Increasing skeletal muscle carnitine availability does not alter the adaptations to high-intensity exercise training

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Running head: Muscle carnitine loading and high intensity training

Abstract

Increasing skeletal muscle carnitine availability alters muscle metabolism during steady-state exercise in healthy humans. We investigated whether elevating muscle carnitine, and thereby the acetyl-group buffering capacity, altered the metabolic and physiological adaptations to 24 weeks of high-intensity training (HIT) at 100% maximal exercise capacity ($Watt_{max}$). Twenty-one healthy male volunteers (age 23±2 years; BMI 24.2±1.1 kg/m^2) performed 2x3 minute bouts of cycling exercise at 100% $Watt_{max}$, separated by five minutes rest. Fourteen volunteers repeated this protocol following 24 weeks of HIT and twice-daily consumption of 80g carbohydrate (CON) or 3g L-carnitine+carbohydrate (CARN). Before HIT, muscle phosphocreatine (PCr) degradation ($P<0.0001$), glycogenolysis($P<0.0005$), PDC activation ($P<0.05$), and acetylcarnitine($P<0.005$) were 2.3, 2.1, 1.5 and 1.5-fold greater, respectively, in exercise bout two compared to bout one, whilst lactate accumulation tended ($P<0.07$) to be 1.5-fold greater. Following HIT, muscle free carnitine was 30% greater in CARN vs CON at rest and remained 40% elevated prior to the start of bout two ($P<0.05$). Following bout two, free carnitine was no different, and PCr degradation, glycogenolysis, lactate accumulation, and PDC activation were all similar between CON and CARN, albeit markedly lower than before HIT. VO_{2max}, $Watt_{max}$ and work-output were similarly increased in CON and CARN, by 9, 15 and 23% ($P<0.001$), respectively. In summary, increased reliance on non-mitochondrial ATP resynthesis during a second bout of intense exercise is accompanied by increased carnitine acetylation. Augmenting muscle carnitine availability during 24 weeks of HIT did not alter this, nor enhance muscle metabolic adaptations or performance gains beyond those with HIT alone.

Keywords: Repeated-bout exercise, training adaptation, carnitine supplementation, pyruvate dehydrogenase complex

Introduction

The reversible esterification of skeletal muscle free carnitine with acetyl-CoA, by the mitochondrial enzyme carnitine acetyltransferase (CAT), maintains a viable pool of free coenzyme A and thereby facilitates pyruvate dehydrogenase complex (PDC) and TCA cycle fluxes under conditions of elevated ATP demand (Childress & Sacktor, 1966; Constantin-Teodosiu *et al.*, 1991a). This is evidenced by the linear relationship that exists between acetyl-CoA and acetylcarnitine accumulation across sub-maximal exercise intensities (Carlin *et al.*, 1990; Constantin-Teodosiu *et al.*, 1991a), as well as the finding that increasing skeletal muscle free carnitine availability in healthy humans resulted in a greater acetylcarnitine accumulation and PDC activation during exercise at 80% maximal aerobic capacity (VO_{2max}), which was concomitant with a lower rate of phosphocreatine (PCr) degradation and a diversion of glycogenolysis away from lactate accumulation (Wall *et al.*, 2011). However, during very intense exercise there is a plateau in muscle acetyl-group concentration that is associated with a greatly accelerated rate of lactate formation (Harris & Foster, 1990; Stephens *et al.*, 2007a) and yet it is not clear whether the availability of free carnitine is limiting to PDC flux and mitochondrial ATP resynthesis, or simply that TCA cycle flux and PDC flux are maximal and well matched. In support of the latter it was previously demonstrated that during five minutes of leg extension exercise at 100% maximal workload ($Watt_{max}$), in-vitro determination of PDC activation accounted for 90% of the estimated TCA cycle flux from leg O_2 uptake (Gibala *et al.*, 1998). Moreover, using the same model Bangsbo and colleagues (2001) reported that mitochondrial ATP delivery was enhanced in a second three minute bout of single leg-extension at 100% $Watt_{max}$, when free carnitine availability would presumably have been reduced (Putman *et al.*, 1995). However, the limitations to mitochondrial ATP delivery during single leg exercise are not entirely analogous with two-

legged cycling (Andersen & Saltin, 1985; Boushel *et al.*, 2011) and skeletal muscle carnitine metabolism was not determined in these studies.

Exercise training robustly enhances the capacity for mitochondrial ATP resynthesis and is paralleled by reciprocal reductions in the reliance on PCr degradation and glycolytic ATP delivery (Vollaard *et al.*, 2009). These metabolic adaptations reflect a better matching of glycolysis, PDC and TCA cycle fluxes and underpin the functional improvements observed in power output and endurance performance following classical endurance exercise (Holloszy *et al.*, 1977). Low volume, high-intensity interval exercise (HIT) is much promoted as a time-efficient strategy to stimulate metabolic and functional adaptations comparable to continuous endurance-type exercise (Burgomaster *et al.*, 2008) and is well tolerated when practical workloads (i.e. ~100% VO_{2max}) are employed (Little *et al.*, 2010). This type of exercise paradigm markedly increases glycolytic and mitochondrial ATP demand but the acetyl-group buffering role of carnitine under these conditions has not been investigated. If carnitine availability is indeed limiting to PDC flux during intense, repeated-bout exercise, it is plausible that increasing skeletal muscle carnitine availability as in (Wall *et al.*, 2011) during a prolonged period of HIT could influence the adaptations to this type of training. The current study was conducted to gain further insight into the role of skeletal muscle acetyl-group buffering, PDC flux and mitochondrial ATP production during two consecutive bouts of fixed-workload cycling exercise at $Watt_{max}$. Moreover, it was subsequently investigated whether increasing muscle carnitine could lower the reliance on non-mitochondrial ATP production, particularly during a second bout of intense exercise and consequently improve training-induced gains in exercise capacity (VO_{2max}, $Watt_{max}$) and performance (work output).

