Increased Cardiorespiratory Stress During Cycling After Ketone Monoester Ingestion

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ABSTRACT

Nutritional ketosis refers to a state in which blood ketone bodies are elevated above normal basal levels, typically corresponding to a beta-hydroxybutyrate concentration ([β-HB]) of >0.5 mM. Acute ketone supplement ingestion rapidly induces nutritional ketosis without otherwise altering diet, and there is growing interest in the effect of this practice on exercise responses and performance. The limited studies to date have yielded equivocal data, likely due in part to differences in supplement type and dose, increase in [β-HB], exercise intensity, participant training status, and study blinding. ***Purpose:*** We examined the effects of a ketone monoester (KE) supplement on exercise responses and performance in endurance-trained adults (n=10 males, n=9 females; VO_{2peak} = 57±8 ml/kg/min). ***Methods:*** Participants completed two trials in a randomized, double-blind, counterbalanced manner. A commercial KE solution (600 mg/kg body mass) or flavour-matched placebo was ingested 30 min before a 30-min cycling bout performed at individual ventilatory threshold intensity (71±3% VO_{2peak}), followed 15 min later by a 3 kJ/kg body mass time-trial. ***Results:*** KE versus placebo ingestion increased plasma [β-HB] before exercise (3.9±1.0 vs 0.2±0.3 mM, $p<0.0001$, $d_z=3.4$), mean ventilation (77±17 vs. 71±15 L/min, $p<0.0001$, $d_z=1.3$) and heart rate (155±11 vs 150±11 beats/min, $p<0.001$, $d_z=1.2$) during exercise, and rating of perceived exertion at the end of exercise (15.4±1.6 vs 14.5±1.2, $p<0.01$, $d_z=0.85$). Plasma [β-HB] remained higher after KE vs placebo ingestion prior to the time trial (3.5±1.0 vs 0.3±0.2 mM, $p<0.0001$, $d_z=3.1$) but subsequent performance was not different (KE: 16:25±2:50 vs placebo: 16:06±2:40 min:s, $p=0.20$; $d_z=0.31$). ***Conclusion:*** KE supplementation, per the conditions described, increased markers of cardiorespiratory and perceived stress during submaximal exercise but did not affect time-trial performance in endurance-trained participants.

40 Key words: Nutritional ketosis; supplement; exercise; time-trial performance; beta-

41 hydroxybutyrate.

INTRODUCTION

The ketone bodies beta-hydroxybutyrate (β-HB) and acetoacetate can serve as both fuels and signalling molecules, with the potential to alter tissue-specific and whole-body substrate metabolism (1). The venous blood concentration of the major circulating ketone body, β-HB, is <0.2 mM in healthy individuals consuming a mixed diet (1,2). Nutritional ketosis typically refers to a state in which blood [β-HB] is increased to >0.5 mM through dietary intervention (3). This can be achieved by severely restricting carbohydrate intake, and occurs during fasting or adherence to a ketogenic diet (2,4). Alternately, commercial ketone supplement ingestion acutely induces nutritional ketosis without otherwise changing diet (5,6). There is considerable emerging interest in the effects of this practice on exercise responses and performance (7–13).

Acute ketone supplementation has been reported to enhance (14), impair (15,16), and have no effect on exercise performance (17–20), as determined by various measures including time trials and time to fatigue. Studies have also reported varying effects on cardiorespiratory variables including heart rate, ventilation, oxygen uptake and respiratory exchange ratio during constant load exercise (14,19,21–27). The limited and equivocal data to date are likely attributable in part to differences in research design, including specific ketone supplement and dose, participant training status, and exercise intervention. Ketone monoester (KE) ingestion has been reported to elicit larger increases in blood β-HB as compared to other supplements (28), and it is the only supplement type that has been reported to improve exercise performance (14). Endurance-trained participants may also have a greater capacity to utilize ketone bodies during exercise (7).

The present study examined the effects of acute ketone monoester (KE) ingestion on exercise responses and performance in endurance-trained participants, using a randomized, double-blind, counterbalanced design. We assessed markers of cardiorespiratory and perceived

stress during constant load cycling performed at individual ventilatory threshold intensity and subsequent 3-kJ/kg body mass time-trial performance. We tested the hypothesis that KE ingestion would induce nutritional ketosis, reduce indices of cardiorespiratory and perceived stress, and improve time-trial performance, as compared to a flavour-matched placebo. A secondary purpose was to compare point-of-care and lab assay determinations of β-HB in various blood fractions.

METHODS

Participants. The study inclusion criteria were: age 18-50 years; regularly engaged in endurance-type exercise for >3 h/wk; VO_{2peak} in the 90_{th} percentile for age and sex (29); habitually consuming >50 g/d of carbohydrate (30); and deemed safe to engage in physical activity. An *a priori* power calculation determined that N=14 was required to detect changes in time-trial performance, based on a paired t-test (d_z=0.82, α=0.05, power=80%) and data reported by Cox *et al.* (14). To preserve power, N=20 individuals were recruited. One participant sustained a leg injury unrelated to the study and dropped out, and therefore data are reported for the remaining N=19 (25±5 y, 70±10 kg, 57±8 ml/kg/min; n=10 males, 25±3 y, 77±9 kg, 61±7 ml/kg/min, 70±8 ml/kg fat-free mass/min; n=9 females, 26±7 y, 62±6 kg, 53±6 ml/kg/min, 70±8 ml/kg fat-free mass/min). All participants were informed of the study requirements and potential risks before written informed consent was obtained. The project was approved by the Hamilton Integrated Research Ethics Board (#7209).

Study Overview. A randomized, crossover, double-blind, counterbalanced design was employed. It initially involved two screening visits to determine eligibility, and subsequently two familiarization trials and two experimental trials. Treatment order following study enrolment was determined by a coin flip (block size of 2; stratified by sex). The investigators who interacted with the participants during the experimental trials and collected cardiorespiratory and exercise performance data was blinded to treatment condition. A separate investigator who did not interact

with participants performed the randomization and analyses of fingerprick blood samples at the time of sampling. Experimental trials were separated by 7±2 d and performed at the same time of day within 1 h. Female participants taking oral contraceptives were tested while receiving the same dose of synthetic hormones. The others who were not taking contraceptives were tested in the early follicular phase of their menstrual cycle, except for one participant who was amenorrhoeic.

