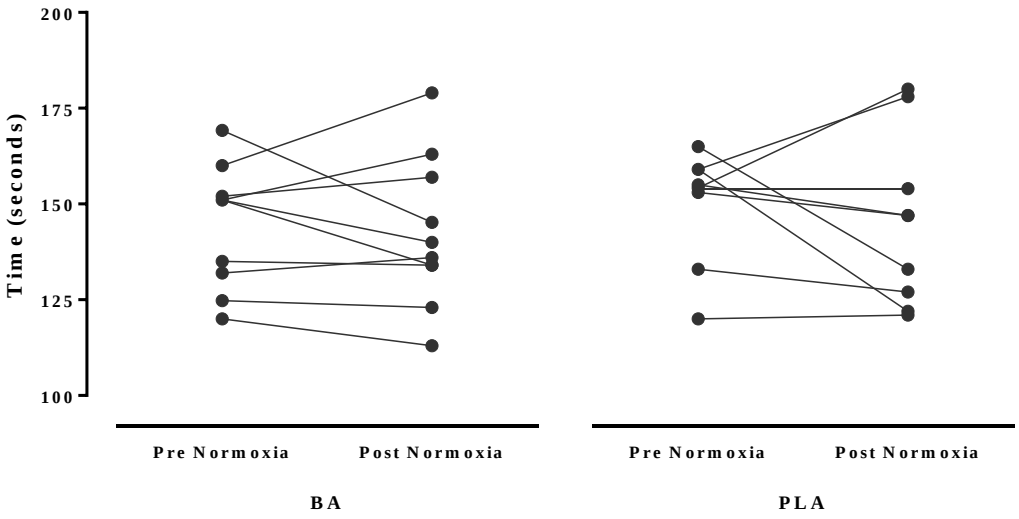

Fig. 2. Individual participants comparison between pre-normoxic and post-normoxic $CCT_{110\%}$ trials in BA group (grey) and PLA group (black). Mean ± SD are shown.

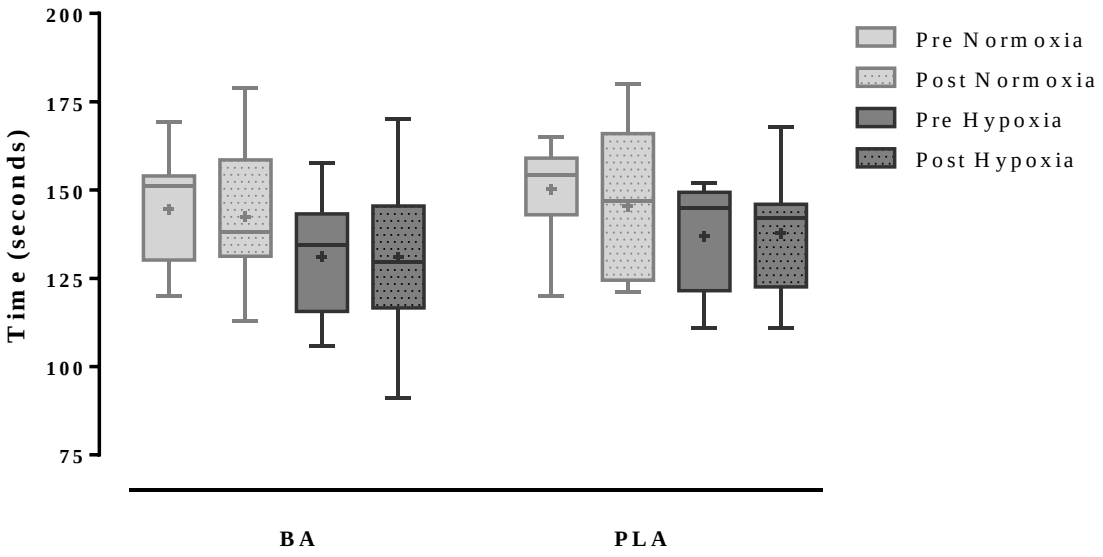

Fig. 3. TTE in $CCT_{110\%}$ hypoxic and normoxic trials pre- and post-supplementation, subdivided by supplementation groups (BA and PLA). The + denotes the mean, the horizontal line within the box denotes the median, the box shows the interquartile range, and the 'whiskers' denote the full range.

Blood analysis

There was no interaction effect of supplementation on blood pH (Group x Trial x Time, $P = 1.000$), lactate (Group x Trial x Time, $P = 0.437$), base excess (Group x Trial x Time, $P = 0.358$) or HCO_3^- (Group x Trial x Time, $P = 0.400$) during exercise across trials. There was no Group x Trial effect for pH, lactate, base excess and HCO_3^- (all $P > 0.05$). There was no Trial effect throughout exercise on pH, base excess or HCO_3^- (Trial x Time, all $P > 0.05$). A significant Trial x Time interaction ($P = 0.016$) and a significant main effect of Trial ($P < 0.001$) were shown for lactate. Blood pH, lactate, base excess and HCO_3^- all showed a significant main effect of time ($P \leq 0.001$; Table 2).

Table. 2. Blood pH, lactate, base excess (BE) and HCO_3^- during pre- and post- BA and PLA supplementation $CCT_{110\%}$ trials. Mean (SD).

	Pre-Hypoxia			Post-Hypoxia			Pre-Normoxia			Post-Normoxia		
	Before	After	+5min	Before	After	+5min	Before	After	+5min	Before	After	+5min
pH												
BA	7.41 (0.02)	7.28 (0.02)	7.21 (0.04)	7.42 (0.02)	7.29 (0.05)	7.23 (0.04)	7.40 (0.03)	7.25 (0.03)	7.21 (0.03)	7.39 (0.02)	7.25 (0.04)	7.21 (0.05)
PLA	7.41 (0.03)	7.25 (0.04)	7.19 (0.05)	7.41 (0.02)	7.23 (0.04)	7.20 (0.05)	7.41 (0.01)	7.24 (0.04)	7.19 (0.06)	7.40 (0.02)	7.23 (0.03)	7.20 (0.04)
Lac												
BA	2.07 (0.49)	13.29 (1.88)	15.81 (2.45)	1.63 (0.33)	11.17 (2.04)	13.08 (3.43)	2.01 (0.41)	13.61 (1.94)	16.69 (2.00)	1.49 (0.41)	10.51 (2.08)	13.65 (2.58)
PLA	1.78 (0.37)	12.79 (2.98)	15.88 (3.67)	1.54 (0.29)	12.27 (1.81)	13.50 (3.28)	1.96 (0.39)	13.2 (2.53)	16.62 (3.68)	1.43 (0.38)	12.46 (2.73)	14.73 (3.66)
BE												
BA	1.30	-9.45	-14.87	1.58	-8.85	-14.22	1.13	-10.42	-15.63	0.96	-10.05	-15.21

	(1.13)	(1.72)	(2.06)	(1.37)	(2.73)	(2.34)	(1.45)	(1.53)	(1.92)	(1.25)	(2.63)	(3.30)
PLA	0.94	-10.30	-15.39	1.56	-11.70	-15.24	1.77	-10.09	-15.86	1.2	-10.80	-15.39
	(1.33)	(3.88)	(4.25)	(1.23)	(2.46)	(3.36)	(1.64)	(2.79)	(3.95)	(1.36)	(2.40)	(2.94)
HCO_3^-												
BA	25.20	17.59	14.45	25.49	17.98	14.82	25.05	16.98	14.10	24.89	17.16	14.28
	(0.84)	(1.09)	(1.26)	(1.07)	(1.84)	(1.45)	(1.05)	(1.03)	(1.12)	(0.81)	(1.67)	(1.88)
PLA	24.91	16.92	14.04	25.34	16.07	14.16	25.59	17.04	13.89	25.28	16.60	14.08
	(0.92)	(2.22)	(2.22)	(0.96)	(1.47)	(1.89)	(1.20)	(1.66)	(2.18)	(1.06)	(1.43)	(1.62)

HR, RPE and questionnaires

A mixed model ANOVA was used to analyse HR and RPE data collected at exhaustion. There were no interaction effects of supplementation across trial conditions throughout exercise on HR (Group x Condition x Time, P = 0.405; Group x Condition, P = 0.168; Condition x Time, P = 0.824) or RPE (Group x Condition x Time, P = 0.175; Group x Condition, P = 0.175; Condition x Time, P = 0.941). There was no significant main effect of time on HR (P = 0.161) or RPE (P = 0.175). There was also no significant main effect of condition on HR (P = 0.235) or RPE (P = 0.941). Analysis of POMS and PSQI did not show any main effects (all P values > 0.05; Table 3).