Materials and Methods

Volunteers

Twenty-one healthy, non-vegetarian, untrained males (age 23 ± 2 years; BMI 24.2 ± 1.1 kg/m^2; VO_2max 45 ± 3 ml/kg/min) gave written informed consent and attended a routine medical screening prior to participating in the present study which was approved by the University of Nottingham Medical School Ethics Committee.

Protocol

Baseline testing: The overall study protocol is depicted in **Figure 1A**. All volunteers completed a continuous, incremental exercise test to volitional exhaustion on an electronically-braked cycle ergometer (Lode Excalibur; Lode, Groningen, The Netherlands) to determine their VO_{2max} (SensorMedics, USA) and the constant workload that would elicit this ($Watt_{max}$). Both parameters were confirmed on a separate occasion and volunteers were subsequently familiarised with the experimental protocol one week prior to the experimental visit. On the day of the main experimental visit, volunteers arrived at the laboratory (~8.30 am) following an overnight fast and having abstained from alcohol, caffeine or strenuous exercise for 48 hours. Following a 30 minute period of supine rest, a single muscle biopsy was obtained (Bergstrom, 1975) and volunteers completed a three minute warm-up period on the cycle ergometer at an intensity (25% $Watt_{max}$) known to have a negligible impact upon muscle acetyl-group concentrations (Constantin-Teodosiu *et al.*, 1991a; Howlett *et al.*, 1998). Volunteers subsequently cycled for three minutes at their pre-determined $Watt_{max}$ workload and, upon cessation of exercise, a second biopsy was immediately obtained from the same leg as the first. Volunteers then rested for five minutes before completing a second three minute bout of exercise at the same absolute workload, with further biopsies obtained from the contralateral leg immediately before and after the second bout (**Figure 1B**).

Following the initial baseline experimental visit, fourteen volunteers were randomised to receive either L-carnitine (n=7) or placebo (n=7) supplementation during a 24 week period of supervised HIT (see below), with the absolute training workload adjusted to reflect any changes in $Watt_{max}$ which, along with VO_{2max}, was reassessed after 4, 8, 12, 18 and 24 weeks. The main experimental study visit was then repeated at 24 weeks and an additional resting muscle biopsy was obtained at 12 weeks.

High-intensity training: Volunteers trained three times per week (normally Monday, Wednesday and Friday) and were required to complete >85% of all training sessions over 24 weeks for inclusion in the final data set. All training sessions involved a three minute warm-up at 25% $Watt_{max}$ followed by 3 x 3 minute exercise bouts at $Watt_{max}$ and a fourth bout to exhaustion, each separated by five minute passive recovery periods. During the fourth bout of each training session, volunteers received standardised verbal encouragement to exercise as long as they could (typically less than six minutes). The absolute training workload was adjusted, in line with the reassessment of $Watt_{max}$ every four weeks (**Figure 1A**), to maintain a constant relative intensity of $Watt_{max}$. Additionally, when volunteers were able to exercise for six minutes in the final bout during more than two consecutive training sessions, the workload was increased by a further 1% for the subsequent training session. This approach maximised total work output, whilst restricting exercise duration to 15 minutes, in every training session.

Supplementation: Volunteers were assigned to receive twice-daily beverages of either 80 g carbohydrate (Maldex 180, SYRAL Belgium; CON) or 80 g carbohydrate plus 2.25 g L-carnitine tartrate (1.5 g L-carnitine; Nutramet, UK; CARN) in a randomised, double-blinded fashion. The quantities of L-carnitine and carbohydrate provided were identical to those used in a prior study (Wall *et al.*, 2011). Drinks were made up in 500 ml cold water from sachets of powder and were matched for flavour, appearance and carbohydrate type. Volunteers were

instructed to consume one beverage first thing in the morning, and the second beverage four hours later to maximise the time period over which plasma carnitine and serum insulin concentrations were simultaneously elevated (Stephens *et al.*, 2007b; Shannon *et al.*, 2016).

Sampling and analysis

Muscle biopsies were rapidly frozen (~10 seconds) in liquid nitrogen-cooled isopentane and a ~50 mg (wet weight; [ww]) portion was freeze-dried for the determination of free, acetyl and total carnitine (Cederblad *et al.*, 1990), as well as ATP, PCr, lactate and glycogen, corrected (excluding lactate) for muscle total creatine (Harris *et al.*, 1974). A second portion (~15 mg ww) was used for the determination of PDC activation status (Constantin-Teodosiu *et al.*, 1991b). Total carnitine was also determined in the 12 week muscle biopsy and in plasma samples.

Calculations and statistical analysis

$Watt_{max}$ was calculated as W'+(30·T/180) where W' (watts [joules/sec]) is the highest workload completed and T (secs) is the time attained in the final (incomplete) stage at exhaustion during the incremental exercise test. The theoretical rate of non-mitochondrial ATP resynthesis, attributed to ATP breakdown, PCr degradation and lactate accumulation (Constantin-Teodosiu *et al.*, 2009), was estimated from the equation: $\Delta[PCr]+1.5\cdot\Delta[lactate]+2\cdot\Delta[ATP]$. In non-occluded muscle, this equation ignores lactate efflux and intermediary metabolite concentrations, but still provides a relative index reflective of the overall matching of oxidative ATP production to total ATP demand. Glycogenolysis was calculated as Δ[glycogen]. Baseline metabolite concentrations and delta values were compared using two-way ANOVA (exercise x bout) or paired t-test, respectively. Post-training data were compared separately using two-way ANOVA (exercise x

supplementation), with Bonferoni-corrected t-test post-hoc. All data are presented as mean ± SE, with absolute metabolite concentrations expressed in mmol·kg dry weight $(dw)^{-1}$.

