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RESEARCH PAPER

Delaying post-exercise carbohydrate intake impairs next-day exercise capacity but not muscle glycogen or molecular responses

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Abstract

Aim: To investigate how delayed post-exercise carbohydrate intake affects muscle glycogen, metabolic- and mitochondrial-related molecular responses, and subsequent high-intensity interval exercise (HIIE) capacity.

Methods: In a double-blind cross-over design, nine recreationally active men performed HIIE (10 × 2-min cycling, ~94% $\dot{W}_{peak}$) in the fed state, on two occasions. During 0–3 h post-HIIE, participants drank either carbohydrates ("Immediate Carbohydrate" [IC], providing 2.4 g/kg) or water ("Delayed Carbohydrate" [DC]); total carbohydrate intake over 24 h post-HIIE was matched (~7 g/kg/d). Skeletal muscle (sampled pre-HIIE, post-HIIE, +3 h, +8 h, +24 h) was analyzed for whole-muscle glycogen and mRNA content, plus signaling proteins in cytoplasmic- and nuclear-enriched fractions. After 24 h, participants repeated the HIIE protocol until failure, to test subsequent HIIE capacity; blood lactate, heart rate, and ratings of perceived effort (RPE) were measured throughout.

Results: Muscle glycogen concentrations, and relative changes, were similar between conditions throughout ($p > 0.05$). Muscle glycogen was reduced from baseline (mean ± SD mmol/kg dm; IC: 409 ± 166; DC: 352 ± 76) at post-HIIE (IC: 253 ± 96; DC: 214 ± 82), +3 h (IC: 276 ± 62; DC: 269 ± 116) and +8 h (IC: 321 ± 56; DC: 269 ± 116), returning to near-baseline by +24 h. Several genes (*PGC-1a*, *p53*) and proteins (p-ACC^{Ser79}, p-P38 MAPK$^{Thr180/Tyr182}$) elicited typical exercise-induced changes irrespective of condition. Delaying carbohydrate intake reduced next-day HIIE capacity (5 ± 3 intervals) and increased RPE (~2 ratings), despite similar physiological responses between conditions.

Conclusion: Molecular responses to HIIE (performed in the fed state) were not enhanced by delayed post-exercise carbohydrate intake. Our findings support immediate post-exercise refueling if the goal is to maximize next-day HIIE capacity and recovery time is ≤24 h.

Javier Díaz-Lara and Matthew J. Lee contributed equally to this work.

KEYWORDS
carbohydrates, exercise, mRNA, muscle glycogen, nutrition, signaling

1 | INTRODUCTION

In recent decades, manipulating nutrient provision—particularly carbohydrates—with the goal of enhancing the post-exercise molecular responses that underpin skeletal muscle training adaptations, has gained significant interest.[1] Experiments in cell culture and rodent skeletal muscle have shown that nutrient deprivation causes transcription factors to translocate from the cytoplasm into the nucleus to regulate mitochondrial biogenesis,[2–4] a hallmark adaptation to cardiorespiratory exercise.[5] In contrast, subsequent nutrient provision in these models causes these transcription factors to translocate out of the nucleus, reducing the transcriptional activity of their target genes.[3,4] These experiments may be considered analogous to human exercise studies in which restricting carbohydrate intake during recovery augmented exercise-induced increases in the mRNA content of metabolic- and mitochondrial-related genes, compared with a high-carbohydrate diet.[6,7] However, studies involving prolonged low-carbohydrate intakes during recovery have also demonstrated reduced muscle glycogen resynthesis,[6] which could negatively affect performance in subsequent training sessions (e.g., through a reduction in self-selected power output[8] and exercise capacity[9]).

One post-exercise nutrition strategy that has been seldom researched involves a brief, but deliberate, delay in post-exercise carbohydrate intake. This approach might offer the "best of both worlds"; restricting exogenous carbohydrate availability for a few hours post-exercise may provide an opportunity to enhance adaptive molecular responses, while subsequent carbohydrate intake could promote muscle glycogen resynthesis to support recovery for future exercise sessions. Indeed, it seems the potential negative effects of low carbohydrate intake on muscle glycogen resynthesis[6] can be negated if enough carbohydrate is consumed later in the recovery period (i.e., from 8 to 24 h after exercise[10]). However, while some studies that investigated acute carbohydrate restriction (3–4 h post-exercise) did not report greater exercise-induced content of various metabolic and mitochondrial genes during recovery,[11–13] none assessed mRNA content between 4 and 12 h post-exercise—a time period in which we have shown post-exercise changes in several metabolic and mitochondrial-related genes to peak,[14] and the most significant effects of carbohydrate restriction on mRNA content were reported by Pilegaard et al.[6]

We, and others, have also demonstrated that exercise increases the phosphorylation and accumulation of signaling kinases in nuclear-enriched fractions (e.g., AMPK[15,16] and p38 MAPK[17]), where they may directly interact with transcription factors and coactivators (reviewed recently in[18]) to increase their stability and/or transcriptional activity. However, the effects of manipulating carbohydrate intake around exercise on the subcellular localization of these proteins—particularly AMPK—have yielded contrasting findings.[15,19,20] Furthermore, despite the suggested effects of nutrient availability on transcription factor location and activity in cell culture and rodent models,[2–4] acute "recover low" studies have primarily used whole-muscle lysate, with either limited[11] or no analyses[13] of subsequent protein abundance or the phosphorylation of key signaling kinases and transcription factors in different cellular subfractions. Thus, whether acute post-exercise carbohydrate restriction alters the exercise-induced activity and cellular localization of key molecular markers of mitochondrial biogenesis in human skeletal muscle requires further investigation.

Much of the literature on this topic has involved prolonged, moderate-intensity aerobic exercise alone[6,7,11,21] or in combination with repeated maximal sprints[13]; however, both recreational exercisers and athletic populations also incorporate high-intensity interval exercise (HIIE) into their workout routines. For the former group, HIIE offers a time-efficient training method to achieve many of the benefits of traditional aerobic exercise.[22] Meanwhile, integrating HIIE into the periodized training programs of athletes can improve performance via enhanced adaptations of cardiopulmonary, metabolic, and skeletal muscle characteristics.[22] Accordingly, the aim of this study was to explore how a short (3-h) delay in carbohydrate intake after HIIE affects adaptive responses (i.e., protein and mRNA content associated with mitochondrial biogenesis) and recovery (i.e., muscle glycogen restoration and subsequent HIIE capacity). Based on literature involving prolonged, submaximal exercise, we hypothesized that compared with immediate post-exercise carbohydrate consumption, delaying carbohydrate intake would augment protein signaling (e.g., AMPKα, ACC, p38MAPK signaling) and mRNA content (e.g., *PGC-1α*, *PDK4*, *UCP3*, *CD36*, *CPT1*) associated with metabolism and mitochondrial biogenesis, during 3–8 h post-exercise. However, we hypothesized that delaying carbohydrate intake would have limited influence on muscle glycogen concentrations and subsequent exercise capacity.

2 | RESULTS

2.1 | Muscle glycogen concentration

There were no main effects of condition ($p = 0.179$) or condition × time interaction ($p = 0.987$) for absolute muscle glycogen concentrations; there was a main effect of time ($p < 0.001$, Figure 1A). Compared to pre-HIIE, muscle glycogen was reduced immediately post-HIIE ($p < 0.001$; immediate carbohydrate [IC]: −36%, delayed carbohydrate [DC]: −36%), and at +3 h ($p < 0.001$; IC: −27%, DC: −22%) and +8 h ($p = 0.026$; IC: −11%, DC: −14%). By +24 h, muscle glycogen concentrations were not different from pre-HIIE ($p = 0.160$; IC: −3%, DC: −6%). There were differences between post-HIIE and +3 h ($p = 0.026$), +8 h ($p < 0.001$), and +24 h ($p < 0.001$), and between +3 h and +24 h ($p < 0.014$). The relative changes in muscle glycogen over the 24-h period were also similar between conditions ($p > 0.05$), as were resynthesis rates estimated between biopsy timepoints (0–3 h post-HIIE: IC = 8 ± 15 mmol/kg dm/h, DC = 19 ± 18 mmol/kg dm/h; +3–8 h post-HIIE: IC = 9 ± 12 mmol/kg dm/h, DC = 3 ± 16 mmol/kg dm/h; +8–24 h post-HIIE: IC = 2 ± 3 mmol/kg dm/h, DC = 1 ± 5 mmol/kg dm/h).

2.2 | Plasma glucose and lactate

There was a main effect of time for both plasma glucose ($p < 0.001$, Figure 1B) and lactate ($p < 0.001$, Figure 1C) measured during the 24-h recovery period. Plasma glucose increased significantly immediately post-HIIE and was greater than all other timepoints ($p \leq 0.006$), before returning to pre-HIIE levels at 3 h. Plasma lactate also increased significantly immediately post-HIIE ($p < 0.001$ vs. all timepoints). There was also a significant difference between plasma lactate at +8 h and pre-HIIE ($p < 0.001$), +3 h ($p = 0.044$) and +24 h ($p < 0.001$), and between +3 h and + 24 h ($p = 0.014$). However, there were no main effects of condition or condition × time interactions for either variable ($p > 0.05$).

2.3 | Protein abundance and phosphorylation status

Fold-changes within each condition are displayed in Figure 2 and are available in the Supplementary Data files. The relative abundance of phosphorylated proteins was normalized to their respective total target protein content.

