Skeletal muscle adaptation and performance responses to once a day versus twice every second day endurance training regimens.

Running title: Glycogen availability and training adaptation

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ABSTRACT

We determined the effects of a cycle training program in which selected sessions were performed with low muscle glycogen content on training capacity and subsequent endurance performance, whole-body substrate oxidation during submaximal exercise, and several mitochondrial enzymes and signaling proteins with putative roles in promoting training adaptation. Seven endurance-trained cyclists/triathletes trained daily (HIGH) alternating between 100 min steady-state aerobic rides (AT) one day, followed by a high-intensity interval training session (HIT; 8 x 5 min at maximum self-selected effort) the next day. Another 7 subjects trained twice every second day (LOW), first undertaking AT, then 1-2 hr later, the HIT. These training schedules were maintained for 3 wk. Forty-eight hr before and after the first and last training sessions, all subjects completed a 60-min steady-state ride (60SS) followed by a 60-min performance trial. Muscle biopsies were taken before and after 60SS and rates of substrate oxidation were determined throughout this ride. Resting muscle glycogen concentration (412 ± 51 vs. 577 ± 34 µmol·g⁻¹ d.w.), rates of whole-body fat oxidation during 60SS (1261 ± 247 vs. 1698 ± 174 µmol·kg⁻¹·60 min⁻¹), the maximal activities of citrate synthase (45 ± 2 vs. 54 ± 1 mmol·kg⁻¹·min⁻¹ d.w) and β-hydroxyacyl-CoA-dehydrogenase (18 ± 2 vs. 23 ± 2 mmol·kg⁻¹·min⁻¹ d.w) along with the total protein content of cytochrome c oxidase subunit IV were increased only in LOW (all p<0.05). Mitochondrial DNA content and PGC-1α protein levels were unchanged in both groups after training. Cycling performance improved by ~10% in both LOW and HIGH. We conclude that compared to training daily, training twice every second day compromised high-intensity training capacity. While selected markers of training adaptation were enhanced with twice-a-day training, the performance of a 1 hr time-trial undertaken after a 60 min steady-state ride was similar after once daily or twice every second day training programs.

Key words: AMPK, citrate synthase, mitochondrial DNA, fat-oxidation, PGC-1α, train-low, high intensity interval training, mitochondria.
Introduction

Training capacity (the ability to complete strenuous training sessions over days and weeks) and nutrition are highly interrelated and it is generally assumed that optimal adaptation to the demands of repeated training sessions requires a diet that can sustain muscle energy reserves (7, 13). However, evidence is accumulating to suggest that commencing endurance exercise with low muscle glycogen content increases the transcription rate of several genes and proteins involved in the training response/adaptation (15, 16, 30, 38, 41, 49). Indeed, in recent years it has become evident that commencing exercise in the face of low muscle glycogen stores amplifies the activation of a number of signaling proteins, including the AMP-activated protein kinase (AMPK) and the p38 mitogen-activated protein kinase (MAPK), two enzymes with direct roles in controlling the expression and activity of several transcription factors involved in mitochondrial biogenesis and promoting training adaptation (for review see 3, 25). Accordingly, the notion that commencing training with low muscle glycogen levels (‘train-low’) to maximize the physiological response/adaptation to endurance training has gained popularity (3).

Only one study has tested the hypothesis that undertaking a training program in which a portion of exercise sessions are deliberately commenced with low muscle glycogen may be beneficial for training adaptation and subsequent performance. In that investigation, Hansen et al. (18) recruited 7 untrained males to undertake a 10 wk program of leg knee extensor ‘kicking’ exercise. In an ingenious experimental design, both of the subject’s legs were trained according to a different daily schedule but the total amount of work undertaken by each leg over the study duration was the same: subjects trained one leg twice-a-day, every second day, whereas the contra lateral leg was trained daily. Compared to the leg that performed daily training with normal glycogen reserves, the leg that commenced half of the training sessions with low muscle glycogen levels had a more pronounced increase in resting glycogen content and citrate synthase activity (18).
Remarkably, this ‘train-low’ approach resulted in an almost two-fold greater training-induced increase in one-leg exercise time to fatigue compared to when participants commenced all training sessions in a glycogen replete state. The results of Hansen et al. (18) demonstrate that under specific experimental conditions, training adaptation may be augmented by a relative lack of glycogen availability and this, in turn, leads to an enhancement in endurance.

