


Post-exercise ketone supplementation improves endurance performance and mitochondrial adaptations during an 8-week endurance training intervention

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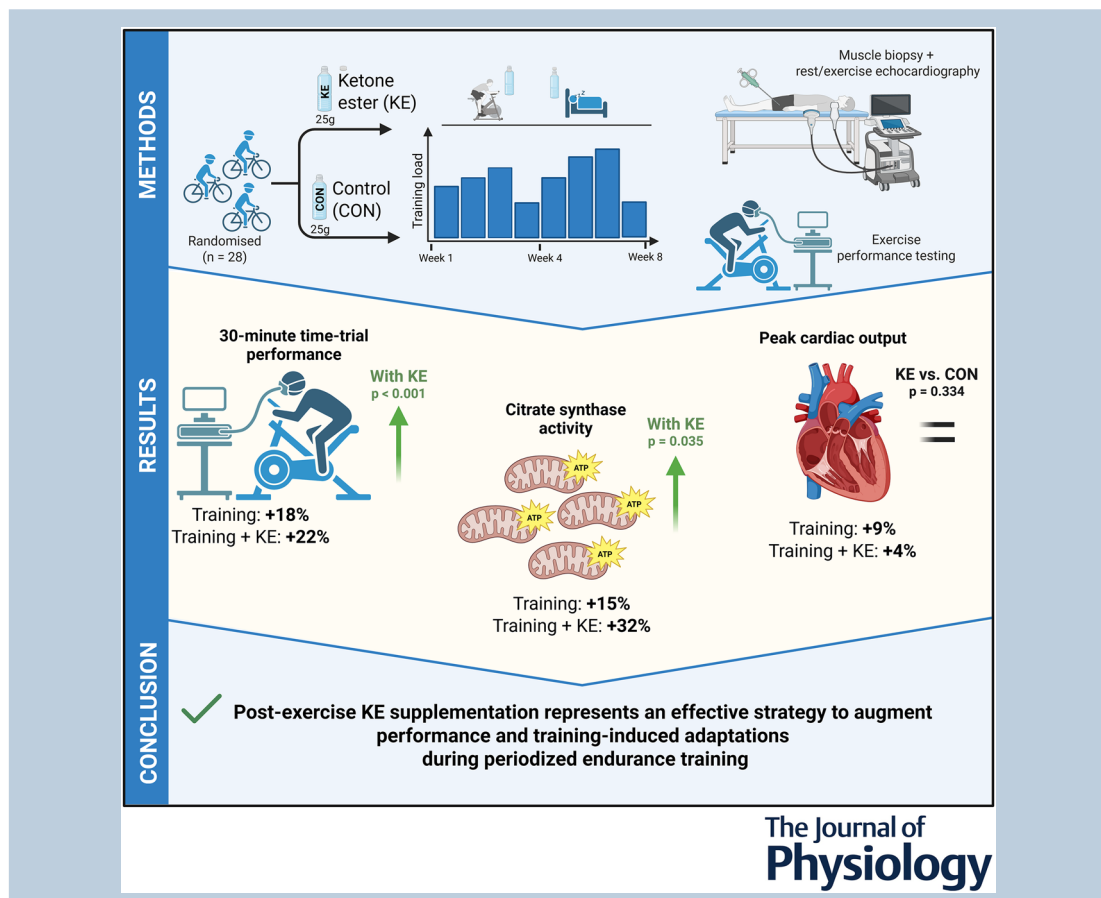
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Abstract figure legend Twenty-eight trained males completed 8 weeks of supervised indoor cycling training, receiving post-exercise and pre-sleep ketone monoester (KE, $n = 14$) or isocaloric placebo (CON, $n = 14$). Both groups improved 30 min time-trial performance with training, but KE achieved a 4% higher power output than CON at the end of the training program. This KE-induced improvement in cycling performance was supported by a greater increase in citrate synthase activity, a mitochondrial mass marker, compared to CON. Peak cardiac output remained stable in both groups. These findings establish post-exercise KE supplementation as an effective nutritional strategy to augment endurance performance and mitochondrial adaptations during periodised endurance training.

Abstract Post-exercise ketone supplementation (PEKS) previously improved endurance performance and muscular adaptations during overload training. However, whether and how PEKS improves endurance performance during a periodised endurance training period remains unclear. Twenty-eight trained males completed 8 weeks of supervised cycling, receiving either 25 g of the ketone monoester (*R*)-hydroxybutyl (*R*)-hydroxybutyrate (KE, $n = 14$) or isocaloric placebo (CON, $n = 14$) post-exercise and before sleep. Outcomes included exercise performance and muscular/cardiac adaptations assessed at baseline (PRE), week 3 (MID) and 7 (POST), and following a taper week (POST_{+1week}). The training intervention improved 30 min time trial performance (TT_{30min}), absolute and relative $\dot{V}_{O_{2peak}}$, peak power output during the $\dot{V}_{O_{2peak}}$ test (PPO $\dot{V}_{O_{2peak}}$), citrate synthase (CS) activity, and peak cardiac output (all $P < 0.05$ PRE vs. POST). Notably, TT_{30min} (CON: 291 ± 27 W vs. KE: 302 ± 28 W, $P < 0.001$, Hedges' $g = 0.40$) was 4% higher in KE compared to CON at POST. Although peak cardiac output was similar, relative $\dot{V}_{O_{2peak}}$ increased more in KE (+12%) than CON (+6%, $\Delta P < 0.001$), suggesting enhanced peripheral oxygen utilisation. Accordingly, CS activity was at POST 14% higher in KE (9.37 ± 1.36 mol h⁻¹ kg protein⁻¹) compared to CON (8.21 ± 0.97 mol h⁻¹ kg protein⁻¹, $P = 0.035$, Hedges' $g = 0.98$) and OXPHOS complex II muscle protein content remained unaltered in CON while increasing by 25% in KE ($P = 0.030$ vs. PRE). In conclusion, these findings establish PEKS as a nutritional strategy to enhance endurance performance and mitochondrial adaptations during periodised endurance training.

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Key points

- Previous research showed that post-exercise ketone ester (KE) supplementation improves endurance performance and muscle adaptations during 3 weeks of excessive endurance exercise. Whether KE ingestion enhances performance, muscular and cardiac adaptations during a periodised endurance training intervention remained unknown.
- We demonstrate that KE supplementation post-exercise and before sleep during an 8 week periodised endurance training period enhances endurance performance and peak oxygen uptake rate in trained male participants.
- These performance improvements were mediated by muscular adaptations (e.g. increased mitochondrial content), while cardiac adaptations were not affected by KE.
- Our findings show that KE enhances endurance performance primarily by muscular adaptations, indicating that post-exercise KE supplementation is effective to amplify endurance exercise training adaptations.

Introduction

Endurance exercise training drives co-ordinated physiological adaptations across multiple organ systems that are

not only critical for improving exercise performance, but also for maintaining cardiovascular and metabolic health (Moreira et al., 2020; Thyfault & Bergouignan, 2020). The magnitude of these functional improvements is dictated

not only by the training stimulus itself, but also by the capacity to recover from each training session (Skorski et al., 2019). In this respect, nutritional strategies that can improve post-exercise recovery (e.g. carbohydrate and protein intake) have been shown to be effective in augmenting the adaptive response to exercise training (Lin et al., 2021; Moore, 2015). Recent evidence from our laboratory indicates that ingestion of the ketone monoester (*R*)-3-hydroxybutyl (*R*)-3-hydroxybutyrate (KE), may also enhance post-exercise recovery (Poffé et al., 2019, 2023). Yet, whether this translates into an improved training response remains to be determined.

KE ingestion is a viable approach to intermittently elevate the concentration of the ketone bodies *D*- β -hydroxybutyrate (β HB) and acetoacetate (AcAc) in the blood and other tissues (Robberechts & Poffé, 2024). Interestingly, emerging evidence suggests that post-exercise KE ingestion may enhance exercise recovery, most probably as a result of the ability of ketone bodies to act as signalling molecules that affect a broad range of physiological processes (Robberechts & Poffé, 2024). In this context, we have demonstrated that consistent post-exercise (and pre-sleep) KE ingestion attenuates the development of overreaching symptoms induced by a 3-week endurance training (ET) overload program (Poffé et al., 2019). Furthermore, the KE group showed increased skeletal muscle angiogenesis and elevated circulating erythropoietin (EPO) levels (Poffé et al., 2023). These physiological adaptations coincided with a 15% increase in manageable training load and enhanced endurance performance during the final week of the overload training period in the KE group (Poffé et al., 2019).

Despite these findings, it remains unclear whether post-exercise ketone supplementation (PEKS) enhances training adaptations and performance during a periodised ET program that does not induce overreaching symptoms. Additionally, the presence of dietary intake differences between groups and the development of other overreaching symptoms in our previous study (Poffé et al., 2019) did not allow for disentangling whether improved functional responses were either directly caused by PEKS or resulted from the ability of ketone bodies to counter-

act the development of overreaching symptoms thereby enabling a normal training response.

In favour of the latter, earlier studies indicated that excessive exercise training impairs mitochondrial function and only increases skeletal muscle oxidative capacity in athletes who do not exhibit overreaching symptoms (Bellinger et al., 2020). Conversely, studies suggest that ketone bodies can both impact central and muscular adaptations to ET. At the muscular level, *in vitro* and *in vivo* experiments showed that ketone bodies enhance mitochondrial function (Parker et al., 2018), attenuate the activity of catabolic pathways (Chen et al., 2022), stimulate myogenesis (Qiu et al., 2025) and activate satellite cells (Zou et al., 2016). However, direct evidence in human muscle is limited, with our study reporting an increase in muscular capillarisation (Poffé et al., 2023) and another study reporting that PEKS activates downstream effectors of mTORC1 as associated with enhance muscle protein synthesis (Vandoorne et al., 2017).

Ketone bodies also have the potential to improve central adaptations to endurance exercise. This is supported by a recent study showing that intermittent KE supplementation for 14 days improved resting cardiac output in patients with a reduced ejection fraction under fasted conditions (Berg-Hansen et al., 2024). PEKS may also stimulate blood EPO concentrations, which, in the longer term, may enhance oxygen transport. Studies have reported an increase in circulating EPO levels with PEKS following both acute exercise (Evans et al., 2023) and after a 3 week overload training period (Poffé et al., 2023). However, other studies found that acute KE ingestion does not further enhance EPO levels during acute hypoxic exposure (Stalmans et al., 2025), or immediately following a short (<1 h) bout of endurance exercise (Howard et al., 2024).