Screening and Familiarization Visits. Participants were initially deemed safe to engage in physical activity, as determined by a standard questionnaire (Get Active Questionnaire, Canadian Society for Exercise Physiology). They subsequently performed a ramp exercise test to volitional exhaustion on an electronically braked cycle ergometer (Excalibur Sport version 2.0; Lode, Groningen, The Netherlands) to determine VO_{2peak} and ventilatory threshold. Briefly, the ramp test began with a 2-min warm-up at 50 or 100 W and workload was increased 1 W every 2 s until the subject's cadence was <60 rpm. Expired gasses and ventilation were continuously recorded with a metabolic cart (Quart CPET, COSMED Inc., Concord, CA). VO_{2peak} was determined as the mean VO_2 during the last 30 s of the test, and ventilatory threshold as described by Gaskill et al. (2001). Participants who satisfied the inclusion criterion for VO_{2peak} completed additional questionnaires and were recruited into the study if they met all inclusion criteria. Body composition was determined during a subsequent visit using an air displacement plethysmograph (Bodpod, COSMED Inc., Concord, CA). For this measure, participants arrived in the overnight fasted state, and after abstaining from strenuous exercise and water in the morning.

Participants returned to the laboratory on two occasions for familiarization procedures and to become accustomed with the experimental protocol. Briefly, participants cycled for 30 min at their pre-determined individual ventilatory threshold intensity, rested for 15 min, and completed a 3 kJ/kg body mass time-trial. During the first familiarization trial, minor adjustments were made

111 to exercise workload so the measured VO_2 during the 30-min steady-state cycle period elicited the
112 predicted ventilatory threshold. For the time trial, the alpha factor on the cycle ergometer was
113 initially set such that a cadence of 80 rpm corresponded to 70% peak power output and adjusted
114 after the first familiarization trial based on participant preference.

115 **Experimental protocol.** Participants were instructed to maintain their habitual diet and
116 physical activity habits throughout the study. On the day before each experimental trial,
117 participants were instructed to avoid strenuous exercise, ingest the same foods that corresponded
118 to their habitual diet, and avoid alcohol. Caffeine was not restricted, provided the timing and
119 amount of was the same before each trial. Compliance was assessed by a 1-d dietary recall.
120 Subjects ingested a standardized breakfast provided by the researchers 2 h before exercise onset.
121 It consisted of a commercial energy bar (250 kcal, 5-g fat, 44-g carbohydrate, 4-g fibre, 10-g
122 protein; Clif Bar & Company, CA, USA,), and commercial sports drink powder (Gatorade;
123 PepsiCo Canada, Mississauga, Ontario, Canada) mixed into 500 ml of water, such that total
124 carbohydrate intake was 1 g/kg body mass.

125 Participants arrived at the laboratory ~45 min prior to exercise and provided a mid-stream
126 urine sample. They were also asked whether they were currently experiencing any gastrointestinal
127 symptoms assessed on the questionnaire. All fluid ingested from this point until after the time trial
128 was measured, and fluid ingested during the first trial was matched in the second trial. Starting 35
129 min prior to exercise, participants were allowed 5 min to ingest either 600 mg/kg body mass of the
130 ketone monoester (KE) supplement (120 kcal/25 g KE; Pure ΔG Ketone Ester; HVNM, CA, USA)
131 or a ketone-free, flavour-matched placebo (PL) (0 kcal; HVNM). Both KE and PL were mixed
132 with 25 g of the commercial sports drink powder (Gatorade) and dissolved in water such that total
133 beverage volume was 500 ml. Two participants required ~15 min to drink the beverages but were

allowed to remain in the study since this was consistent between their trials. Approximately 5 min before exercise, another urine sample was collected, body mass was measured, venous and capillary blood samples were obtained, and a supplement tolerability questionnaire was completed. The questionnaire contained ten questions pertaining to perceptions of gastrointestinal symptoms, including stomach cramps and burning; nausea; dizziness; flatulence; urge to urinate, defecate, and vomit; and gastric reflux. For each question, participants indicated their perception by drawing an arrow on a 10-cm line. The 0-, 3.3-, 6.6- and 10-cm points corresponded to the ratings "not present", "mild", "moderate" and "severe", respectively.

The exercise protocol involved a 5-min warm-up at 50 W, followed by 30 min at the predetermined workload corresponding to individual ventilatory threshold (71±3% VO_{2peak}, 53±6% peak power output). Cycle cadence and ergometer seat and handlebar configuration were consistent within a participant over experimental trials. During the 30-min exercise period, RPE was obtained every 5 min with the 20-point Borg scale (32), expired gases were collected for ~5 min over two periods starting at ~7.5 and ~25 min (Quark CPET; Cosmed Inc., Concord, CA, USA), and heart rate was collected continuously throughout (Polar Electro, Model A300; Kempele, Finland). The coefficient of variation for heart rate and RPE, determined in pilot testing on five endurance-trained adults, was 2.6±0.5% and 2.1±0.5% respectively.

After a 15-min rest, participants performed a 3 kJ/kg body mass time-trial. During the rest period, venous and capillary blood and urine samples were collected in the same order as before exercise, and participants completed the supplement tolerability and fatigue questionnaires. The fatigue questionnaire contained five questions pertaining to perceptions of fatigue, including light-headedness, winded, muscles cramping, leg and whole-body fatigue. It assessed perceptions in the same way as the supplement tolerability questionnaire. During the time-trial, participants could

view work completed, but no other feedback was provided. Heart rate was collected continuously and peak and overall RPE were obtained immediately after time-trial completion. To incentivize and motivate participants, they were made aware during the consent process that a prize (commercial gift card) was given to the participant who achieved the highest mean percentage of their maximum heart rate in the two time-trials. After the time trial, subjects were weighed and completed a questionnaire to assess blinding effectiveness, supplement tolerability, and fatigue. To assess the effectiveness of blinding participants were first asked if they thought they could distinguish between PL and KE, and if so, which drink they thought they received.

Biochemical Analyses

Urine. Urine specific gravity and ketone concentration were semi-quantitively determined by the same researcher who submerged a urinalysis dipstick (Cat no. 2161, Bayer Diagnostics; Mississauga, ON, Canada) into the urine sample promptly after the sample was provided.