While the results of that study (18) are intriguing, several issues currently preclude exercise scientists from making firm recommendations to athletes with respect to undertaking endurance training for performance enhancement in a low glycogen state. First, the subjects in the investigation of Hansen et al. (18) were untrained and it is not currently known whether undertaking training sessions in a lowered glycogen state will translate into improved training adaptation and performance in already well-trained athletes. Second, the training sessions undertaken by subjects in that study were ‘clamped’ at a fixed submaximal intensity for the duration of the training program: athletes typically periodize their programs to incorporate a ‘hard-easy’ pattern to the overall organization of training, as well as progressive overload (21, 42) rather than training to a fixed (submaximal) intensity. Third, the mode of training (one-legged knee extensor kicking) and the exercise ‘performance’ task (submaximal kicking to exhaustion) bear little resemblance to the whole-body training modes and performance tasks undertaken by the majority of competitive athletes. Fourth, training schedules that induce chronically low muscle glycogen levels may increase the risk for the overtraining syndrome (37) and actually lead to a reduced training capacity (1). Finally, the vast majority of athletes are reluctant to take complete rest days, and training every second day would be considered an extreme practice amongst this cohort. Accordingly, the aims of the present study were to determine the effects of undertaking selected training sessions with low muscle glycogen content on 1) training capacity and endurance performance, 2) whole-body substrate metabolism during submaximal exercise and 3) several mitochondrial enzymes and
signaling proteins with putative roles in promoting training adaptation. We selected well-trained subjects for this investigation: we hypothesized that these athletes would already have maximized their training adaptation and that further gains would be minimal, irrespective of whether they trained with low- or normal levels of muscle glycogen.

Methods

Subjects and preliminary testing.

Eighteen endurance-trained male cyclists or triathletes volunteered to participate in this study after they were informed about the possible risks of all procedures. All subjects gave written consent. This study was approved by the RMIT University Human Research Ethics Committee. Of the eighteen subjects (Table 1), 14 took part in the chronic training study while 12 subjects (which included 8 subjects from the chronic study plus an additional 4 subjects who met the same criteria for age, fitness level and training history) participated in the acute phase of the investigation (described in detail subsequently). In the 6 wk prior to commencement of the study, subjects were riding 300-500 km wk$^{-1}$ but were not undertaking any interval training. Subjects had a history of $>3$ yr of endurance-based training. One week prior to an experimental testing session, each subject undertook an incremental cycling test to exhaustion on an electromagnetically braked cycle ergometer (Lode, Groningen, The Netherlands). The testing protocol has been described in detail previously (22). During the maximal test and the subsequently described experimental trials, subjects breathed through a Hans Rudolph two way non-rebreathing valve and mouthpiece attached to a calibrated online gas system (Parvomedics, Utah, U.S.A) interfaced to a computer, that calculated the instantaneous rates of O$_2$ consumption (VO$_2$), CO$_2$ production (VCO$_2$), minute ventilation (VE$_{STPD}$), and the respiratory exchange ratio (RER) every 15 s from conventional equations (36). Before each maximal test and all experimental trials, the analyzers were calibrated with commercially available gases of known O$_2$ and CO$_2$ content. Peak VO$_2$ was defined as the highest O$_2$ uptake a subject
attained during any 60 s of the test while PPO was calculated from the last completed work rate plus the fraction of time spent in the final noncompleted work rate multiplied by 25 W. This value was used to determine the power output corresponding to 70% of each subject’s VO\textsubscript{2} peak (63% of PPO) to be used in the subsequently described experimental trials and training sessions. The maximal test and all experimental trials were conducted under standard laboratory condition (18-22\textdegree C, 40-50% relative humidity) and subjects were fan cooled during all exercise sessions.

**Experimental Design.**

An overview of the experimental design is shown in Figure 1. In brief, the subjects were divided into two groups (matched for age, VO\textsubscript{2} peak and training history) and undertook 18 laboratory training sessions during a 3 wk intervention period. The control group (HIGH) trained 6 days per week (rest on day-7) for 3 wk, alternating between 100-min steady state aerobic training (AT; ~70% VO\textsubscript{2} peak, 63% of PPO) on the first day and high intensity interval training (HIT; 8 x 5-min work bouts at maximal effort with 1-min recovery in between work bouts at ≤ 100W) the next day. In contrast, the experimental group (LOW) trained twice per day, every second day, performing the AT in the morning, followed by 1-2 hr of rest and then HIT (Figure 1). During the time between these two training sessions, subjects rested in the lab and were given ad libitum access to water. Subjects groups were designated as HIGH or LOW: HIGH completed all HIT sessions at a time when muscle glycogen levels were restored whereas LOW undertook these HIT sessions at a time when muscle glycogen stores were partially depleted, or lower than normal. Accordingly, the 100-min AT was used in this study because it has previously been reported that endurance-trained cyclists comparable in training status to those in the current investigation utilized ~60% of their muscle glycogen content after 105 min of steady-state cycling at ~70% of individual VO\textsubscript{2} peak. (14). The HIT session utilized in this study has been described in detail previously (45). In brief, this training session involves 8
repetitions of 5-min work bouts at ~75-80% of PPO, separated by 1 min of recovery at ≤100W. We have previously reported that this workout utilizes ~50% of muscle glycogen stores when the session is commenced with normal glycogen content (~500 µmol·g⁻¹ d.w) (45). All the training sessions were performed using the subject’s own bicycle attached to a stationary trainer (Kinetic, MN, USA) and training intensities were monitored using PowerTap power meters (CycleOps, Madison, USA). Before every training session, the PowerTap was zeroed according to the manufacturer’s instructions. These devices have previously been shown as a valid and reliable tool to accurately monitor power output in both lab and field settings (4, 17, 35). All HIT sessions in the HIGH group and all training sessions in the LOW group were performed in the laboratory under supervision of the principal investigator. Subjects in the HIGH group performed their AT at home on an indoor stationary trainer. Power output for all these sessions were recorded to ensure that the subjects trained at the prescribed intensity (63% PPO) and for the required duration (100 min).