Table 3. Final HR, RPE, POMS and PSQI data during pre- and post- BA and PLA supplementation $CCT_{110\%}$ trials. Mean (SD).

	Pre-Hypoxia	**Post-Hypoxia**	**Pre-Normoxia**	**Post-Normoxia**
HR				
BA	185 (10)	186 (12)	184 (13)	184 (10)
PLA	186 (11)	185 (8)	183 (10)	184 (11)
RPE				
BA	20.0 (0.0)	20.0 (0.0)	19.8 (0.6)	20.0 (0.0)
PLA	20.0 (0.0)	19.8 (0.7)	20.0 (0.0)	20.0 (0.0)
POMS				
BA	0.1 (11.9)	4.2 (11.6)	0.3 (12.5)	3.8 (12.6)
PLA	12.8 (28.3)	16.4 (28.3)	14.0 (20.3)	17.2 (29.4)
PSQI				
BA	4.6 (2.9)	3.8 (2.3)	5.0 (2.5)	4.2 (2.9)
PLA	4.1 (2.1)	3.7 (3.1)	3.6 (2.2)	3.9 (3.4)

Discussion

This is the first study to investigate effects of BA supplementation on high intensity cycling capacity in hypoxia when compared with normoxia. The hypothesis was that BA supplementation would improve high intensity cycling capacity in both normoxia and hypoxia, with greater improvements in hypoxia. Our findings suggest, however, that BA supplementation had no ergogenic effect on TTE in normoxia or hypoxia, contrary to our hypothesis.

These main findings, particularly those showing no significant effect of BA supplementation on high-intensity cycling capacity in normoxia, go against the results of previous studies using the same exercise protocol (Hill et al. 2007; Sale et al. 2011; Danaher et al. 2014; Saunders et al. 2017a). Our results do, however, concur with Gross et al. (2014), who also showed that BA supplementation did not affect high intensity cycling performance. Our supplementation protocol was similar to that of previous studies that have shown increases of around 60% in intramuscular carnosine content (Harris et al. 2006) following BA supplementation and that this improves exercise capacity (Hill et al. 2007; Sale et al. 2011; Danaher et al. 2014; Saunders et al. 2017a). It is unclear why our results

are in contrast to these previous studies, given that our participants underwent the same supplementation regime of 6.4g·d^{-1} of BA as Saunders et al. (2017a) and followed similar exercise protocols to many studies showing an effect, as described above. It could be speculated that supplementation for a more prolonged period of time may have resulted in performance benefits. Indeed, Saunders et al. (2017a) supplemented participants with BA for 24 weeks and carried out performance tests and muscle biopsies every 4 weeks to determine any effects. After the first 4 weeks, muscle carnosine content increased (+11.37 ± 7.03 mmol·kg^{-1} dm) and exercise capacity improved (BA +5.0% *vs* PLA +1.8%). Upon closer inspection, however, *post hoc* analyses revealed that exercise capacity was only significantly improved at week 20 compared to week 0, coinciding with peak muscle carnosine content. Since Saunders et al. (2017a) showed a large variability in muscle carnosine increases with BA supplementation, it could be speculated that not all individuals in our study increased muscle carnosine content to a sufficient degree to improve exercise capacity. Indeed, Figure 2 shows the individual responses in exercise capacity to BA supplementation in our participants, however upon further examination of our data we can find no commonalities between individuals to explain our findings. Further research is necessary to elucidate the minimum increase in muscle carnosine necessary to impact exercise capacity or performance.

The $CCT_{110\%}$ has previously been shown to be susceptible to changes in buffering capacity via BA (Hill et al. 2007; Sale et al. 2011; Danaher et al. 2014; Saunders et al. 2017a) or sodium bicarbonate supplementation (Saunders et al. 2014b; Dias et al. 2015). Interestingly, Dias et al. (2015) showed that performance improvements with supplementation were inconsistent across four separate occasions, despite the mechanism for improvement (blood alkalosis) always being present. The physically active, but not highly trained, population recruited for this study and by Dias et al. (2015) may have also contributed to the results; less well-trained individuals generally display greater variation in performance during exercise tests, rendering it more difficult to determine the true effect of the intervention. It is also possible that the exercise response could be influenced by factors beyond buffering capacity, such as mood (McNair et al. 1971), sleep quality (Buysse et al. 1989) and nutrition (Burke et al. 2019; Stecker et al. 2019). In the current study, however, neither mood state nor sleep quality were likely to have influenced the outcome of the study, given that we accounted for these factors using pre-validated questionnaires, and no differences between the test conditions were recorded in either parameter. Participants were also asked to standardise their diet and physical activity for 24h prior to each test and adherence was verbally confirmed at each testing session. Participants were provided a standardised breakfast 2h prior to all testing sessions, which differs from several (Danaher et al. 2014; Sale et al. 2011), though not all (Saunders et al. 2017a) previous BA supplementation studies employing the CCT110% exercise test. Since this was standardised prior to all testing sessions it is unlikely to influence the exercise response between trials.

Hypoxia resulted in a 9.1% decrease in cycling capacity compared to normoxia, as supported in the wider literature (Deb et al. 2018a). Interestingly, exercise in hypoxia led to a similar reduction in blood pH, bicarbonate and base excess, and increase in blood lactate, as following exercise in normoxia, despite the significantly shorter exercise duration. This demonstrates the greater requirement for energy supply from anaerobic sources in hypoxia, leading to an earlier attainment of critical levels of metabolites resulting in fatigue (Kent-Braun et al. 2012). Given that this was the basis for our hypothesis (that increases in the muscle carnosine content through BA supplementation would result in significantly improved cycling capacity in hypoxic conditions compared with normoxic conditions) it is somewhat surprising that our results showed no significant effects on cycling capacity or on blood responses. Similarly, Saunders et al. (2014a) showed no effect of BA supplementation on repeated sprints in hypoxia, while Wang et al. (2019) showed no additional benefits of BA to a repeated-sprint training protocol performed in hypoxia. In contrast to the current study however, those previous studies employed exercise tests that were unlikely to be significantly influenced by BA supplementation (Hobson et al. 2012; Saunders et al. 2017b). As it stands, there is little evidence to suggest that the benefits of BA supplementation shown by several authors under normal conditions (*i.e.*, normoxia), are evident when exercise is performed in hypoxia. It should, however, be noted that a recent study has suggested the mechanism of fatigue when exercising in a hypoxic environment may not be due to impaired motor function as a result of peripheral or central fatigue, but could be related to the hypoxia affecting areas within the brain (Mira et al. 2020). More research is warranted to determine the mechanism of fatigue in hypoxia, and whether increased muscle carnosine content via BA supplementation

can lead to improved exercise capacity in hypoxia and whether these changes are similar or greater than those shown in normoxia.