Results

Baseline repeated-bout exercise metabolism

Skeletal muscle carnitine metabolism and PDCa

Muscle free carnitine concentration declined by 28% ($P<0.05$) during bout one and remained unchanged during recovery, before declining a further 39% ($P<0.01$) during bout two (**Figure 2A**). Acetylcarnitine concentrations mirrored the decline in free carnitine, increasing by 2.3-fold and 1.7-fold during bout one and bout two, respectively (both $P<0.05$; **Figure 2A**), whilst absolute acetylcarnitine accumulation (Δ[acetylcarnitine]) was similar between bout one and two (4.1 ± 0.9 and 4.2 ± 0.9 mmol·kg dw^{-1}, respectively). PDCa increased 2.1-fold ($P<0.01$) during bout one before returning to basal values prior to the start of bout two. During bout two, PDCa increased 3.3-fold ($P<0.001$), to a greater extent than during bout one (interaction effect $P<0.05$; **Figure 2B**).

Non-mitochondrial ATP delivery and glycogenolysis

ATP concentration declined progressively during bout one (from 26.1 ± 0.7 to 23.5 ± 0.8; $P<0.05$) and bout two (from 24.0 ± 1.0 to 20.7 ± 0.9 mmol·kg dw^{-1}; $P<0.01$) with a similar Δ[ATP] in each bout ($P=0.5$; **Figure 2C**, black bars). PCr concentration declined during bout one from 76.5 ± 2.3 to 52.9 ± 4.6 mmol·kg dw^{-1} ($P<0.001$) before returning to resting values prior to the start of the second bout. During bout two, PCr concentration fell to 23.4 ± 3.4 mmol·kg dw^{-1}, such that Δ[PCr] was 2.3-fold greater than during bout one ($P<0.001$; **Figure 2C**, white bars). Muscle lactate increased from a resting value of 6.0 ± 0.8 to 49.0 ± 9.8 mmol·kg dw^{-1} ($P<0.01$) during bout one and remained elevated above basal values ($P<0.05$)

prior to the start of the second bout (26.6 ± 5.4 mmol·kg dw^{-1}). During bout two, muscle lactate increased further to 92.9 ± 7.4 mmol·kg dw^{-1}, with Δ[lactate] tending to be 1.5-fold greater during bout two than bout one (P=0.07; **Figure 2C**, dotted bars). The total calculated ATP delivery from non-mitochondrial routes was 1.7-fold greater during bout two versus bout one (159.4 ± 12.0 vs. 93.3 ± 19.5 mmol ATP·kg dw^{-1}; P<0.01; **Figure 2C**, summed bars). Resting muscle glycogen concentration was 396 ± 18 mmol·kg dw^{-1} and declined by 15% and 32% during bout one and bout two, respectively (both P<0.001), such that Δ[glycogen] was 2.1-fold greater during bout two than bout one (P<0.001; **Figure 2D**).

Post-supplementation

Following randomisation, CON and CARN groups were well matched for baseline exercise capacity (**Figure 3A-C**) and skeletal muscle metabolism during repeated-bout exercise (**supporting Table 1**).

Exercise capacity and work performance

Twenty-four weeks of HIT increased VO_{2max} by ~9% (**Figure 3A**), $Watt_{max}$ by ~15% (**Figure 3B**) and work output during training by ~23% (**Figure 3C**) in CON and CARN (all P<0.001), with no differences between groups. These adaptations occurred primarily between 0-12 weeks, with no further improvements observed between 12-24 weeks (**Figure 3A-C**). Relative changes were not influenced by normalising values to body weight.

Post-supplementation skeletal muscle carnitine metabolism and PDCa

Skeletal muscle total carnitine in the CON group decreased from baseline to 12 weeks (17.8 ± 1.0 vs 15.7 ± 1.2; P<0.05) but was no different from baseline by 24 weeks (16.9 ± 1.0; P=0.17 vs baseline). In contrast, total carnitine in the CARN group was unchanged from baseline at 12 weeks (19.4 ± 129 vs. 19.5 ± 1.9; P=0.9) but tended to increase at 24 weeks

(20.9 ± 1.8; P=0.06 vs baseline), such that the absolute change from baseline was significantly greater in CARN than CON (1.5 ± 0.7 vs -0.9 ± 0.6 mmol·kg dw^{-1}; P<0.05). This resulted in a 30% greater resting free carnitine availability in CARN versus CON (P<0.05) and, following an initial exercise bout, free carnitine remained 28% greater in CARN vs CON (P<0.05; **Figure 4A**). However, following the second exercise bout, free carnitine values were similar between groups (**Figure 4A**). Post-exercise PDCa was no different between CON and CARN for either exercise bout (**Figure 4B**).

Post-supplementation non-mitochondrial ATP delivery and glycogenolysis

ATP, PCr degradation and lactate accumulation following HIT were all similar between CON and CARN treated groups in both exercise bouts (**Figure 4C**). There was a main effect of HIT for reducing Δ[PCr] and Δ[lactate] across both groups in bout one (P<0.05) and bout two (P<0.01). Resting muscle glycogen increased 1.5- and 1.4-fold in CON (P<0.01) and CARN (P<0.05), respectively, following HIT but remained similar between groups. There were no differences in Δ[glycogen] between CON and CARN during bout one or bout two (**Figure 4D**), although Δ[glycogen] in bout two was lower for both groups following HIT (main effect of HIT; P<0.01).

Discussion

The principle finding of the present study was that increasing skeletal muscle free carnitine content and the capacity to buffer excess acetyl groups during repeated 3 min bouts of high-intensity exercise at 100% $Watt_{max}$ did not further influence skeletal muscle metabolism during this exercise paradigm, nor improve training-induced changes in muscle metabolism, VO_{2max}, $Watt_{max}$, or work output over 24 weeks of high-intensity interval training (HIT). This would suggest that either acetylcarnitine formation, or PDC flux, is not limiting to mitochondrial ATP delivery during repeated bouts of exercise of this duration and intensity, or that the adaptations to HIT outweighed any benefit of increasing free carnitine availability. Indeed, a novel finding of the present study was that during a repeated bout of intense cycling exercise at $Watt_{max}$, where free carnitine availability was reduced, the reliance on phosphocreatine degradation increased, which is indicative of a declining contribution from mitochondrial ATP delivery. However, following 24 weeks of HIT non-mitochondrial ATP production and acetylcarnitine accumulation during a second bout of exercise were blunted to such an extent that they were lower than the pre-training first bout. Taken together these data suggest that 24 weeks of HIT *per se* results in a better matching of PDC and TCA cycle flux during repeated bouts of exercise, lowering the dependence on mitochondrial acetyl-group buffering.