**Dietary control.**

Our goal was to provide a similar dietary treatment to that received by subjects in the Hansen study (17). We used a variety of methods to achieve this in our free-living subjects. Dietary intake was ‘clamped’ 24 hr prior to an experimental trial (0.21 MJ·kg⁻¹ BM; 8 g·kg⁻¹·d⁻¹ and 65% of energy from CHO; 2.0 g·kg⁻¹·d⁻¹ protein and 1.0 g·kg⁻¹·d⁻¹ fat). All meals and snacks were supplied with diets being individualized for food preferences and BM. Subjects received their food in pre-prepared packages and were required to keep a food checklist to note their compliance to the dietary instructions and their intake of any additional food or drinks. This method was also used during the day(s) preceding the training sessions that were chosen for examination of the acute response to exercise (see below). During the remainder of the training period, subjects were given dietary instructions by a sports dietitian, including sample diets and check-lists of carbohydrate-
rich foods to ensure that they were consuming 8-9 g·kg\(^{-1}\)·d\(^{-1}\) BM of CHO. Every 2 days, subjects were required to submit records of their dietary intake and morning BM to the sports dietitian to ensure adherence to carbohydrate intake targets and to fine tune energy intake to maintain energy balance.

*Acute responses to AT and HIT sessions*

Twelve subjects (including 8 from the experiments described previously and 4 additional subjects who met the same criteria for age, VO\(_2\) peak and training history) gave their informed consent to complete one session of AT and one session of HIT according to the training schedule for their designated group. One week prior to the commencement of a training session, the 4 additional recruits reported to the laboratory to complete preliminary testing (described previously). Subjects were rested and provided with packaged diets (described above) for the 24 hr prior to these training sessions. Subjects performed the training sessions on their own bikes attached to a stationary trainer with power meters attached (described previously). On arrival in the laboratory, a single leg was prepared for skeletal muscle biopsy sampling through two incisions 2–3 cm apart along the *vastus lateralis* muscle. A resting muscle sample was then taken using the percutaneous biopsy technique with suction applied while a second biopsy was obtained immediately before the HIT. All muscle biopsies were rapidly frozen in liquid N\(_2\) within ~15 s and stored at -80°C and later analyzed for muscle glycogen content. In the case of the subjects who were concurrently involved in the chronic training study, these invasive sessions were performed mid-way through the 3 wk training program (Figure 3).

*Experimental trial.*

Forty-eight hr before the first training session and 48 hr after the last training session, subjects reported to the laboratory after a 12- to 14-h overnight fast to undertake an experimental trial comprising of a 60-min steady state ride at 70% of VO\(_{2\text{peak}}\) (60SS)
followed by a 60-min performance trial. Skeletal muscle biopsies (described previously) were performed at rest upon subjects’ arrival in the laboratory and immediately after the 60SS. The subjects were given 10 min of rest after the resting biopsy before began the 60SS and after 10, 30 and 50 min, expired gas was collected (for 5-min) to estimate the instantaneous rates of substrate oxidation. Fifteen min after the completion of 60SS, the subjects began the performance ride. Endurance performance was determined as the average power maintained (W/kg BM) during the 60-min period.

*Analytical Procedures*

*Rates of whole body fat oxidation.*

Rates of whole body fat oxidation (g·min⁻¹) were calculated from the respiratory exchange ratio (RER) data collected for 5 min at 10, 30 and 50 min of the 60SS. The calculations were made from VCO₂ and VO₂ measurements, assuming a nonprotein RER value, according to the following equation (36).

\[
\text{Fat oxidation} = 1.695 \text{ VO}_2 - 1.701 \text{ VCO}_2
\]

Rates of fatty acid oxidation (µmol·kg⁻¹·min⁻¹) were determined by converting the rate of triglycerol oxidation (g·min⁻¹) to its molar equivalent assuming the average molecular weight of human triglycerol to be 855.3 g·mol⁻¹ and multiplying the molar rate of triglycerol oxidation by 3, because each molecule contains three molecules of fatty acids. Total fat oxidation during the 60SS was estimated by calculating the area under the oxidation versus time curves for each subject.

*Muscle glycogen concentration.*

Approximately 10-15 mg of muscle was freeze-dried and powdered with all visible blood and connective tissue removed under magnification. The freeze-dried muscle sample was
then incubated in 250 µL of 2 M hydrochloric acid at 100 ºC for 2 hr before being neutralized with 750 µL of 0.67 M sodium hydroxide. Glycogen concentration was determined via enzymatic analyses (50 mM Tris, 25 mM HCL, 1 mM MgCl2, 0.5 mM DTT, 0.3 mM ATP, 0.05 mM NADP and 1 U/ml HK, 0.1 U/ml G-6-PDH) with glucose standards by fluorometric detection and was expressed as millimoles of glycogen⋅kg⁻¹d.w.