Blood. Fingerpick samples were analyzed at the time of collection for [β-HB] using a point-of-care analyzer (Freestyle Precision Neo; Abbott Laboratories, IL, USA). A total of ~10 ml of venous blood for all other measures were collected in appropriate BD-vacutainer tubes. A small aliquot of heparinized blood was immediately analyzed for [β-HB] using a point-of-care analyzer by a researcher unblinded to condition, and the remainder was promptly centrifuged for 10 min at 3000 rpm, except for serum samples which were left at room temperature for 30 min and allowed to clot before centrifugation. All samples were kept on ice for ≤60 min until frozen at -80°C for subsequent analysis. Commercial colorimetric assay kits were used for the measurements of β-HB and acetoacetate (Abnova, cat no KA1630; Fisher Scientific, Ottawa, Ontario).

Calculations. Cardiorespiratory data were first averaged into 30-s bins, and then averaged over the recording period. Respiratory exchange ratio (RER) was calculated as VCO_2/VO_2 (33)

and the coefficient of variation determined in pilot testing was 2.3±0.9% (same piloting as above). Arterial CO_2 partial pressure (P_aCO_2) was calculated using tidal volume and end-tidal CO_2 as per (34). Individual time-trial performance was expressed as % change for KE vs. PL relative to individual time-trial coefficient of variation, to account for inter-subject repeatability differences and expressed as mean [95% confidence intervals] as per Burke & Peeling (35). The coefficient of variation for mean time-trial power between the second familiarization and PL trials was 3.9±3.0%. Total ketone bodies are β-HB + acetoacetate via calorimetric assay. A gastrointestinal symptom incidence was a score >0 cm. Gastrointestinal symptom load and fatigue element score were the measured distance on the scale. Total condition symptom load/incidence was determined for all time points and symptoms, time-point symptom load/incidence for all symptoms at one time point, and symptom-specific load/incidence for one symptom at the time points specified.

Statistical analyses. Urinalysis was not performed in two subjects due to analytical issues, therefore urine data represent n=17. Venous blood could not be obtained in two participants and due to missing samples and technical factors a total of 12 complete datasets were obtained. Fatigue and gastrointestinal questionnaire analysis were only performed on complete datasets, n=18 and 19 respectively. Continuous variables were first tested for normality using a Shapiro-Wilks test, and if not normal, then tested for lognormality. If lognormal, then data were log transformed before being tested, and if not, non-parametric tests were performed. Cardiorespiratory and time-trial data were analyzed using paired t-tests (condition). Time-trial data were also assessed for time-period effect (trial 1 vs 2) with a paired t-test. RPE, mean fatigue element scores, and urine and blood data were assessed for differences with a repeated measure by both factors two-way analysis of variance (ANOVA) (time x condition). Differences in β-HB obtained in different blood fractions and techniques were tested with a repeated measure ANOVA (measurement x time). Significant

F-tests were followed by a Sidak's *post-hoc* test (both condition and time). A correlation matrix was applied between all ketone measurements. The % change in all variables obtained during the 30-min cycle and blood β-HB were tested for correlation to individual change in time-trial performance. Significantly correlated variables were then tested for relationship by linear regression. Bland-Altman plots assessed method agreement (β-HB in method 1 – method 2) in data from the KE condition only. Differences in total condition, time-point, and symptom-specific gastrointestinal symptom incidence were tested with a Chi-squared test (condition x yes/no). Differences in total condition, time-point, and symptom-specific gastrointestinal symptom load and fatigue element scores were tested with a Wilcoxon test (condition). Statistics were performed with Prism 8 (Graphpad, San Diego, CA, USA). Significance was accepted at $p=0.0167$ for time-point symptom incidence and load, and $p<0.05$ for everything else. Normal data are presented as mean±SD, non-normal data as median [95% confidence intervals], and effect size as Cohen's d_z.

RESULTS

Urine and blood data. Urine specific gravity was similar between conditions upon arrival (PL=1.015±0.007, KE=1.013±0.006, $p=0.33$) and no sample contained acetoacetate. Acetoacetate was not detectable in all but three PL samples and a value of 0 was used in these instances. Urine acetoacetate was higher in KE vs PL before exercise and the time trial (Interaction, $p=0.04$; *post-hoc*, $p=0.01$ for both, $d_z=0.54$; Table 1). β-HB, acetoacetate, and total ketone bodies were higher in KE vs. PL prior to exercise and remained higher before the time trial, for all blood fractions and analytical methods ($p<0.0001$; Figure 1, Table 1). β-HB was lower before the time trial vs. before exercise only in the KE condition for all blood fractions and analytical methods (Interaction $p<0.05$, *post-hoc* $p<0.05$, $d_z=-0.42–1.2$). Acetoacetate was higher before the time trial vs before exercise only in the KE condition in plasma (Interaction $p=0.01$; *post-hoc* $p<0.0001$, $d_z=1.6$) but

not serum (Interaction p=0.32). Plasma, but not serum (Time p=0.56), total ketone bodies were significant greater before the time trial vs before exercise (Time p=0.04, d_z=0.59).

Exercise responses. Ventilation (71±15 vs. 77±17 L/min, p<0.0001, d_z=1.3; Figure 2A), heart rate (150±11 vs. 155±11, p<0.001, d_z=1.3; Figure 2B), breathing frequency and tidal volume (Table 2), were higher during the 30-min exercise period in the KE vs PL condition. End-tidal CO_2 and P_aCO2 were lower in the KE vs PL condition, while end-tidal O_2, VO_2, VCO_2, and RER were unaffected by condition (Table 2). RPE was higher in the KE vs. PL condition at the end of the 30-min bout (15.4±1.6 vs 14.5±1.2; Interaction p<0.01; *post-hoc*, p<0.01, d_z=0.85).

Time-trial performance. Mean time-trial power was not different between conditions (PL=201 [174–279], KE=196 [176–295] W, p=0.21, d_z=-0.32, Figure 3A) nor was the time-trial duration (16:25±2:50 vs 16:06±2:40 min:s, p=0.20; d_z=0.31). Individual time-trial change was -0.34 [-0.94 to 0.26]% (Figure 3B), which was correlated to the % change in RPE during the 30-min cycle (Figure 3C) but not any blood ketone measure (p>0.12) or other variable obtained in the 30-min cycle (p>0.09). Neither peak (PL=18.4±1.2, KE=18.2±1.1, p=0.99) or overall (PL=17.0±1.5, KE=17.1±1.7, p=0.33) time-trial RPE were affected by condition. There was no time-period effect for mean time-trial power (p=0.27).