In contrast to previous reports that reliance on non-mitochondrial ATP delivery is lessened over repeated bouts of intense exercise (Putman *et al.*, 1995; Bangsbo *et al.*, 2001), here we observed a two-fold greater PCr degradation during the second exercise bout relative to the first. Resynthesis of PCr, via the mitochondrial creatine kinase shuttle, relies on mitochondrial ATP provision (Perry *et al.*, 2012) and thus the extent of PCr degradation during exercise can be considered a sensitive marker of mitochondrial ATP delivery. Indeed, total non-mitochondrial ATP production, a reciprocal index of oxidative ATP delivery, was

71% greater during the second exercise bout. Assuming a negligible contribution from fat oxidation at this intensity (Venables *et al.*, 2005) and taken together with the continued rise in muscle lactate concentrations and the doubling of glycogenolysis, these data strongly contend that mitochondrial ATP provision was compromised during the second bout relative to the first. The discrepancy between this finding and data from previous studies probably relates to methodological differences. For example, Bangsbo and colleagues (2001) assessed rates of PCr degradation during two bouts of single-leg knee extension, which allows a greater blood flow per unit muscle mass and likely imposes more modest restrictions on mitochondrial ATP delivery (Boushel *et al.*, 2011). On the other hand, the evaluation of repeated-bout exercise metabolism using maximal two-legged cycle sprints precludes complete PCr resynthesis between bouts and is further confounded by the inability to maintain a constant work output across successive bouts (Putman *et al.*, 1995). With fixed-workload, two-legged cycling, we postulated that despite similar or greater PDC activation, the continued decline of free carnitine availability during a second exercise bout may restrict acetyl-group delivery to the TCA cycle by impairing PDC flux (Wall *et al.*, 2011), which thus provides a rationale for attempting to manipulate muscle carnitine content during chronic HIT.

Using the same L-carnitine supplementation strategy as that employed previously in our lab (Wall *et al.*, 2011), here we were able to increase resting skeletal muscle free carnitine availability following a period of HIT. Importantly, and in line with previous findings of Wall *et al.,* (2011), this post-supplementation elevation of free carnitine persisted throughout, and during recovery from, the first exercise bout at 100% $Watt_{max}$. The latter was hypothesised to prevent the apparent decline in mitochondrial ATP production during a second exercise bout, as observed prior to supplementation, by facilitating a greater flux through the PDC. However, post-supplementation neither PDC activation, nor carnitine acetylation were appreciably different between CON and CARN following bout two, which would suggest

that PDC flux during the second bout was comparable between groups. Thus, it would appear that the increased availability of free carnitine in CARN did not translate into an enhancement of mitochondrial acetyl-group delivery or any further sparing of PCr degradation or lactate accumulation compared to CON. This is somewhat surprising, considering the robust physiological and gene network adaptations previously reported in carbohydrate and lipid metabolism following carnitine supplementation (Wall *et al.*, 2011; Stephens *et al.*, 2013). It is possible that the three minute exercise bouts employed in the current study were of insufficient duration to allow the plateau in acetyl-group accumulation typically observed within 10 minutes of exercise at 75-90% VO_{2max} (Hiatt *et al.*, 1989; Constantin-Teodosiu *et al.*, 1992), such that the maximal capacity of carnitine to buffer mitochondrial acetyl-group accumulation becomes less critical. Indeed, carnitine acetylation during bout two, following HIT, in the current study reached only ~35% of the free + acetylcarnitine pool. This value is approximately half that observed following 20 minutes of exercise at 80% VO_{2max}, whereupon increasing skeletal muscle carnitine availability did indeed influence anaerobic energy production (Wall *et al.*, 2011). In this respect, the lower intensity and greater duration of the exercise protocol employed by Wall and colleagues (2011) likely permitted a greater contribution of lipids to acetyl-group delivery than in the current study (van Loon *et al.*, 2001), which may augment the dependence of mitochondrial ATP production on free carnitine availability. Ultimately, despite substantial reductions in lactate accumulation and PCr utilisation compared to baseline, reliance on non-mitochondrial ATP delivery was still markedly greater in a second exercise bout relative to the first. Increasing free carnitine availability prior to the start of the second bout did not alter this, suggesting that factors other than the mitochondrial acetyl-group buffering capacity (e.g. electron transport chain oxygen availability (Boushel & Saltin, 2013)) are limiting oxidative ATP delivery under these conditions. In view of these data, it seems unlikely that metabolic

flux was appreciably altered in CARN during training sessions, where four successive exercise bouts were performed at $Watt_{max}$, particularly as work output was equivalent between groups throughout the 24 week HIT period, and it is thus perhaps unsurprising that the gains in VO_{2max} and $Watt_{max}$ were comparable.

Limited data exist on the plasticity of the muscle carnitine stores in response to exercise training. Here we found 12 weeks HIT to be associated with a transient 12% decline in skeletal muscle total carnitine content (in CON), before partial restoration after 24 weeks continued training. Importantly, and consistent with one previous report on the impact of L-carnitine supplementation during training (Arenas *et al.*, 1991), the carnitine loading strategy used here was able to avert the observed decline in muscle carnitine content. The reason for the initial decline in muscle carnitine in CON is unclear, but given the prospective influence of habitual diet on the transcriptional regulation of tissue carnitine content (Stephens *et al.*, 2011; Zhou *et al.*, 2014a; Zhou *et al.*, 2014b), could be an adaptation to the 160 g/day carbohydrate load, which we have previously shown to influence transcription factor (e.g. PPARα) expression in skeletal muscle differentially to carbohydrate plus L-carnitine feeding (Stephens *et al.*, 2013). Alternatively, the decline in muscle carnitine content could reflect an adaptation to the highly glycolytic nature of the training paradigm, during the initial 8-12 weeks of the intervention when the gains in work output (and presumably metabolic adaptations) were greatest. In relation to this, the temporal nature of the adaptations observed here raises the question as to whether the widely-reported health benefits of short-term HIT (Gillen & Gibala, 2013; Sloth *et al.*, 2013) can continue to improve beyond 12 weeks.