**Citrate synthase and β-hydroxyacyl-CoA dehydrogenase activity.**

Freeze-dried, powdered muscle (2-3 mg) was homogenized in 100 mM potassium phosphate buffer (pH 7.3, 1:400 dilution), and citrate synthase was assayed spectrophotometrically at 25ºC by the reduction of DNTB, as published previously (40) with slight modifications (5). β-hydroxyacyl-CoA dehydrogenase (β-HAD) activity was assayed spectrophotometrically at 25ºC using the same homogenates, based on the disappearance of reduced NADH (32).

**Western blotting**

Approximately 30 mg of wet muscle was homogenized (50 mM Tris HCl, pH 7.5, 1 mM EDTA, 1 mM EGTA, 1 mM dithiothreitol, 50 mM NaF, 5 mM Na pyrophosphate, 10% glycerol, 1% Triton X-100, 10 µg/ml trypsin inhibitor, 2 µg/ml aprotinin, 1 mM benzamidine, and 1 mM phenylmethylsulfonyl fluoride) and the homogenates were then centrifuged at 20,000 g for 30 min at 4ºC. The supernatant was aliquoted and stored at -80ºC until further analysis. The total protein concentration in the aliquots was determined by the bicinchoninic acid method (Pierce, IL, USA).

Muscle lysates containing 60 µg (pAMPK<sup>Thr172</sup>, Total AMPK and PGC-1α) and 30 µg (Cytochrome C oxidase subunit II and IV [COX II and COX IV respectively]) of total protein were electrophoresed on 10% (pAMPK<sup>Thr172</sup>, Total AMPK and PGC-1α) and 14%
(COX II and COX IV) SDS-PAGE and detected by immunoblotting with antibodies specific for PGC-1α (Santa Cruz Biotechnology, Santa Cruz, CA; sc13067), α-tubulin (Cell Signaling, Danvers, MA; #2144), pAMPK \textsuperscript{Thr172} and Total AMPKα (a gift from Professor Bruce Kemp), COX II (Mitosciences, Oregon; MS 405) and COX IV (Mitosciences, Oregon; MS 407). Since most analyses required completion of multiple gels due to the large number of samples, an identical internal standardised human skeletal muscle sample, designated “Control”, was used in all gels to account for variability between exposures of different membranes. PGC-1α, COX II and COX IV protein content was expressed relative to total α-tubulin, whereas pAMPK \textsuperscript{Thr172} was expressed relative to total AMPK. The immunoreactive proteins were detected with enhanced chemiluminescence (Amersham Biosciences) on a BioRad Chemidoc XRS system (BioRad, CA) and quantified by densitometry (Quantity one, BioRad, CA).

\textit{Mitochondrial DNA determination}

Mitochondrial DNA (mtDNA) was quantified by real-time PCR using methods described previously (33), with modification and optimization to allow multiplex PCR. Briefly, total mitochondrial and nuclear DNA was isolated from ~20 mg of snap-frozen muscle tissue using a commercially available kit (Qiagen, Victoria, Australia), and the concentration of each sample was determined using a Nanodrop ND-1000 spectrophotometer (ThermoFisher Scientific, DE, USA). Ten ng of DNA from each sample was amplified in a BioRad iCycler PCR detection system using BioRad iQ Supermix (BioRad, NSW, Australia), 900 nM of forward and reverse primers for mtDNA and β-globin as a housekeeping gene (Sigma Genosys, NSW, Australia), and 225 nM of FAM-labelled Taqman probe for mtDNA and VIC-labelled probe for β-globin (Applied Biosystems, Victoria, Australia). Reaction volume was 25 µl, and primer and probe sequences were as
previously reported (33). Data were quantified using the delta-Ct method to quantitate fold changes in mtDNA compared to pre-training values.

Statistical Analysis

Treatment effects were analysed using two-factor (treatment and time) repeated measure analysis of variance (ANOVA) with post-hoc analysis performed using the Holm-Sidak method. Data were analysed using SigmaStat 3.1.1 (Systat Software, Inc., CA) and all values are expressed as means ± standard error (SE), with significance reported as p<0.05.

Results

Muscle glycogen concentration before AT and HIT training sessions

Figure 2 displays muscle glycogen content before AT and HIT in the subgroup of 12 subjects who participated in the acute phase of the study. As intended, muscle glycogen stores in HIGH were similar before both AT and HIT (~400 µmol·g⁻¹ d.w). In LOW, however, muscle glycogen stores were significantly reduced after AT (p<0.05, Figure 2) such that LOW commenced the HIT with ~50 % less glycogen than before the AT.

Training intensity during HIT

During week 1 (HIT 1-3), LOW trained at a significantly lower average percentage of PPO compared to HIGH (p<0.05, Figure 3). During the second week (HIT sessions 4-6) of intervention, there was a strong trend for the average training intensity to be lower (p=0.06, Figure 3) but by the third week (HIT sessions 7-9), the average training intensities between LOW and HIGH were not different (Figure 3).
Resting muscle glycogen concentration

After 3 wk of training, resting muscle glycogen concentration increased significantly from $412 \pm 51 \mu\text{mol} \cdot \text{g}^{-1} \text{d.w}$ to $577 \pm 34 \mu\text{mol} \cdot \text{g}^{-1} \text{d.w}$ ($p < 0.05$, Figure 4) in LOW. While glycogen levels increased in HIGH, such changes failed to reach statistical significance.