A limitation of the current study was that intramuscular carnosine content was not measured, which could have provided insight into the effectiveness of BA supplementation on elevating muscle carnosine content. Our participants ingested ~190 g of BA, similar to previous work consistently showing increases of approximately 10 mmol·kg^{-1}dm or a 60% increase (Harris et al. 2006; Hill et al. 2007). The majority of previous literature shows muscle carnosine content increases post BA supplementation (Harris et al. 2006; Derave et al. 2007; Hill et al. 2007; Saunders et al. 2017a). Indeed, a recent meta-analysis showed that 99.3% of individuals increase muscle carnosine concentration with BA supplementation (Rezende et al. 2019). These results suggest that, although intramuscular carnosine content was not measured herein, it would have almost certainly been elevated post-supplementation. Another possible limitation is the use of recreationally trained individuals, who display a greater variance in exercise test performance than elite athletes, although the CCT110% is a reliable exercise test in recreationally active men (CV 4.43%; Saunders et al. 2013). Another experimental consideration, though not necessarily a limitation, was the recruitment of participants with a diverse range of different ethinic backgrounds. Within the BA group of the current study there were three participants of Indian heritage, five Caucasians, one Hispanic and one mixed race participant. The PLA group consisted of two participants of Indian heritage, four Caucasians, one Afro-Caribbean, and two mixed race participants. There is no suggestion from our data that any one ethnic group responded differently to supplementation compared to the others, although given the potential racial differences in relevant physiological traits, such as muscle fibre type distribution (Ceaser & Hunter, 2015) and possibly, by implication, the demand upon physicochemical buffering, there is justification for future research in this area.

In conclusion, exercise capacity was negatively influenced by hypoxia compared to normoxia, but 4 weeks of supplementation with BA had no effect on high intensity cycling capacity in either condition.

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Dear Reviewers

Many thanks for taking the time to review our manuscript. Please find below the comments you made along with our responses (in red for ease), which we believe have improved the manuscript.

During our revisions we have come across a very recently published article looking for the mechanism of fatigue when cycling in hypoxic conditions. (Mira et al 2020 doi: 10.1249/MSS.0000000000002331). We feel that we should include a comment on this article, and so have added the following text to the discussion: It should, however, be noted that a recent study has suggested the mechanism of fatigue when exercising in a hypoxic environment may not be due to impaired motor function as a result of peripheral or central fatigue, but could be related to the hypoxia affecting areas within the brain (Mira et al. 2020). More research is warranted to determine the mechanism of fatigue in hypoxia, and whether increased muscle carnosine content via BA supplementation can lead to improved exercise capacity in hypoxia…

Comments:

Reviewer #1:
The manuscript entitled "The effect of β-alanine supplementation on high intensity cycling capacity in normoxia vs hypoxia" investigated the influence of most likely improved intramuscular carnosine content after ~28 days of B-alanine supplementation on supramaximal cycling test until exhaustion in two distinct condition: normoxia and hypoxia. The authors' hypothesis suggests that the supplementation of B-alanine and consequently increment on intramuscular carnosine content would mitigate the acidosis effects induced by high intensity exercise and contribute to the improvement of the cycling performance, especially in the hypoxia condition. Surprisingly, the results showed no difference on cycling performance when compared pre- and post-supplementation, as well as between B-alanine and placebo groups.
The study was conducted in a counter-balanced double-blinded experimental design, which, despite simple, perfectly fit to test the hypothesis, and that must be emphasized. Also, the manuscript is well written, denoting the authors expertise in this particular field of sport science.
The following topics highlighted here, are minor details intending to improve the manuscript quality.
Many thanks for this positive summary.

Title
I would suggest changing "normoxia vs hypoxia" to "normoxia and hypoxia". The term "versus (vs)", in my point of view, might induce to the interpretation that the normoxia condition may influenced the hypoxia condition and vice-versa, however those are two independent conditions in the present experimental design.
Yes, we agree completely and have changed the title to reflect this.

Introduction
As well as the entire manuscript, the introduction section is really well written and easy to follow the "train of thought" since it goes right to the point.
Thank you

My concern it's regarding two points that must improve this section. First, the final aim for B-alanine supplementation is improve the sport performance and despite the best results of this ergogenic aid has been verified during maximum exercises lasting 60-240 seconds, a couple of others papers has showed positive effects of B-Alanine supplementation on a wide range of sports/exercises modalities. A small note regarding this topic would strengthen the motivation to choose B-Alanine as ergogenic aid instead any other. Ergogenic Effects of β-Alanine Supplementation on Different Sports

Modalities: Strong Evidence or Only Incipient Findings? Brisola GMP, Zagatto AM. J Strength Cond Res. 2019 Jan;33(1):253-282.

Thank you for this suggestion. In response, we have updated our exisiting sentence realting to the two full systematic reviews and meta analyses already cited, which both demonstrate the efficacy of B-alanine. The sentence now reads: The efficacy of BA to improve a range of high-intensity exercise outcomes, particularly those lasting between 1 and 10 minutes, indicates the potential for BA to have a positive effect over a wide range of sports/exercises modalities, and has been demonstrated using meta-analytical data (Hobson et al. 2012; Saunders et al. 2017b). Research on its efficacy in alternate environmental conditions, such as hypoxia, is, however, lacking.

My second, and more important point is about the promising findings linking B-Alanine supplementation and attenuation of neuromuscular fatigue induce by muscle acidosis, which affect the excitation-contraction coupling and cross-bridge interactions. This are in line with the authors hypothesis and I think that must be mentioned. Effect of β-alanine supplementation during high-intensity interval training on repeated sprint ability performance and neuromuscular fatigue. Milioni F, de Poli RAB, Saunders B, Gualano B, da Rocha AL, Sanchez Ramos da Silva A, Muller PTG, Zagatto AM. J Appl Physiol (1985). 2019 Dec 1;127(6):1599-1610.

Thank you for this suggestion, but we are not sure that there is direct evidence to show that B-alanine attenuates neuromuscular fatigue induced by metabolic acidosis through an influence on excitation-contraction coupling or cross-bridge interactions. This is certainly not directly shown in the suggested manuscript, where there was a lack of an effect of increased muscle carnosine content on muscle buffering capacity. In the study suggested, participants performed maximal voluntary contractions pre- and post-exercise before and after 4 weeks of HIIT, with and without B-alanine. The B-alanine group showed some improvements from pre- to post-supplementation that were not shown with the placebo, but there is nothing here to show that these improvements in maximum voluntary contraction were in anyway as a result of the mechanisms suggested above.

Methods

What was the cadence maintained during the powermax and CCT tests?

We did not record cadence during the powermax or CCT tests. The exercise bike was set in a linear mode such that the resistance is automatically adjusted in line with the cadence to maintain power. Participants were fully familiarised with this setting. The rules of the tests were simply to maintain a cadence above 60rpm. If cadence fell below this then the test was stopped.

Why the powermax test was not carried out at the post-test? The powermax might be different at post supplementation, affecting the results. That should be discussed.

We have interpreted this reviewer query in two ways, and have therefore responded to both potential meanings.

1) Potential changes in the fitness level of the particpants *per se* over the supplementation period*:*

Our participants were recreationally active, and all maintained their activity levels over the duration of the supplementation period. This was verbally checked with the participants at the post supplementation trials, and where this was not the case the participants were excluded, as detailed in the methodology. This decision was taken to avoid excessive testing and laboratory visits as the protocol already involved 7, and therefore improve participant retention. We have added the following to the relevant section in the methods: 'Participants were asked to maintain their usual physical activity routines during the supplementation period to avoid changes in fitness; this was confirmed at the first post supplementation trial and where this had not been adhered to they were excluded from the trial as detailed above.'

2) Potential changes in the powermax post-supplemetation due to the supplement:

The workload for all the CCT110% tests was set relative to the initial powermax test. If we introduced another powermax test after the supplementation period, this would change our baseline and we would no longer be able to test our hypothesis. If the BA supplementation improved powermax, and we used the improved powermax score to reset the CCT110% workload, this would make the test harder and therefore make it less likely to see any effect of the BA, which was the reason the test would have been made harder. It essential to compare changes to the baseline in order to test our hypothesis and therefore no post-supplemetation powermax was included.

What was the equipment/method used to induce hypoxia condition? This must be better described.
We have added the following detail to the manuscript to clarify this. 'the tests were performed in an environmental chamber (WIR52-20HS, Design Environmental Ltd., Gwent, Wales, U.K)'

What was the supplement used as placebo?
The beta-alanine group took SR CarnoSyn® beta-alanine tablets, which are patented sustained release tablets containing beta-alanine, cellulose, and excipients. The placebo group used matched placebo tablets also manufactured by the same company, wherein beta-alanine is replaced by celluloses, with all other ingredients being the same. We have clarified this within the supplementation section of the methods.