Questionnaires. There were zero gastrointestinal symptoms incidences at arrival. Total condition symptom incidence was greater in the KE vs PL condition (50% vs 37%, p<0.0001). Time-point symptom incidence was similar between conditions before exercise (PL=41%, KE=47%, p=0.28) but significantly different during the 30-min bout (PL=33%, KE=51%, p<0.01) and time trial (PL=35%, KE=50%, p=0.01). Symptom-specific incidence was unaffected by condition in the 30-min bout (p>0.06) and time trial (p>0.15).

Total condition symptom load was greater in the KE vs PL condition (0.8 [0.3-1.3] vs 0.3 [0.3-1.0 cm, $p<0.01$). Time-point symptom load was higher in the KE vs PL condition during the 30-min bout (0.9 [0.4-1.3] vs 0.4 [0.0-0.6] cm, $p<0.001$) and time trial (0.7 [0.2-1.3] vs 0.3 [0.0-0.6] cm, $p=0.02$), but not pre-exercise ($p=0.56$). Symptom-specific load averaged over the 30-min bout and time-trial was greater in the KE vs PL condition for stomach cramps (0.1 [0.0-1.4] vs 0.0 [0.0-0.3] cm, $p<0.05$), dizziness (1.4 [0.0-3.3] vs 0.2 [0.0-1.7] cm, $p<0.01$), gastric reflux (0.2 [0.0-1.7] vs 0.0 [0.0-0.4] cm, $p<0.05$), and urge to vomit (0.3 [0.0-1.5] vs 0.0 [0.0-0.4] cm, $p<0.01$), but not stomach burning ($p=0.10$), nausea ($p=0.12$), flatulence ($p=0.94$), or urge to urinate ($p=0.33$) or defecate ($p=0.13$).

The mean of all fatigue elements was unaffected by condition (PL=3.8±1.4, KE=4.1±1.4 cm; $p=0.21$), but was greater during the time trial vs 30-min bout ($p=0.001$). In the KE vs placebo condition, significantly greater scores were observed for lightheadedness during the 30-min bout (2.3±2.1 vs 1.1±0.9 cm, $p=0.01$, $d_z=0.64$) and muscles cramping during the time trial (3.1 [0.0–5.1] vs 1.5 [0.0–3.3] cm, $p=0.01$, $d_z=0.63$). No other fatigue elements were significantly affected by condition at either time ($p>0.11$).

Blood marker correlations and regressions. Plasma and serum β-HB assessed by point-of-care analyzer were not different from each other but were higher than plasma and serum β-HB determined by colorimetric assay, and capillary and whole blood via point-of-care analyzer ($p<0.0001$ for all, Table 1). Bland-Altman plots revealed poor method agreement between average of plasma and serum β-HB via colorimetric assay and β-HB in capillary, whole blood, and plasma analyzed with point-of-care analyzer (Figure 4A). Log(β-HB) in plasma and serum via colorimetric assay were generally correlated to all venous blood fractions at a similar strength and total ketone bodies were correlated to all β-HB measurements. Urine acetoacetate was not

correlated to any ketone measurement and nothing measured was correlated to acetoacetate in either blood fraction. Log(β-HB), averaged from plasma and serum via colorimetric assay, was linearly regressed to capillary (R^2=0.20, p=0.01; y=4.3x + 1.4), whole blood (R^2=0.43, p<0.0001; y=5.6x + 0.82), and plasma β-HB via point-of-care analyzer (R^2=0.42, p=0.0001; y=3.9x + 2.9). Plasma total ketone bodies was linearly regressed to capillary, whole blood and plasma β-HB via point-of-care analyzer, and average of plasma and serum log(β-HB) via colorimetric assay (Figure 4B).

DISCUSSION

The major novel finding of this study was that acute ingestion of 600 mg/kg body mass of a KE supplement increased markers of cardiorespiratory stress during subsequent constant load cycling performed at ventilatory threshold intensity in endurance-trained individuals, as compared to a flavour-matched placebo. Additionally, after KE compared to placebo ingestion, perceived effort was higher at the end of exercise and there was a small but significant increase in perceived gastrointestinal symptoms. The effect of KE on cardiorespiratory responses was quite consistent, with 17/19 participants having a higher ventilation and 16/19 having a higher heart rate, as compared to the placebo condition. Performance in a 3 kJ/kg body mass time-trial (~16 min), assessed 15 min after the constant load cycling bout, was not different between conditions on average but individual variability existed. The individual differences in performance between conditions were related to the differences in perceived effort, such that greater perceived effort was associated with reduced performance. Overall, these findings are contrary to our hypothesis, and suggest that acutely ingesting a relatively large bolus dose of KE does not favourably affect exercise responses or performance in endurance-trained participants.

This is the first study to report that KE ingestion elevated heart rate during exercise as others reported KE ingestion did not alter exercising heart rate compared to placebo (23,25,26). Another study found that ketone salt ingestion increased heart rate during subsequent exercise compared to water (22), whereas the present study used a flavour-matched placebo. Similar to the present study, Dearlove *et al.* (23) showed that ingesting 330 mg/kg body mass KE compared to a placebo increased ventilation at maximal exercise workload and this was associated with reduced blood pH and $P_{ET}CO_2$ (23). We also observed a higher ventilation and reduced $P_{ET}CO_2$ in the KE compared to placebo condition, which were associated with a reduced P_aO_2. Therefore, it is possible that the higher cardiorespiratory stress observed in the KE condition in the present study was a related to acidosis. The metabolism of β-HB includes dissociation of H^+ and could increase CO_2 production as predicted by the Henderson-Hasselbach equation, which would serve to increase ventilation in an effort to restore blood pH. It was suggested that plasma total ketone bodies must exceed 7 mM to induce acidosis (1), but blood pH after KE ingestion was lower when blood β-HB was ~2-4 mM (23,28). Therefore, the acidosis threshold may be lower after ingesting a bolus of exogenous β-HB compared to endogenous ketosis. Alternately, the elevated cardiorespiratory stress may be explained by a response to counter an increase in blood acetone that would result from acetoacetate degradation (36). There could also be direct effects on cardiac muscle (1) or catecholamine effects (20), but more direct measurements are required to better study the cardiac responses to ketone bodies in humans.