Fat oxidation during 60-min steady-state ride

Estimated rates of fat oxidation during the 60SS after 10, 30 and 50 min of the ride are shown in Figure 5. Fat oxidation during the 60SS, measured as the total area under the fat oxidation vs. time curve (AUC) showed a strong trend ($P=0.051$) to be greater in LOW after the 3 wk training period ($1698 \pm 174$ vs. $1261 \pm 247 \mu\text{mol} \cdot \text{kg}^{-1} \cdot 60 \text{ min}^{-1}$). The AUC measured as the last 40 min of the 60SS was significantly greater in LOW after training ($1220 \pm 100$ vs. $867 \pm 166 \mu\text{mol} \cdot \text{kg}^{-1} \cdot 40 \text{ min}^{-1}$, $p<0.05$).

Citrate Synthase and β-HAD activity

Maximal citrate synthase activity in LOW increased from $45 \pm 2 \text{mmol} \cdot \text{kg}^{-1} \cdot \text{min}^{-1} \text{d.w}$ to $54 \pm 1 \text{mmol} \cdot \text{kg}^{-1} \cdot \text{min}^{-1} \text{d.w}$ (Figure 6A; $p<0.05$) after the 3 wk training period such that the post-training maximal activity in LOW was significantly higher than the post-training maximal activity in HIGH (Figure 6A; $p<0.05$). The maximal citrate synthase activity remained unchanged in HIGH ($39 \pm 5 \text{mmol} \cdot \text{kg}^{-1} \cdot \text{min}^{-1} \text{d.w}$ to $43 \pm 3 \text{mmol} \cdot \text{kg}^{-1} \cdot \text{min}^{-1} \text{d.w}$, Figure 6A) after the 3 wk training period. β-HAD activity increased in LOW ($18 \pm 2 \text{mmol} \cdot \text{kg}^{-1} \cdot \text{min}^{-1} \text{d.w}$ to $23 \pm 2 \text{mmol} \cdot \text{kg}^{-1} \cdot \text{min}^{-1} \text{d.w}$; $p<0.05$; Figure 6B) such that the post-training maximal activity in LOW was significantly higher than the similar time point in HIGH (Figure 6A; $p<0.05$). The maximal β-HAD activity remained unchanged in HIGH after the 3 wk training period ($16 \pm 1 \text{mmol} \cdot \text{kg}^{-1} \cdot \text{min}^{-1} \text{d.w}$ to $17 \pm 1 \text{mmol} \cdot \text{kg}^{-1} \cdot \text{min}^{-1} \text{d.w}$).
Cytochrome c oxidase subunits II and IV

The total protein content of COX II (relative to total α-tubulin content) remained unchanged after the 3 wk training intervention in both groups (Figure 6C). However, the total protein content of COX IV (relative to total α-tubulin content) was significantly higher only in LOW after the 3 wk training intervention (p<0.05, Figure 6D).

Mitochondrial DNA

There was no effect of either training program on skeletal muscle mtDNA content after the 3 wk training interventions (data not shown).

pAMPK Thr172 and PGC1α

Phosphorylation of AMPK at threonine-172 measured in skeletal muscle biopsy samples was unchanged after 1 hr of submaximal cycling both before and after training (Figure 7A). Total protein content of PGC-1α (relative to total α-tubulin) content was also unchanged after the 3 wk training intervention (Figure 7B).

Endurance performance

Figure 8 displays the average power output (corrected for BM) maintained during the 60-min performance trial before and after the 3 wk training intervention, along with individual changes for each subject. After the 3 wk training intervention, performance during the 60-min time trial was significantly higher for both groups (12.2 ± 2.3 % vs. 10.2 ± 3.1 % for LOW and HIGH respectively, p<0.01; Figure 8). The magnitude of increase in performance was not different between groups.

DISCUSSION

The novel findings of the present study were that in skeletal muscle of trained individuals 1) resting glycogen content, 2) the maximal activities of citrate synthase and β-
hydroxyacyl-CoA-dehydrogenase, 3) the content of the electron transport chain component cytochrome C oxidase subunit IV, and 4) rates of whole-body fat oxidation during submaximal exercise were enhanced to a greater extent by training twice every second day compared to training daily for 3 weeks. These findings are in direct contrast to our original research hypothesis, namely that well-trained athletes would have maximized their training adaptation, and that further gains would be minimal, independent of whether they trained daily or twice every second day. Nevertheless, despite metabolic and enzymatic changes resulting in an enhanced training adaptation profile after twice every second day ‘low glycogen’ training, we were unable to detect a clear advantage to endurance performance compared to when subjects undertook daily workouts with normal glycogen stores.

The present investigation was undertaken to further examine the hypothesis that commencing exercise sessions with low muscle glycogen levels would result in a more pronounced training adaptation compared to training in a normal glycogen condition (15, 18). Accordingly, subjects performed either two exercise bouts on the same day (100 min of submaximal cycling followed by 1-2 hr rest and then an intense interval training session) or the same training sessions separated by 24 hr. The former training protocol (training twice every second day) resulted in a marked decrease in muscle glycogen concentration after the first exercise session, such that subjects commenced the second bout of training with significantly lower muscle glycogen content than before the first session of the day (Figure 2). In contrast, when subjects performed the prolonged cycling bout and had 24 hr recovery, they began the intense interval training bouts with normal glycogen stores (Figure 2). While the term ‘train low, compete high’ has been used to describe the twice every second day protocol (18), a fact often overlooked is that subjects in that study (and the present investigation) only performed 50% of their training sessions with low muscle glycogen content.