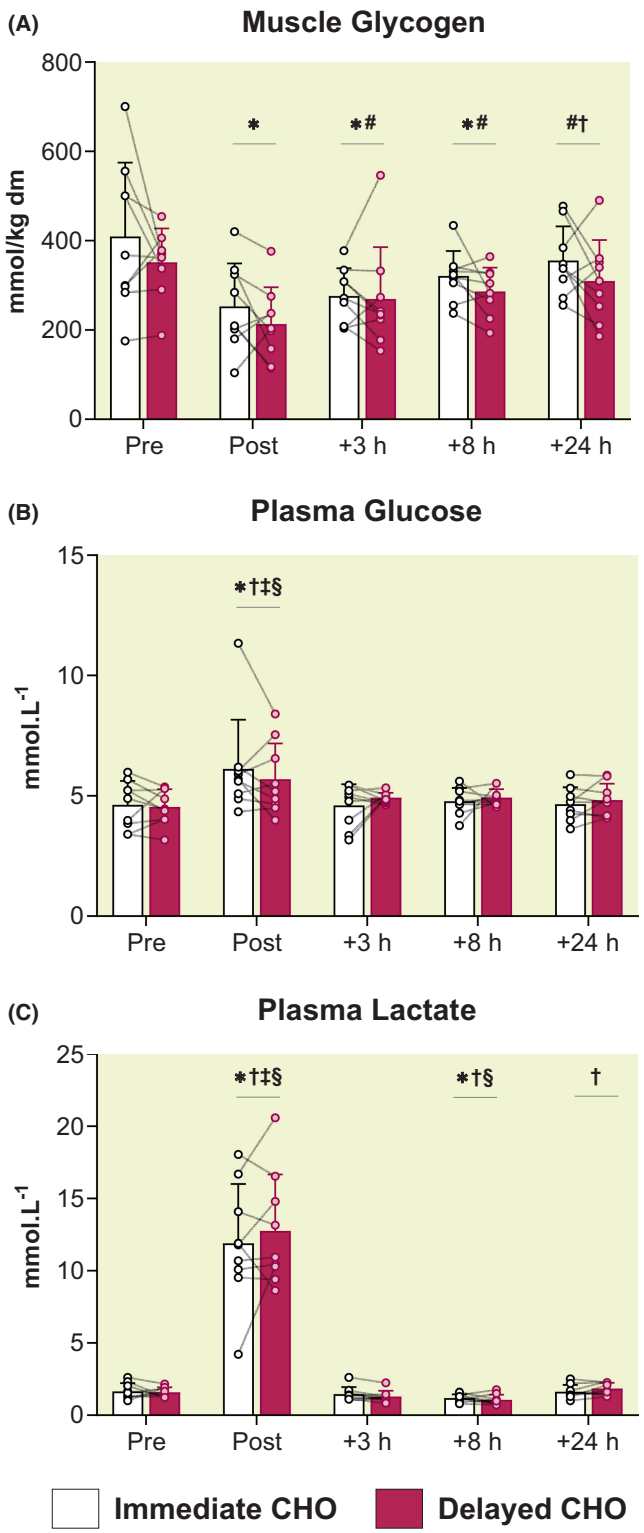

FIGURE 1 Absolute skeletal muscle glycogen (A), plasma glucose (B), and plasma lactate (C) concentrations over the 24-h measurement period. Data are mean ± standard deviation, plus individual data points ($n = 9$, all timepoints). HIIE, high-intensity interval exercise. $p < 0.05$: *versus pre; #versus post; †versus +3 h; ‡versus +8 h; §versus +24 h. Lines indicate the same participant between conditions.

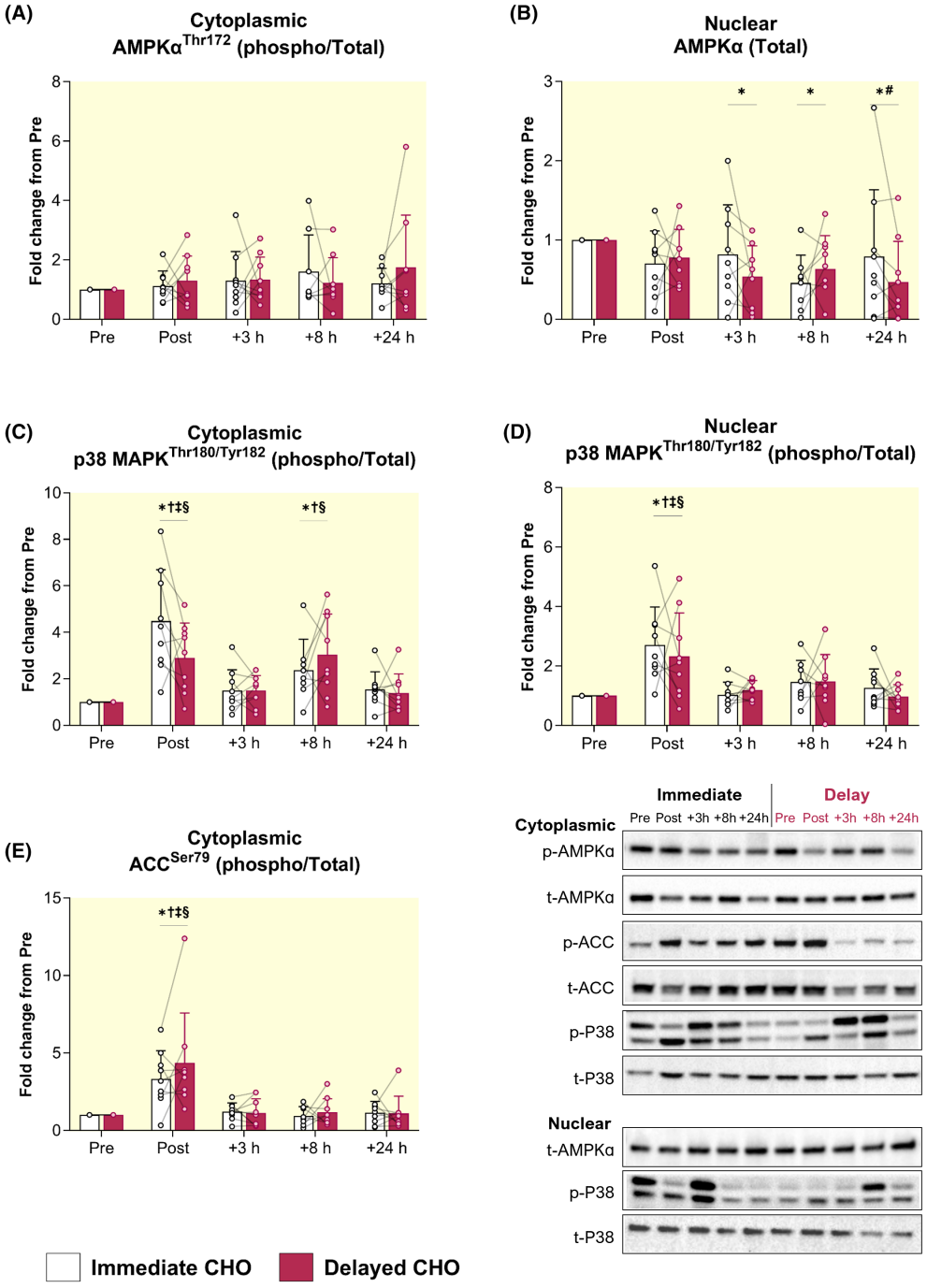

FIGURE 2 Changes in protein content over the 24-h measurement period for (A) cytoplasmic p-AMPKα^{Thr172}, (B) total nuclear AMPKα, (C) cytoplasmic p-P38 MAPK$^{Thr180/Tyr182}$, (D) nuclear p-P38 MAPK$^{Thr180/Tyr182}$, and (E) cytoplasmic acetyl-CoA carboxylase (p-ACC^{Ser79}). Data are mean ± standard deviation, plus individual data points, and representative western blot images. All targets are $n = 9$ per group per timepoint, except in the Immediate Carbohydrate trial at +8 h, which is $n = 8$ (due to insufficient muscle lysate). We could not adequately detect p-AMPKα in our nuclear fractions. Thus, excluding nuclear AMPKα, all other data were derived by calculating the ratio of phosphorylated to corresponding total target protein (in arbitrary units), then each time point was divided by its resting value to provide a fold-change from pre-HIIE (high-intensity interval exercise). $p < 0.05$: *versus pre; #versus post; †versus +3 h; ‡versus +8 h; §versus +24 h. Lines indicate same participant between conditions.

2.3.1 | Cytoplasmic-enriched fraction

There were main effects of time ($p < 0.001$) for both cytoplasmic p-P38 MAPK$^{Thr180/Tyr182}$ abundance and p-ACC^{Ser79} abundance (Figure 2C,E). Cytoplasmic p-ACC^{Ser79} abundance (relative to total ACC) was higher immediately post-HIIE than all other time-points ($p < 0.001$). Likewise, cytoplasmic p-P38

MAPK$^{Thr180/Tyr182}$ abundance (relative to total P38 MAPK) was higher immediately post-HIIE ($p \leq 0.001$) and +8 h post-HIIE ($p \leq 0.001$) than pre-HIIE, and with all other timepoints ($p \leq 0.048$). There was no effect of time for p-AMPKα^{Thr172} abundance ($p = 0.890$; Figure 2A). There were no main effects of condition, nor condition × time interactions, for any variable ($p > 0.05$).

2.3.2 | Nuclear-enriched fraction

We were unable to detect p-AMPKα^{Thr172} in our nuclear fractions; however, there was a main effect of time ($p = 0.008$) for the nuclear abundance of total AMPKα, which was lower than pre-HIIE at +3 h ($p = 0.050$), +8 h ($p = 0.026$), and +24 h ($p = 0.005$; Figure 2B). There was also a difference between post and +24 h ($p = 0.050$). There was a main effect of time ($p < 0.001$) for nuclear p-P38 MAPK$^{Thr180/Tyr182}$ abundance; immediately post-HIIE, p-P38 MAPK$^{Thr180/Tyr182}$ abundance (relative to total P38 MAPK) was higher than all other timepoints ($p \leq 0.001$; Figure 2D). There were no main effects of condition, nor condition × time interactions, for any variable ($p > 0.05$).

2.4 | Whole-muscle mRNA content

Fold-changes within each condition are displayed in Figures 3 and 4 and are available in the supplementary data files. There were several genes that did not respond to either the exercise or nutrition intervention, including *β-HAD* (Figure 4D), *CHCHD4*, *COXIV*, *citrate synthase*, *cytochrome C*, *PFK*, *SDH*, *TFam*, *TFEB*, and *GLUT4* (data not shown but are available via the link in the appendices).