To investigate the impact of PEKS during periodised ET on muscular and cardiac adaptations, we conducted an 8 week randomised, placebo-controlled trial. Based on previous research, we hypothesised that PEKS would augment both cardiac (e.g. cardiac output) and muscular adaptations (e.g. skeletal muscle metabolites and mitochondrial function), leading to greater improvements in endurance performance compared to placebo.

Ruben Robberechts is a postdoctoral researcher at the REVAL research group of the University of Hasselt (Belgium) under the supervision of Professor Chiel Poffé. His research investigates how ketone bodies impact exercise performance, training adaptations and recovery in athletes. His broader aim is to optimise human performance through targeted nutritional and training interventions. **Youri Bekhuis** is a cardiologist in training at University Hospital Leuven (Belgium) and is finalising his joint PhD at KU Leuven and the University of UHasselt under supervision of Professor Guido Claessen. His research focuses on how the heart performs under physical stress, using advanced exercise cardiac imaging to better understand how the heart adapts to exercise.



Table 1. Baseline participant characteristics.

	CON	KE
Age (years)	24.3 ± 4.3	25.9 ± 4.2
Height (m)	1.81 ± 0.04	1.82 ± 0.05
Body mass (kg)	73.5 ± 5.90	73.9 ± 5.61
BMI (kg m ⁻²)	22.5 ± 1.8	22.3 ± 1.4
$\dot{V}_{O_2\text{peak}}$ (mL kg ⁻¹ min ⁻¹)	59.4 ± 6.0*	55.9 ± 4.9*
Cycling training volume (h week ⁻¹)	4.1 ± 2.1	3.9 ± 2.1

Data are presented as the mean ± SD and represent baseline characteristics of participants receiving either post-exercise and pre-sleep control (CON, $n = 14$) or ketone ester supplements (KE, $n = 14$). * $P < 0.05$ for CON vs. KE.

Methods

Ethical approval

The study was approved by the Ethics Committee of UZ/KU Leuven (B3222021000725) and conforms to the latest revision of the *Declaration of Helsinki*. The study was registered in a clinical trial database (clinicaltrials.gov: NCT05932420). All participants were informed about the potential risks of the study and signed the informed consent form prior to enrollment in the study.

Participant characteristics

Thirty trained male participants (Tier 2) (McKay et al., 2021) were included in the study. Prior to inclusion, all participants were screened by a physician using a medical questionnaire, a resting electrocardiogram, and by measuring body weight and blood pressure. Participants were aged between 18 and 35 years, had a body mass index (BMI) between 18 and 25 kg m⁻², $\dot{V}_{O_2\text{peak}} > 50$ mL kg⁻¹ min⁻¹, performed at least two cycling training sessions per week, were non-smokers, and did not take supplements or medication that could have prolonged impact on exercise performance or adaptation (e.g. β -alanine, creatine and ketone supplements) in the 3 months preceding the study or during the experimental period. From the initial 30 participants, one participant of the control group and one participant of the KE group did not complete the study because of illness during the experimental period. Twenty-eight participants eventually completed the full study protocol and were included in the final data analyses. Participant characteristics are outlined in Table 1.

Preliminary testing and participants randomisation

Two weeks prior to the start of the study, participants completed two preliminary sessions that were separated

by a 72 h period. During the first visit, they performed a dual graded exercise test to determine their lactate threshold (LT) and whole-body peak oxygen uptake ($\dot{V}_{O_2\text{peak}}$), as well as a 30 min time trial (TT_{30min}) on a calibrated cycling ergometer (Cyclus 2; RBM elektronik-automation GmbH, Leipzig, Germany). To determine LT, participants started at a power output of 100 W which increased by 40 W every 8 min. Every 4 min, a capillary blood sample was obtained to determine blood lactate concentration (Lactate Pro2; Arkray, Amstelveen, The Netherlands) and LT was defined as the lowest power output corresponding to a 1 mM increase in blood lactate within the same stage. Upon reaching LT, participants actively recovered (cycling for 15 min at 100 W) before starting a second graded exercise protocol to determine their $\dot{V}_{O_2\text{peak}}$. This protocol started at a power output of 100 W, which incremented every 30 s by 25 W until volitional exhaustion. During the entire test, \dot{V}_{O_2} and \dot{V}_{CO_2} were measured continuously by a breath-by-breath spirometry system (Metalyzer 3B; Cortex Biophysik GmbH, Leipzig, Germany) and $\dot{V}_{O_2\text{peak}}$ was determined as the highest oxygen uptake rate averaged over a 30 s period. The highest power output attained during the $\dot{V}_{O_2\text{peak}}$ test before termination of the test (cadence <70 rpm) was defined as the peak power output $\dot{V}_{O_2\text{peak}}$ (PPO $\dot{V}_{O_2\text{peak}}$). Following a 15 min recovery period at 100 W, participants started the TT_{30min} during which they had to aim for the highest possible mean power output. The initial power output was set at the participant's LT, and, from $t_{5\text{min}}$ to $t_{25\text{min}}$, participants were allowed to adjust the power output every 5 min, and, from $t_{25\text{min}}$ to $t_{29\text{min}}$, every minute to facilitate complete exhaustion. During the second visit, TT_{30min} was familiarised for a second time. First, participants warmed up by 5 min of cycling at 75% and 5 min of cycling at 85% of their mean power output of TT_{30min} obtained during their first visit. Immediately following the warming up, they started TT_{30min} at their mean power output achieved during the TT_{30min} of the first session and followed an identical procedure.

Participant randomisation and general experimental design

Following the preliminary sessions, participants were pair-matched based on TT_{30min} and were subsequently randomly split into a control group and experimental group by a researcher who was not involved in any of the experimental measurements. Following randomisation, the researcher verified that the pairs did not differ significantly in other key characteristics including PPO $\dot{V}_{O_2\text{peak}}$, cycling training history and BMI. The participants subsequently engaged in a double-blind, parallel group study design, during which they followed an 8 week cycling training program that was designed to improve

endurance exercise performance. During this period, participants received either a ketone ester (KE, $n = 14$) or an isocaloric placebo (CON, $n = 14$) drink immediately after each training session and 30 min before sleep.

Training intervention

The 8 week training intervention consisted of two 4 week mesocycles with a 3:1 work to recovery balance. During each mesocycle, the training load increased during the first 3 weeks, followed by a taper week during which the training load was reduced by 40–50% to facilitate recovery and prevent the development of non-functional overreaching. Except for the taper weeks, the participants trained 5 days per week, including one or 2 days with double training sessions. The training program consisted of a combination of ET, intermittent endurance training (IET), high-intensity interval training (HIIT) and sprint interval training (SIT). During the first mesocycle, exercise intensity was set relative to the starting power output of $TT_{30\text{mi}}$ at PRE, whereas, during the second mesocycle, exercise intensity was set relative to the starting power output of $TT_{30\text{min}}$ at MID. This starting power output was identical between paired participants (see section below on ' $TT_{30\text{min}}$ ').

All training sessions were performed in an indoor, exercise laboratory under supervision of the researchers. The participants performed the training sessions on their own bicycle that was mounted on a cycling ergometer (Tacx Neo Smart, Wassenaar, The Netherlands), which was calibrated every other day using an SRM Science power meter (SRM GmbH, Jülich, Germany). ET sessions consisted of constant load cycling at 70% of $TT_{30\text{min}}$, whereas, during IET, the intensity varied between 60% and 110% of $TT_{30\text{min}}$. HIIT sessions consisted of 3 min of cycling at 120% of $TT_{30\text{min}}$ interspersed by 4 min of cycling at 50%. During the SIT sessions, the participants performed 6–12 all-out 30 s sprints followed by active recovery intervals of 4 min 30 s at 50% of $TT_{30\text{min}}$. The participants were verbally encouraged during each sprint to ensure maximum effort. The training load was adjusted by modifying the number of training sessions, as well as their duration, intensity and number. Whenever a participant was unable to perform a training session because of illness or injury, or when the training load needed to be reduced because of physical fatigue, this was recorded in an individual training diary.

Nutritional intervention

During each training session that lasted longer than 1 h, participants received ~60 g of carbohydrates per hour through a 500 mL isotonic sports drink and an energy bar (6D Sports Nutrition, Antwerp, Belgium).

Water consumption was allowed *ad libitum*. Participants consumed 25 g of a ketone monoester (KE) drink or an isocaloric control drink (CON) immediately post-exercise and 30 min before bedtime on all training days except the first day, which was designated as a baseline sleep measurement day (reported in a separate manuscript). This dosing strategy was based on previous studies of our research group (Poffé et al., 2019, 2023; Robberechts et al., 2023). Post-exercise supplements were always administered on-site by the present researcher to ensure compliance. For pre-sleep supplements on training days and all supplements on non-training days, participants received a weekly supply bag containing the exact number of supplement tubes needed for that week. Empty tubes were collected and returned at each subsequent training session to monitor adherence. The pure (R)-3-hydroxybutyl (R)-3-hydroxybutyrate ketone monoester was purchased from KetoneAid Inc. (Falls Church, VA, USA). The control drink consisted of 16.4 g of pure medium-chain triglycerides mixed with 1 mM bitter sucrose octaacetate (Sigma0-Aldrich, Bornem, Belgium) to match the taste of KE, comprising a procedure that has previously been shown to be effective in making the KE and CON indistinguishable (Poffé et al., 2019, 2023). The supplements were always provided in non-transparent tubes, aiming to avoid potential visual identification. The total energy content of both supplements was 489 kJ per dose of 25 g. Five minutes after each training session, the participants also consumed a 300 mL recovery shake delivering 64 g of carbohydrates and 30 g of proteins (6D Sports Nutrition).

Overview experimental sessions

Participants completed experimental sessions at four timepoints: baseline (PRE), after 3 weeks (MID), 7 weeks (POST) and following the tapering week ($\text{POST}_{+1\text{WEEK}}$). Sessions lasted either one day ($\text{POST}_{+1\text{WEEK}}$) or two consecutive days (PRE, MID and POST) and aimed to evaluate endurance performance and training adaptations.

Day 1. The evening before the experimental session, participants consumed a standardised carbohydrate-rich dinner (5400 kJ; 69% carbohydrate, 16% fat, 15% protein). Following an overnight fast, they arrived at the laboratory where venous (antecubital) and capillary earlobe blood samples were obtained. Body composition was assessed by dual energy X-ray absorptiometry (DXA) at PRE and POST only. Next, participants consumed a standardised breakfast (2700 kJ; 71% carbohydrate, 15% fat, 14% protein) and rested for 90 min during completion of the RESTQ-76 sports questionnaire (Kallus et al., 2001). Thereafter, they performed a 10 min warm-up, which was

immediately followed by $TT_{30\text{min}}$. Muscle biopsies from the vastus lateralis were collected before and immediately after $TT_{30\text{min}}$ at PRE and POST. Following $TT_{30\text{min}}$, participants received a 300 mL recovery shake consisting of 64 g of carbohydrates and 30 g of proteins.