Although KE ingestion increased markers of cardiorespiratory stress relative to placebo, whole-body VO_2 and VCO_2 were not different ($p=0.09$, $d_z=0.42$ and 0.41 respectively). Other studies also reported non-significant effects of ketone supplement ingestion on VO_2 and VCO_2 during subsequent exercise (16,19,21–23,25,26). Butanediol ingestion that increased capillary β-

316 HB to ~0.5 mM augmented VO_2 and VCO_2 (d_z=0.32 and 0.29, respectively) 20-min into a bout of
317 cycling at ~73% VO_{2peak} as compared to placebo (21). In contrast, KE relative to placebo ingestion
318 elevated plasma β-HB to ~3.5 mM and reduced VO_2 only at 300 W during an incremental exercise
319 test (23). RER was also not different between the KE and placebo trials, but this may not be
320 reflective of unchanged substrate oxidation. Interpreting RER was complicated by potential
321 changes in CO_2 production due to acid-base buffering and ketone contribution to energy
322 expenditure, both which would be expected to elevate RER. Additional work is necessary to clarify
323 the potential effects of KE ingestion on expired gases during exercise, as well as the application
324 of more sophisticated techniques such as stable isotopic tracers to clarify underlying physiological
325 processes.

326 RPE was higher at the end of constant load cycling in the KE condition compared to
327 placebo, a difference not reported in previous studies that have included this measure (16,19–
328 22,24–26,37). One investigation, however, found that ingesting 330 mg/kg body mass of KE
329 increased perceptions of leg discomfort and anxiety of breathing and leg discomfort during
330 subsequent exercise compared to a placebo (37). In the present study, participants also reported a
331 slightly elevated gastrointestinal distress score in the KE compared to placebo condition which
332 may have contributed to augmenting perceived fatigue, albeit the magnitude of symptoms
333 indicated that distress was less than "mild". Previous work has reported that ingesting 700 mg/kg
334 body mass KE split equally before and throughout exercise induced more incidences of
335 gastrointestinal symptoms during intermittent but not steady-state running compared to a placebo
336 (25,26). Ingesting 65 g of KE before a 3 h bout of cycling and multiple performance measures,
337 including a 15-min time-trial and an all-out sprint, elevated the incidence of upper abdominal
338 gastrointestinal discomfort compared to placebo, but lower abdominal and systemic

gastrointestinal discomfort were not different (20). Also, ingesting 395 and 600 mg/kg body mass of KE before and during 3 h of cycling respectively induced a similar magnitude of gastrointestinal symptoms as compared to a carbohydrate control (38). Thus, gastrointestinal symptoms following KE ingestion may be slightly yet statistically elevated compared to a placebo, perhaps relating to the bolus volume of ketones ingested and/or exercise modality; however, the magnitude of gastrointestinal symptoms observed are not practically meaningful.

Time-trial performance was not different between the KE and placebo conditions. This occurred despite plasma β-HB being >2 mM in all participants, a hypothesized ergogenic threshold (39). Whereas cardiorespiratory stress responses during constant-load cycling were relatively consistent, individual variability in time-trial performance was apparent. Performance change in the KE compared to placebo condition exceeded individual day-to-day variability in nine participants, and of those, performance was impaired in six and improved in three participants (Figure 3B). These diverse responses may be related in part to perceived effort, as well as the magnitude of nutritional ketosis. A circulating β-HB of ~1-3 mM was also previously suggested for optimal endurance performance (7). The blood β-HB in most participants in the present study prior to the time trial exceeded the upper range, with the mean value being ~3.5 mM. However, in the five participants whose pre-time-trial β-HB was <3 mM, performance in the KE vs PL condition was within day-to-day variability in two and impaired in three. Two previous studies found that when KE or a placebo was ingested before and throughout a 1-h submaximal exercise and subsequent ~30-min time-trial, KE improved mean power in endurance-trained cyclists when plasma β-HB was 2.5-3.0 mM (27) but had no effect in endurance-trained runners when plasma β-HB was ~1.0-1.5 mM (26). Similarly, the recent study that examined multiple performance measures in endurance-trained cyclists found no effect on performance when 65 g of KE was

ingested, which increased capillary β-HB to ~0.8 mM before the first test (20). Performance in the present study may have also been influenced by the preceding constant-load exercise, because measures of cardiorespiratory stress and perceived effort were different between conditions. Also, the supplements were not matched for total energy, which may have been preferable to assess potential differences in constant-load exercise responses but not performance. Overall, the effects of KE ingestion on endurance performance remains unclear, with dose likely being an important consideration in this regard. Future research should test endurance performance following KE ingestion at a dose that does not alter blood pH, co-ingesting KE with an antacid, in combination with other nutritional strategies, and during longer duration exercises.

Both β-HB and acetoacetate were increased after KE ingestion compared to placebo and remained elevated before the time trial. The increase was expected based on the pharmacokinetics of acute KE ingestion (5,6,28), but the magnitude of such was greater than previous KE and performance studies (14,23,25,26). While remaining elevated in the KE compared to the placebo condition, β-HB decreased over the course of the exercise bout and acetoacetate increased. The KE supplement did not contain acetoacetate, suggesting that some of the ingested β-HB may have been metabolized to acetoacetate, in addition to potentially being oxidized by active tissues (40). Ketone metabolism is also complicated by endogenous production, as suggested by total ketone bodies increasing over the exercise bout in both conditions. This time effect may also be explained by gut ketone absorption in the KE trial. Overall, direct measures of ketone gut absorption, kinetics, and oxidation are required to assess metabolic effects after KE ingestion.

The method used to determine blood β-HB concentration is also important. Typically, measuring serum ketones is avoided because acetoacetate spontaneously degrades into acetone (36) and our results provide some evidence for this. Thus, acetoacetate and total ketone bodies are

best presented in blood plasma. β-HB was similar in plasma and serum fractions, but the point-of-care analyzer yielded higher β-HB compared to colorimetric assay and between-method agreement was poor. Likewise, β-HB quantified in capillary and whole venous blood via point-of-care analyzer showed poor method agreement with the colorimetric assay despite similar mean values. Therefore, β-HB obtained with the point-of-care analyzer should be interpreted with caution but may be an economic method, perhaps with adjustment to lab-based assays using the presented linear regressions, to evaluate circulating plasma β-HB in a population sample.