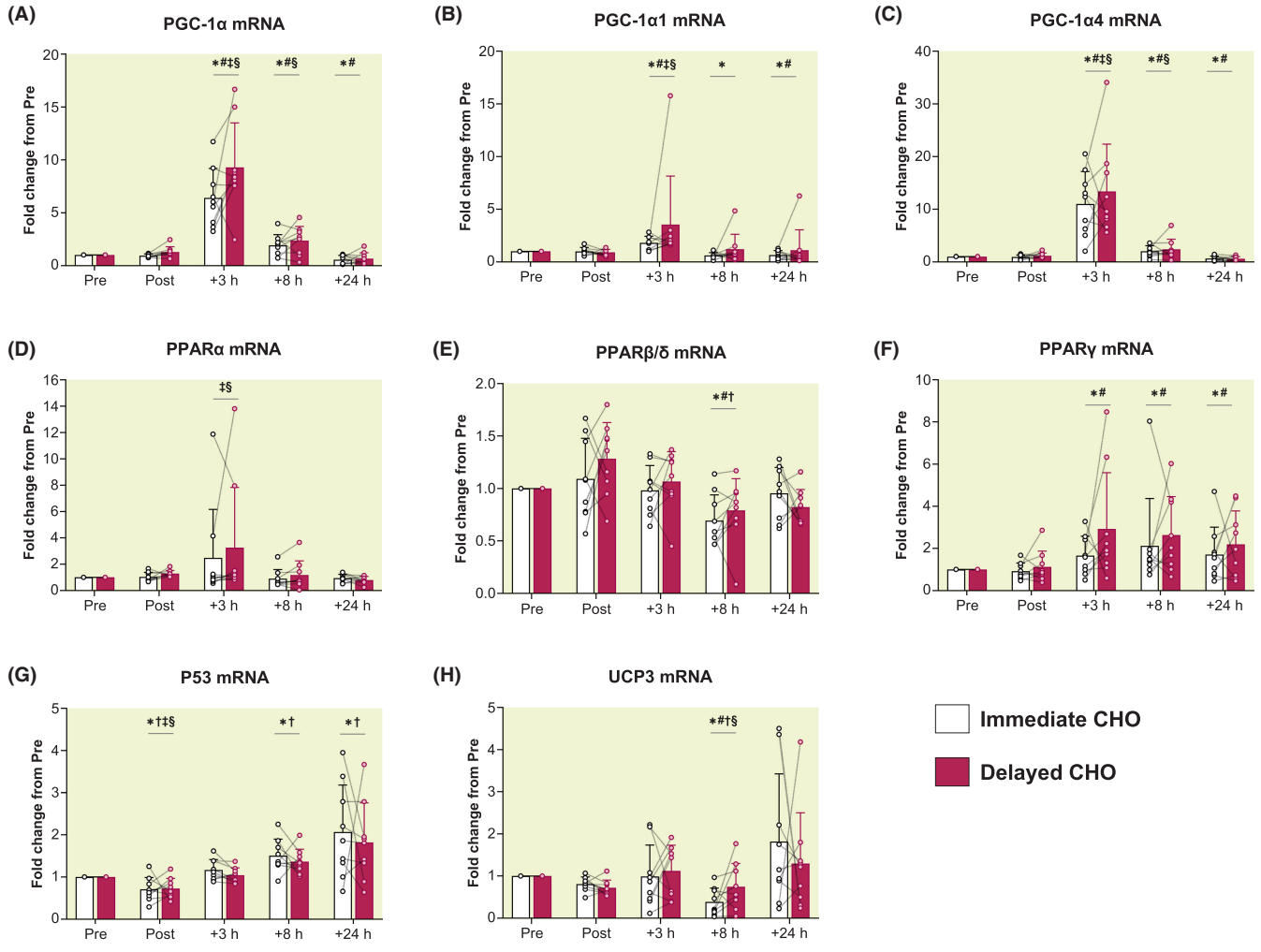

FIGURE 3 Changes in whole-muscle mRNA content over the 24-h measurement period, for (A) *PGC-1a*, (B) *PGC-1a1*, (C) *PGC-1a4*, (D) *PPARa*, (E) *PPARβ*, (F) *PPARγ*, (G) *p53*, and (H) *UCP3 mRNA*. Data are mean ± standard deviation, plus individual data points ($n = 9$ all timepoints). For each participant, data were derived by dividing the arbitrary unit of mRNA content at each time point by its resting value, to provide a fold-change from pre-HIIE (high-intensity interval exercise). $p < 0.05$: *versus pre; #versus post; †versus +3 h; ‡versus +8 h; §versus +24 h. Lines indicate the same participant between conditions.

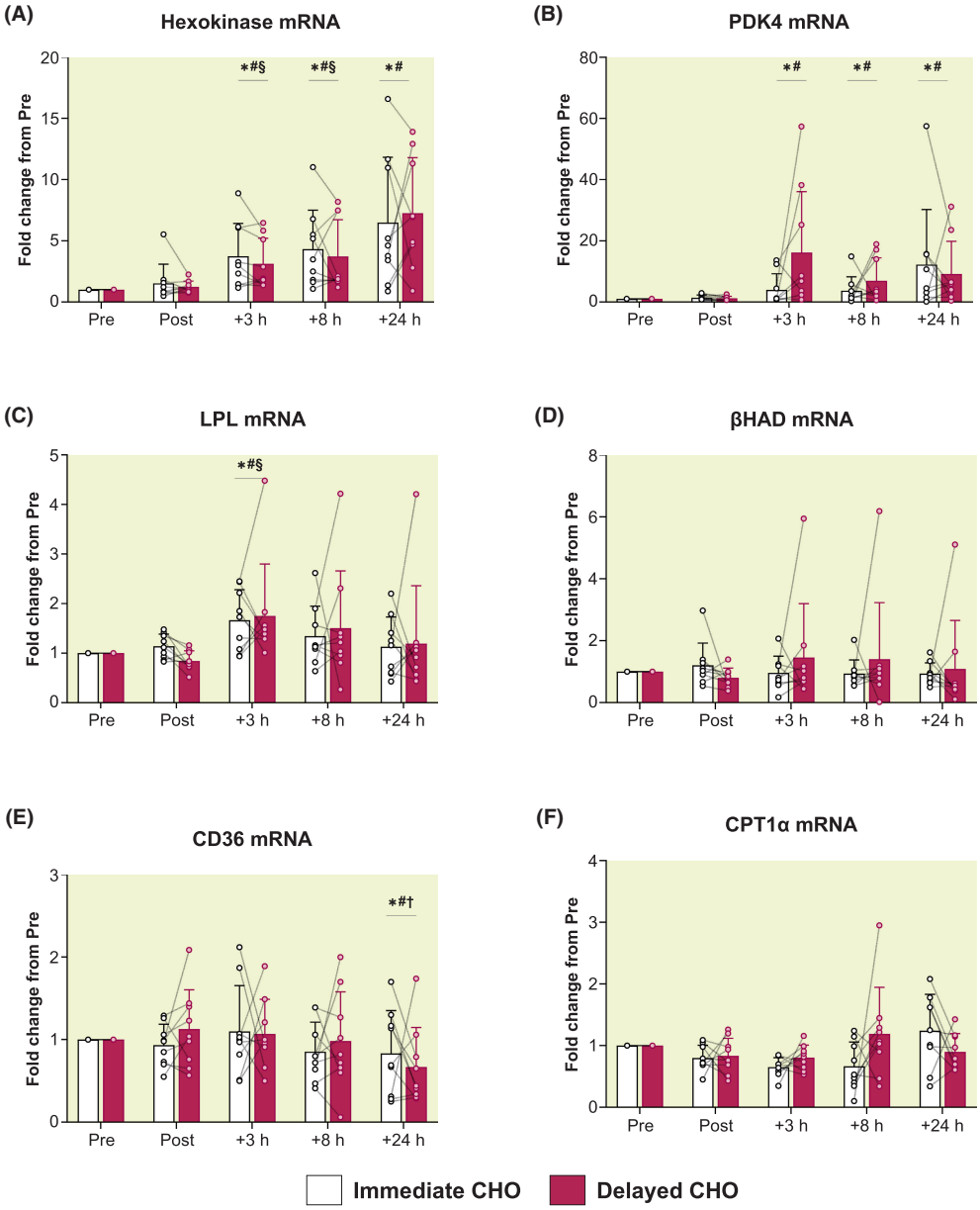

FIGURE 4 Changes in whole-muscle mRNA content over the 24-h measurement period, for (A) *Hexokinase*, (B) *PDK4*, (C) *LPL*, (D) *β-HAD*, (E) *CD36*, and (F) *CPT1α mRNA*. Data are mean ± standard deviation, plus individual data points ($n = 9$ all timepoints). For each participant, data were derived by dividing the arbitrary unit of mRNA content at each time point by its resting value, to provide a fold-change from pre-HIIE (high-intensity interval exercise). $p < 0.05$: *versus pre; #versus post; †versus +3 h; ‡versus +8 h; §versus +24 h. Lines indicate the same participant between conditions.

2.4.1 | Mitochondria-related genes, transcription factors, and coactivators

Main effects for time were evident for the mRNA content of *PGC-1α*, *PGC-1α1*, and *PGC-1α4* (all $p < 0.001$), as well as *PPARα* ($p = 0.007$), *PPARβ* ($p = 0.001$), *PPARγ* ($p < 0.001$), *P53* ($p < 0.001$), and *UCP3* ($p < 0.001$). Immediately post-HIIE, *P53 mRNA* was reduced ($p < 0.001$). At +3 h, the mRNA content of all three *PGC-1α* isoforms (all $p < 0.001$), and *PPARγ* ($p < 0.001$) were elevated from pre-HIIE. At +8 h, *P53* ($p = 0.003$), all *PGC-1α* isoforms ($p \leq 0.039$), and *PPARγ* ($p = 0.002$) remained higher than pre-HIIE, while *PPARβ* ($p = 0.003$) and *UCP3 mRNA* ($p < 0.001$) were lower than pre-HIIE. At +24 h, *P53 mRNA* remained elevated from pre-HIIE ($p < 0.001$), while all three isoforms of *PGC-1α* were lower than pre-HIIE (all $p \leq 0.001$). Other significant comparisons between timepoints are displayed in Figure 3. There were no main effects of condition, nor condition × time interactions, for any variable ($p > 0.05$).

2.4.2 | Metabolic genes

Main effects for time were observed for mRNA content of *HKII* ($p < 0.001$), *PDK4* ($p < 0.001$), *LPL* ($p = 0.003$), *CD36* ($p = 0.010$), and *CPT1a* ($p = 0.048$). At +3 h *mRNA* content of *HKII* ($p < 0.001$), *LPL* ($p = 0.005$) and *PDK4* ($p = 0.001$) were elevated from pre-HIIE. At +8 h, *PDK4 mRNA* ($p = 0.010$) and *HKII* ($p < 0.001$) were elevated from pre-HIIE. At +24 h, *PDK4* ($p < 0.003$) and *HKII mRNA* ($p < 0.001$) remained elevated, while *CD36 mRNA* content was lower than pre-HIIE ($p = 0.013$). Other significant comparisons between timepoints are displayed in Figure 4. Despite a main effect of time, there were no significant pairwise comparisons between timepoints for *CPT1a*. There were no main effects of condition, nor condition × time interactions, for any variable ($p > 0.05$).