Day 2. Participants consumed a standardised lunch at home (2050 kJ; 54% carbohydrates, 28% fat, 18% protein). Two hours later, cardiac function was assessed at rest and during exercise by echocardiography at PRE and POST. The $\dot{V}_{O_{2\text{peak}}}$ test was performed 4 h post-lunch at PRE, MID and POST using procedures identical to those employed during the preliminary session.

$TT_{30\text{min}}$

$TT_{30\text{min}}$ started with a 10 min warm-up consisting of 5 min of cycling at 70% and 5 min of cycling at 85% of their mean power output achieved during $TT_{30\text{min}}$ in the final preliminary session. The warming-up was immediately followed by $TT_{30\text{min}}$, which started at the mean power output of the last preliminary session using a procedure identical to that employed during the preliminary sessions. At MID, the relative intensities of the warm-up and $TT_{30\text{min}}$ were based on the mean power output at PRE, whereas, at POST and $POST_{+1\text{WEEK}}$, these were based on the mean power output at MID. When a difference existed within pairs (i.e. maximal within-pair difference was 17 W), the average power output of the CON and KE participant of the previous $TT_{30\text{min}}$ was used for the starting power output of the paired-participants. This ensured that the starting power output for the time trial remained identical between paired participants (i.e. KE vs. CON) at all testing sessions.

Throughout $TT_{30\text{min}}$, participants received standardised verbal encouragement, real-time feedback on elapsed time and water *ad libitum* (volume kept identical across sessions for each participant). Heart rate was continuously registered using a chest strap monitor (Polar H10; Polar Electro, Kempele, Finland). Capillary blood samples were collected from the earlobe at 0, 10, 20 and 30 min for lactate analysis (Lactate Pro2; Arkray, Kyoto, Japan). Immediately upon completion, participants rated their perceived exertion using the 6–20 Borg scale (Borg, 1982).

Body composition, resting heart rate and blood pressure measurement

Body composition was evaluated by a whole-body DXA scan (Discovery W, Hologic Inc., Bedford, MA, USA). The densitometer was calibrated daily against a spinal phantom, and the measurements were performed by the same certified technician to reduce day-to-day variability

between the measurements. The parameters evaluated were whole-body bone mineral content (BMC) and density (BMD), body fat percentage, and lean tissue mass.

Assessment of subjective stress and recovery state

The RESTQ-76 Sport questionnaire was each time administered immediately following breakfast to assess the participant's perceived stress-recovery state (Kallus et al., 2001). The total stress score was computed as the sum of the 10 subscales evaluating stress, whereas the total recovery score was calculated as the sum of the nine subscales assessing recovery. Next, the recovery stress-state was determined by subtracting the total stress score from the total recovery score. Higher scores indicate adequate physical and psychological recovery, whereas lower scores indicate more perceived physical and psychological stress.

Rest and cardiopulmonary exercise test (CPET)-echocardiography

Cardiac function at rest and during exercise was evaluated with CPET-echocardiography according to a standardised protocol as previously described (de Wilde et al., 2023; Verwerft et al., 2023). Three-dimensional (3D) left-ventricular volumes were obtained at rest in the left decubitus position. Exercise was performed on a semi-supine bicycle ergometer (Ergoline GmbH, Bitz, Germany) using a ramp protocol ($20 + 20 \text{ W min}^{-1}$) aiming for a total exercise duration of 10–12 min ($60\text{--}65 \text{ rotations min}^{-1}$). Images were acquired at rest, during low-intensity exercise (heart rate between 90 and $100 \text{ beats min}^{-1}$, before fusion of E and A waves) and at peak exercise (respiratory exchange ratio >1.05 or severe exhaustion) by a qualified physician. The power output was held constant during image acquisition. Resting and peak cardiac output were calculated from the velocity-time integral of the flow through the aortic valve in the apical five-chamber view via pulsed wave Doppler, left ventricular outflow tract diameter and heart rate. Left ventricular global longitudinal strain was calculated as the average peak systolic strain from all segments in apical four-, three- and two-chamber views (Voigt et al., 2015). To account for the expected decline in acoustic quality as a result of hyperventilation, loop registration of at least 10 beats was recorded. Measurement were always performed by the same physician. Analyses were performed offline using EchoPAC software, version 204 (GE Vingmed, Horten, Norway) in accordance with contemporary international guidelines (Lang et al., 2015; Nagueh et al., 2016). Peak cardiac output and stroke volume data of five individuals (3 KE and 2 CON) were not included in the final analysis because of missing data caused by aliasing or breathing artefacts.

Analysis of venous and capillary blood samples

Fasted venous blood samples from an antecubital vein were collected into vacuum tubes containing either EDTA, Silica Clot Activator (BD Vacutainer; Becton Dickinson, Franklin Lakes, NJ, USA) or heparin. Subsequently, the tubes were centrifuged, and the supernatant was stored at -80°C for later analysis. Commercially available high-sensitive enzyme-linked immunosorbent assay kits were used to determine serum EPO and GDF15 concentration (EPO: DY286-05, GDF15: DGD150; R&D, Minneapolis, MN, USA). In addition to the capillary blood samples collected at the experimental sessions, capillary blood samples were collected (i) before, (ii) after and (iii) 30 min after training session 7 (week 1), training session 16 (week 3) and training sessions 43 (week 7) for determination of βHB concentration (GlucoMen areo 2K-meter with β -ketone sensor strips; A. Menarini Diagnostics, Firenze, Italy).

Dietary control

Nutritional intake was monitored through an online food diary (Mijn Eetmeter, Stichting Voedingscentrum Nederland; <https://mijn.voedingscentrum.nl>). The participants completed the food diary during the first, third and seventh training week. The food diaries were always completed on the same days of the week, involving each time two weekdays and one weekend day.

Muscle biopsy procedure

Immediately before and after completion of $\text{TT}_{30\text{min}}$ at PRE and POST, a biopsy was obtained by a medical doctor from the vastus lateralis under local anaesthesia (2% xylocaine without adrenaline, 1 mL s.c. injection) using a Bergström type needle. Muscle biopsies were each time obtained from the same incision of the right leg with the needle pointing respectively proximal and distal, before and after $\text{TT}_{30\text{min}}$. At POST, the incision was made ~ 2 cm distal and ~ 1 cm medial from the previous incision at PRE. The muscle biopsy was immediately frozen in liquid nitrogen and stored at -80°C for biochemical analyses.

Western blot

As previously described (Poffé et al., 2023), frozen muscle tissue samples 15–20 mg were homogenised (4×20 s) in ice-cold lysis buffer using a FastPrep (MP Biomedicals, Santa Ana, CA, USA). The lysis buffer was composed of 1:10 w/v: 50 mM Tris-HCl (pH 7.0), 270 mM sucrose, 5 mM EGTA, 1 mM EDTA, 1 mM sodium orthovanadate, 50 mM glycerophosphate, 5 mM sodium pyrophosphate, 50 mM sodium fluoride, 1 mM dithiothreitol, 0.1% Triton

X-100 and one protease inhibitor tablet (Roche Applied Science, Vilvoorde, Belgium). Next, the homogenates were centrifuged (10 min at 10 000 g) and the supernatants were stored at -80°C . The detergent-compatible (DC protein assay kit (Bio-Rad Laboratories, Nazareth, Belgium) was used for protein quantification of the supernatants. Fifteen micrograms of the proteins was loaded and separated by SDS-PAGE on 4%–20% Criterion TGX stain-free gels (Bio-Rad, Hercules, CA, USA) and subsequently transferred to polyvinylidene difluoride membranes. Next, the membranes were blocked for 1 h in Tris-buffered saline Tween 20 containing 5% non-fat milk. Thereafter, the membranes were incubated overnight at 4°C with the total OXPHOS Rodent Antibody Cocktail (dilution 1:250, AB_110413, 45-8099; Thermo Fisher Scientific, Waltham, MA, USA) or antibodies against peroxisome proliferator-activated receptor γ coactivator 1- α (PGC-1 α ; AB_2755043, sc-517380; Santa Cruz Biotechnology, Santa Cruz, CA, USA), sirtuin 3 (SIRT3; D22A3; Cell Signaling Technology, Danvers, MA, USA) and acetylated lysine (9441; Cell Signaling Technology). The next morning, membranes were incubated (dilution 1:5000) for 45 min with the appropriate horseradish peroxidase-conjugated secondary antibody (Sigma-Aldrich) at room temperature. Thereafter, the membranes were analysed with Genesnap and Genetools Software (Syngene, Cambridge, UK). Results are displayed as the protein of interest relative to the total protein content.

Citrate synthase (CS) activity

CS activity was assessed using the same homogenates prepared for western blotting. These homogenates were diluted to a protein concentration of 3 mg mL^{-1} with the same lysis buffer as that used in our western blot procedure. Subsequently, CS activity was assessed in triplicate in accordance with the protocol described by Kuang et al. (2022). After addition of 5 μL of the muscle homogenates, 235 μL of a solution consisting of 0.5 mM acetyl-CoA (A2180; Sigma-Aldrich), 0.1 mM 5,5'-dithiobis (2-nitrobenzoic acid) (D8130; Sigma-Aldrich) and 45 mM Tris buffer (pH 8.3) was added. Next, baseline reaction activity was measured spectrophotometrically at 412 nm every 15 s for a total of 180 s using a microplate reader. Thereafter, the CS reaction was initiated by adding 15 μL of 10 mM oxaloacetic acid (O4126; Sigma-Aldrich) and absorbance readings were obtained every 15 s following the same protocol. Next, CS activity ($\Delta_{\text{A}412} \text{ min}^{-1}$) was calculated by subtracting the baseline activity from the total activity. Thereafter, $\Delta_{\text{A}412} \text{ min}^{-1}$ was multiplied by a constant factor to report as $\text{mol h}^{-1} \text{ kg}^{-1}$ (Brischigliaro et al., 2022). The coefficient of variation of the triplicates was $5.2 \pm 3.7\%$.