In conclusion, these data demonstrate that daily L-carnitine and carbohydrate feeding can be used to manipulate skeletal muscle free carnitine content during a sustained period of HIT. However, elevating muscle free carnitine availability during HIT does not appear to influence the training-induced increase in mitochondrial ATP provision across repeated bouts of high-

intensity cycling exercise, suggesting that the skeletal muscle adaptations to HIT are not restricted by free carnitine availability or the mitochondrial acetyl-group buffering capacity. As a result, the whole-body gains in exercise performance and capacity following HIT were not augmented by the manipulation of muscle carnitine content. Nevertheless, the previously reported enhancement of exercise metabolism by skeletal muscle carnitine loading (Wall *et al.*, 2011) may still be of benefit to training paradigms that employ steady-state exercise intensities lower than 100% $Watt_{max}$.

Perspectives

Augmenting skeletal muscle carnitine content can demonstrably alter steady-state exercise metabolism, body adiposity and the expression of related gene-networks (Wall *et al.*, 2011; Stephens *et al.*, 2013). More recent observational concepts on the acetyl-group buffering role of carnitine in human metabolism and health (Davies *et al.*, 2016) lack longitudinal support. Here we increased free carnitine availability during a 24 week period of high-intensity exercise training and show that the skeletal muscle acetyl-group buffering capacity is unlikely limiting to the metabolic and physiological adaptations to this type of training paradigm.

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Figure legends

Figure 1 Schematic outlining study visit protocols A: At baseline, volunteers completed an exercise study visit (see **B**) and, following randomisation into either CON (n=7) or CARN (n=7), completed 24 weeks of HIT and supplementation before a second exercise study visit. **B:** For exercise study visits, volunteers performed two 3 minute bouts of cycle ergometer exercise at 100% of their predetermined $Watt_{max}$, separated by a five minute passive recovery period. Muscle biopsies (arrows) were obtained before and after each exercise bout. A three minute warm-up period at 25% $Watt_{max}$ was completed prior to the first exercise bout (following the resting muscle biopsy). *The first three exercise bouts lasted three minutes, the fourth bout was open-ended to allow exercise until exhaustion but generally lasted 0-minutes. Dotted arrow indicates that only a resting biopsy was taken.

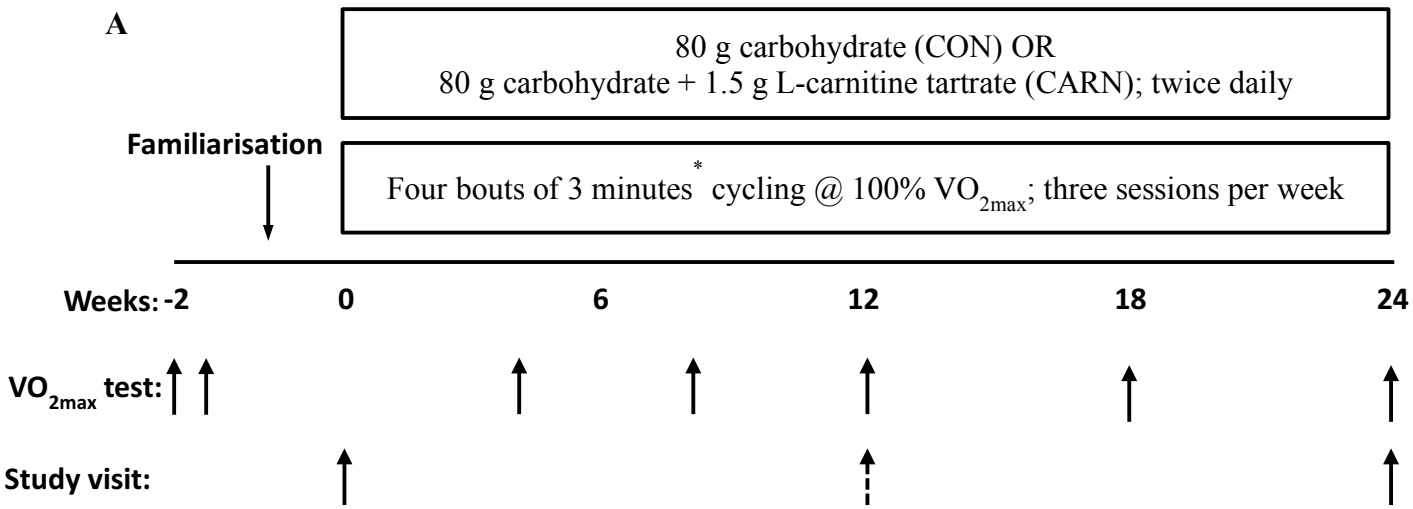
Figure 2 Repeated-bout exercise metabolism Free carnitine (black bars) and acetylcarnitine (dotted grey bars) concentration (**A**) and PDC activation status (**B**) before and after two 3 minute cycle ergometer exercise bouts at 100% $Watt_{max}$ separated by five mintues passive recovery. The contribution of ATP degradation (black bars), PCr degradation (white bars) and lactate accumulation (dotted bars) to total non-mitochondrial ATP production (NMAP; **C**); and glycogen utilisation (**D**) during each exercise bout. ** $P<0.01$, *** $P<0.001$ versus pre-exercise value for same bout; † $P<0.05$, †† $P<0.01$, ††† $P<0.001$ versus corresponding bout one value. Values are mean ± SE (n=21).

Figure 3 Influence of L-carnitine supplementation on exercise performance following 24 weeks HIT VO_{2max} (A), $Watt_{max}$ (B) and work output during training sessions (C) determined at baseline and following 12 and 24 weeks of HIT with twice daily beverages of either 80g carbohydrate (CON; black bars) or 80g carbohydrate + 1.5g L-carnitine (CARN; white bars).