2.5 | HIIE to task failure (HIIE-TF): Exercise capacity and physiological measures

Participants performed more intervals to task failure in the IC condition than in the DC condition ($p = 0.001$) (Figure 5A). For ratings of perceived effort (RPE), there were main effects for condition ($p < 0.001$) and time ($p < 0.001$), and a condition×time interaction ($p = 0.011$) (Figure 5B). Throughout both trials, RPE increased from the 1st interval, at each timepoint (all $p < 0.001$); RPE was also ~2 units higher in the DC condition than the IC condition, after both the 5th ($p = 0.004$) and 10th intervals ($p < 0.001$). Heart rate (Figure 5C) and blood lactate (Figure 5D) both elicited main effects for time ($p < 0.001$), increasing similarly throughout the HIIE-TF in both trials; for both variables, there were no main effects for condition (heart rate: $p = 0.298$; lactate: $p = 0.724$) or condition × time interactions (heart rate: $p = 0.379$; lactate: $p = 0.219$).

3 | DISCUSSION

Our HIIE protocol reduced muscle glycogen concentrations and altered the mRNA and protein content of several genes associated with mitochondrial biogenesis and metabolism over the 24-h recovery period. However, delaying post-exercise carbohydrate intake by 3 h did not augment these exercise-induced molecular responses, nor did it alter muscle glycogen resynthesis compared to immediate refueling. Contrary to our hypothesis, delaying post-exercise carbohydrate intake compromised HIIE capacity and increased the perception of effort during an HIIE session performed 24 h later.

Like Cochran et al.[23]—who used a similar HIIE protocol and an identical carbohydrate refueling strategy to ours—muscle glycogen concentrations, and resynthesis rates over 3 h post-HIIE were similar between conditions, despite participants consuming 2.4 g/kg of carbohydrate in the IC feeding trial. Muscle glycogen resynthesis rates were below those previously reported when 1.2 g of carbohydrate/kg/h is consumed post-exercise (i.e., ~20–40 mmol/kg dm/h), which is proposed to be sufficient to maximize resynthesis rates.[24] However, as discussed by Cochran et al.[23] muscle glycogen resynthesis rates early in recovery are inversely related to pre-exercise carbohydrate availability[25] and the magnitude of glycogen depletion[26]; glycogen synthase activity and its affinity to glucose are also inversely related to muscle glycogen content.[26,27] Our participants commenced exercise ~1 h after a high-carbohydrate breakfast, and the glycogen depletion post-HIIE (~36%, in both conditions) is modest compared to other glycogen-depleting protocols or prolonged, exhaustive exercise models, which are often performed in the fasted state (see[24]). These factors may have prevented differences in post-exercise muscle glycogen content and resynthesis between conditions during recovery (and in the previous study by Cochran et al.[23]). In both conditions in the present study, muscle glycogen had returned to near-basal levels by +24 h (Figure 1A). By using an HIIE protocol, our study extends previous work involving prolonged, moderate-intensity exercise[11]—another common training approach. Together, these studies suggest that if cardiorespiratory exercise is conducted in the fed-state, restricting carbohydrate intake for 3–4 h post-exercise does not compromise muscle glycogen resynthesis over extended recovery durations (i.e., 24 h) if sufficient carbohydrate is subsequently consumed.

Exercise-induced changes in cytoplasmic p-ACC^{Ser79} and p-P38 MAPK$^{Thr180/Tyr182}$, and in nuclear p-P38 MAPK$^{Thr180/Tyr182}$ and total AMPKα, were similar between conditions (Figure 2B–E). Other studies that acutely delayed post-exercise carbohydrate intake did not also measure these targets; however, studies investigating carbohydrate availability *during* exercise suggest signaling responses may only be affected by exogenous carbohydrate availability if muscle glycogen utilization differs between conditions.[28–30] Furthermore, recent studies involving exhaustive[9] and non-exhaustive HIIE[15,19] have shown signaling responses are not augmented if post-exercise muscle glycogen concentrations are similar between conditions, regardless of differences in *pre-exercise* muscle glycogen. Thus, the similar muscle glycogen utilization and concentrations between conditions may have contributed to the lack of differences in cell signaling proteins.

Many genes associated with mitochondrial biogenesis (*PGC-1a*, *P53*, *UCP3*, *PPARs α/ß/ɣ*) and metabolism

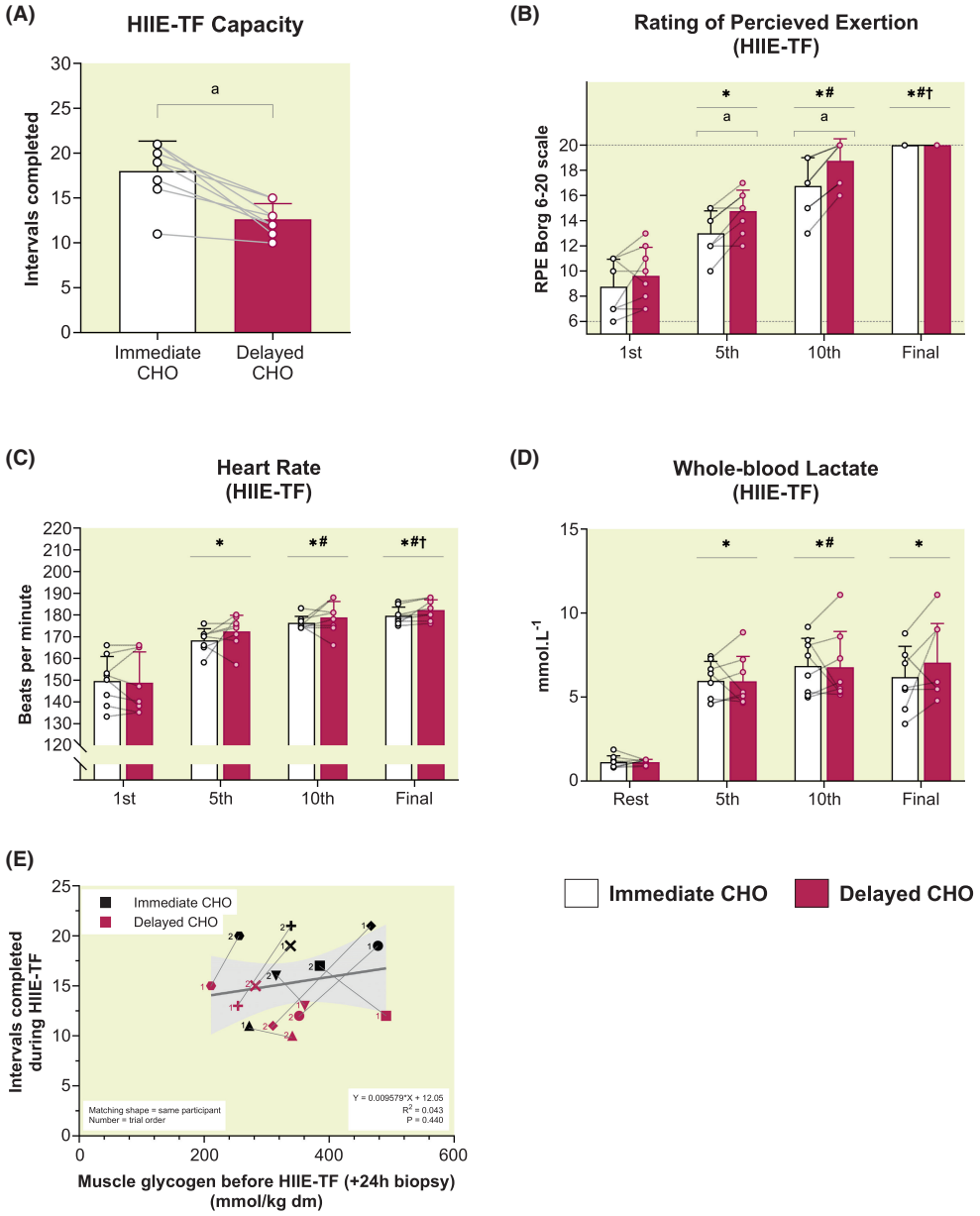

FIGURE 5 Exercise capacity, physiological, and subjective measures taken during the HIIE session to task failure (HIIE-TF). This was performed 24 h after the initial high-intensity interval exercise (HIIE) session. Data are mean ± standard deviation, plus individual data points ($n = 8$). (A) Number of 2-min HIIE intervals completed until task failure, (B) rating of perceived exertion, (C) heart rate during the 1st, 5th, 10th, and final HIIE intervals, (D) whole blood lactate concentrations at rest, and after the 5th, 10th, and final intervals, (E) correlation between muscle glycogen concentrations at +24 h post-HIIE and the number of 2-min HIIE intervals performed to task failure (HIIE-TF). $p < 0.05$: [a] between conditions; *versus "Rest" (or "1st" interval), # versus "5th" interval, †versus "10th" interval. Lines indicate the same participant between conditions.

(*HKII*, *PDK4*, *LPL*, *CD36*, *CPT1a*) also elicited exercise-induced changes that were not significantly different between conditions (Figures 3 and 4). While our data align with other studies that did not observe greater *PGC-1a* mRNA 2 to 5 h after cardiorespiratory exercise when carbohydrate intake was restricted,[6,7,11–13] we acknowledge that individual variability may have prevented the detection of statistically significant effects in *PGC-1a mRNA* (Figure 3A) and other targets (see *PDK4* mRNA at +3 h, Figure 4B; DC: ~16-fold increase vs. IC: ~4-fold increase). Despite our initial sample size estimation (calculated for differences in *PGC-1a* mRNA between conditions), each target gene (and protein) may have necessitated a unique sample size, and the varied individual responses may confound our interpretation of potentially meaningful effects that were not statistically significant.

One notable difference to the earlier studies that reported greater exercise-induced mRNA content after

delayed post-exercise carbohydrate intake is that participants in those studies exercised after an overnight fast.[6,7] In our study, and others that did not observe greater changes in exercise-induced mRNA content,[11] participants were provided a pre-exercise meal. Thus, it is possible the greater, and more sustained duration of nutrient deprivation experienced in the earlier studies (i.e., pre-, during, and post-exercise[6,7]) may have provided a more potent stimulus to enhance mRNA transcription events (like previous findings in cell culture and rodent skeletal muscle[2–4]); however, more research in humans is needed to explore this hypothesis.