In the present study we chose to incorporate high intensity interval training sessions (HIT) in combination with prolonged, steady-state aerobic riding as competitive cyclists
typically employ such workouts in their race preparation (for review see 24). We have consistently demonstrated substantial performance enhancements after just 3 weeks of HIT in already well trained athletes (31, 44, 47, 48). Furthermore, we have shown that when subjects commence HIT sessions with normal glycogen levels (~450-500 mmol·kg⁻¹ d.w.), they deplete ~50% of resting stores (45). Hence, we were not surprised to observe that when subjects performed self-selected HIT sessions at maximal effort following 100 min of cycling, at a time when glycogen stores were already 50% reduced, relative power output (the percentage of PPO sustained for the 8 x 5 min work bouts) was significantly lower compared to when the same sessions were undertaken in the glycogen replete state (Figure 3). Of note was that while self-selected maximal power output was significantly lower for the first 6 HIT sessions (i.e., the first two weeks of the training program), during the last week of training there were no differences in average power output whether or not subjects commenced the workouts with low or normal glycogen stores (Figure 3). We are confident that subjects produced maximal efforts for all HIT sessions. Indeed, in order to ensure compliance, attractive financial incentives were provided to the subject from each training group who performed the most overall work (when work was corrected for PPO and BM).

Resting muscle glycogen stores are typically increased by 20-30% when untrained subjects complete short term (i.e. < 6 weeks) endurance training programs (for review see 19). Resting muscle glycogen levels in endurance-trained subjects are consistently elevated (i.e. 450-550 mmol·kg⁻¹) above untrained individuals (23). Hence, a surprising finding in the present study was that training twice every second day resulted in a further increase in muscle glycogen stores (Figure 4). Our results are in agreement with those of Hansen et al. (18) who only found elevated resting muscle glycogen content after their ‘train-low’ intervention.

In order to determine whether the training adaptation could be amplified by training twice every second day, we measured indirect markers of tricarboxylic acid cycle flux
(citrate synthase), β oxidation (β-HAD) and electron transport chain activity (cytochrome c oxidase subunits). The increase in the activity/content of these mitochondrial enzymes only reached statistical significance in the group that trained twice every second day (Figure 6). It may seem surprising that 3 weeks of training is sufficient to elevate maximal enzyme activities in already trained subjects. However, we have previously observed increases in β-HAD activity after just 5 days of intense training incorporating two sessions of HIT in well-trained athletes ingesting a low-carbohydrate, high-fat diet (10). The potential mechanism for the increased mitochondrial enzyme activity after training twice every second day is hard to define, and it is possible that exercising with low glycogen stores could promote training adaptations through perturbation in homeostasis (i.e. increased systemic factors) and not directly through low glycogen availability per se (3). Although we did not measure catecholamine levels during training in the present study, Hansen et al. (18) have reported that the catecholamine response to exercise performed with low muscle glycogen levels are higher than when exercise undertaken with normal glycogen stores, demonstrating a higher stress response.

In contrast to the increases in mitochondrial enzyme activities induced by the twice every second day training regimen, mitochondrial DNA content and PGC-1α protein content (Figure 7B) were unchanged in response to both training interventions. Mitochondrial DNA is a marker of mitochondrial volume and is increased in trained compared to untrained individuals (39). Furthermore, mtDNA typically parallels increases in mitochondrial density, although not necessarily by the same magnitude as mitochondrial volume (39). Consequently the ~20% increases in maximal enzyme activity observed after training twice every second day could coincide with a small increase in mitochondrial volume that is not detectable through mtDNA analysis. PGC-1α plays a role in regulating the expression of genes encoding mitochondrial proteins in skeletal muscle (27). Importantly, PGC-1α can also co-activate the transcription factor nuclear respiratory factor-1 to regulate a nuclear encoded protein (mitochondrial transcription factor A or Tfarm) that
controls mtDNA replication and transcription (29). Although no previous study has examined the effect of twice every second day training on PGC-1α protein content, Mortensen et al. (34) have previously reported that this training regimen does not increase the mRNA expression of the PGC-1α family of transcriptional coactivators. While an acute bout of high intensity training increases PGC-1α content to a greater extent than low intensity exercise (46), and chronic short duration interval training elevates PGC-1α to the same extent as more traditional endurance workouts (6), it is unlikely that the changes in power output observed between the twice every second day and once-a-day interval training sessions were large enough to increase PGC-1α protein levels. In addition, since we measured PGC-1α content, but not transcriptional co-activity, we cannot speculate as to whether or not completion of high intensity training in the LOW group transiently increased PGC-1α activity, thereby contributing to the changes in mitochondrial enzymes we measured.