Please, improve the description of the pills used. It was used gastro-resistant capsule or tablets?

The tablets used were SR CarnoSyn® beta-alanine tablets, which are patented sustained release tablets containing beta-alanine, cellulose and excipients. We have updated the supplementation section of the methods.

Results
Figure 3 - Since the candle stick graphics are not regularly used in sports science manuscripts, an improvement of the figure legend would benefit the readers' comprehension. Please, describe what means the box and the symbols and horizontal lines inside the box.
We have now changed the second sentence of the figure legend to read as follows: The + denotes the mean, the horizontal line within the box denotes the median, the box shows the second and third quartiles, and the 'whiskers' denote the full range.

Discussion
The results are well discussed and make clear the strengths and weakness of the present manuscript; however, one particular topic should be discussed deeper (despite the author already have addressed). As state by the authors the heterogeneity and fitness level of the sample were limiting factors, how beneficial would be extend the supplementation period for more than ~28 days?
We have noted the possibility that an increased supplementation period could have altered our findings. The section now reads as follows: It is unclear why our results are in contrast to these previous studies, given that our participants underwent the same supplementation regime of 6.4g·d^{-1} of BA as Saunders et al. (2017a) and followed similar exercise protocols to many studies showing an effect, as described above. It could be speculated that supplementation for a more prolonged period of time may have resulted in performance benefits. Indeed, Saunders et al. (2017a) supplemented participants with BA for 24 weeks and carried out performance tests and muscle biopsies every 4 weeks to determine any effects. After the first 4 weeks, muscle carnosine content increased (+11.37 ± 7.03 mmol·kg^{-1} dm) and exercise capacity improved (BA +5.0% *vs* PLA +1.8%).

Line 297 - CCT110%
Thank you for spotting the typo, we have now corrected this.

Reviewer #2:

The current manuscript investigates the influence of beta-alanine supplementation on an exercise capacity test conducted in both a normoxic and a hypoxic environment. Exercise capacity was reduced in the hypoxic condition in comparison to normoxia, but BA supplementation did not influence exercise capacity in either condition. The study addresses an interesting question, and uses an appropriate design. The null result is interesting as it contrasts with what was hypothesized based on available data, but the authors do a good job of discussing this, and I believe the publication of null results are essential to advance our estimates of the true effects of various supplementation strategies. I have made some suggestions below on aspects that could be clarified within the manuscript.

Thank you for taking the time to review our manuscript.

Line 57 - 58: While certainly acidosis may impact contractile function as indicated here, it may be interesting to highlight that it may also impact a number of other processes that may be relevant for exercise performance, e.g., reducing the rate of creatine rephosphorylation and impacting oxidative enzyme activity. Perhaps consider expanding this explanation to include these other processes?

We have added these mechanisms, with supporting references, to this section to hopefully provide a more rounded explanation.

Line 62: It would be useful to highlight here whether supplementation similarly improved performance in both conditions, or whether it exerted a greater influence in hypoxia, as may be expected based on the heightened challenge to pH regulation that a hypoxic environment may create.

We have addressed this comment by rewriting the section and including two further references to clarify the existing data. It now reads: Thus, high-intensity exercise performed in hypoxia will incur even greater metabolic stress and increase the demands on the body's buffering capacity. Nutritional supplementation methods to increase both extracellular (Flinn et al. 2014; Saunders et al. 2014a; Deb et al. 2018b; Haussirth et al. 1995) and intracellular (Saunders et al. 2014a) buffering capacity have been explored as a means to buffer exercise-induced acidosis in normoxia and/or hypoxia. Some studies showed similar performance improvements with these supplements in both hypoxia and normoxia (Deb et al., 2017; Hausswirth et al. 1995), while others showed no improvements in either condition (Flinn et al., 2014). Saunders et al. (2014) did not perform exercise in normoxia but supplementation with beta-alanine and/or sodium bicarbonate was ineffective to improve repeated sprints in hypoxia.

Line 69 - 73: I found this sentence to be quite long and a little difficult to follow and suggest rephrasing to enhance clarity.

We agree, this was very long, and we have now rewritten it for clarity. It now reads: 'Specifically, BA supplementation has been shown to be effective during high-intensity cycling capacity at 110% of previously determined peak power output ($CCT_{110\%}$; Hill et al. 2007; Sale et al. 2011; Danaher et al. 2014; Saunders et al. 2017a). Such exercise results in high levels of blood lactate and reductions in blood pH (Saunders et al. 2013), likely due to increasing muscle acidosis.'

Line 104: What do you mean by a supplement disclaimer? It would be useful to be more precise on what information was obtained from the participants.

The supplement disclaimer is a document which we ask participants to sign to inform and remind them that, whilst every care is taken to source uncontaminated supplements, the individual is responsible for what supplements they consume. This is, of course, particularly pertinent in those athletes who might need to undertake a doping test, although in the case of the current study on

recreational participants this is extremely unlikely to be of direct relevance. As such, we have removed reference to this document from the methods to avoid confusion.

Line 152 - 156: Although it becomes clearer later in the discussion, it is not immediately clear what the purpose of the POMS and PSQI is, and so it may be useful to clarify this within the methods
Yes, we agree an explanation would be beneficial. A sentence has been added to explain their inclusion. It reads: These questionnaires were undertaken to confirm that participants had been having similar sleep quality and were in a similar mood state for each testing session.

Line 163: The justification for undertaking both an independent sample t-test based on changes in performance in the BA and PLA groups, as well as a mixed model ANOVA was not clear to me. What information does the t-test provide that the ANOVA does not?
Thanks for pointing this out. We did all our analysis separately initially to clearly investigate the effect of the hypoxia, the effect of the BA, and then the effect of the BA in the environmental conditions. But as you rightly point out the independent sample t-test comparison was within the Mixed Model ANOVA. Therefore, we have removed reference to the independent sample t-test in the methodology and thank you for highlighting this.

Line 246: It is not clear to me how you are defining "clearly improving" or "performing worse". How did you distinguish between typical variation and performance improvements/decrements? The identification of "responders" and "non-responders" has been the topic of much discussion recently, and is very difficult to determine in practice. Recent articles have discussed this in more detail and may be useful, e.g., Atkinson et al. 2019 (https://eur03.safelinks.protection.outlook.com/?url=https%3A%2F%2Fdoi.org%2F10.1113%2FEP087712&data=01%7C01%7Cruth.james%40ntu.ac.uk%7Cf258d90a100d476c332708d86d46e04b%7C8acbc2c5c8ed42c78169ba438a0dbe2f%7C0&sdata=LSemIbT2IyN8PtyGhO8QWft5G4m5HqV1jCPRPpRLEvg%3D&reserved=0) or Swinton et al. 2019 (https://eur03.safelinks.protection.outlook.com/?url=https%3A%2F%2Fdoi.org%2F10.3389%2Ffnut.2018.00041&data=01%7C01%7Cruth.james%40ntu.ac.uk%7Cf258d90a100d476c332708d86d46e04b%7C8acbc2c5c8ed42c78169ba438a0dbe2f%7C0&sdata=LdibbVwTQYU44XgmfZrCaJ6jSbJPXGlYdPyxqOCnq9c%3D&reserved=0).
Thanks for highlighting this point; as it was not our intention to in anyway denote or define ‘responders’ or ‘non-responders’, or to apply any kind of analysis to this simple interpretation we have removed the leading/misleading terms from the manuscript. The section now reads: Indeed, Figure 2 shows the individual responses in exercise capacity to BA supplementation in our participants, however upon further examination of our data we can find no commonalities between individuals to explain our findings.