Throughout recovery, muscle glycogen was maintained between ~200 and 350 mmol/kg dm in both conditions (Figure 1A). These values correspond with a proposed "glycogen threshold," within which post-exercise molecular responses are hypothesized to be enhanced.[1] As such, this may also have contributed to the similar post-exercise molecular responses between conditions (i.e., restricting exogenous carbohydrate availability does not further enhance molecular responses to exercise, if post-exercise muscle glycogen concentrations already fall within this threshold). This has been observed elsewhere[13,31]; however, more research is needed to explore the notion of a 'glycogen threshold', particularly with acute post-exercise carbohydrate restriction models like the present study, as there are other studies that do not fit this hypothesis.[11] However, the present results do support other evidence that suggests post-exercise muscle glycogen depletion and content may dictate the potential for observing enhanced molecular responses to exercise.[9,15,32] It is also important to consider that exercise-induced changes in mRNA and protein levels may not follow a linear temporal relationship,[33] and that "snapshot" measurements of transient molecular pathways at distinct post-exercise timepoints may not predict subsequent training adaptations.[34] As such, training interventions involving acute "recover low" paradigms are also warranted to better understand their effect on training outcomes.

High-intensity interval exercise is popular among recreational exercisers and athletes alike, as a time-efficient way to enhance many performance- and health-related outcomes.[22] Therefore, we included an extra HIIE session, performed 24 h later, to investigate how delayed carbohydrate intake affects next-day HIIE capacity. A novel finding was that delaying carbohydrate intake after the initial HIIE session reduced the time to task failure (by ~30%, or ~10 min) and increased perceptions of effort during the subsequent session; this occurred despite participants consuming the same total carbohydrate intake over the 24 h recovery period in each condition. Heart rate and blood lactate were similar between conditions, suggesting a similar physiological response to the exercise. Before commencing the HIIE-TF session there was a small, non-significant difference in mean muscle glycogen concentrations between conditions (~46 mmol/kg dm, Figure 1A). This may have been sufficient to compromise subsequent exercise capacity, as others have reported that graded reductions in pre-exercise muscle glycogen (~100 mmol/kg dm) impaired subsequent HIIE capacity (1-min intervals until exhaustion, at 80% $\dot{W}_{peak}$) by ~20–50% (≈8–26 min).[9] However, although 5/8 individuals exhibited greater muscle glycogen content before the HIIE-TF in their IC trial, muscle glycogen concentrations at +24 h did not correlate with the number of HIIE-TF intervals completed (Figure 5E). An important consideration is that muscle glycogen was determined in mixed muscle samples and that differential glycogen utilization within specific sub-cellular locations could have affected contractile function and capacity between trials. Indeed, intramyofibrillar glycogen depletion is correlated with impaired sarcoplasmic Ca^{2+} release rate[35] and endurance capacity,[36] and glycogen utilization rates among different subcellular locations (i.e., intramyofibrillar, intermyofibrillar, and subsarcolemmal) may differ during exercise.[36] However, our existing design limits our ability to further explore this, and the significant effect on next-day HIIE capacity and RPE, as our primary focus was to study the adaptive molecular responses within the 24-h recovery period. Others reported higher RPE scores during prolonged (3 h) fasted cycling, and that exercise-induced hypoglycaemia attenuated CNS activation and reduced voluntary force production, compared to ingesting carbohydrate *during* exercise.[37] However, in our study, plasma glucose was similar between trials throughout and unchanged from pre-HIIE between +3 and +24 h post-HIIE. Furthermore, given the 24-h recovery period, plus the short duration and high intensity of our HIIE cycling protocol, central fatigue mechanisms may not have been limiting factors.[38,39] Future studies would benefit from incorporating additional measures to elucidate potential factors affecting subsequent exercise capacity (e.g., substrate oxidation, plasma insulin and free fatty acid concentrations, liver glycogen concentrations) and additional markers of fatigue (e.g., non-subjective measures of central and peripheral fatigue). Nonetheless, our results show a clear reduction in next-day HIIE capacity, and increased perception of effort, when post-exercise carbohydrate intake was delayed by 3 h the day before. This has important implications for highly active populations who may routinely undergo periods of low carbohydrate availability throughout a training day and be required to train and perform at high intensities after limited recovery durations (≤24 h).

We acknowledge this study has some limitations. First, variation is evident both between and within participants

for pre-HIIE muscle glycogen concentrations (and in other molecular targets at other time points). This may have been improved by implementing a muscle glycogen-depleting protocol before a longer pre-trial dietary control period (e.g., 48 h), as demonstrated in other studies that manipulated carbohydrate intake around exercise,[9,15,29] to induce more similar pre-trial muscle glycogen concentrations. Second, while we adhered to the manufacturer's guidelines for the fractionation protocol, these fractions are crude cytoplasmic- and nuclear-enriched fractions, and we cannot discount potential effects of between-sample variability in fraction purity and/or contamination (as highlighted in a previous study[40]). We also acknowledge that only immunoblotting subcellular fractions may not identify dynamic protein translocation events between cellular compartments.[41] Thirdly, we could not sample additional muscle between 8 and 24 h post-HIIE; however, our recent work suggests that our chosen biopsy time points were appropriate to capture the peak changes in most of our target genes.[14] Finally, while we did not systematically record the efficiency of our participant blinding, conversations with participants after the study revealed they could not detect differences between the two beverages (*Diaz-Lara*, *written communication*, *2022*). Thus, it is unlikely their knowledge of the trial conditions provided a placebo/nocebo effect on subsequent HIIE capacity.

4 | MATERIALS AND METHODS

4.1 | Ethical approval

This study was approved by the Victoria University Human Research Ethics Committee (HRE19-011) and conducted in the Exercise Physiology Laboratory at Victoria University, Footscray Park Campus, in accordance with the Declaration of Helsinki (except pre-registration before recruitment). All participants were given comprehensive written and verbal information about the study, before providing written consent to participate.

4.2 | Participants

Participants were healthy (i.e., non-smokers, with no pre-existing medical conditions or musculoskeletal injuries) and recreationally active (i.e., exercising >150 min/week, not following a prescribed training program; "Tier 1"—see Mackay et al.[42]). Using published data of *PGC-1α* mRNA content in response to low carbohydrate availability,[6] an effect size was estimated for an anticipated difference between our conditions (Cohen's $d = 0.79$). A priori sample size was then estimated in G*Power (v3.1.9.2, Heinrich-Heine-University Düsseldorf, Germany), based on performing ANOVA analyses with five repeated measurements, and an assumed α of 0.05, and $1-\beta$ of 0.8. The estimated sample size ($n = 8$) was consistent with other studies that detected significant effects of carbohydrate restriction on mRNA content.[6,7] To account for attrition, 10 participants were recruited. Nine participants completed all procedures in both conditions (Table 1); one was unable to perform the HIIE-TF in both conditions; as such, data for this test is $n = 8$.

4.3 | Experimental design

In a repeated-measures, double-blind, cross-over design, participants completed two trials in a randomized and counterbalanced order. Each trial was separated by a 2-week washout period. During each trial, participants performed a high-intensity interval exercise (HIIE) session on a cycle ergometer. In the 12 h preceding each trial, and during the 24-h recovery period post-HIIE, participants consumed a standardized, high-carbohydrate diet (Table 2). However, during the initial 3 h post-HIIE, participants consumed either a carbohydrate drink (IC, providing 2.4 g/kg) or a taste-matched, non-energetic placebo, thereby delaying post-exercise carbohydrate intake (DC). Both drinks were consumed as small boluses every 20 min (IC provided 0.4 g/kg/bolus CHO). Skeletal muscle was sampled from the *vastus lateralis* immediately before and after HIIE, plus 3, 8, and 24 h post-HIIE. After the +24-h biopsy, participants repeated as many HIIE intervals as possible, to task failure (HIIE-TF), to assess HIIE capacity. The muscle samples were analyzed for glycogen concentration, whole-muscle mRNA content, plus protein phosphorylation and abundance in cytoplasmic- and nuclear-enriched fractions (Figure 6).

TABLE 1 Characteristics of the participants who completed this study ($n = 9$); data are mean ± standard deviation.

Age (years)	24 ± 2
Body mass (kg)	75 ± 11
Height (m)	1.80 ± 0.06
BMI (kg/m^2)	23.1 ± 2.6
Peak oxygen uptake, $\dot{V}O_{2peak}$ (mL/kg/min)	46.0 ± 6.2
Peak oxygen uptake, $\dot{V}O_{2peak}$ (L/min)	3.46 ± 0.55
Lactate threshold, $\dot{W}_{LT}$ (watts)	164 ± 49
Peak aerobic power, $\dot{W}_{peak}$ (watts)	240 ± 38
HIIE and HIIE-TF, intensity (watts)	225 ± 39
HIIE and HIIE-TF, intensity (% $\dot{W}_{peak}$)	94 ± 3

Abbreviations: HIIE, high intensity interval exercise; HIIE-TF, HIIE to task failure.

4.4 | Experimental procedures

Two weeks before their first trial, participants visited the lab on three occasions, each separated by 48–72 h. In the first visit, participants performed a graded exercise test (GXT). On the second and third visits, they were familiarized with the HIIE protocol. For at least 48 h before each visit, participants were instructed to avoid alcohol and caffeine consumption, and strenuous physical activity; this was confirmed verbally before each visit.