In conclusion, ingesting 600 mg/kg body mass of a KE supplement before constant-load cycling at ventilatory threshold intensity increased markers of cardiorespiratory stress during exercise as well as perceived effort at the end of the bout in endurance-trained participants. Subsequent performance during a 3 kJ/kg body mass time trial was not significantly different between KE and placebo conditions. Additional research is required to clarify the effect of ketone supplementation on endurance performance and exercise responses, including studies that directly assess ketone body metabolism and oxidation and that focus on intra- and extra-muscular substrate metabolism.

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CONFLICT OF INTEREST

The authors do not have any professional relationships with companies or manufacturers who will benefit from the results of the present study. The results of the present study do not constitute endorsement by ACSM. The authors declare that the results of the study are presented clearly, honestly, and without fabrication, falsification, or inappropriate data manipulation.

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507

FIGURE CAPTIONS

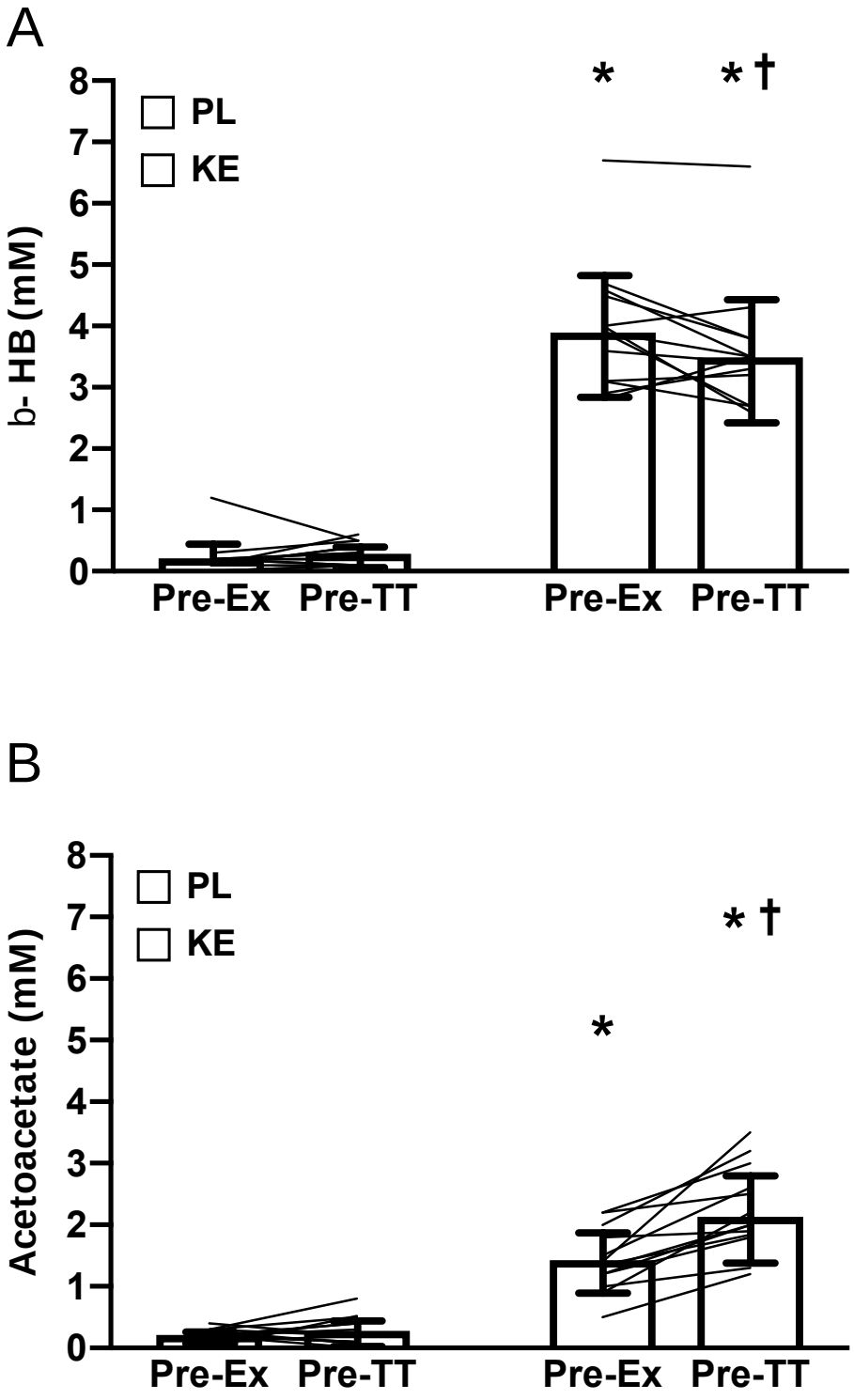
Figure 1 – Plasma beta-hydroxybutyrate (β-HB) (A) and acetoacetate (B) before a 30-min bout of cycling at ventilatory threshold intensity (Pre-Ex) and before a 3-kJ/kg body mass time-trial (Pre-TT) in the placebo (PL) and ketone monoester (KE) conditions. Values are mean ± SD (n=12); squares indicate males and circles females. * indicates *post-hoc* $p<0.0001$ vs PL within a time; †, $p<0.05$ vs Pre-Ex within a condition.

Figure 2 – Expired minute ventilation (A) and heart rate (B) during a 30-min bout of cycling at ventilatory threshold intensity in the placebo (PL) and ketone monoester (KE) conditions. Values are mean ± SD (n=19), lines connect individual data points (males are solid, females dashed). * indicates $p<0.05$ vs PL (paired t-test).

Figure 3 – Performance during a 3-kJ/kg body mass time trial (TT) performance in the placebo (PL) and ketone monoester (KE) conditions: mean power (A), % change KE vs. PL relative to individual TT CV (B), and linear regression between % change TT power and % change in RPE during the preceding 30-min bout of cycling at ventilatory threshold intensity (C). Bars are mean ± SD (n=19) for A and mean ± 95% confidence interval for B, lines connect individual data points, open circles and dashed lines represent female participants and squares and solid lines males.

Figure 4 – Comparison of blood ketone analytical methods. (A) Bland-Altman plot comparing capillary, plasma or whole blood beta-hydroxybutyrate (β-HB) determined via point-of-care analyzer to the mean of plasma and serum β-HB determined via colorimetric assay. Symbols with error bars represent mean bias ± 95% confidence intervals. (B) Linear regressions between plasma total ketone bodies (β-HB + acetoacetate) determined via colorimetric assay and capillary, whole-blood, and plasma β-HB determined via point-of-care analyzer and log(β-HB)

averaged in plasma and serum samples determined via colorimetric assay. Lines indicate line of best fit ± 95% confidence bands.