Targeted metabolomics

Targeted metabolomic analyses were performed on the vastus lateralis biopsies that were obtained before and after exercise on 26 participants (13 CON and 13 KE). In two participants (1 CON and 1 KE), analyses could not be performed because of insufficient muscle tissue sample volume. Approximately 10 mg of frozen muscle tissue was weighed and added to 400 μ L of extraction buffer (methanol). Subsequently, the samples were homogenised twice for 30 s at 6500 rpm using an automated tissue homogeniser (Precellys 24; Bertin Technologies, Paris, France). Next, the samples were centrifuged at $17\,000 \times g$ for 10 min and 400 μ L of the supernatant was transferred to a fresh Eppendorf tube, to which 400 μ L chloroform was added. A volume of 10 μ L of each sample was loaded into a Dionex UltiMate 3000 LC System (Thermo Fisher Scientific, Bremen, Germany). This system was equipped with a C-18 column (Acquity UPLC-HSS T3, 1.8 μ m; 2.1×150 mm, Waters, Milford, MA, USA) connected to a Q Exactive Orbitrap mass spectrometer (Thermo Fisher Scientific) functioning in the negative ion mode. The separation process involved a step gradient using two solvents: Solvent A comprising 10 mM tert-butyl alcohol and 15 mM acetic acid, and Solvent B, which comprised pure methanol. Initially, the gradient was set at 100% Solvent A, and 0% Solvent B, maintaining this composition for 2 min. Subsequently, a linear gradient increase to 37% Solvent B was implemented for 5 min. The gradient further increased to 41% Solvent B at minute 14. The gradient was further elevated to 100% Solvent B from minutes 14–26 and this gradient was maintained for an additional 4 min. At minute 30, the gradient was reverted to 0% Solvent B, and the chromatography procedure was stopped after 40 min. Throughout the whole analysis, the flow rate was maintained constant at $250 \mu\text{L min}^{-1}$ with a constant column temperature of 25°C . The mass spectrometer was operated in full scan mode, covering a mass-to-charge ratio (m/z) of 70–1050, with the parameters set at a spray voltage of 3.2 kV, a capillary temperature of 320°C , sheath gas at 10.0 and auxiliary gas at 5.0. The automatic gain control target (AGT) was set at 3×10^6 , with a resolution of 140 000 and a maximum ion trap fill time of 512 ms. Data acquisition was conducted using Xcalibur software (Thermo Fisher Scientific) and involved peak area integration using EI-Maven – Polly software (Elucidata, Boston, MA, USA).

Assessment of supplement blinding

Upon completion of the intervention, participants completed a questionnaire to assess the success of blinding. Participants were asked to identify which supplement they believed they had received (KE or CON)

and to rate their certainty on a scale from 0 (not at all certain) to 10 (completely certain).

Statistical analysis

Statistical analysis were performed using Prism, version 10.1.2 (GraphPad Software Inc., San Diego, CA, USA). Baseline participant characteristics were assessed by a two-sided paired t test. To assess differences between groups and over time, two-way repeated measures ANOVA (group \times time) was used. In case of significant time or interaction effects, *post hoc* analyses with Šidák correction were performed. Data with missing values (i.e. peak cardiac output and stroke volume) were analysed with a mixed-model in Prism. Effect sizes for primary outcomes were calculated using Hedges' g . Metabolite levels assessed by targeted metabolomics were analysed with two-way repeated measures ANOVA using the Pingouin statistical package, version 0.5.4, in Python 3 (Vallat, 2018). Obtained P values were adjusted for multiple comparisons using the false-discovery rate method of Benjamini–Hochberg with the Statsmodels package in Python. If a significant time or interaction effect was detected, Bonferroni *post hoc* analyses were performed using the Pingouin package. Whenever a significant time or interaction effect was observed, all reported P values refer to the *post hoc* analyses. Otherwise, they refer to the P value of the interaction effect. For all analyses, $P < 0.05$ was considered statistically significant. Data are presented as the mean \pm SD, unless otherwise indicated. An *a priori* sample size calculation (G*Power 3.1.9.7; <http://www.gpower.hhu.de>) was conducted to obtain a significant interaction effect ($P < 0.05$) by a two-way ANOVA on $\text{TT}_{30\text{min}}$ performance, with a statistical power of 0.8, four measurements and a correlation among repeated measures of 0.5. The effect size (partial eta squared = 0.05) used for the sample size calculation was derived from the $\text{TT}_{30\text{min}}$ data from Poffé et al. (2019). This yielded a required sample size of 14 participants per group.

Results

Blood β HB levels

Fasted blood β HB concentrations were low (~ 0.3 – 0.5 mM) in both KE and CON during each experimental session. In agreement, β HB concentrations were also similar between KE and CON both before (~ 0.1 – 0.3 mM) and immediately after the training sessions (~ 0.5 mM, $P < 0.001$ vs. pre-ex) (Fig. 1A). Post-exercise KE ingestion markedly elevated β HB levels to 2.6 ± 0.9 mM within 30 min ($P < 0.001$ vs. pre-ex),

whereas the levels in CON remained stable at 0.5 ± 0.3 mM ($P < 0.001$ vs. KE).

Training load

Training load was equal between groups, with no significant group \times time interaction ($P = 0.122$) or group effect ($P = 0.398$) detected (Fig. 1B). Training load during the first week was 5739 ± 646 kJ in CON and 5773 ± 618 kJ in KE. Subsequently, training load increased by 7% in week 2 ($P = 0.177$) and 19% in week 3 ($P < 0.001$) compared to week 1. This was followed by a 39% reduction in training load during the first taper week ($P < 0.001$ vs. week 1). In the first week of the second mesocycle (week 5), the training load was 6297 ± 1040 kJ in CON and 6362 ± 929 kJ in KE. Relative to week 5, the training load increased by 26% in week 6 ($P < 0.001$) and by 44% in week 7 ($P < 0.001$). During the second taper week, training load was reduced by 54% compared to week 5 ($P < 0.001$). Four participants in both CON and KE missed one or more training sessions because of illness or injury [one missed session: $n = 1$ (CON)/1 (KE); two missed sessions: $n = 0$ (CON)/2 (KE); three missed sessions: $n = 3$ (CON)/1 (KE)]. Furthermore, training intensity needed to be reduced in at least one session for five participants in CON and four participants in KE [one session: $n = 1$ (CON)/0 (KE); two sessions: $n = 1$ (CON)/2 (KE); three sessions: $n = 3$ (CON)/2 (KE)].

Exercise performance and $\dot{V}O_{2\text{peak}}$

A group \times time effect was detected for mean absolute power output in $TT_{30\text{min}}$ ($P = 0.027$) (Fig. 2A and B),

PPO $\dot{V}O_{2\text{peak}}$ ($P = 0.013$) (Fig. 2C and D) and relative $\dot{V}O_{2\text{peak}}$ ($P = 0.044$) (Fig. 2E and F). At PRE, mean power output in $TT_{30\text{min}}$ was similar between groups (~ 250 W), with both groups demonstrating a comparable $\sim 10\%$ increase to ~ 275 W at MID. However, from MID to POST, mean power output increased more in KE compared to CON resulting in an absolute power output at POST that was 4% higher in KE (302 ± 28 W) than in CON (291 ± 27 W; mean difference: 11 W [95% confidence interval (CI) = 4–18 W], $P < 0.001$, Hedges' $g = 0.40$). This between-group difference persisted at $POST_{+1\text{WEEK}}$ [mean difference: 9 W (95% CI = 2–15 W), $P = 0.006$, Hedges' $g = 0.28$]. RPE remained consistent between groups across all sessions at 18 ± 1 (range: 14–20).

PPO $\dot{V}O_{2\text{peak}}$ was 433 ± 47 W in CON and 422 ± 41 W in KE at PRE, with both groups showing a 5% increase at MID to respectively 455 ± 38 W and 443 ± 37 W ($P < 0.001$ vs. PRE). At POST, PPO $\dot{V}O_{2\text{peak}}$ stagnated in CON ($P = 0.978$ vs. MID), whereas it improved with an extra 7% in KE to 473 ± 41 W ($P < 0.001$ vs. MID). From PRE to POST, the increase in PPO $\dot{V}O_{2\text{peak}}$ was greater in KE ($+52 \pm 34$ W) compared to CON [$+25 \pm 38$ W, mean difference 27 W (95% CI = 13–41 W), $P < 0.001$, Hedges' $g = 0.75$]. However, absolute values at POST were not different between KE and CON ($P = 0.091$).

Despite a baseline difference for relative $\dot{V}O_{2\text{peak}}$ [CON: 56.8 ± 5.6 mL $\text{kg}^{-1} \text{min}^{-1}$ vs. KE: 54.4 ± 5.4 mL $\text{kg}^{-1} \text{min}^{-1}$, mean difference 2.4 mL $\text{kg}^{-1} \text{min}^{-1}$ (95% CI = 0.2–4.6 mL $\text{kg}^{-1} \text{min}^{-1}$), $P = 0.028$], both groups showed a similar $\sim 3\%$ increase at MID ($P < 0.001$ vs. PRE). However, relative $\dot{V}O_{2\text{peak}}$ plateaued at POST in CON ($P = 0.285$ vs. MID), whereas KE showed further improvement of 7% to

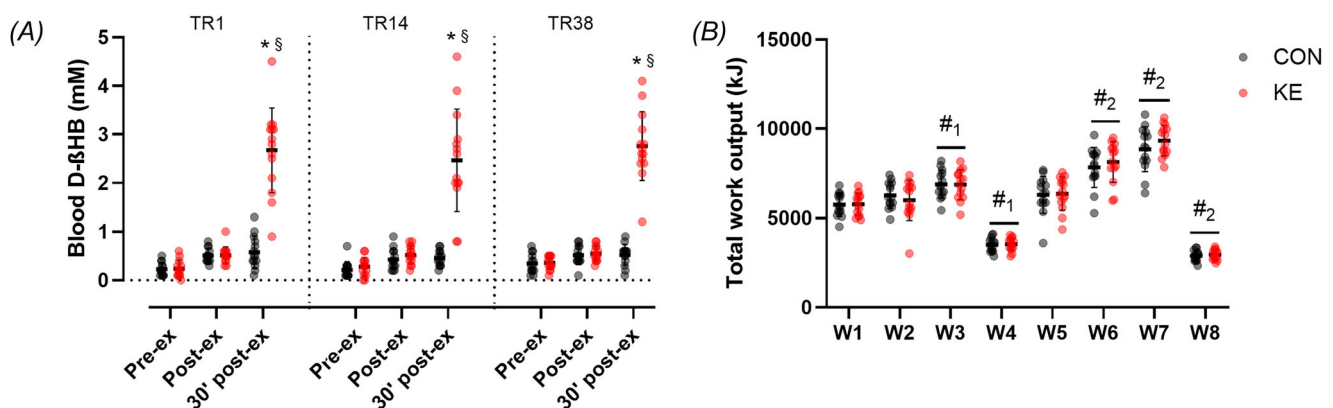


Figure 1. Effect of ketone ester supplementation on blood D-βHB concentrations and training workload

Data are the mean \pm SD for (A) D-βHB concentration pre- and post-training session (TR) 7 (week 1), 16 (week 3) and 43 (week 7) and (B) the work output of every training week. During the 8 week training intervention, the participants received either 25 g of a ketone ester (KE, $n = 14$) or isocaloric control drink (CON, $n = 14$) post-exercise and 30 min before sleeping time each day. # $P < 0.05$ for both groups vs. PRE, * $P < 0.05$ for CON vs. KE for the indicated timepoint; § $P < 0.05$ for the indicated group vs. pre-ex; # $P < 0.05$ for both groups vs. week 1; # $P < 0.05$ for both groups vs. week 5.