**P<0.01, ***P<0.001 main effect of HIT vs baseline. Values are mean ± SE (n=7 per group).

Figure 4 Influence of L-carnitine supplementation on repeated bout exercise metabolism following 24 weeks HIT Post-exercise free carnitine (solid bars) and acetylcarnitine (dotted bars) concentrations (**A**) and PDC activation status after two 3 minute cycle ergometer exercise bouts at 100% $Watt_{max}$ following 24 weeks of HIT with twice daily beverages of either 80g carbohydrate (CON; black bars) or 80g carbohydrate + 1.5g L-carnitine (CARN; white bars). The contribution of ATP degradation (black bars), PCr degradation (white bars) and lactate accumulation (dotted bars) to total non-mitochondrial ATP production (NMAP; **C**); and glycogen utilisation (**D**) during each exercise bout following HIT and supplementation. * P<0.05 versus CON. Values are mean ± SE (n=7 per group).

Figure 1


A
80 g carbohydrate (CON) OR
80 g carbohydrate + 1.5 g L-carnitine tartrate (CARN); twice daily
Familiarisation
Four bouts of 3 minutes* cycling @ 100% VO2max; three sessions per week
Weeks: -2
0
6
12
18
24
VO2max test:
Study visit:

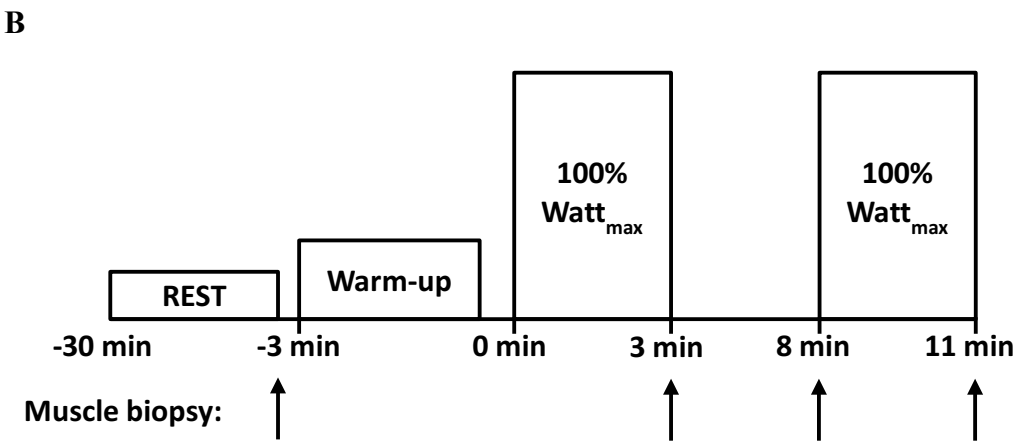

B
100% Wattmax
100% Wattmax
REST
Warm-up
-30 min
-3 min
0 min
3 min
8 min
11 min
Muscle biopsy:

Figure 2

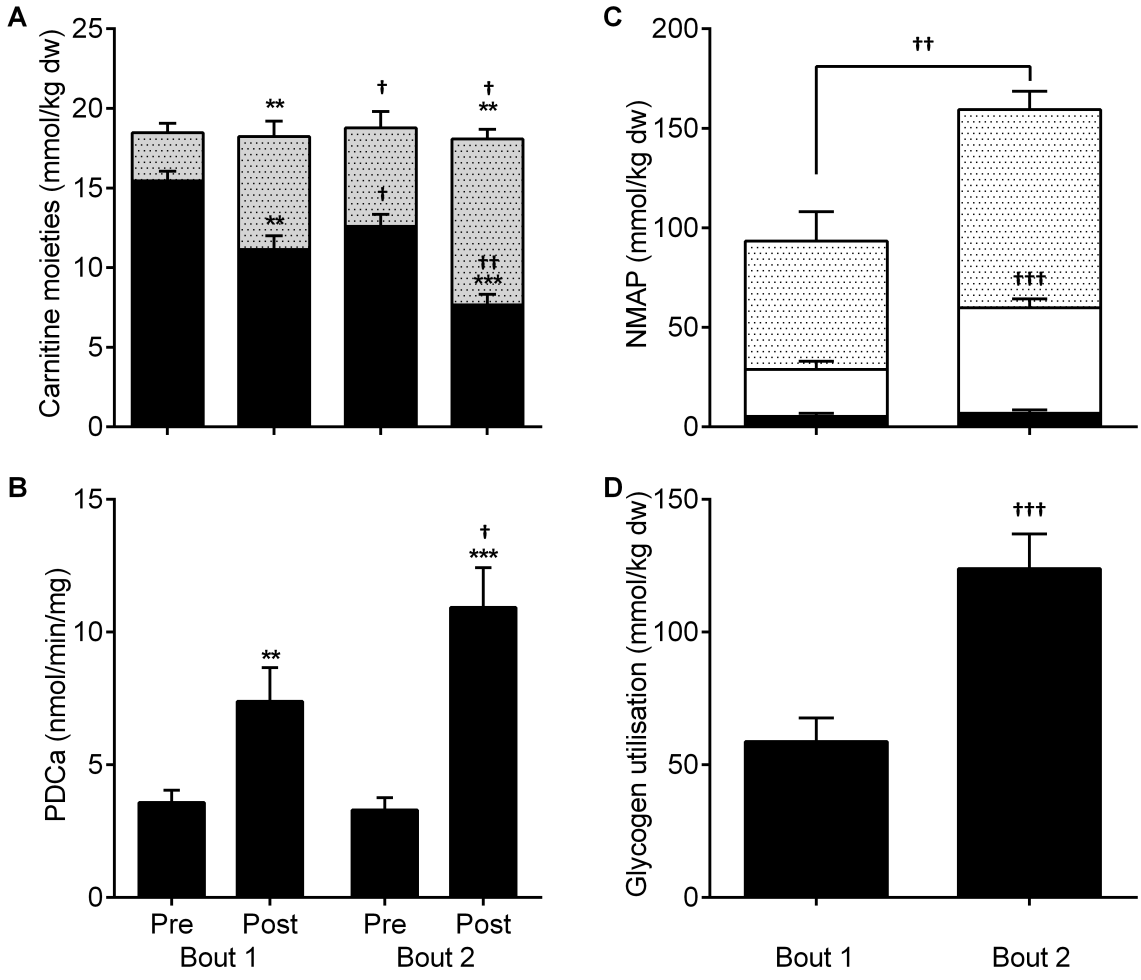

A
Carnitine moieties (mmol/kg dw)
25
20
15
10
5
0
**
**
†
†
†
**
††
***
B
PDCa (nmol/min/mg)
15
10
5
0
**
†
***
Pre
Post
Pre
Post
Bout 1
Bout 2
C
NMAP (mmol/kg dw)
200
150
100
50
0
††
†††
D
Glycogen utilisation (mmol/kg dw)
150
100
50
0
†††
Bout 1
Bout 2