Article Title: The effect of β-alanine supplementation on high intensity cycling capacity in normoxia and hypoxia

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Acknowledgements

Conflict of interest

Abstract

The availability of dietary beta-alanine (BA) is the limiting factor in carnosine synthesis within human muscle due to its low intramuscular concentration and substrate affinity. Carnosine can accept hydrogen ions (H^+), making it an important intramuscular buffer against exercise-induced acidosis. Metabolite accumulation rate increases when exercising in hypoxic conditions, thus an increased carnosine concentration could attenuate H^+ build-up when exercising in hypoxic conditions. This study examined the effects of BA supplementation on high intensity cycling capacity in normoxia and hypoxia. In a double-blind design, nineteen males were matched into a BA group (n=10; $6.4 g \cdot d^{-1}$) or a placebo group (PLA; n=9) and supplemented for 28 days, carrying out two pre- and two post-supplementation cycling capacity trials at 110% of powermax, one in normoxia and one in hypoxia (15.5% O_2). Hypoxia led to a 9.1% reduction in exercise capacity, but BA supplementation had no significant effect on exercise capacity in normoxia or hypoxia ($P>0.05$). Blood lactate accumulation showed a significant trial x time interaction post-supplementation ($P=0.016$), although this was not significantly different between groups. BA supplementation did not increase high intensity cycling capacity in normoxia, nor did it improve cycling capacity in hypoxia even though exercise capacity was reduced under hypoxic conditions.

Keywords: Exercise capacity – Carnosine – Hypoxia – Supplementation – Buffering

Introduction

Training at high altitude has been used in many sports as it provides an ergogenic benefit to physical performance upon return to sea level (Millet et al. 2010). However, the physiological adaptations and performance benefits from this strategy require many weeks of acclimation and training (Saunders et al. 2019). Acute hypoxic conditions increase metabolic demand and trigger the anticipation of fatigue; there is an increase in heart rate and respiratory rate at rest (Burtscher et al. 2006) and an overload of the body's acid-base systems, since pCO_2 is lower than at sea level (Kayser et al. 1993). Consequently, performing exercise in these conditions poses a substantial challenge, especially for high-intensity exercise characterised by large accumulation of metabolites such as hydrogen ions (H^+), ADP and Pi in skeletal muscles. These cause a reduction in muscle pH, as well as a reduction in arterial and venous blood pH, thereby stressing the body's buffering systems. At rest, the standard intramuscular pH is around 7.0 and arterial and venous blood pH is around 7.4, however, during high-intensity exercise, muscle pH can drop to as low as 6.0 and blood pH can decline to ~7.0 (Pan et al. 1991). Reductions in pH levels during exercise can limit performance by reducing the rate of creatine rephosphorylation (Hogan et al. 1999), by negatively impacting oxidative enzyme activity (Jubrias et al. 2003) and by negatively impacting the excitation-contraction coupling mechanism and cross-bridge interactions (Knuth et al. 2006; Debold et al. 2008). Thus, high-intensity exercise performed in hypoxia will incur even greater metabolic stress and increase the demands on the body's buffering capacity. Nutritional supplementation methods to increase both extracellular (Flinn et al. 2014; Saunders et al. 2014a; Deb et al. 2018b; Haussirth et al. 1995) and intracellular (Saunders et al. 2014a) buffering capacity have been explored as a means to buffer exercise-induced acidosis in normoxia and/or hypoxia. Some studies showed similar performance improvements with these supplements in both hypoxia and normoxia (Deb et al., 2017; Hausswirth et al. 1995), while others showed no improvements in either condition (Flinn et al., 2014). Saunders et al. (2014) did not perform exercise in normoxia but supplementation with beta-alanine and/or sodium bicarbonate was ineffective to improve repeated sprints in hypoxia.

Carnosine is a naturally occurring cytoplasmic dipeptide found in many human tissues and in high concentrations within the skeletal muscle (Harris et al. 2006; Hill et al. 2007; Saunders et al. 2017a; Carvalho et al. 2018) and has the ability to accept H^+ (Smith 1938; Tanokura et al. 1976). Previous research has shown that BA supplementation can augment intramuscular carnosine content after four weeks of supplementation (Harris et al. 2006; Derave et al. 2007; Hill et al. 2007; Saunders et al. 2017a) which can improve high-intensity exercise capacity and performance (Saunders et al. 2017a). Specifically, BA supplementation has been shown to be effective during high-intensity cycling capacity at 110% of previously determined peak power output ($CCT_{110\%}$; Hill et al. 2007; Sale et al. 2011; Danaher et al. 2014; Saunders et al. 2017a). Such exercise results in high levels of blood lactate and reductions in blood pH (Saunders et al. 2013), likely due to increasing muscle acidosis. The efficacy of BA to improve a range of high-intensity exercise outcomes, particularly those lasting between 1 and 10 minutes, indicates the potential for BA to have a positive effect over a wide range of sports/exercises modalities, and has been demonstrated using meta-analytical data (Hobson et al. 2012; Saunders et al. 2017b). Research on its efficacy in alternate environmental conditions, such as hypoxia, is, however, lacking.

The literature suggests that BA supplementation has ergogenic potential to improve exercise performance under acute hypoxic conditions. Exercise in acute hypoxia leads to a reduction in oxygen at the mitochondrial level and a consequent increase in the participation of the glycolytic pathway, thereby increasing the rate of metabolite accumulation (Levine et al. 2008). It causes an earlier onset of muscle acidosis for the same exercise load compared with sea level, and the effects of acute hypoxia reduce performance by 10% of cycling peak power output and 18% of VO_{2max} (Deb et al. 2018b). The increase in H^+ when exercising in hypoxia strain the buffering systems of the body in order to maintain exercise performance. Only two studies have investigated the effects of BA on exercise in hypoxia. Saunders et al. (2014a) used a sprint based, football specific intermittent treadmill task and showed no significant effect of supplementation on 5 x 6s repeated sprint performance at simulated 2500 m (15.5% O_2). Wang et al. (2019) showed that BA supplementation maintained anaerobic working capacity in normobaric hypoxia, but it did not attenuate the onset of fatigue nor enhance repeated sprint performance. However, as indicated by meta-analyses on the topic (Hobson et al. 2012; Saunders et al. 2017b), BA supplementation does not influence performance in exercise tasks lasting <60s. The $CCT_{110\%}$ has been used

extensively in previous BA studies (Hill et al. 2007; Sale et al. 2011; Danaher et al. 2014; Saunders et al. 2017a) and been shown to be malleable to improvements from increased buffering capacity. Furthermore, the $CCT_{110\%}$ is an exercise test of the type and duration which appears to be influenced by acute exposure to hypoxia (Deb et al. 2018a).

Therefore, the aim of the current study was to examine the effects of BA supplementation on high intensity cycling capacity, using the $CCT_{110\%}$, in normoxia and hypoxia. It was hypothesised that BA supplementation would improve high intensity cycling capacity in normoxia and hypoxia, with greater improvements in hypoxia.

Methods

Participants

Twenty-two recreationally active males volunteered to take part in this study (age: 21 ± 2 y, height: 1.78 ± 0.07 m, body mass: 74.40 ± 11.47 kg, Mean ± SD). Three participants were excluded due to injury, illness and difficulties in data collection. Ethical approval for the study was granted by #### (application number: 342). Prior to testing, participants completed a health screen and provided written informed consent. Participants were excluded from the trial if they had surgical history or pain in the lower limbs, were vegan/vegetarian, or had consumed BA supplements within the last three months. Participants were asked to refrain from vigorous exercise and alcohol consumption for 24 hours prior to each trial, which were separated by a minimum of 24 hours and took place at the same time of day. Participants were asked to complete a food diary 24 hours prior to their first trial and to replicate their food and fluid intake prior to subsequent trials. A standardised breakfast, consisting of a cereal bar and a sports drink (total energy content 270 kcal), was also provided for participants to consume two hours prior to attending a trial. Participants were asked to maintain their usual physical activity routines during the supplementation period to avoid changes in fitness; this was confirmed at the first post supplementation trial and where this had not been adhered to, they were excluded from the trial as detailed above.

Table 1. Participant characteristics. Mean (SD).