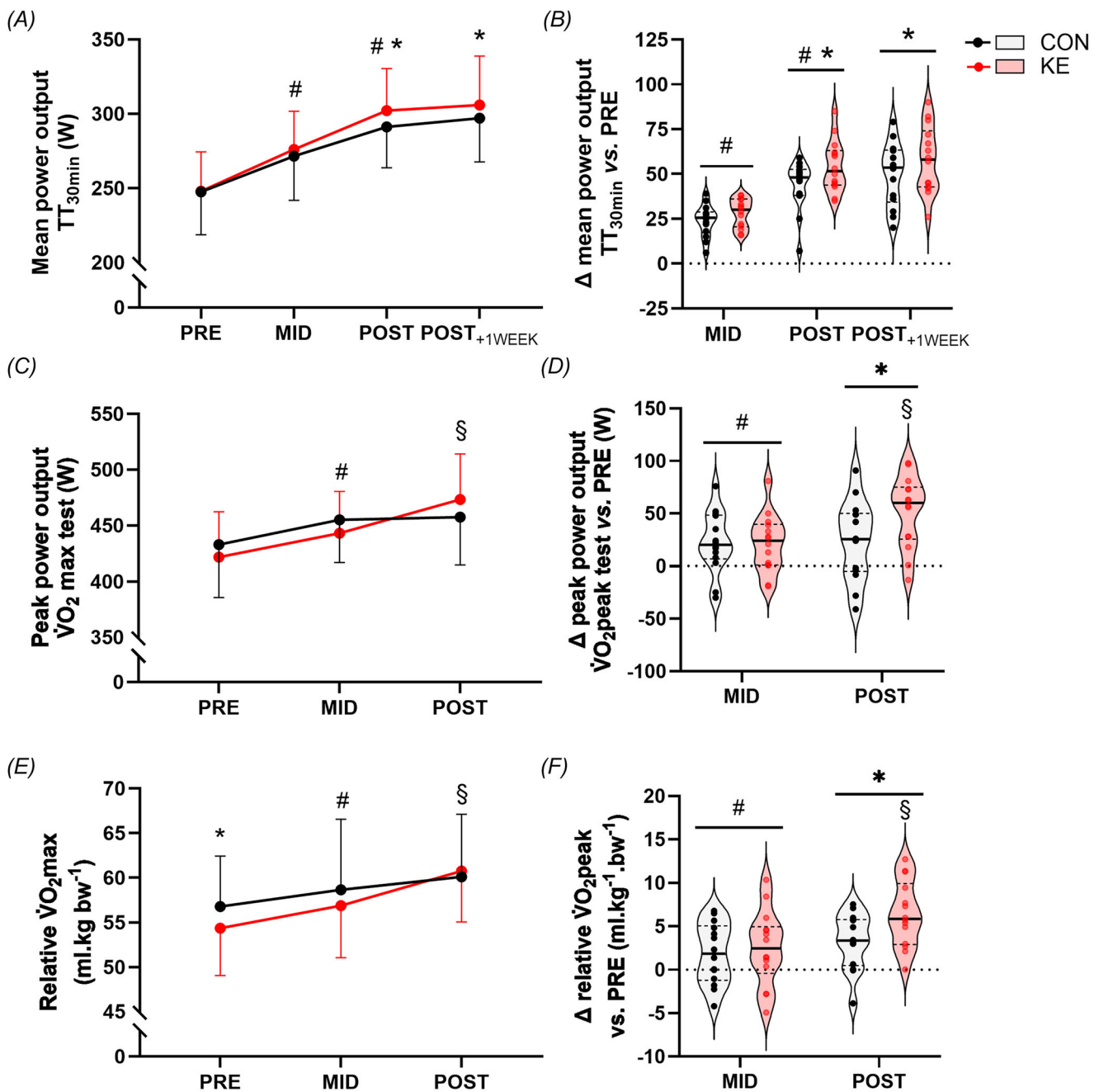


Figure 2. Effect of training and KE supplementation on 30-min time trial performance, peak power output during the $\dot{V}O_{2peak}$ test and relative $\dot{V}O_{2peak}$

Data are the mean \pm SD (line plots) or as distributions of individual differences (vs. PRE) with median and interquartile range indicated (violin plots) for (A) average power output during 30-min time trial (TT_{30min}); (B) individual differences in average power output during TT_{30min} vs. PRE; (C) peak power output during the $\dot{V}O_{2peak}$ test; (D) individual differences in peak power output during the $\dot{V}O_{2peak}$ test vs. PRE; (E) relative $\dot{V}O_{2peak}$; and (F) individual differences in relative $\dot{V}O_{2peak}$ vs. PRE. During the 8 week training intervention, the participants received either 25 g of a ketone ester (KE, $n = 14$) or isocaloric control drink (CON, $n = 14$) post-exercise and 30 min before sleeping time each day. # $P < 0.05$ for both groups vs. previous measurement; § $P < 0.05$ for the indicated group vs. previous measurement; * $P < 0.05$ for CON vs. KE at the indicated timepoint.

$60.8 \pm 5.7 \text{ mL kg}^{-1} \text{ min}^{-1}$ ($P < 0.001$ vs. MID). Hence, from PRE to POST, relative $\dot{V}_{\text{O}_2\text{peak}}$ increased more in KE ($+6.4 \pm 3.8 \text{ mL kg}^{-1} \text{ min}^{-1}$) than in CON [$+3.3 \pm 3.2 \text{ mL kg}^{-1} \text{ min}^{-1}$, mean difference $3.1 \text{ mL kg}^{-1} \text{ min}^{-1}$ (95% CI = $1.5\text{--}4.7 \text{ mL kg}^{-1} \text{ min}^{-1}$), $P < 0.001$, Hedges' $g = 0.88$].

Skeletal muscle CS activity and content of proteins implicated in mitochondrial adaptations

Group \times time effect was observed for CS activity ($P < 0.001$) (Fig. 3A and B) and OXPHOS complex II protein expression ($P = 0.036$) (Fig. 4B). Baseline CS activity was similar between both groups at $7.14 \pm 1.29 \mu\text{mol h}^{-1} \text{ kg protein}^{-1}$. From PRE to POST, CS activity increased by 15% ($8.21 \mu\text{mol h}^{-1} \text{ kg protein}^{-1}$) in CON and 32% ($9.37 \mu\text{mol h}^{-1} \text{ kg protein}^{-1}$) in KE (both $P < 0.001$ vs. PRE). The higher increase in CS activity in KE resulted in a higher absolute value at POST compared to CON [mean difference $1.16 \mu\text{mol h}^{-1} \text{ kg protein}^{-1}$ (95% CI = $0.07\text{--}2.25 \mu\text{mol h}^{-1} \text{ kg protein}^{-1}$), $P = 0.035$, Hedges' $g = 0.98$]. Complex II [succinyl dehydrogenase (SDH)] protein content remained unaltered in CON at the same time as increasing by 25% in KE ($P = 0.030$ vs. PRE). Complex I protein expression showed a similar 20% increase in both groups ($P < 0.05$ vs. PRE), whereas complexes III ($P = 0.203$) and V ($P = 0.124$) remained unchanged in both groups (Fig. 4A, C and D). PGC-1 α protein expression equally increased (+30%) in both groups ($P = 0.004$ for PRE vs. POST) (Fig. 4E). By contrast, SIRT3 protein expression ($P = 0.899$) (Fig. 4F) and acetylated lysine ($P = 0.925$) (Fig. 4G) were not affected by either the training intervention or KE supplementation.

Targeted metabolomics on skeletal muscle

In total, 57 metabolites were identified. A selection of the most significant altered metabolites is presented in Fig. 5. A graph of each metabolite is shown in the Supplementary information (Fig. 1–7). Univariate analyses was conducted to identify whether specific metabolites were impacted by (i) KE supplementation, (ii) acute exercise (i.e. TT_{30min}) and (iii) periodised ET. However, only interaction effects were detected when not corrected for multiple testing for phosphoenolpyruvic acid, citric acid, AMP, hexose biphosphate, GMP and Ile-Leu. However, there was only a significant difference between groups *post hoc* test for citric acid ($P = 0.003$). At POST, muscle citric acid content was $\sim 35\%$ higher in KE compared to CON post-exercise ($P = 0.003$). When corrected for multiple testing, the following metabolites increased (+) or decreased (–) in response to TT_{30min} at PRE and/or POST: L-lactic acid (+), phosphoenolpyruvic acid (–), pyruvic acid (–), citric acid (+), L-cysteine (+), glutamic acid (–), L-phenylalanine (+), L-serine (+), L-threonine (+), UDP (+) and acetyl-CoA (+). Furthermore, the training intervention affected the abundance of 10 metabolites: hexose (–), phosphoenolpyruvic acid (–), succinic acid (+), glycine (–), L-arginine (–), L-glutamic acid (+), L-glutamine (–), L-lysine, L-proline (–) and NADPH (+).

Rest and CPET-echocardiography

Cardiac responses to the training intervention were comparable between CON and KE, with no group \times time effects observed for peak cardiac output ($P = 0.334$), peak stroke volume ($P = 0.428$) or other cardiac parameters at rest or during exercise (Table 2) (all $P > 0.05$). Peak exercise cardiac output increased following the training

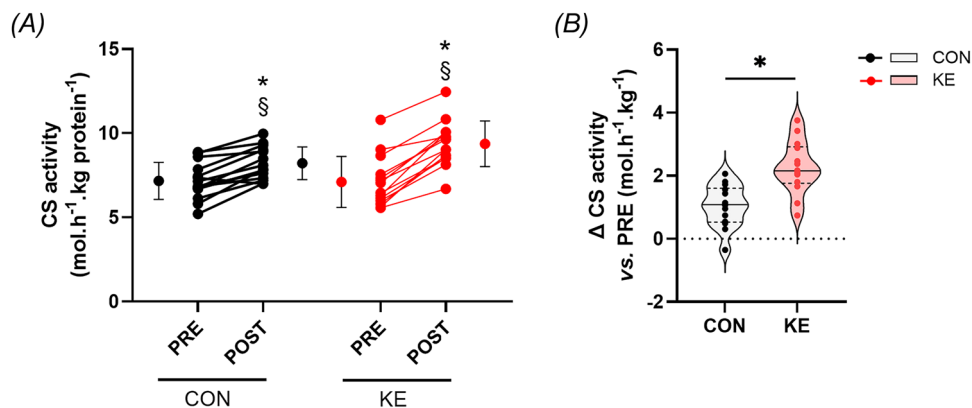


Figure 3. Effect of the training intervention on citrate synthase activity

A, individual dot plots together with mean (circle) \pm SD (whiskers) for citrate synthase (CS) activity. B, the distribution of individual differences (vs. PRE) with median and interquartile range indicated (violin plots). During the 8 week training intervention, the participants received either 25 g of a ketone ester (KE, $n = 14$) or isocaloric control drink (CON, $n = 14$) post-exercise and 30 min before sleeping time each day. * $P < 0.05$ for CON vs. KE at the indicated timepoint; § $P < 0.05$ for the indicated group vs. PRE.

intervention in both groups by $\sim 6\%$ ($P = 0.046$ vs. PRE), whereas cardiac output at rest remained stable. In both groups, the increase in peak cardiac output resulted from an 8% increase in stroke volume at peak exercise intensity ($P = 0.005$ for PRE vs. POST), whereas peak heart rate remained unaffected ($P = 0.21$ for PRE vs. POST). Left ventricular 3D volumes at rest and left ventricular global longitudinal strain during submaximal exercise

remained unaltered by either the training intervention or KE supplementation.