4.4.1 | Graded exercise test (GXT)

The GXT was performed on an electronically braked cycle ergometer (Excalibur, V2.0; Lode, Groningen, The Netherlands), to determine peak oxygen uptake ($\dot{V}O_{2peak}$), peak aerobic power ($\dot{w}_{peak}$), and the power at the second lactate threshold ($\dot{w}_{LT}$; modified D_{max}[43]). Antecubital blood (~1 mL) was sampled via intravenous cannulation at rest and in the last 15 seconds of each stage, then immediately analyzed in duplicate for lactate (YSI 2300 STAT Plus; YSI Inc., Yellow Springs, OH, USA). The $\dot{w}_{LT}$ was determined using https://shiny.fmattioni.me/lactate/. After a standardized warm-up (5 min cycling at 30 W), the test began at 50 W, and increased by 25 W every 4 min, until participants either reached task failure or could not maintain a cadence >60 rpm. Heart rate (Polar, Kempele, Finland) and ratings of perceived exertion (RPE,[44]) were recorded throughout. A metabolic analyzer (Quark Cardiopulmonary Exercise Testing, Cosmed, Italy) was calibrated immediately before each test as per the manufacturer's instructions. Breath-by-breath $\dot{V}O_2$ data were interpolated on a second-by-second basis, averaged into 5-second bins; $\dot{V}O_{2peak}$ was determined by as the highest 20-s rolling average.[43] $\dot{w}_{peak}$was defined using the below formula:

$$\dot{W}_{peak} = W_{com} + ((t / 240) \times 25)$$

where W_{com} is the last completed stage, and t is the time spent in the next stage in which task failure was reached.[45]

4.4.2 | High-intensity interval exercise and task failure sessions

HIIE

On an electromagnetically braked cycle ergometer (Velotron, Racer-Mate, Seattle, WA, USA), participants completed 10 × 2-min intervals (separated by 1-min rest periods), at 80% of the difference between $\dot{w}_{LT}$ and $\dot{w}_{peak}$ (mean ± SD, 225 ± 39 W, ≈94 ± 3% $\dot{w}_{peak}$), and a cadence of 90–100 rpm. Heart rate and RPE were recorded at the end of each interval. Intravenous blood was sampled at rest, and after the 1st, 5th, and 10th intervals, then analyzed immediately for lactate, as described. This acute HIIE protocol has previously been shown to reduce muscle glycogen by ~23%–50% post-HIIE ([19,46,47]), and increase mRNA and/or protein content of some metabolic and mitochondrial-related genes and proteins, in whole-muscle,[47] and subcellular fractions.[19] When implemented in various training interventions, we have shown this type of training improves cardiorespiratory fitness ($\dot{V}O_{2peak}$, $\dot{w}_{LT}$, and $\dot{w}_{peak}$[48,49]), endurance performance (20 km time trial,[48]), and mitochondrial characteristics (e.g., mitochondrial content and respiratory function[48]). Both $\dot{w}_{LT}$ and $\dot{w}_{peak}$ were used as anchors to prescribe exercise intensity, with the aim of inducing more consistent metabolic and physiological responses between participants (discussed in[50]). We also increased the relative exercise intensity from our previous studies [46,49], with the aim of inducing greater muscle glycogen utilization.

HIIE-TF

After the +24 h muscle biopsy, participants were instructed to complete as many HIIE intervals as possible (separated by 1-min rest periods) until task failure. The prescribed HIIE intensity was the same as in the first HIIE session. Blood lactate, heart rate, and RPE were recorded as described, with an additional measurement taken after each participant's final interval.

4.4.3 | Diet intervention

The diet (Table 2) was based on existing guidelines for endurance athletes[51] and provided ~7 g of carbohydrate (CHO) per kg of body mass per day (g/kg/d), ~2 g/kg/d of protein, and ~1 g/kg/d of fat. The diet consisted of pre-prepared meals, beverages, and snacks, provided by the researchers. One hour before each muscle biopsy, the participants did not consume any food or fluids. During the first 2-h post-HIIE, the participants immediately consumed either a commercially available CHO drink (Lucozade Original, UK) to provide 2.4 g/kg CHO, or a taste-matched, non-energetic placebo; both drinks were consumed as smaller boluses every 20 min (IC: 0.4 g/kg/bolus CHO). After the +3 h biopsy, all participants consumed the same standardized lunch. After the +8 h biopsy, all participants consumed the alternate beverage, as per their trial order (thereby balancing total daily CHO intake), plus an additional bolus of protein and fat. Participants were also given dinner and breakfast to consume at home (~11.5 and ~22.5 h post-HIIE, respectively; Figure 6; Table 2). All participants verbally

confirmed they had consumed all food and drink provided to them to consume at home.

4.4.4 | Blood collection and plasma analyses

At each biopsy timepoint, intravenous blood (~10 mL) was also sampled into vacutainers containing EDTA and serum separation gel (BD vacutainer, USA). Samples were centrifuged (1500 *g* for 15 min at 4°C), then serum and plasma aliquots were stored at −80°C for later analysis. Plasma glucose and lactate were determined using an automated analyzer as described. The researchers analyzing the blood (and muscle) samples were blind to the tissue sample conditions until all samples had been analyzed.

4.4.5 | Muscle biopsies

Participants were asked to avoid exercise, plus alcohol and caffeine consumption, for 48 h before all resting biopsies. *Vastus lateralis* muscle was sampled under local anesthesia (Xylocaine, 1%) and sterile conditions, using a suction-modified Bergström needle. New incisions were made for each timepoint (~1 cm below the previous site), and muscle was sampled from alternating legs between trials (i.e., Trial A = left, Trial B = right). All samples were immediately frozen in liquid nitrogen and stored at −80°C until analysis.

4.4.6 | Muscle glycogen assay

Frozen muscle samples (~10–15 mg) were freeze-dried for 24 h (Heto PowerDry LL1500 Freeze Dryer, Thermo Electron Corporation), dissected of non-muscle tissue and weighed, then homogenized in 250 μL of 2 M HCl for 2 h at 100°C (agitated gently every 20 min), then neutralized in 750 μL of 0.66 M NaOH. Muscle glycogen content was subsequently determined using an enzymatic assay with fluorometric detection[52] modified for a microplate reader.[53] Muscle glycogen values are expressed as mmol/kg dm (dry mass). All samples from the same participant were analyzed in duplicate on the same plate; intra-assay typical error of measurement (TEM) between duplicates was 12 mmol/kg dm, and the coefficient of variation (CV%) was 3.9%.

4.4.7 | Quantitative RT-PCR

Frozen muscle (~20 mg) was homogenized in TRIzol™ reagent (Life Technologies) and using an automated homogenizer (Tissue Lyser II, Qiagen). RNA extraction, quantification, sample integrity analysis, and reverse transcription (iScript™ Reverse transcription supermix, BioRad) were performed using our previously described methods.[54] The relative mRNA content of *β-HAD*, *CD36*, *CHCHD4*, *CPT1α*, *citrate synthase*, *cytochrome c*, *GLUT4*, *HKII*, *LPL*, *p53*, *PDK4*, *PFK*, *PGC-1α*, *PGC-1α1*, *PGC-1α4*, *PPARα*, *PPARβ*, *PPARγ*, *SDH*, *Tfam*, *TFEB*, and *UCP3*, as well as reference genes *18S*, *β2M*, *Cyclophilin*, *GAPDH*, *ACTB*, and *TBP*, were measured via qPCR (QuantStudio 7 Flex, Applied Biosystems, Foster City, CA). The primer sequences were previously published.[19,21] All reactions were performed in duplicate, on 384-well MicroAmp optical plates (Applied Biosystems™, ThermoFisher Scientific) using an epMotion M5073 automated pipetting system (Eppendorf AG, Hamburg, Germany). The relative content of target genes were quantified using the $2^{-\Delta\Delta CT}$ method[55]; values were normalized to the geometric mean[56] of the three most stable housekeeping genes (*ACTB*, *cyclophilin* and *18S*), which were determined using both *BestKeeper*[57] and *NormFinder* software.[58]

4.4.8 | Western blotting

Subcellular fractionation

Fractionation into cytoplasmic- and nuclear-enriched fractions was achieved using a commercially available kit (NE-PER™, ThermoFisher Scientific) according to the manufacturer's instructions. Briefly, frozen muscle (~40 mg) was washed in 250 μL of phosphate-buffered saline (PBS) containing a protease-phosphatase inhibitor cocktail diluted 1:600 (#5872, Cell Signaling Technology [CST], Danvers, MA, USA), and manually minced into small portions, then centrifuged for 10 min at 500 *g* and 4°C. The supernatant was discarded; using a hand-held homogenizer and vortex, the was pellet resuspended in ice-cold cytoplasmic extraction reagent (CER1) with added inhibitors (diluted 1:100), then placed on ice for 10 min. The CER2 buffer then was added to the sample, vortexed, and centrifuged for 10 min at 10 000 *g* and 4°C. The supernatant was collected as the crude cytoplasmic-enriched fraction. The pellet was washed five times in 250 μL of PBS (with inhibitors) and centrifuged for 1 min at 16 000 *g* between washes, to minimize cytoplasmic contamination. The nuclear proteins were extracted by manually resuspending the pellet in the nuclear extraction reagent (NER, with inhibitors [1:100]). Each sample was then vortexed for 15 s, every 10 min, four times, before being centrifuged for 20 min at 21 100 *g* and 4°C. The supernatant was collected as the nuclear-enriched fraction. The protein concentration of the fractionated lysates was then determined in triplicate using a commercial colorimetric Bradford assay (Protein Assay kit-II; Bio-Rad, Gladesville, NSW,

TABLE 2 Absolute and relative energy and macronutrient intakes prescribed for both conditions, consumed before the high-intensity interval exercise (HIIE) session and during the subsequent 24-h recovery (data are mean ± SD).