	BA (n = 10)	PLA (n = 9)	*P*
Age (y)	21 (3)	20 (1)	0.114
Height (m)	1.80 (0.08)	1.75 (0.04)	0.355
Body Mass (kg)	73.77 (11.02)	75.09 (12.57)	0.779
Powermax (W)	279 (66)	267 (40)	0.279
Supplement Compliance (%)	99 (2)	95 (6)	0.001
Total supplement consumed (g)	189.7 (4.0)	182.9 (12.0)	0.001

Experimental design

Participants carried out seven separate trials: 1 powermax test, 2 familiarisation $CCT_{110\%}$ trials (normoxia followed by hypoxia), and 4 main trials; 2 pre-supplementation $CCT_{110\%}$ and 2 post-supplementation $CCT_{110\%}$ trials. During the four main $CCT_{110\%}$ trials, chamber conditions were randomised and carried out in a single blind, crossover and counterbalanced design. For the allocation to BA or PLA supplementation groups, subjects were pair-matched for body mass and Powermax, where the first participant of each pair was randomly allocated to one supplement group, with their pair-match being subsequently allocated to the other group (see Table 1). This process was done by only the lead investigator to maintain the double-blind nature of the study.

Experimental protocol

Powermax test. Using a Lode cycle ergometer (Lode B.V., Groningen, The Netherlands) participants were asked to cycle to exhaustion with exercise intensity increasing by 6W every 15s. Exhaustion was defined as when the participant could no longer continue to cycle above 60 rpm despite verbal encouragement from the experimenter. This test was used to determine the relative exercise intensity for participants in the $CCT_{110\%}$ trials.

$CCT_{110\%}$. The reliability of a $CCT_{110\%}$ was assessed by Saunders et al. (2013) who showed the test to be a reliable measurement of performance in recreationally active males (CV of 4.43% for TTE) and suited to a nutritional intervention study. In the current study, the tests were performed in an environmental chamber (WIR52-20HS, Design Environmental Ltd., Gwent, Wales, U.K), in both normoxic (20.9% O_2, 50% humidity and 18 °C) and hypoxic conditions (15.5% O_2, 50% humidity and 18 °C). Participants first rested in the chamber for 10 min before carrying out a 5 min warm-up working at 60% of age predicted max heart rate. The $CCT_{110\%}$ began by first starting at 80% for 15s, followed by 95% for 15s and finally reaching 110%, which was then maintained until exhaustion, again defined as when a participant could no longer cycle above 60rpm. Heart rate (HR; Polar heart rate monitor, Kempele, Finland) and rating of perceived exertion (RPE; Borg 1982) were recorded every minute of the test and immediately upon completion. Finger prick capillary blood samples (70µL) were taken at rest (before the warm-up), immediately after and 5 min after the $CCT_{110\%}$ and analysed for blood pH, blood lactate concentration, base excess and bicarbonate (HCO_3^-), using a blood gas analyser (ABL FLEX 90, Radiometer, Ireland).

Supplementation. Supplementation with either daily BA (patented sustained release tablets (Carnosyn^{SR}, Natural Alternatives International, USA) 6.4 $g \cdot d^{-1}$) or PLA (matched with the CarnoSyn^{SR} tablets, with the BA replaced by celluloses) commenced once a participant completed their last pre- $CCT_{110\%}$ trial. Supplementation was provided in the form of pills and the dosage required participants to take two tablets, four times per day. Supplementation lasted for 29 ± 1 day before the first post-trial commenced. Participants recorded their intake on a supplementation log and were contacted on a weekly basis to try to optimise compliance. Participants continued to supplement up to the last post-trial and returned any left-over pills, along with their supplementation log so that supplement compliance could be calculated (see Table 1).

Questionnaires. The Profile of Moods State questionnaire (POMS; McNair et al. 1971) was used to evaluate the mood of the volunteers prior to every trial. It is composed of 65 clauses rated as "Not at All", "A Little", "Moderately", "Quite a Lot" or "Extremely", which is then summed to give the outcome of Total Mood Disturbance. The Pittsburgh sleep quality index (PSQI; Buysse et al. 1989) was used to assess the quality of sleep by global PSQI score. These questionnaires were undertaken to confirm that participants had been having similar sleep quality and were in a similar mood state for each testing session.

Statistical analysis

The Statistical Package for Social Sciences (IBM SPSS Statistics 24) was used to analyse the data collected. Data were first checked for normality of distribution using the Shapiro-Wilk test and all data were shown to be normal. A paired-samples t-test was executed to examine any differences between pre-normoxic and pre-hypoxic $CCT_{110\%}$ trials, to determine the effect of the environmental conditions on participants exercise capacity. Mixed model ANOVA's were conducted to determine whether or not there were differences in exercise capacity, relevant blood markers and questionnaires (POMS and PSQI) pre- to post-supplementation, between groups, and across environmental conditions. Statistical significance was accepted at the $P \leq 0.05$ level. Effect size was calculated using Hedges g for small sample sizes, and effect sizes of about 0.20, 0.5, and 0.8 are considered small, medium, and large, respectively. All data are presented as mean ± 1SD.

Results

Performance analysis

There was a significant reduction in exercise capacity in hypoxia compared to normoxia pre-supplementation ($P < 0.001$; Effect Size -0.85; **Figure 1**). There was no significant difference in ΔTTE between BA and PLA, *i.e.* the effect of BA supplementation ($P = 0.387$; Effect Size 0.05; **Figure 2**). The ANOVA showed no significant effect of BA on TTE in hypoxic or normoxic conditions(Time, $P = 0.578$; Group x Condition x Time, $P = 0.747$; Group x Condition, $P = 0.566$; Condition x Time, $P = 0.506$; Effect Sizes BA hypoxia -0.02, BA normoxia -0.12, PLA hypoxia 0.05, PLA normoxia -0.24; **Figure 3**).

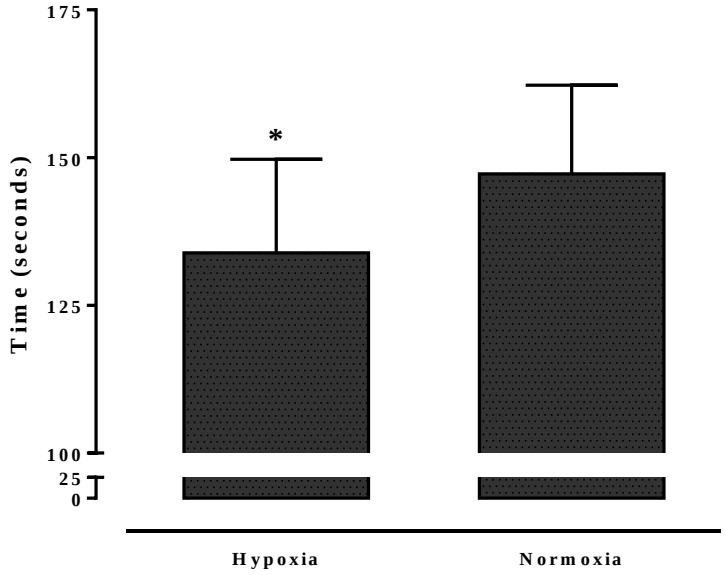

Fig. 1. Comparison of TTE between pre-supplementation hypoxic and normoxic $CCT_{110\%}$ trials. Mean ± SD are shown. * denotes a significant difference between pre-supplementation trials at P <0.001.

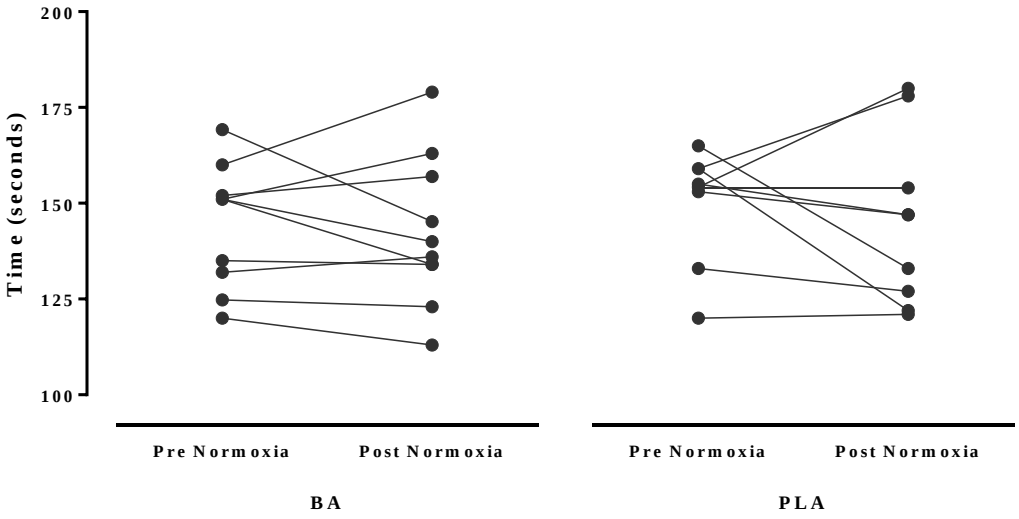

Fig. 2. Individual participants comparison between pre-normoxic and post-normoxic $CCT_{110\%}$ trials in BA group (grey) and PLA group (black). Mean ± SD are shown.