Figure 3

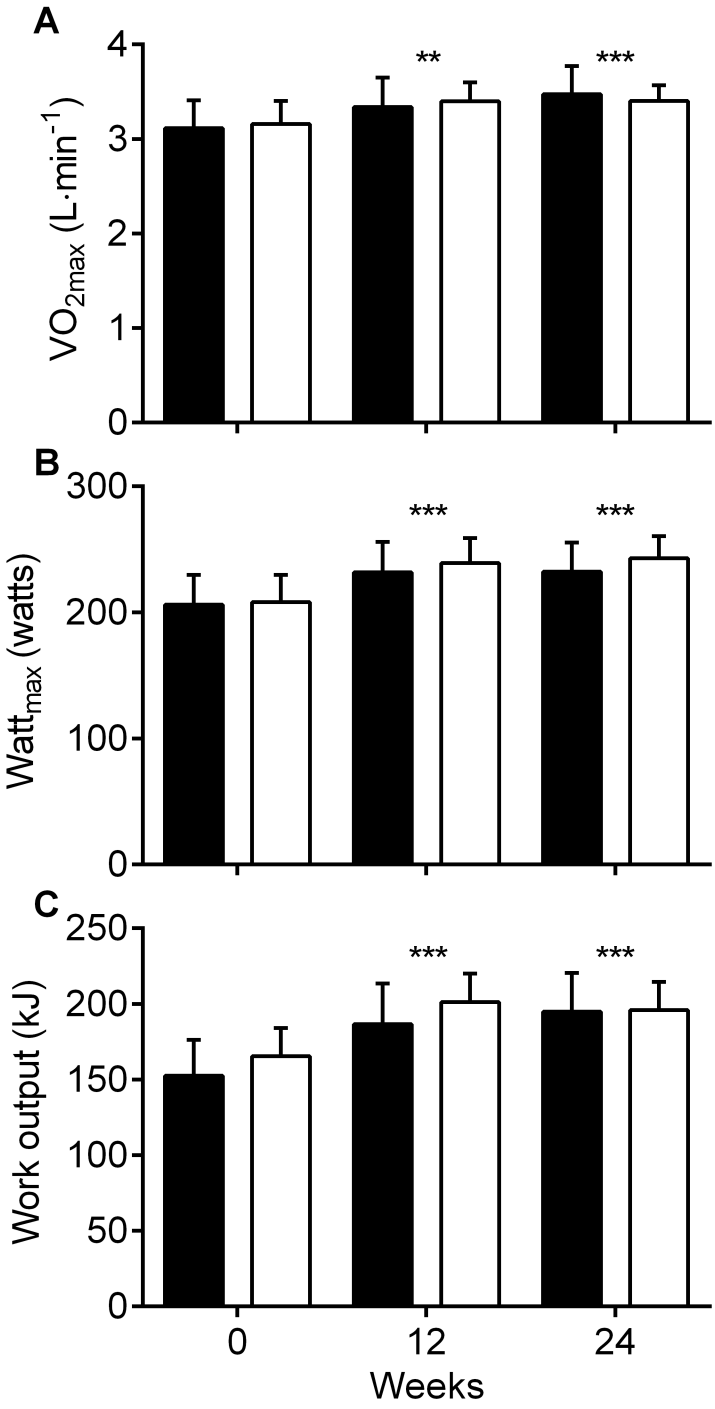
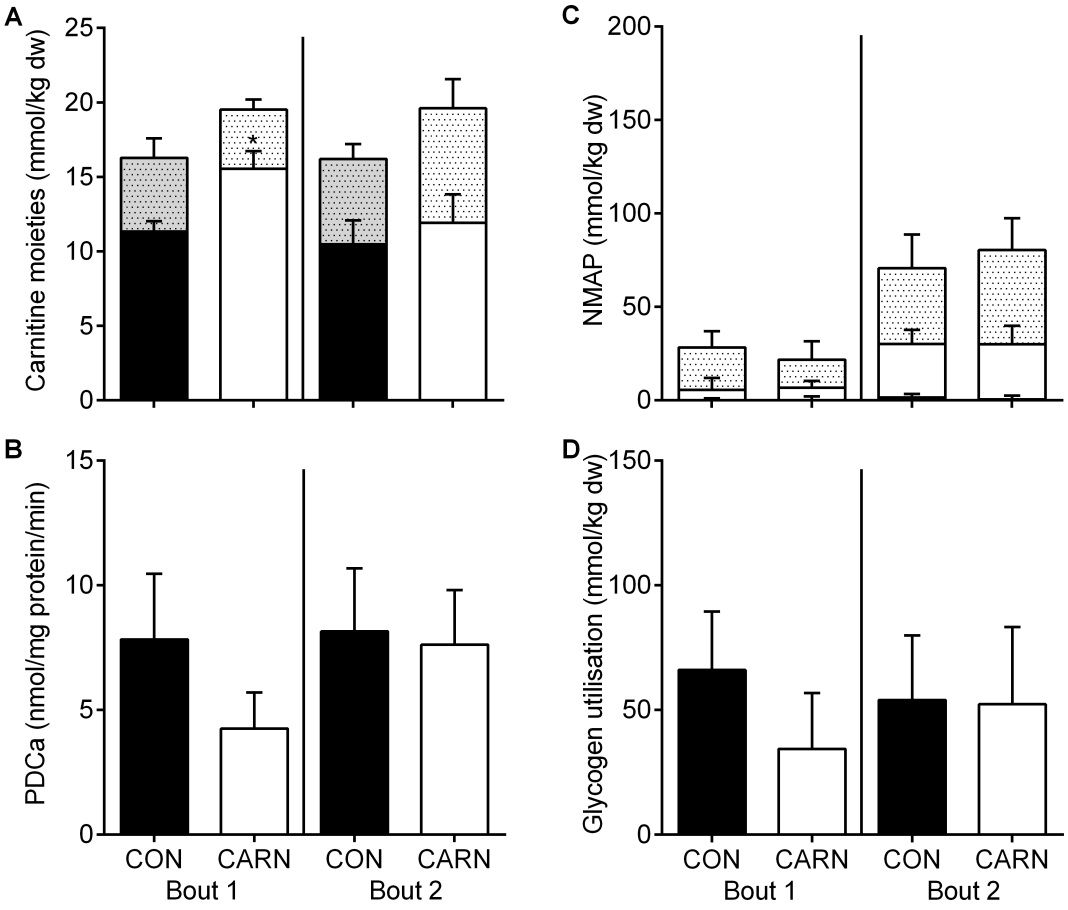