		Immediate carbohydrate trial							Delayed carbohydrate trial						
Meal	Time (relative to HIIE)	Energy (kcal)	Carbohydrate (g)	Carbohydrate (g/kg)	Protein (g)	Protein (g/kg)	Fat (g)	Fat (g/kg)	Energy (kcal)	Carbohydrate (g)	Carbohydrate (g/kg)	Protein (g)	Protein (g/kg)	Fat (g)	Fat (g/kg)
Pre-exercise dinner	−12 h	845 ± 88	146 ± 21	2.0 ± 0.1	25 ± 0.4	0.34 ± 0.04	18 ± 0.4	0.24 ± 0.03	845 ± 88	146 ± 21	2.0 ± 0.1	25 ± 0.4	0.34 ± 0.04	18 ± 0.4	0.24 ± 0.03
Pre-exercise breakfast	−1 h	610 ± 84	104 ± 18	1.4 ± 0.08	20 ± 1.4	0.27 ± 0.02	13 ± 1.0	0.17 ± 0.02	610 ± 84	104 ± 18	1.4 ± 0.08	20 ± 1.4	0.27 ± 0.02	13 ± 1.0	0.17 ± 0.02
Post-exercise drink/ placebo	+0 h	713 ± 104	178 ± 26	2.4 ± 0.0	0	0	0	0	0	0	0	0	0	0	0
Post-exercise lunch	+3 h	535 ± 34	96 ± 8.0	1.3 ± 0.1	22 ± 0.3	0.3 ± 0.04	7.0 ± 0.1	0.1 ± 0.01	535 ± 34	96 ± 8.0	1.3 ± 0.1	22 ± 0.3	0.3 ± 0.04	7.0 ± 0.1	0.1 ± 0.01
Post-exercise snack	+8 h	436 ± 11	27 ± 0.4	0.37 ± 0.05	18 ± 0.1	0.24 ± 0.03	28 ± 1.0	0.38 ± 0.04	436 ± 11	27 ± 0.4	0.37 ± 0.05	18 ± 0.1	0.24 ± 0.03	28 ± 1.0	0.38 ± 0.04
Post-exercise placebo/ drink	+8 h	0	0	0	0	0	0	0	713 ± 104	178 ± 26	2.4 ± 0.0	0	0	0	0
Post-exercise dinner	+11.5 h	834 ± 99	148 ± 24	2.0 ± 0.2	25 ± 0.5	0.34 ± 0.04	16 ± 0.7	0.22 ± 0.03	834 ± 99	148 ± 24	2.0 ± 0.2	25 ± 0.5	0.34 ± 0.04	16 ± 0.7	0.22 ± 0.03
Post-exercise breakfast	+22.5 h	610 ± 84	104 ± 18	1.4 ± 0.08	20 ± 1.4	0.27 ± 0.02	13 ± 1.0	0.17 ± 0.02	610 ± 84	104 ± 18	1.4 ± 0.08	20 ± 1.4	0.27 ± 0.02	13 ± 1.0	0.17 ± 0.02
Estimated totals during 24-h recovery post-HIIE		3128 ± 310	553 ± 71	7.4 ± 0.3	84 ± 2.2	1.1 ± 0.1	64 ± 2.3	0.9 ± 0.1	3128 ± 310	553 ± 71	7.4 ± 0.3	84 ± 2.2	1.1 ± 0.1	64 ± 2.3	0.9 ± 0.1
Estimated totals during 36-h experimental trial		3972 ± 392	699 ± 91	9.4 ± 0.4	110 ± 2.5	1.5 ± 0.2	82 ± 2.6	1.1 ± 0.1	3972 ± 392	699 ± 91	9.4 ± 0.4	110 ± 2.5	1.5 ± 0.2	82 ± 2.6	1.1 ± 0.1

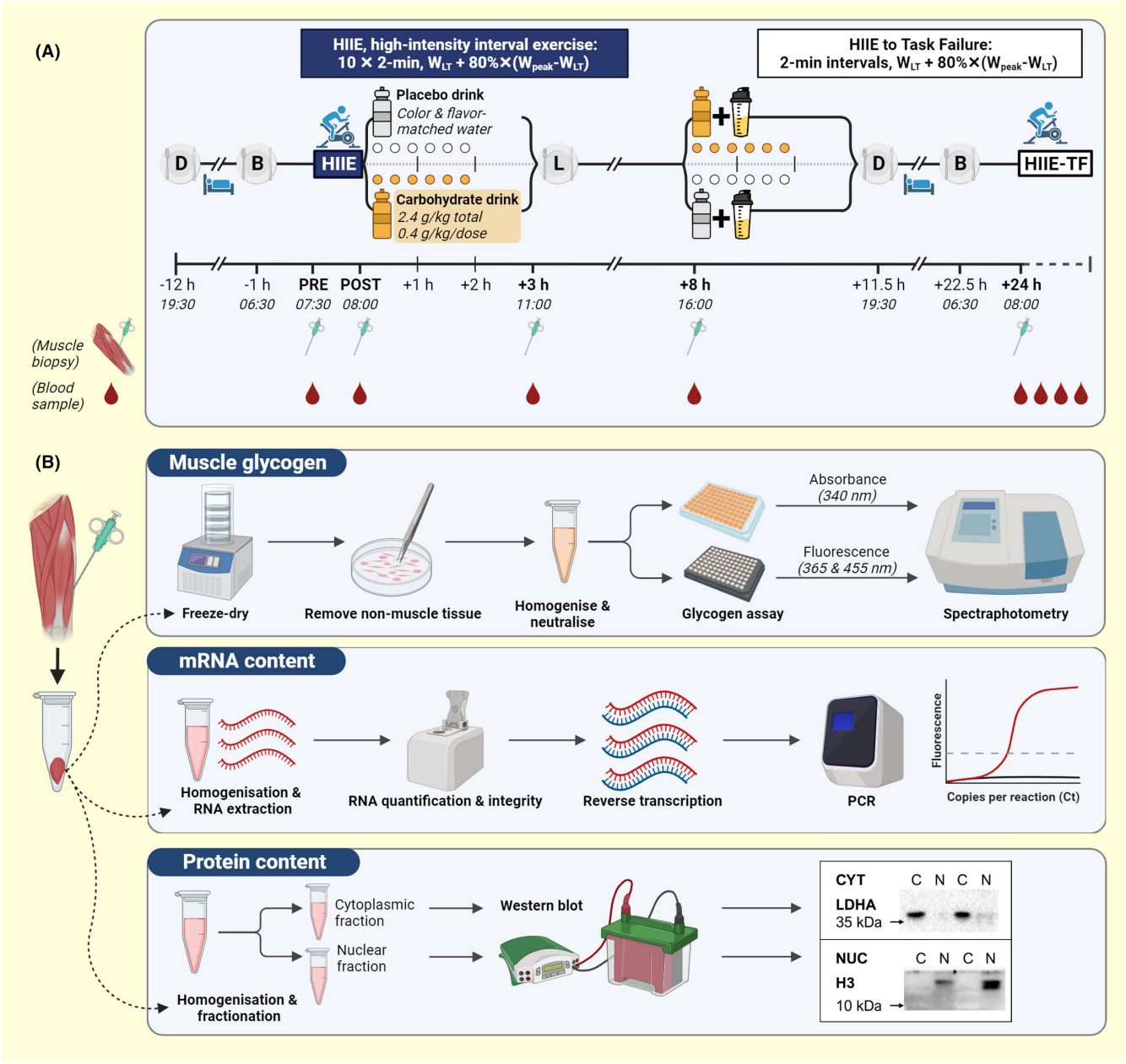

FIGURE 6 Schematic overview of (A) the experimental design and (B) muscle analyses. Muscle samples for protein content analysis were separated into cytoplasmic- (CYT) and nuclear-enriched (NUC) subfractions, lactate dehydrogenase A (LDHA), and Histone 3 (H3) were used as indicators of cytoplasmic "*C*" and nuclear "*N*" enrichment, respectively. *D*, dinner; *B*, breakfast; *L*, lunch; *g/kg*, grams per kg of body mass; $\dot{W}_{peak}$, peak aerobic power; $\dot{W}_{LT}$, power at the lactate threshold. *Created in BioRender. Bishop, D. (2024) BioRender.com/i88n308*
[Correction added on September 18, 2024, after first online publication: Acknowledgment for BioRender has been added.]

Australia). The fractionation process was confirmed by blotting both nuclear and cytoplasmic lysates in antibodies for predominantly nuclear (Histone 3, #4499, CST) and cytoplasmic proteins (LDHA, #2012, CST) (Figure 6).

Immunoblotting

The fractionated lysates were diluted to the same concentration (2 μg/μL) in 4× Laemmli buffer plus either NER or CER1 buffers. Samples were loaded in equal quantities of total protein (20–30 μg, target-dependant) into pre-cast 4–20% Criterion™ TGX Stain-Free™ gels (Bio-Rad). All samples per participant were loaded into the same gel, in adjacent wells. Every gel also contained at least three internal standards (comprised of equal quantities of every sample collected), loaded in increasing protein volumes. These standards were used to form a calibration curve of density versus total protein loaded. Proteins were separated via SDS-PAGE, then transferred onto polyvinylidene fluoride membranes (Bio-Rad) using a commercially available, semi-dry

transfer system (Trans-Blot® Turbo™ Transfer System, Bio-Rad). Membranes were then blocked with either 5% BSA for phospho-proteins (Sigma-Aldrich) or skimmed milk powder, diluted in Tris-buffered saline with 0.1% Tween-20 (TBST). Membranes were then washed in TBST (4 × 5 min vigorous rocking) and incubated overnight in primary antibodies at 4°C with gentle rocking. Primary antibodies (purchased from Cell Signaling Technology, Danvers, MA, USA), include p-ACC^{Ser79} (#3661), ACC (#3662), p-AMPKα (#2535), AMPKα (#2532), p-P38 MAPK$^{Thr180/Tyr182}$ (#9211), and P38 MAPK (#9212). The membranes were washed in TBST and incubated in horseradish peroxidase-conjugated secondary antibody (#7074, CST) diluted in either 5% BSA or skimmed milk powder. Membranes were then washed in TBST before being treated with a chemiluminescent solution (either Clarity™ Western ECL Substrate [Bio-Rad], or SuperSignal™ West Femto Maximum Sensitivity Substrate [ThermoFisher Scientific]). Images were taken using ChemiDoc™ MP imaging system (Bio-Rad), and proteins were quantified via densitometry (Image Lab 5.0 software, Bio-Rad). Total protein content from stain-free images was used for sample normalization.

4.5 | Statistical analyses

Raw data were checked for outliers, defined as ±3 SD outside the mean at each timepoint; all data fit within this threshold. Residuals were assessed for normality using the Shapiro–Wilk test. Analyses were conducted on log-transformed (log10) data (excluding HIIE-TF intervals, which were normally distributed, and RPE which is on a fixed scale). HIIE-TF intervals were analyzed using a paired t-test, and other HIIE-TF variables (i.e., heart rate, RPE, whole-blood lactate) were analyzed using a two-way repeated measures ANOVA, where the within factors were condition (*two levels*: immediate vs. delayed carbohydrate) and time (*four levels*: Rest/1st, 5th, 10th, and final interval). Where appropriate, a Greenhouse–Geisser correction was used to adjust for sphericity. Variables measured in recovery from HIIE (i.e., muscle glycogen, plasma glucose and lactate concentrations, mRNA content, protein phosphorylation, and abundance) were analyzed using a linear mixed model (*fixed effects*: condition [immediate vs. delayed carbohydrate] and time [pre, post, +3 h, +8 h, +24 h], *random effects*: participant identity). Western blot data contained one missing datapoint, due to insufficient muscle lysate for one participant, at +8 h in the IC condition (tissue sample sizes for each variable are noted in figure legends). Due to the number of statistical tests on HIIE recovery variables, *P* values of all main effects of condition, time, and interactions, were, respectively, collated and adjusted for multiple comparisons using the False Discover Rate (FDR) method. For all variables, where significant main effects of condition, time, or interactions remained evident after correction, post-hoc pairwise comparisons were conducted, and FDR correction was applied. Pearson's correlation was used to assess the association between +24 h muscle glycogen and HIIE-TF interval number. Exact *P* values are presented unless $p < 0.001$ or >0.999. T-tests and ANOVA were conducted in SPSS (v. 29), linear mixed models were conducted using R (*R* v4.4.0 and RStudio v2024.04.1), and Pearson's correlation and figures were generated in GraphPad Prism 10 (v. 10.2.3).