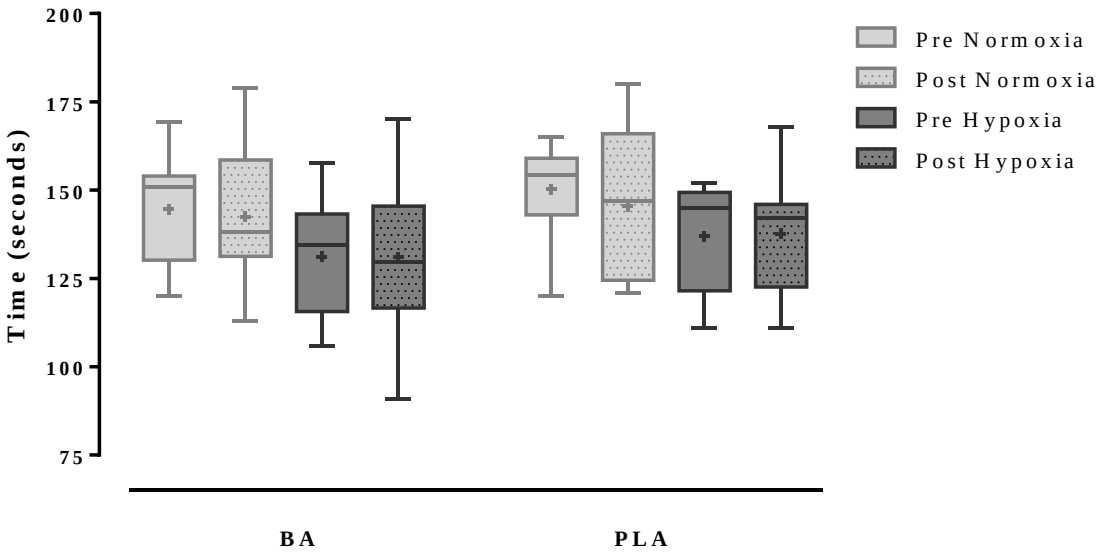

Fig. 3. TTE in $CCT_{110\%}$ hypoxic and normoxic trials pre- and post-supplementation, subdivided by supplementation groups (BA and PLA). The + denotes the mean, the horizontal line within the box denotes the median, the box shows the interquartile range, and the 'whiskers' denote the full range.

Blood analysis

There was no interaction effect of supplementation on blood pH (Group x Trial x Time, $P = 1.000$), lactate (Group x Trial x Time, $P = 0.437$), base excess (Group x Trial x Time, $P = 0.358$) or HCO_3^- (Group x Trial x Time, $P = 0.400$) during exercise across trials. There was no Group x Trial effect for pH, lactate, base excess and HCO_3^- (all $P > 0.05$). There was no Trial effect throughout exercise on pH, base excess or HCO_3^- (Trial x Time, all $P > 0.05$). A significant Trial x Time interaction ($P = 0.016$) and a significant main effect of Trial ($P < 0.001$) were shown for lactate. Blood pH, lactate, base excess and HCO_3^- all showed a significant main effect of time ($P \leq 0.001$; Table 2).

Table. 2. Blood pH, lactate, base excess (BE) and HCO_3^- during pre- and post- BA and PLA supplementation $CCT_{110\%}$ trials. Mean (SD).

	Pre-Hypoxia			Post-Hypoxia			Pre-Normoxia			Post-Normoxia		
	Before	After	+5min	Before	After	+5min	Before	After	+5min	Before	After	+5min
pH												
BA	7.41 (0.02)	7.28 (0.02)	7.21 (0.04)	7.42 (0.02)	7.29 (0.05)	7.23 (0.04)	7.40 (0.03)	7.25 (0.03)	7.21 (0.03)	7.39 (0.02)	7.25 (0.04)	7.21 (0.05)
PLA	7.41 (0.03)	7.25 (0.04)	7.19 (0.05)	7.41 (0.02)	7.23 (0.04)	7.20 (0.05)	7.41 (0.01)	7.24 (0.04)	7.19 (0.06)	7.40 (0.02)	7.23 (0.03)	7.20 (0.04)
Lac												
BA	2.07 (0.49)	13.29 (1.88)	15.81 (2.45)	1.63 (0.33)	11.17 (2.04)	13.08 (3.43)	2.01 (0.41)	13.61 (1.94)	16.69 (2.00)	1.49 (0.41)	10.51 (2.08)	13.65 (2.58)
PLA	1.78 (0.37)	12.79 (2.98)	15.88 (3.67)	1.54 (0.29)	12.27 (1.81)	13.50 (3.28)	1.96 (0.39)	13.2 (2.53)	16.62 (3.68)	1.43 (0.38)	12.46 (2.73)	14.73 (3.66)

BE												
BA	1.30	-9.45	-14.87	1.58	-8.85	-14.22	1.13	-10.42	-15.63	0.96	-10.05	-15.21
	(1.13)	(1.72)	(2.06)	(1.37)	(2.73)	(2.34)	(1.45)	(1.53)	(1.92)	(1.25)	(2.63)	(3.30)
PLA	0.94	-10.30	-15.39	1.56	-11.70	-15.24	1.77	-10.09	-15.86	1.2	-10.80	-15.39
	(1.33)	(3.88)	(4.25)	(1.23)	(2.46)	(3.36)	(1.64)	(2.79)	(3.95)	(1.36)	(2.40)	(2.94)
HCO_3^-												
BA	25.20	17.59	14.45	25.49	17.98	14.82	25.05	16.98	14.10	24.89	17.16	14.28
	(0.84)	(1.09)	(1.26)	(1.07)	(1.84)	(1.45)	(1.05)	(1.03)	(1.12)	(0.81)	(1.67)	(1.88)
PLA	24.91	16.92	14.04	25.34	16.07	14.16	25.59	17.04	13.89	25.28	16.60	14.08
	(0.92)	(2.22)	(2.22)	(0.96)	(1.47)	(1.89)	(1.20)	(1.66)	(2.18)	(1.06)	(1.43)	(1.62)

HR, RPE and questionnaires

A mixed model ANOVA was used to analyse HR and RPE data collected at exhaustion. There were no interaction effects of supplementation across trial conditions throughout exercise on HR (Group x Condition x Time, P = 0.405; Group x Condition, P = 0.168; Condition x Time, P = 0.824) or RPE (Group x Condition x Time, P = 0.175; Group x Condition, P = 0.175; Condition x Time, P = 0.941). There was no significant main effect of time on HR (P = 0.161) or RPE (P = 0.175). There was also no significant main effect of condition on HR (P = 0.235) or RPE (P = 0.941). Analysis of POMS and PSQI did not show any main effects (all P values > 0.05; Table 3).

Table 3. Final HR, RPE, POMS and PSQI data during pre- and post- BA and PLA supplementation $CCT_{110\%}$ trials. Mean (SD).