Heart rate and lactate concentration

No group \times time effects were detected for resting heart rate ($P = 0.901$), average heart rate during $TT_{30\text{min}}$

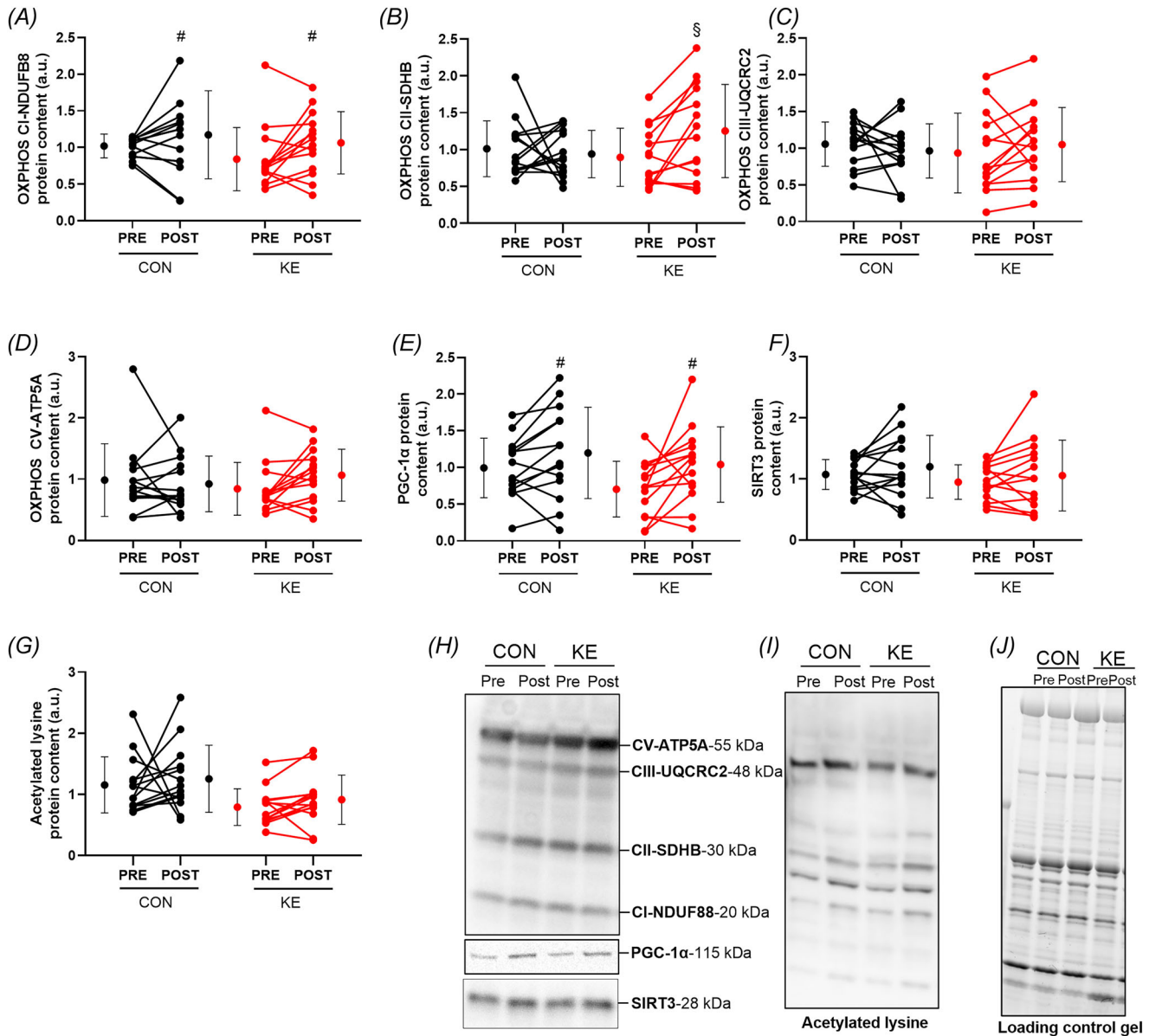


Figure 4. Effect of the training intervention and KE supplementation on OXPPOS, PGC-1 α , SIRT3 and acetylated lysine protein expression

A–G, individual dot plots together with mean (circle) \pm SD (whiskers) for OXPPOS complex I, OXPPOS complex II, OXPPOS complex III, OXPPOS complex V, PGC-1 α , SIRT3 and acetylated lysine protein expression measured in skeletal muscle. During the 8 week training intervention, the participants received either 25 g of a ketone ester (KE, $n = 14$) or isocaloric control drink (CON, $n = 14$) post-exercise and 30 min before sleeping time each day. A muscle biopsy of the vastus lateralis was obtained before exercise at the pre-test (PRE) and post-test (POST) and analysed by western blotting. Representative western blot and gel staining images are shown in (H) to (J). # $P < 0.05$ for both groups vs. PRE; § $P < 0.05$ for the indicated group vs. PRE.

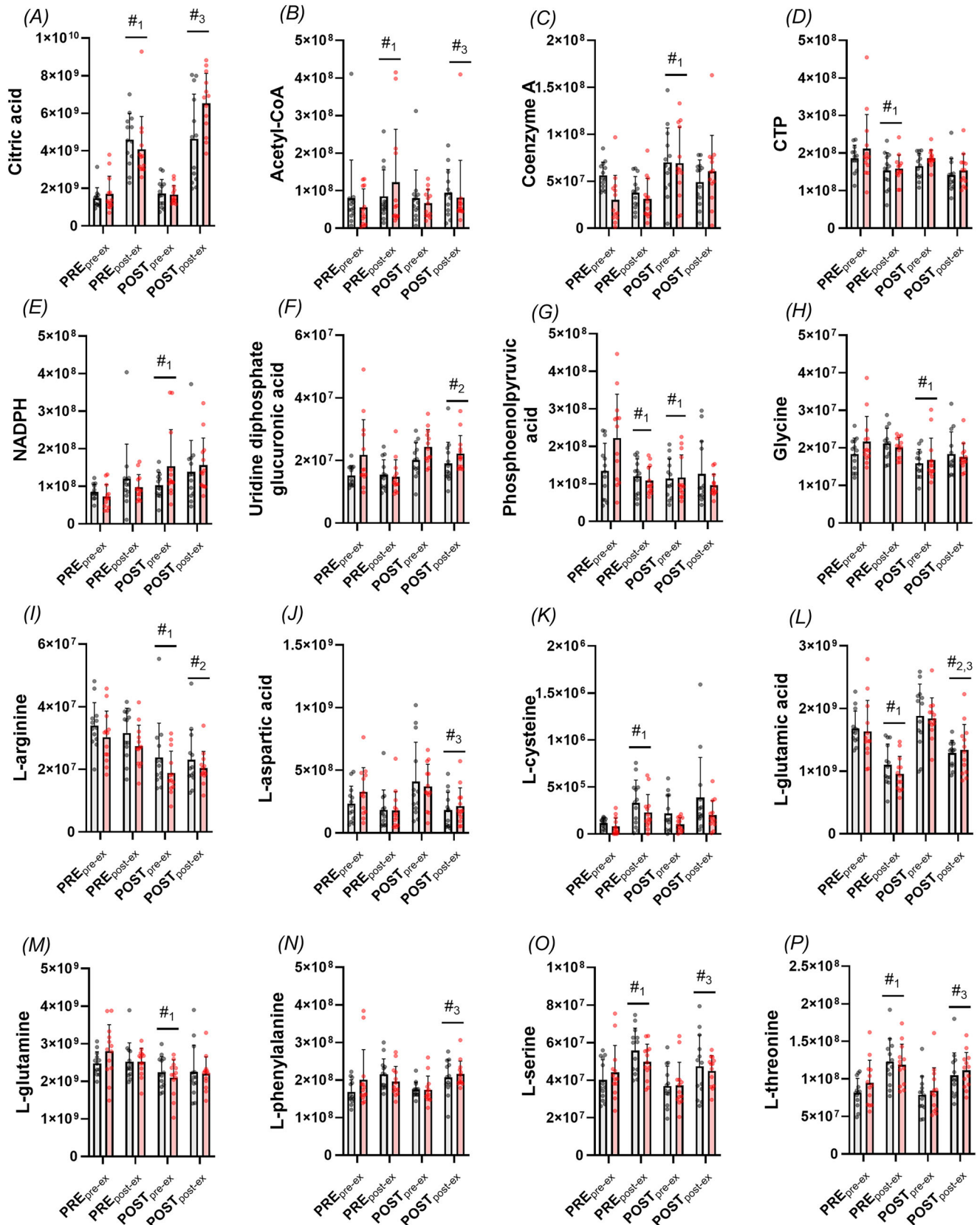


Figure 5. Selection of most significantly altered muscle metabolites

A-P, selection of the most significantly altered muscle metabolites (mean ± SD) throughout the training intervention is shown. During the 8 week training intervention, the participants received either 25 g of a ketone ester

(KE, $n = 13$) or isocaloric control drink (CON, $n = 13$) post-exercise and 30 min before sleeping time each day. A muscle biopsy of the vastus lateralis was obtained before (pre-ex) and immediately after a 30 min timetrial (post-ex) at the pre-test (PRE) and post-test (POST) and analysed by targeted metabolomics. #₁ $P < 0.05$ for both conditions vs. PRE_{pre-ex}; #₂ $P < 0.05$ for both conditions vs. PRE_{post-ex}; #₃ $P < 0.05$ for both conditions vs. POST_{pre-ex}.