Analogous to PGC-1α, the AMPK has been shown to have important regulatory roles in both the responses to an acute bout of exercise and also chronic training adaptations (for review see 2). The AMPK is an important sensor of decreased energy charge in cells and subsequently acts to increase catabolic reactions and decrease anabolic reactions, one of which is the direct phosphorylation and subsequent increased transcriptional co-activity of PGC-1α (28). In this regard, Wojtaszewski et al. (49) have previously reported that AMPK activity in resting human muscle and the degree of activation during an acute exercise bout are dependent on the fuel status of the muscle cells (i.e., AMPK activity is elevated in muscle with low glycogen stores). In the present investigation AMPK phosphorylation and total AMPK protein content was similar before and after both training interventions. Thus, our results indicate that our 3 week training program (in which subjects performed 50% of their training sessions with low starting muscle glycogen content) was insufficient to increase AMPK protein levels and/or activation in already well-trained individuals. This finding is in agreement with Clark et al.
who previously reported that 3 weeks of intensified training in well-trained athletes does not alter AMPK signaling in skeletal muscle in response to a submaximal exercise bout.

In order to assess the effect of the different training protocols on the metabolic responses to submaximal exercise, subjects performed 60 min of steady-state cycling pre- and post intervention. In accord with the elevated \( \beta \)-HAD activity after the twice every second day training program, we observed a robust increase in rates of whole-body fat oxidation compared to once-a-day training (Figure 5). Yet, despite creating metabolic conditions that should, in theory, enhance endurance capacity, training twice every second day failed to increase the performance of a 1 hour time-trial (a performance measure similar to road cycling time-trials conducted at major championships and Tours) undertaken after 1 hr of submaximal cycling, to a greater extent than one-a-day training. We (8, 9, 11) and others (19, 25) have repeatedly demonstrated the ability of well-trained subjects to further improve their ability to oxidize fat and ‘spare’ carbohydrate after short-term (< 7 days) dietary/training periodization. Part of the reason why nutrient/training interventions that enhance fat-oxidative capabilities do not confer concomitant performance benefits is that the observed ‘carbohydrate sparing’ is more likely to be an impairment of carbohydrate oxidation due to a down-regulation of the multi-enzyme complex pyruvate dehydrogenase (PDH) (43). Unfortunately, in the present study we did not have sufficient muscle tissue to assess PDH activity, but such a measure would provide useful mechanistic insight into training response/adaptation in future studies that manipulate the nutrient/training environment.

Our finding of no difference in a whole-body endurance performance task contrasts that of Hansen et al. (18) who reported that training twice every second day resulted in superior endurance capacity compared to training daily. Several major differences in the training protocols between the current study and that of Hansen et al. (18) are likely to be responsible for the contrasting effects on performance. First we chose whole-body exercise
(cycling) versus a one-legged kicking model as our training mode. Second, we incorporated intense interval training sessions into our 3 week training programs. Third, we allowed subjects to self-select the highest sustainable power output during the HIT sessions, whereas Hansen et al. (18) ‘clamped’ the training intensity. Fourth, we utilized athletes who had completed a base of aerobic training before entering the study, compared to previously healthy, but untrained subjects chosen by Hansen et al. (18). Notwithstanding such differences, one might offer an alternative perspective on the results from our investigation. Namely, despite compromised high-intensity training capacity, the twice every second day training regimen elicited a comparable increase in endurance performance to that attained after training every day. Thus for an athlete unable to train daily but who can perform two workouts in close proximity, with the second session performed under conditions of low starting muscle glycogen, ‘train-low’ may offer a time efficient method of maintaining training adaptations and performance.

In conclusion, compared to training daily, training twice every second day compromised high-intensity training capacity but augmented selected markers of training adaptation (i.e., resting muscle glycogen content, the maximal activities of several mitochondrial enzymes and the protein content of COX IV). However, despite creating conditions that, in theory, should enhance endurance performance capacity, performance of a one hour time-trial undertaken after a 60 min steady-state ride was similar after daily or twice every second day training. Further studies will be needed to determine whether low muscle glycogen stores per se or perturbation in systemic or other intramuscular factors are responsible for the amplified training response observed after twice every second day versus daily training schedules.
Acknowledgements

This study was supported by a research grant from Glaxo SmithKline (U.K.) to JAH, the Australian Sports Commission (JAH) and the National Sports Council of Malaysia (WKY). ALC is supported by a Peter Doherty post doctoral fellowship from the National Health and Medical Research Council. The authors would like to thank Professor Bruce Kemp for his kind gift of the AMPK antibody. The authors would also like to acknowledge the commitment of the subjects in completing a difficult and strenuous study.
References


Table 1. Characteristics of the subjects that participated in the 3-week chronic training study and the acute study. PPO, Peak sustained power output. Values are means ±SE.

<table>
<thead>
<tr>
<th>Subject characteristics</th>
<th>Age (yr)</th>
<th>Body Mass (kg)</th>
<th>VO\textsubscript{2peak} (L·min\textsuperscript{-1})</th>
<th>PPO (W)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Chronic study (n=14)</td>
<td>30.0 ± 1.8</td>
<td>77.7 ± 2.7</td>
<td>4.7 ± 0.1</td>
<td>382.1 ± 9.3</td>
</tr>
<tr>
<td>Acute study (n=12)</td>
<td>28.1 ± 2.0</td>
<td>77.4 ± 3.3</td>
<td>4.7 ± 0.2</td>
<td>365.5 ± 7.9</td>
</tr>
</tbody>
</table>
FIGURE LEGENDS

Figure 1. Overview of the study design and experimental trial. Q, AT, Aerobic training. ●, HIT, High intensity interval training. R, Rest. Ex. Trial, experimental trial.