	Pre-Hypoxia	**Post-Hypoxia**	**Pre-Normoxia**	**Post-Normoxia**
HR				
BA	185 (10)	186 (12)	184 (13)	184 (10)
PLA	186 (11)	185 (8)	183 (10)	184 (11)
RPE				
BA	20.0 (0.0)	20.0 (0.0)	19.8 (0.6)	20.0 (0.0)
PLA	20.0 (0.0)	19.8 (0.7)	20.0 (0.0)	20.0 (0.0)
POMS				
BA	0.1 (11.9)	4.2 (11.6)	0.3 (12.5)	3.8 (12.6)
PLA	12.8 (28.3)	16.4 (28.3)	14.0 (20.3)	17.2 (29.4)
PSQI				
BA	4.6 (2.9)	3.8 (2.3)	5.0 (2.5)	4.2 (2.9)
PLA	4.1 (2.1)	3.7 (3.1)	3.6 (2.2)	3.9 (3.4)

Discussion

This is the first study to investigate effects of BA supplementation on high intensity cycling capacity in hypoxia when compared with normoxia. The hypothesis was that BA supplementation would improve high intensity cycling capacity in both normoxia and hypoxia, with greater improvements in hypoxia. Our findings suggest, however, that BA supplementation had no ergogenic effect on TTE in normoxia or hypoxia, contrary to our hypothesis.

These main findings, particularly those showing no significant effect of BA supplementation on high-intensity cycling capacity in normoxia, go against the results of previous studies using the same exercise protocol (Hill et

al. 2007; Sale et al. 2011; Danaher et al. 2014; Saunders et al. 2017a). Our results do, however, concur with Gross et al. (2014), who also showed that BA supplementation did not affect high intensity cycling performance. Our supplementation protocol was similar to that of previous studies that have shown increases of around 60% in intramuscular carnosine content (Harris et al. 2006) following BA supplementation and that this improves exercise capacity (Hill et al. 2007; Sale et al. 2011; Danaher et al. 2014; Saunders et al. 2017a). It is unclear why our results are in contrast to these previous studies, given that our participants underwent the same supplementation regime of 6.4g·d^{-1} of BA as Saunders et al. (2017a) and followed similar exercise protocols to many studies showing an effect, as described above. It could be speculated that supplementation for a more prolonged period of time may have resulted in performance benefits. Indeed, Saunders et al. (2017a) supplemented participants with BA for 24 weeks and carried out performance tests and muscle biopsies every 4 weeks to determine any effects. After the first 4 weeks, muscle carnosine content increased (+11.37 ± 7.03 mmol·kg^{-1} dm) and exercise capacity improved (BA +5.0% *vs* PLA +1.8%). Upon closer inspection, however, *post hoc* analyses revealed that exercise capacity was only significantly improved at week 20 compared to week 0, coinciding with peak muscle carnosine content. Since Saunders et al. (2017a) showed a large variability in muscle carnosine increases with BA supplementation, it could be speculated that not all individuals in our study increased muscle carnosine content to a sufficient degree to improve exercise capacity. Indeed, Figure 2 shows the individual responses in exercise capacity to BA supplementation in our participants, however upon further examination of our data we can find no commonalities between individuals to explain our findings. Further research is necessary to elucidate the minimum increase in muscle carnosine necessary to impact exercise capacity or performance.

The $CCT_{110\%}$ has previously been shown to be susceptible to changes in buffering capacity via BA (Hill et al. 2007; Sale et al. 2011; Danaher et al. 2014; Saunders et al. 2017a) or sodium bicarbonate supplementation (Saunders et al. 2014b; Dias et al. 2015). Interestingly, Dias et al. (2015) showed that performance improvements with supplementation were inconsistent across four separate occasions, despite the mechanism for improvement (blood alkalosis) always being present. The physically active, but not highly trained, population recruited for this study and by Dias et al. (2015) may have also contributed to the results; less well-trained individuals generally display greater variation in performance during exercise tests, rendering it more difficult to determine the true effect of the intervention. It is also possible that the exercise response could be influenced by factors beyond buffering capacity, such as mood (McNair et al. 1971), sleep quality (Buysse et al. 1989) and nutrition (Burke et al. 2019; Stecker et al. 2019). In the current study, however, neither mood state nor sleep quality were likely to have influenced the outcome of the study, given that we accounted for these factors using pre-validated questionnaires, and no differences between the test conditions were recorded in either parameter. Participants were also asked to standardise their diet and physical activity for 24h prior to each test and adherence was verbally confirmed at each testing session. Participants were provided a standardised breakfast 2h prior to all testing sessions, which differs from several (Danaher et al. 2014; Sale et al. 2011), though not all (Saunders et al. 2017a) previous BA supplementation studies employing the CCT110% exercise test. Since this was standardised prior to all testing sessions it is unlikely to influence the exercise response between trials.

Hypoxia resulted in a 9.1% decrease in cycling capacity compared to normoxia, as supported in the wider literature (Deb et al. 2018a). Interestingly, exercise in hypoxia led to a similar reduction in blood pH, bicarbonate and base excess, and increase in blood lactate, as following exercise in normoxia, despite the significantly shorter exercise duration. This demonstrates the greater requirement for energy supply from anaerobic sources in hypoxia, leading to an earlier attainment of critical levels of metabolites resulting in fatigue (Kent-Braun et al. 2012). Given that this was the basis for our hypothesis (that increases in the muscle carnosine content through BA supplementation would result in significantly improved cycling capacity in hypoxic conditions compared with normoxic conditions) it is somewhat surprising that our results showed no significant effects on cycling capacity or on blood responses. Similarly, Saunders et al. (2014a) showed no effect of BA supplementation on repeated sprints in hypoxia, while Wang et al. (2019) showed no additional benefits of BA to a repeated-sprint training protocol performed in hypoxia. In contrast to the current study however, those previous studies employed exercise tests that were unlikely to be significantly influenced by BA supplementation (Hobson et al. 2012; Saunders et al. 2017b). As it stands, there is little evidence to suggest that the benefits of BA supplementation shown by several authors under normal conditions (*i.e.*, normoxia), are evident when exercise is performed in hypoxia. It should, however, be

noted that a recent study has suggested the mechanism of fatigue when exercising in a hypoxic environment may not be due to impaired motor function as a result of peripheral or central fatigue, but could be related to the hypoxia affecting areas within the brain (Mira et al. 2020). More research is warranted to determine the mechanism of fatigue in hypoxia, and whether increased muscle carnosine content via BA supplementation can lead to improved exercise capacity in hypoxia and whether these changes are similar or greater than those shown in normoxia.

A limitation of the current study was that intramuscular carnosine content was not measured, which could have provided insight into the effectiveness of BA supplementation on elevating muscle carnosine content. Our participants ingested ~190 g of BA, similar to previous work consistently showing increases of approximately 10 $mmol \cdot kg^{-1}dm$ or a 60% increase (Harris et al. 2006; Hill et al. 2007). The majority of previous literature shows muscle carnosine content increases post BA supplementation (Harris et al. 2006; Derave et al. 2007; Hill et al. 2007; Saunders et al. 2017a). Indeed, a recent meta-analysis showed that 99.3% of individuals increase muscle carnosine concentration with BA supplementation (Rezende et al. 2019). These results suggest that, although intramuscular carnosine content was not measured herein, it would have almost certainly been elevated post-supplementation. Another possible limitation is the use of recreationally trained individuals, who display a greater variance in exercise test performance than elite athletes, although the CCT110% is a reliable exercise test in recreationally active men (CV 4.43%; Saunders et al. 2013). Another experimental consideration, though not necessarily a limitation, was the recruitment of participants with a diverse range of different ethinic backgrounds. Within the BA group of the current study there were three participants of Indian heritage, five Caucasians, one Hispanic and one mixed race participant. The PLA group consisted of two participants of Indian heritage, four Caucasians, one Afro-Caribbean, and two mixed race participants. There is no suggestion from our data that any one ethnic group responded differently to supplementation compared to the others, although given the potential racial differences in relevant physiological traits, such as muscle fibre type distribution (Ceaser & Hunter, 2015) and possibly, by implication, the demand upon physicochemical buffering, there is justification for future research in this area.

In conclusion, exercise capacity was negatively influenced by hypoxia compared to normoxia, but 4 weeks of supplementation with BA had no effect on high intensity cycling capacity in either condition.

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