Table 2. Effect of ketone ester supplementation on cardiac parameters at rest and during exercise.

	CON		KE	
	PRE	POST	PRE	POST
3D volumes at rest (mL)				
EDV	159 ± 22	161 ± 17	165 ± 23	170 ± 22
ESV	71 ± 17	70 ± 10	71 ± 13	65 ± 12
Heart rate (beats min ⁻¹)				
Rest	66 ± 10	64 ± 7	64 ± 11	60 ± 8
Peak	172 ± 11	169 ± 8	169 ± 13	164 ± 12
Stroke volume (mL)				
Rest	95 ± 18	105 ± 17*	100 ± 19	107 ± 19*
Peak	114 ± 15	126 ± 17*	126 ± 20	133 ± 18*
Cardiac output (L min ⁻¹)				
Rest	6.1 ± 1.0	6.7 ± 1.1	6.5 ± 1.3	6.5 ± 1.3
Peak	19.7 ± 3.0	21.4 ± 2.9*	20.9 ± 2.7	21.8 ± 2.7*
Contractility				
GLS low (± 100 beats min ⁻¹)	-18.8 ± 1.5	-19.2 ± 1.1	-19.4 ± 1.7	-19.4 ± 1.3
Exercise				
Peak RER	1.05 ± 0.03	1.05 ± 0.04	1.06 ± 0.03	1.04 ± 0.03
Peak power output (W)	248 ± 38	277 ± 25*	243 ± 29	266 ± 28*

Data are the mean ± SD for cardiac and exercise parameters measured at rest and during exercise (peak) by CPET-echocardiography. During the 8-week training intervention the participants received either 25 g of a ketone ester (KE, $n = 13$) or isocaloric control drink (CON, $n = 13$) post-exercise and 30 min before sleeping time each day. * $P < 0.05$ for both groups vs. PRE.

($P = 0.114$) and peak heart rate during the $\dot{V}_{O_2\text{peak}}$ test ($P = 0.364$). Resting heart rate demonstrated a progressive decline from 65 ± 7 beats min⁻¹ at PRE to 63 ± 9 bpm at MID ($P = 0.235$ vs. PRE) and 61 ± 8 beats min⁻¹ at POST ($P = 0.018$ vs. PRE) across all participants. Peak heart rate during the $\dot{V}_{O_2\text{peak}}$ test decreased from 189 ± 11 beats min⁻¹ at PRE to 186 ± 12 beats min⁻¹ at MID ($P = 0.002$ vs. PRE) before stabilising at POST ($P = 0.882$ vs. MID). Average heart rate during TT_{30min} remained stable from PRE to POST at 174 ± 11 beats min⁻¹, subsequently increasing to 177 ± 12 beats min⁻¹ at POST_{+1WEEK} ($P = 0.019$ vs. PRE). Both average (12 ± 3.5 mmol L⁻¹) and peak (14.5 ± 3.1 mmol L⁻¹) lactate concentrations during TT_{30min} remained stable across all experimental sessions, with no differences between CON and KE (all $P > 0.5$).

Blood EPO and GDF15 concentrations

No significant group × time effects were observed for fasted serum EPO ($P = 0.705$) or GDF15 concentrations ($P = 0.764$). Baseline EPO concentrations were 5.03 ± 2.08 IU L⁻¹ in CON and 4.33 ± 1.83 IU L⁻¹ in KE, with both groups maintaining stable levels

throughout the intervention period. Although a baseline difference was observed for GDF15 concentrations (CON: 357 ± 105 pg mL⁻¹ vs. KE: 295 ± 109 pg mL⁻¹; $P = 0.029$), both groups demonstrated stable GDF15 levels across all timepoints during the training intervention.

Body composition and energy intake

No group × time interaction effects were detected for any parameter of body composition or energy and macronutrient intake (Table 3). Body weight at the start of the intervention was comparable between groups (CON: 73.2 ± 7.6 kg vs. KE: 72.8 ± 5.4 kg) and remained stable throughout the training intervention ($P = 0.806$). A main effect of time was observed for body fat percentage which decreased from $13.7\% \pm 3.7\%$ to $12.5\% \pm 3.5\%$ ($P < 0.001$ vs. PRE). Similarly, lean mass increased from 62.9 ± 4.5 to 63.6 ± 4.3 kg ($P < 0.001$ vs. PRE). BMC (2679 ± 254 g) and BMD (1.16 ± 0.08 g cm⁻²) remained unchanged throughout the training period. Both groups increased their daily energy intake by ~5% from week 1 to week 3 and ~7% from week 3 to week 7 (all $P < 0.001$) (Table 3). This increase in energy intake was proportional to the increase in training load in week 3 (CON:

Table 3. Effect of ketone ester ingestion on total energy and macronutrient intake.

	CON			KE		
	Week 1	Week 3	Week 7	Week 1	Week 2	Week 7
Energy intake	14 922 ± 895	15 762 ± 926*	16 704 ± 1030*	14 599 ± 710	15 324 ± 685*	16 580 ± 658*
Carbohydrate	7583 ± 349	8305 ± 620*	8782 ± 547*	7582 ± 451	8426 ± 1047*	9022 ± 527*
Protein	2580 ± 454	2592 ± 488	2852 ± 427*	2556 ± 333	2617 ± 345	2802 ± 361*
Fat	3355 ± 389	3440 ± 576	3646 ± 524*	3319 ± 373	3138 ± 888	3613 ± 482*

Data are the mean ± SD (kJ day⁻¹) for total energy and macronutrient intake. During the 8 week training intervention, the participants received either 25 g of a ketone ester (KE, *n* = 13) or isocaloric control drink (CON, *n* = 13) post-exercise and 30 min before sleeping time each day. Macronutrient data are exclusive of the KE and CON drinks. **P* < 0.05 for both groups vs. week 1.

+153 ± 251 kJ day⁻¹; KE: +68 ± 224 kJ day⁻¹) and week 7 (CON: -84 ± 210 kJ day⁻¹; KE: -158 ± 187 kJ day⁻¹). The elevated energy intake was primarily attributable to elevated carbohydrate consumption, which rose by ~10% from week 1 to week 3, and by ~6% from week 3 to week 7 (all *P* < 0.001). Fat and protein intake remained stable during the first part of the training intervention, but increased by respectively ~10% (*P* < 0.013) and ~9% (*P* = 0.006) from week 3 to week 7.

Stress-recovery state

No group × time effects were detected for stress-recovery state (*P* = 0.811), total stress score (*P* = 0.723) or total recovery score (*P* = 0.891), indicating similar responses between groups. Both CON and KE experienced comparable declines in stress-recovery state throughout the training intervention, decreasing from 72 ± 20 at PRE to 58 ± 28 at MID (*P* = 0.043 vs. PRE) and 42 ± 35 at POST (*P* < 0.001 vs. PRE), before partially recovering to 54 ± 28 at POST_{+1WEEK} (*P* = 0.005 vs. PRE). This decline was predominantly driven by increased total stress scores, which rose from 42 ± 13 at PRE to 58 ± 27 at MID (*P* = 0.021 vs. PRE) and peaked at 63 ± 22 at POST (*P* < 0.001 vs. PRE), before returning towards baseline at POST_{+1WEEK} (48 ± 21, *P* = 0.254 vs. PRE). By contrast, total recovery scores remained stable at ~110 throughout all timepoints.

Identification of experimental condition

In CON, seven of 14 participants correctly identified their experimental condition with a confidence level of 65 ± 25%. In KE, eight of 14 participants correctly identified their group assignment with a confidence level of 70 ± 23%.

Discussion

The present study demonstrates that PEKS supplementation potentiates the adaptive response to

a periodised ET program. Specifically, PEKS elicited additional improvements in TT_{30min} performance (+4%), PPO $\dot{V}_{O_2\text{peak}}$ (+4%) and $\dot{V}_{O_2\text{peak}}$ (+6%) beyond the gains induced by training alone (+20%, +6% and +6%, respectively). These ergogenic effects were paralleled by an upregulation of muscle CS activity and SDH (complex II) protein content in KE compared to CON, whereas cardiac parameters such as peak stroke volume and cardiac output did not further improve with PEKS. Collectively, these findings suggest that PEKS augments endurance performance through muscle-based adaptations. Building on our previous work showing that PEKS attenuates overreaching symptoms and improves training tolerance during periods of excessive load (Poffé et al., 2019, 2023), the current data extend its utility to periodised training in at least trained (Tier 2) endurance athletes (McKay et al., 2021), highlighting PEKS as a promising nutritional strategy to amplify mitochondrial remodelling and endurance performance.

In our previous study, PEKS during a 3 week overload training period improved endurance performance as evidenced by higher tolerable training load during the final training week and increased power output during a 30 min time-trial following 2 h of sub-maximal exercise (Poffé et al., 2019). However, these performance improvements coincided with a suppression of overreaching symptoms as well as a higher energy intake with PEKS, making it unclear whether PEKS may also be efficacious during periodised training. In the present study, we found that PEKS improved training-induced increases in TT_{30min} and PPO $\dot{V}_{O_2\text{peak}}$ by 4% and $\dot{V}_{O_2\text{peak}}$ by 6%, whereas there were no indications that participants developed overreaching. The primary criterion for detecting overreaching is a decrement in performance (Bellinger, 2020). However, in the present study, all participants improved their endurance performance. Moreover, other physiological markers of overreaching that were present in our previous study were either unchanged or of smaller magnitude in the present study. In our earlier study (Poffé et al., 2019), the excessive training period elicited multiple

physiological disturbances including a drop in resting (-7 beats min^{-1}) and maximal (-16 beats min^{-1}) heart rate, a steep decrease in stress-recovery state (-85 points) and a progressive increase in blood GDF15 concentrations, which is increasingly recognised as a stress biomarker (Wischnusen et al., 2020). Conversely, in the present study, both resting and maximal heart rate (-3 beats min^{-1}), as well as the stress-recovery state (-30 points), showed a much weaker response, while blood GDF15 concentrations remained stable.

Furthermore, decreased appetite is another symptom of overreaching (Bellinger, 2020), which resulted into a negative energy balance in the control group in our previous study (Poffé et al., 2019). Conversely, energy balance was preserved in the PEKS group through a proportional increase in energy intake with the training load. This suggests that performance enhancements during the final week of overload training period in PEKS may be attributed to enhanced energy availability rather than direct ergogenic effects of ketone bodies. In the present study, spontaneous energy intake increased equally in both groups with the training load, primarily driven by elevated carbohydrate intake. This indicated that PEKS can enhance endurance performance irrespective of preventing an energy deficit. Moreover, in the study by Poffé et al. (2019), PEKS most probably prevented an energy deficit by suppressing the overload-induced increase in circulating GDF15 levels, which suppresses appetite under metabolic stress (Klein et al., 2022). Considering that GDF15 levels remained stable with periodised training, this suggests that PEKS only modulates energy intake under conditions of heightened metabolic stress. Collectively, these findings provide evidence that PEKS enhances endurance performance under conditions, where no overreaching symptoms are developing, supporting its application during periodised training periods.