Figure 2. Muscle glycogen concentration before aerobic training (AT) and before high intensity interval training (HIT) during the acute training study. * Significantly different from Pre-AT (p<0.05).

Figure 3. Training intensity expressed as percentage of peak power output (PPO) during HIT sessions. * Significantly different between HIGH and LOW (p<0.05).

Figure 4. Resting muscle glycogen concentration before and after the 3-week training intervention. * Significantly different from Pre-training (p<0.05).

Figure 5. Rates of whole-body fat oxidation at 10, 30 and 50 min before (Pre) and after (Post) the 3-week training intervention. * Significantly different from Pre-training (p<0.05).

Figure 6. (A) Maximal citrate synthase activity before (Pre) and after (Post) the 3-week training intervention. (B) Maximal β-hydroxyacyl-CoA-dehydrogenase activity before and after 3-week training intervention. (C) Total protein content of cytochrome c oxidase subunit II before and after 3-week training program. (D) Total protein content of cytochrome c oxidase subunit IV before and after the 3-week training program. * Significantly different compared to LOW-Pre-training (p<0.05). # Significantly different compared to HIGH-Post-training (p<0.05).
**Figure 7.** Representative western blots for (A) phosphorylation of AMPK at threonine-172 and total AMPK at rest and after exercise, before (Pre) and after (Post) 3-week training intervention, and (B) Resting total peroxisome proliferator-activated receptor gamma coactivator 1α and α-tubulin protein content before (Pre) and after (Post) 3-week training intervention. Exerc, Exercise. Control, standardised protein sample loaded to account for variation between membranes.

**Figure 8.** Cycling performance as measured by the average power output (W) maintained during the 60 min time trial before (Pre) and after (Post) 3-week training intervention. † Significantly different from Pre (p<0.01).
Figure 1

- Monday: 100 min steady-state ride at 70% VO2peak (AT)
- Tuesday: 8 x 5 min at maximal pace (HIT)

- Repeat for 2 weeks

3-week training

Ex. trial

Muscle Biopsy

Muscle Biopsy

Performance trial

Gas Collection (for 5 minutes)

- 10
- 30
- 50

24 hr dietary control

- Monday
- Tuesday
- Wednesday
- Thursday
- Friday
- Saturday
- Sunday

High
Low

Rest

Repeat for 2 weeks
Figure 2

Muscle glycogen (µmol·g⁻¹ d.w.)

- Pre-AT
- Pre-HIT

HIGH

LOW

*
Figure 3

The figure illustrates a graph showing the percentage of PPO (% PPO) across different HITs (HIT1 to HIT9). The graph compares two conditions labeled HIGH and LOW, indicated by different symbols: filled squares for HIGH and open squares for LOW. The p-value of 0.06 is marked with an asterisk (*) to indicate statistical significance. The trend lines suggest an increasing % PPO with higher HIT numbers.
Figure 4

Muscle glycogen (μmol·g⁻¹ d.w.)

<table>
<thead>
<tr>
<th></th>
<th>Pre</th>
<th>Post</th>
</tr>
</thead>
<tbody>
<tr>
<td>HIGH</td>
<td></td>
<td></td>
</tr>
<tr>
<td>LOW</td>
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</table>

*Significant difference.
Figure 5

Fat-oxidation (μmol·kg⁻¹·min⁻¹)

<table>
<thead>
<tr>
<th>Time</th>
<th>Pre</th>
<th>Post</th>
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<tbody>
<tr>
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<td></td>
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<tr>
<td>50-min</td>
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</tbody>
</table>

HIGH

LOW

p = 0.05

*
Figure 6

A

Citrate synthase activity (mmol·kg⁻¹·min⁻¹ d.w.)

B

β-HAD activity (mmol·kg⁻¹·min⁻¹ d.w.)

C

COX II

α-tubulin

D

COX IV

α-tubulin
Figure 7

A

<table>
<thead>
<tr>
<th></th>
<th>Pre</th>
<th>Post</th>
<th>Pre</th>
<th>Post</th>
<th>Control</th>
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<tbody>
<tr>
<td>Rest</td>
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<td></td>
<td></td>
<td></td>
<td></td>
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<tr>
<td>Exerc</td>
<td></td>
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pAMPK T172

Total AMPK

B

<table>
<thead>
<tr>
<th></th>
<th>Pre</th>
<th>Post</th>
<th>Control</th>
</tr>
</thead>
<tbody>
<tr>
<td>HIGH</td>
<td></td>
<td></td>
<td></td>
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<tr>
<td>LOW</td>
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</tr>
</tbody>
</table>

PGC-1α

α-tubulin

100 kDa

75 kDa

50 kDa
Figure 8

Δ = 10.2 ± 3.1 %

Δ = 12.2 ± 2.3 %