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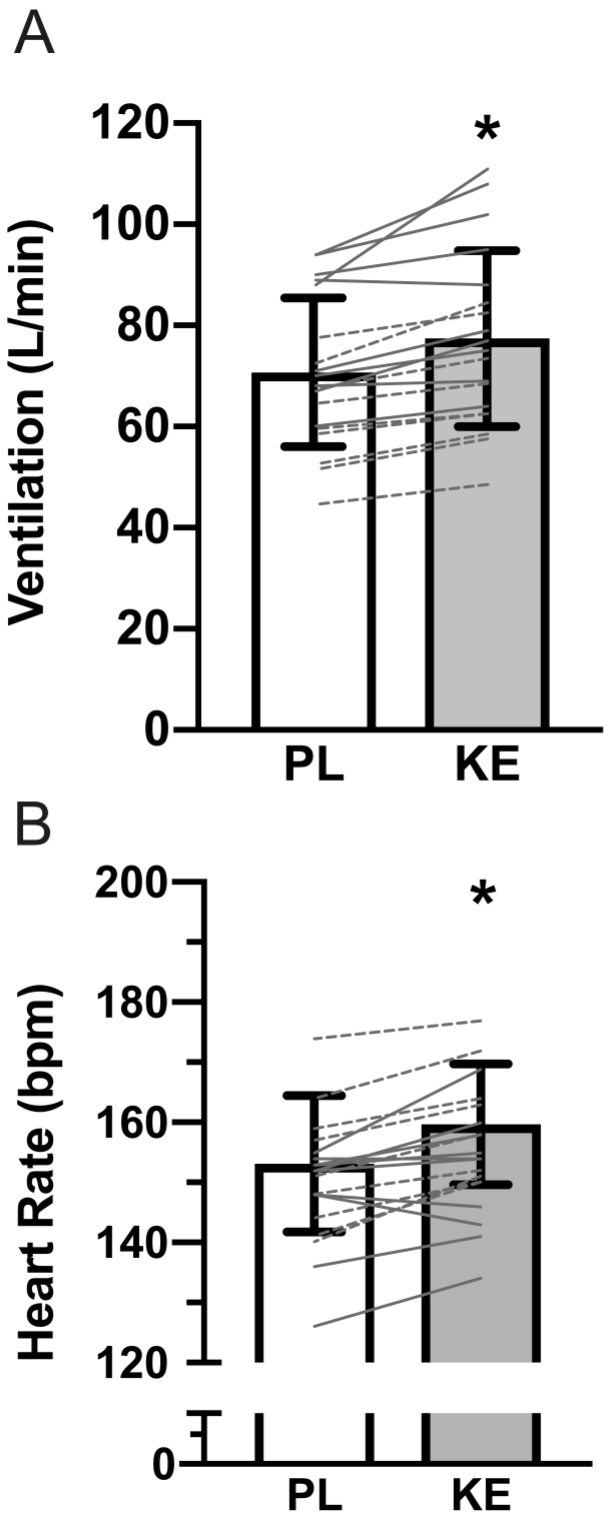

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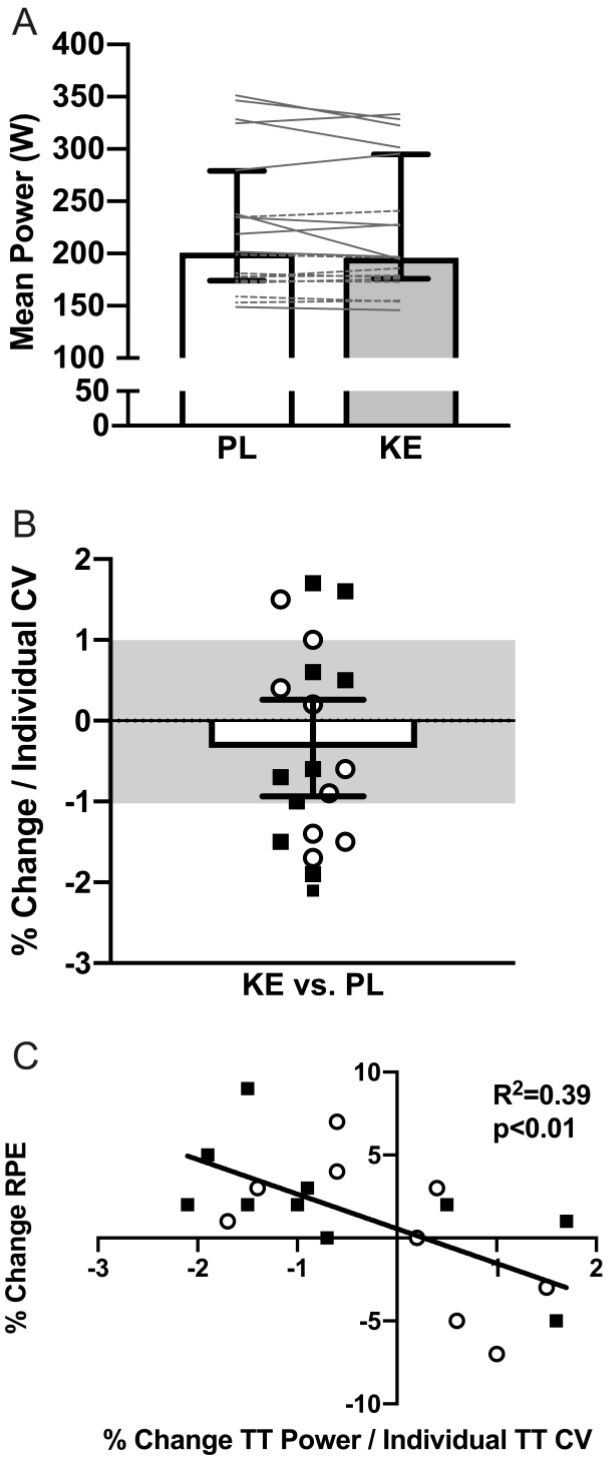