A
B
C
VO_{2max} (L·min^{-1})
$Watt_{max}$ (watts)
Work output (kJ)
**
***
***
***
***
0
1
2
3
4
0
100
200
300
0
50
100
150
200
250
0
12
24
Weeks

Figure 4


A
Carnitine moieties (mmol/kg dw)
25
20
15
10
5
0
*
C
NMAP (mmol/kg dw)
200
150
100
50
0
B
PDCa (nmol/mg protein/min)
15
10
5
0
D
Glycogen utilisation (mmol/kg dw)
150
100
50
0
CON
CARN
CON
CARN
Bout 1
Bout 2
CON
CARN
CON
CARN
Bout 1
Bout 2

Supporting Table 1

Skeletal muscle metabolite concentrations (mmol·kg dw^{-1}) and PDC activation status (nmol acetyl-CoA·mg protein·min^{-1}) before and immediately following two 3 minute exercise bouts at 100% $Watt_{max}$, at baseline and after 24 weeks of high-intensity exercise training and twice daily ingestion of either 80 g carbohydrate (CON; n=7) or 80 g carbohydrate + 1.5 g L-carnitine-tartrate (CARN; n=7). * P<0.05, ** P<0.01 vs baseline; † P<0.05 vs CON.

Supporting table 1

		BOUT ONE				BOUT TWO			
		PRE		POST		PRE		POST	
		CON	CARN	CON	CARN	CON	CARN	CON	CARN
	ATP	25.6 ± 1.3	24.9 ± 0.9	23.5 ± 1.1	22.1 ± 1.7	24.2 ± 2.0	21.7 ± 1.4	20.0 ± 1.8	19.7 ± 1.2
	PCr	72.3 ± 4.0	74.7 ± 3.4	55.4 ± 7.2	50.1 ± 7.7	74.1 ± 3.9	73.4 ± 5.1	25.8 ± 7.3	23.4 ± 3.6
	Lactate	4.5 ± 0.6	7.2 ± 1.7	39.0 ± 10.9	51.2 ± 20.7	15.8 ± 2.1	19.6 ± 7.3	91.3 ± 15.4	83.9 ± 8.2
0	Glycogen	378 ± 28	413 ± 20	318 ± 21	347 ± 15	399 ± 23	375 ± 31	243 ± 13	264 ± 24
	Free carnitine	14.2 ± 0.7	16.9 ± 0.8	11.1 ± 1.1	11.7 ± 1.6	12.0 ± 0.8	13.6 ± 1.6	7.5 ± 1.3	8.4 ± 1.2
	Acetylcarnitine	2.8 ± 0.7	2.3 ± 0.9	5.7 ± 1.2	6.4 ± 1.6	5.0 ± 1.0	5.3 ± 2.0	9.0 ± 0.6	9.8 ± 1.1
	PDCa	3.6 ± 0.9	3.8 ± 0.7	4.8 ± 1.7	5.7 ± 1.5	2.7 ± 0.7	2.8 ± 0.4	7.6 ± 2.3	9.7 ± 1.2
	ATP	23.6 ± 1.5	24.0 ± 1.0	25.0 ± 1.1	24.2 ± 1.6	23.6 ± 1.1	23.8 ± 1.1	22.8 ± 1.1	23.6 ± 1.6*
	PCr	73.9 ± 3.8	78.1 ± 3.2	65.5 ± 5.7	70.8 ± 5.3*	77.6 ± 1.9	78.5 ± 4.1	49.1 ± 7.2*	48.9 ± 9.2*
	Lactate	8.0 ± 2.4	6.7 ± 1.5	23.0 ± 7.3	16.7 ± 6.1*	23.0 ± 3.1	20.0 ± 4.5	50.2 ± 9.7*	53.6 ± 11.7*
24	Glycogen	567 ± 33**	570 ± 46*	501 ± 32**	536 ± 40**	516 ± 48	532 ± 44*	462 ± 55**	479 ± 55**
	Free carnitine	13.5 ± 1.4	17.6 ± 1.6†	11.3 ± 0.7	16.4 ± 1.6*†	11.8 ± 0.8	16.5 ± 1.3†	10.5 ± 1.6	11.9 ± 1.9
	Acetylcarnitine	2.9 ± 0.7	2.8 ± 0.7	4.9 ± 1.2	4.0 ± 0.7	4.2 ± 0.6	3.9 ± 0.9	5.7 ± 0.9	7.7 ± 1.9
	PDCa	3.0 ± 0.7	3.8 ± 0.8	7.8 ± 2.5	3.7 ± 1.4	3.4 ± 1.4	3.3 ± 1.4	8.2 ± 2.3	7.6 ± 2.2