Performance improvements are generally the result of the combined impact on central and peripheral adaptations. Mitochondrial adaptations represent a key peripheral factor because increases in skeletal muscle mitochondrial content and function have been shown to coincide with improvements in endurance performance (Jacobs et al., 2013). Previous research showed that *in-vitro* treatment of myotubes with β HB improves mitochondrial function, as evidenced by reduced H_2O_2 emission and enhanced mitochondrial respiration rate (Parker et al., 2018). Improvements in mitochondrial function increase the reliance on aerobic energy provision during exercise and could result in improved endurance performance (Bassett & Howley, 2000; Jacobs et al., 2013). To assess mitochondrial adaptations, we first measured CS activity, which is considered an appropriate marker of mitochondrial content (Larsen et al., 2012). The training intervention elevated CS activity

in the CON group by 15%. PEKS further augmented this increase in CS activity by an additional 17%, suggesting increased mitochondrial content. This was complemented by a KE-induced increase in SDH content in skeletal muscle. SDH is an important determinant of the oxidative capacity of skeletal muscle, given its role in the tricarboxylic acid cycle and the electron transport chain (Moosavi et al., 2019). However, SDH is also a valid marker of mitochondrial content (Larsen et al., 2012), further supporting our assumption that KE elevated mitochondrial content. When not corrected for multiple testing, our metabolomic analyses also revealed $\sim 35\%$ higher citric acid content in skeletal muscle following $\text{TT}_{30\text{min}}$ in KE vs. CON at the end of the training intervention. This observation suggests that KE also upregulated CS activity, and thus mitochondrial oxidative metabolism, during exercise, potentially contributing to improved endurance performance.

The underlying mechanism by which PEKS upregulate mitochondrial biogenesis have not been fully elucidated. An important transcriptional activator responsible for mitochondrial biogenesis is PGC-1 α (Hood et al., 2016). However, we found that, 14 h after the last training session, PGC-1 α protein content was elevated to a similar extent in both groups. Previous research using a ketogenic diet in aged mice suggested that ketone bodies could potentially upregulate mitochondrial biogenesis and alter the activity of mitochondrial enzymes by increasing SIRT3 activity (Pathak et al., 2022). However, SIRT3 protein content was unaltered in the present study. Another possible mechanism by which ketone bodies could affect the transcription of genes involved in mitochondrial biogenesis is by the inhibition of histone deacetylases (HDACs resulting in increased lysine acetylation levels of histone proteins and enhanced transcription of genes implicated in exercise adaptation (McGee & Hargreaves, 2011; Shimazu et al., 2013). Nonetheless, the degree of lysine acetylation was not affected by PEKS or the training intervention. Given that lysine acetylation is a reversible process with a half-life of 1–2 h (Zheng et al., 2013), it is possible that ketone bodies mainly stimulate this in the first few hours after ingestion, while the muscle biopsy was taken ~ 10 h after the last intake. Therefore, the timing of our measurement may not have captured the transient molecular events responsible for the mitochondrial adaptations. Furthermore, direct measurement of SIRT3 and HDAC enzymatic activity, rather than protein content and acetylation status, respectively, could have provided more mechanistic insight into these regulatory pathways.

Because PEKS also resulted in a greater improvement in $\dot{V}_{\text{O}_2\text{peak}}$, which is determined by both central (e.g. cardiac output and total haemoglobin mass) and peripheral factors (e.g. muscle mitochondrial content and capillarisation), it is plausible that PEKS enhanced central adaptations as well (Bassett & Howley, 2000;

Lee & Zhang, 2021). To address this, we quantified peak cardiac output during exercise before and after the intervention. Peak cardiac output increased to a similar extent in both groups, and neither stroke volume during CPET-echocardiography nor peak heart rate differed between conditions, making it improbable that PEKS further enhanced cardiac output at maximal effort, particularly given the plateau in stroke volume at moderate intensity (Rowland, 2008; Claessen et al., 2019). These observations suggest that the improvement of $\dot{V}_{O_{2peak}}$ with PEKS was predominantly driven by muscular mechanisms, most notably enhanced mitochondrial oxygen consumption, consistent with the observed increases in skeletal muscle mitochondrial content and supported by the strong relationship between training-induced improvements in CS activity and $\dot{V}_{O_{2max}}$ (Vigelsø et al., 2014).

Nevertheless, we cannot exclude the possibility of central contributions because we did not assess total haemoglobin mass. This limitation is particularly relevant given prior evidence that PEKS elevates serum EPO levels by $\sim 20\%$ for at least 4 h after 1 h of endurance cycling (Evans et al., 2023) and increases fasted EPO levels following a 3 week overload training period (Poffé et al., 2023). If such elevation could be sustained for multiple weeks, this could translate into increased total haemoglobin mass. This is evidenced by a study showing that 3 weeks of cobalt supplementation, which increased serum EPO levels by $\sim 30\%$, resulted in a 2% increase in total haemoglobin mass (Hoffmeister et al., 2018). Another possibility is that KE widened the arterio-venous oxygen differences by altering the blood flow to active tissues. Such an effect has already been observed following acute KE ingestion in the heart (Gormsen et al., 2017) and brain (Hasselbalch et al., 1996; Stalmans et al., 2024) and following chronic KE supplementation in the brain of obese individuals (Walsh et al., 2021). Additionally, ketones can improve muscular oxygen extraction by stimulating *de novo* angiogenesis (Poffé et al., 2023).

Our findings indicate that PEKS did not further improve cardiovascular adaptations to endurance exercise training. This contrasts with recent evidence in heart failure patients, where 14 days of KE supplementation increased cardiac output at rest and during low-to-moderate, but not peak, exercise intensities, independent of circulating ketone levels (Berg-Hansen et al., 2024). Taken together, these results suggest that KE supplementation may preferentially improve cardiac function in patients with impaired myocardial performance, but not in healthy, physically active individuals. Beyond their relevance for performance, the present cardiovascular measurements were included to evaluate cardiac safety during short-term supplementation. Importantly, left ventricular global longitudinal strain remained unchanged, stroke volume

adapted normally to ET and no arrhythmias were detected at rest or during exercise. Collectively, these data demonstrate that 8 weeks of daily KE supplementation ($50\text{--}75\text{ g day}^{-1}$) is well tolerated and does not adversely affect cardiac structure or function in healthy individuals.

Limitations

A major limitation of the present study is the inclusion of only male participants. This decision was made to facilitate comparison with previous work (Poffé et al., 2019, 2023) and to improve pair-matching. However, this significantly limits the generalisability of our findings. Considering the limited number of studies that investigated the physiological effects of exogenous ketones in females, future studies should investigate whether PEKS produces similar physiological and ergogenic effects in female athletes. The present study represents the first comprehensive assessment of the short-term impact of PEKS on the skeletal muscle metabolome at rest and following exercise, indicating that long-term PEKS does not substantially modify resting or exercise-induced muscle metabolism. However, because we used a targeted approach to detect the primary metabolites involved in muscle metabolism, our metabolomic analyses was limited to 57 metabolites representing only a fraction of the complete metabolome. In addition, we cannot exclude interference from the pre-exercise biopsy procedure on the measured metabolites of the post-exercise biopsy. Furthermore, metabolomic analyses are a static measurement at a single timepoint, and only provide indirect information on enzymatic activity, which is highly dynamic during exercise. Additionally, because the muscle biopsies were collected ~ 14 h after the last training session, changes in the muscle metabolome might result from the acute effect of the preceding training session rather than indicating short-term training adaptations (i.e. last bout effect). Similarly, most probably, at least a part of the increase in CS activity can be attributed to this last bout effect (Savard et al., 1988). Nevertheless, to mitigate the impact of the last training session on our outcomes, we maintained a consistent time interval between the final training session and the collection of the muscle biopsies and blood samples across all participants. Another limitation is the fact that we assessed peak cardiac output rather than maximal cardiac output to obtain sufficient time at high intensity to obtain the echocardiographic images. This limitation is supported by the lower heart rate during the CPET-echocardiography, as well as that we measured peak cardiac output at an respiratory exchange ratio (RER) of 1.05. However, because stroke volume levels plateau at moderate intensity ($\sim 50\% \dot{V}_{O_{2max}}$) in recreational athletes (Claessen et al., 2019; Rowland, 2008), and because neither

stroke volume nor maximal heart rate was affected by PEKS, PEKS most probably did not alter maximal cardiac output.

In conclusion, the present study demonstrates that PEKS represents an effective strategy for enhancing endurance performance and mitochondrial content in trained athletes during a periodised ET program. Furthermore, PEKS did not impact cardiac adaptations to endurance exercise training, suggesting that muscular adaptations may play an important role in the observed ergogenic effects. Beyond athletic performance, the observed enhancements in mitochondrial content and endurance performance may have broader implications for metabolic health and clinical populations, although this requires further research. Although PEKS may also benefit elite athletes, the magnitude of PEKS-induced adaptations might be attenuated in elite endurance athletes who already possess substantially higher mitochondrial capacity. Importantly, the training intervention itself remained the primary driver of adaptation and performance gains. Therefore, optimising training load and recovery should remain the priority, with PEKS serving as a potential adjunct to further augment training-induced adaptations.

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Additional information

Data availability statement

All data supporting the results of the present study are provided in the published article and the accompanying supporting

information. Requests for further information and resources should be directed to and will be fulfilled by the lead contact: Chiel Poffé (chiel.poffe@uhasselt.be).

Competing interests

The authors declare that they have no competing interests.

Author contributions

R.R., G.C. and C.P. were responsible for the conception and design of the study. R.R., Y.B., W.L., M.S., M.K., K.T., T.R., G.C. and C.P. were responsible for data collection and biochemical analyses. R.R., Y.B., G.C. and C.P. were responsible for data interpretation, manuscript drafting and editing. All authors approved the final version of the manuscript submitted for publication and agree to be accountable for all aspects of the work in ensuring that questions related to the accuracy or integrity of any part of the work are appropriately investigated and resolved. All persons designated as authors qualify for authorship, and all those who qualify for authorship are listed.

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Keywords

beta-hydroxybutyrate, cardiac, endurance performance, exercise, ketone bodies, mitochondria

Supporting information

Additional supporting information can be found online in the Supporting Information section at the end of the HTML view of the article. Supporting information files available:

Peer Review History Appendix