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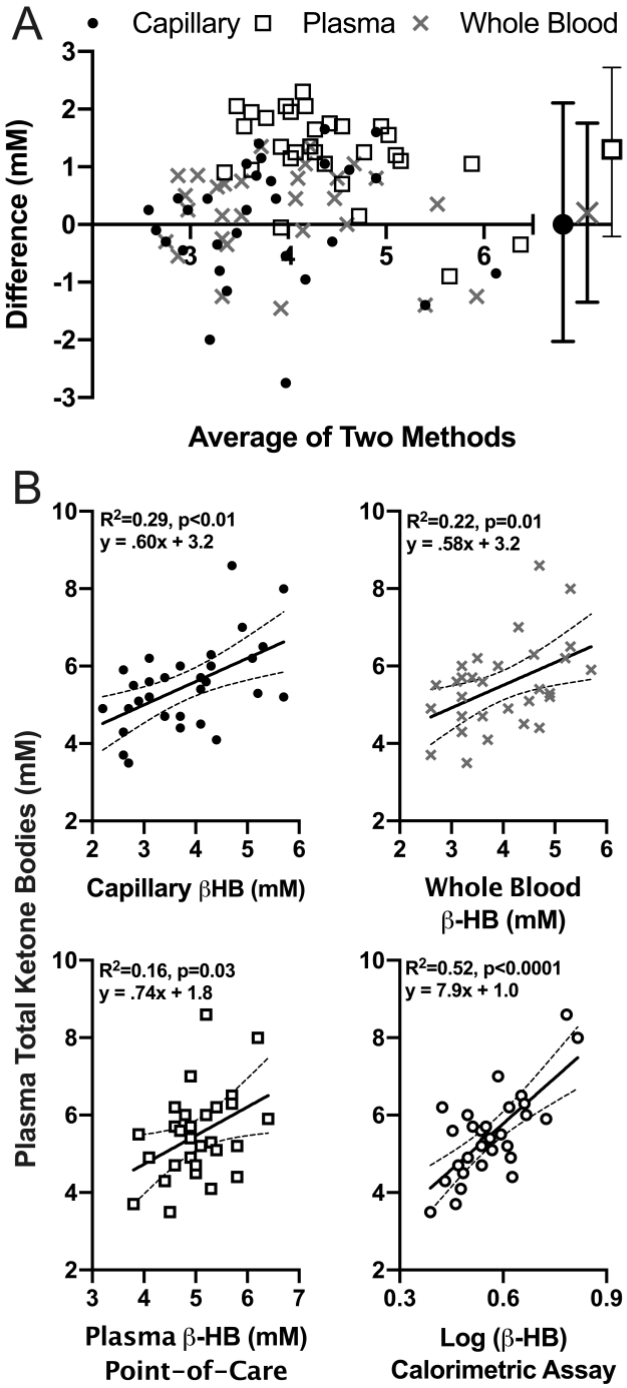

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559 **Table 1.** Blood beta-hydroxybutyrate (β-HB), acetoacetate, and total ketone bodies before exercise
560 (Pre-Ex) and the time trial (Pre-TT) in the placebo (PL) and ketone monoester conditions (KE).

	PL		KE	
	Pre-Ex	**Pre-TT**	**Pre-Ex**	**Pre-TT**
β-HB				
POC analyzer				
Capillary [a]	0.1 ± 0.0	0.2 ± 0.1	4.0 ± 1.7*	3.6 ± 0.8**†
Whole blood [a]	0.1 ± 0.1	0.1 ± 0.1	4.4 ± 0.9 *	3.5 ± 0.6**†
Plasma [b]	0.0 ± 0.0	0.0 ± 0.1	5.3 ± 0.6*	4.7 ± 0.4**†
Serum [b]	0.0 ± 0.0	0.0 ± 0.1	5.3 ± 0.6*	4.8 ± 0.4**†
Colorimetric Assay				
Plasma [a]	0.2 ± 0.3	0.3 ± 0.2	3.9 ± 1.0*	3.5 ± 1.0**†
Serum [a]	0.3 ± 0.2	0.3 ± 0.2	4.3 ± 1.0*	3.4 ± 1.0**†
Acetoacetate				
Plasma	0.2 ± 0.1	0.3 ± 0.2	1.4 ± 0.5*	2.1 ± 0.7**†
Serum **	0.2 ± 0.1	0.3 ± 0.1	1.6 ± 0.6	1.8 ± 0.7
Urine	0.0 ± 0.1	0.0 ± 0.0	6.0 ± 3.7*	7.4 ± 3.9**†
Total ketone bodies				
Plasma **††	0.4 ± 0.2	0.6 ± 0.2	5.3 ± 1.1	5.7 ± 1.2
Serum **	0.5 ± 0.3	0.7 ± 0.2	5.8 ± 1.1	5.3 ± 1.3

561

562 Values are mean ± standard deviation, units are mmol/L, measurements are from venous blood,
563 and total ketone bodies = β-HB + acetoacetate via calorimetric assay. * indicates $p<0.05$ KE vs.
564 PL; †, $p<0.05$ Pre-Ex vs. Pre-TT; different letters, $p<0.05$ between β-HB rows in the KE
565 condition; POC, point-of-care.

Table 2. Metabolic and respiratory data during 30 min of cycling at ventilatory threshold intensity in the placebo (PL) and ketone monoester (KE) conditions.

	PL	KE	p	d_z
Breathing Frequency (breaths/min)	33.2 ± 5.8	34.3 ± 5.4	0.03 *****	0.52
Tidal Volume (L/breath)	2.2 ± 0.5	2.3 ± 0.5	<0.001 *****	1.01
$P_{ET}O_2$ (mmHg)	117 ± 3	119 ± 4	0.01 *	0.41
$P_{ET}CO_2$ (mmHg)	30.5 ± 2.2	28.4 ± 3.2	<0.0001*	-0.97
P_aCO_2 (Torr)	32.9 ± 2.0	31.0 ± 2.9	<0.001*	-1.31
VO_2 (L/min)	2848 ± 659	2879 ± 664	0.09	0.42
VCO_2 (L/min)	2481 ± 600	2519 ± 636	0.09	0.41
RER	0.87 ± 0.04	0.87 ± 0.04	0.79	0.06

Values are mean±SD (n=19). P-values are from paired t-tests. * indicates $p<0.05$ vs. PL; $P_{ET}O_2$, end tidal O_2; $P_{ET}CO_2$, end-tidal CO_2; P_aO_2, arterial O_2 partial pressure; VO_2, oxygen uptake; VCO_2, carbon dioxide expired; RER, respiratory exchange ratio; d_z, effect size.