5 | CONCLUSION

We have shown that our HIIE session provided a sufficient exercise "dose" to increase the content of select proteins and mRNA related to mitochondrial biogenesis and metabolism over 24 h of recovery. Contrary to our hypotheses, delaying carbohydrate intake for 3 h post-exercise did not induce clear differences throughout recovery in post-exercise mRNA content, nor the phosphorylation or abundance of signaling proteins in different subcellular fractions, compared to immediately refueling with carbohydrates post-exercise. However, if post-exercise carbohydrate intake was delayed, the capacity to perform, and the perception of effort during HIIE 24 h later was significantly compromised. As such, for individuals required to perform repeated HIIE sessions after ≤24 h of recovery, our results support the notion to commence refueling immediately after exercise.

AUTHOR CONTRIBUTIONS

Javier Díaz-Lara: Conceptualization; methodology; investigation; project administration; writing – original draft; writing – review and editing. **Elizabeth Reisman:** Investigation; writing – review and editing. **Javier Botella:** Investigation; writing – review and editing. **Bianka Probert:** Funding acquisition; writing – review and editing. **Louise M. Burke:** Funding acquisition; writing – review and editing. **David J. Bishop:** Conceptualization; methodology; funding acquisition; supervision; writing – original draft; writing – review and editing. **Matthew J. Lee:** Formal analysis; investigation; methodology; visualization; writing – original draft; writing – review and editing; project administration.

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CONFLICT OF INTEREST STATEMENT

The authors declare no conflicts of interest.

DATA AVAILABILITY STATEMENT

The data that support the findings of this study are openly available in The Open Science Framework at https://osf.io/p3e8v/.

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REFERENCES

1. Impey SG, Hearris MA, Hammond KM, et al. Fuel for the work required: a theoretical framework for carbohydrate periodization and the glycogen threshold hypothesis. *Sports Med*. 2018;48(5):1031-1048.
2. Mansueto G, Armani A, Viscomi C, et al. Transcription factor EB controls metabolic flexibility during exercise. *Cell Metab*. 2017;25(1):182-196.
3. Martina JA, Chen Y, Gucek M, Puertollano R. MTORC1 functions as a transcriptional regulator of autophagy by preventing nuclear transport of TFEB. *Autophagy*. 2012;8(6):903-914.
4. Settembre C, di Malta C, Polito VA, et al. TFEB links autophagy to lysosomal biogenesis. *Science*. 2011;332(6036):1429-1433.
5. Perry CG, Lally J, Holloway GP, Heigenhauser GJ, Bonen A, Spriet LL. Repeated transient mRNA bursts precede increases in transcriptional and mitochondrial proteins during training in human skeletal muscle. *J Physiol*. 2010;588(Pt 23):4795-4810.
6. Pilegaard H, Osada T, Andersen LT, Helge JW, Saltin B, Neufer PD. Substrate availability and transcriptional regulation of metabolic genes in human skeletal muscle during recovery from exercise. *Metabolism*. 2005;54(8):1048-1055.
7. Cluberton LJ, McGee SL, Murphy RM, Hargreaves M. Effect of carbohydrate ingestion on exercise-induced alterations in metabolic gene expression. *J Appl Physiol*. 2005;99(4):1359-1363.
8. Yeo WK, Paton CD, Garnham AP, Burke LM, Carey AL, Hawley JA. Skeletal muscle adaptation and performance responses to once a day versus twice every second day endurance training regimens. *J Appl Physiol*. 2008;105(5):1462-1470.
9. Hearris MA, Hammond KM, Seaborne RA, et al. Graded reductions in preexercise muscle glycogen impair exercise capacity but do not augment skeletal muscle cell signaling: implications for CHO periodization. *J Appl Physiol*. 2019;126(6):1587-1597.
10. Parkin JA, Carey MF, Martin IK, Stojanovska L, Febbraio MA. Muscle glycogen storage following prolonged exercise: effect of timing of ingestion of high glycemic index food. *Med Sci Sports Exerc*. 1997;29(2):220-224.
11. Jensen L, Gejl KD, Ørtenblad N, et al. Carbohydrate restricted recovery from long term endurance exercise does not affect gene responses involved in mitochondrial biogenesis in highly trained athletes. *Physiol Rep*. 2015;3(2):e12184.
12. Mathai AS, Bonen A, Benton CR, Robinson DL, Graham TE. Rapid exercise-induced changes in PGC-1alpha mRNA and protein in human skeletal muscle. *J Appl Physiol*. 2008;105(4):1098-1105.
13. Ramos C, Cheng AJ, Kamandulis S, et al. Carbohydrate restriction following strenuous glycogen-depleting exercise does not potentiate the acute molecular response associated with mitochondrial biogenesis in human skeletal muscle. *Eur J Appl Physiol*. 2021;121(4):1219-1232.
14. Kuang J, McGinley C, Lee MJC, Saner NJ, Garnham A, Bishop DJ. Interpretation of exercise-induced changes in human skeletal muscle mRNA expression depends on the timing of the post-exercise biopsies. *PeerJ*. 2022;10:e12856.
15. Hearris MA, Owens DJ, Strauss JA, et al. Graded reductions in pre-exercise glycogen concentration do not augment exercise-induced nuclear AMPK and PGC-1α protein content in human muscle. *Exp Physiol*. 2020;105(11):1882-1894.
16. McGee SL, Howlett KF, Starkie RL, Cameron-Smith D, Kemp BE, Hargreaves M. Exercise increases nuclear AMPK alpha2 in human skeletal muscle. *Diabetes*. 2003;52(4):926-928.
17. Granata C, Oliveira RSF, Little JP, Renner K, Bishop DJ. Sprint-interval but not continuous exercise increases PGC-1α protein content and p53 phosphorylation in nuclear fractions of human skeletal muscle. *Sci Rep*. 2017;7:44227.
18. Taylor DF, Bishop DJ. Transcription factor movement and exercise-induced mitochondrial biogenesis in human skeletal muscle: current knowledge and future perspectives. *Int J Mol Sci*. 2022;23(3):1517.
19. Andrade-Souza VA, Ghiarone T, Sansonio A, et al. Exercise twice-a-day potentiates markers of mitochondrial biogenesis in men. *FASEB J*. 2020;34(1):1602-1619.
20. Steinberg GR, Watt MJ, McGee SL, et al. Reduced glycogen availability is associated with increased AMPKalpha2 activity, nuclear AMPKalpha2 protein abundance, and GLUT4 mRNA expression in contracting human skeletal muscle. *Appl Physiol Nutr Metab*. 2006;31(3):302-312.
21. Pilegaard H, Saltin B, Neufer PD. Exercise induces transient transcriptional activation of the PGC-1α gene in human skeletal muscle. *J Physiol*. 2003;546(3):851-858.
22. Gibala MJ, Jones AM. Physiological and performance adaptations to high-intensity interval training. *Nestle Nutr Inst Workshop Ser*. 2013;76:51-60.
23. Cochran AJ, Little JP, Tarnopolsky MA, Gibala MJ. Carbohydrate feeding during recovery alters the skeletal muscle metabolic response to repeated sessions of high-intensity interval exercise in humans. *J Appl Physiol*. 2010;108(3):628-636.
24. Betts JA, Williams C. Short-term recovery from prolonged exercise: exploring the potential for protein ingestion to accentuate the benefits of carbohydrate supplements. *Sports Med*. 2010;40(11):941-959.
25. Price TB, Laurent D, Petersen KF, Rothman DL, Shulman GI. Glycogen loading alters muscle glycogen resynthesis after exercise. *J Appl Physiol*. 2000;88(2):698-704.
26. Zachwieja JJ, Costill DL, Pascoe DD, Robergs RA, Fink WJ. Influence of muscle glycogen depletion on the rate of resynthesis. *Med Sci Sports Exerc*. 1991;23(1):44-48.
27. Lai YC, Stuenæs JT, Kuo CH, Jensen J. Glycogen content and contraction regulate glycogen synthase phosphorylation and affinity for UDP-glucose in rat skeletal muscles. *Am J Physiol Endocrinol Metab*. 2007;293(6):E1622-E1629.
28. Akerstrom TC, Birk JB, Klein DK, et al. Oral glucose ingestion attenuates exercise-induced activation of 5′-AMP-activated

protein kinase in human skeletal muscle. *Biochem Biophys Res Commun*. 2006;342(3):949-955.
29. Fell JM, Hearris MA, Ellis DG, et al. Carbohydrate improves exercise capacity but does not affect subcellular lipid droplet morphology, AMPK and p53 signalling in human skeletal muscle. *J Physiol*. 2021;599(11):2823-2849.
30. Lee-Young RS, Palmer MJ, Linden KC, et al. Carbohydrate ingestion does not alter skeletal muscle AMPK signaling during exercise in humans. *Am J Physiol Endocrinol Metab*. 2006;291(3):E566-E573.
31. Hammond KM, Sale C, Fraser W, et al. Post-exercise carbohydrate and energy availability induce independent effects on skeletal muscle cell signalling and bone turnover: implications for training adaptation. *J Physiol*. 2019;597(18):4779-4796.
32. Rothschild JA, Islam H, Bishop DJ, Kilding AE, Stewart T, Plews DJ. Factors influencing AMPK activation during cycling exercise: a pooled analysis and meta-regression. *Sports Med*. 2021;52:1273-1294.
33. Bishop DJ, Hoffman NJ, Taylor DF, Saner NJ, Lee MJC, Hawley JA. Discordant skeletal muscle gene and protein responses to exercise. *Trends Biochem Sci*. 2023;48(11):927-936.
34. Cochran AJ, Percival ME, Tricarico S, Little JP, Cermak N, Gillen JB. Intermittent and continuous high-intensity exercise training induce similar acute but different chronic muscle adaptations. *Exp Physiol*. 2014;99(5):782-791.
35. Ørtenblad N, Nielsen J, Saltin B, Holmberg HC. Role of glycogen availability in sarcoplasmic reticulum Ca2+ kinetics in human skeletal muscle. *J Physiol*. 2011;589(Pt 3):711-725.
36. Jensen R, Ørtenblad N, Stausholm MLH, et al. Heterogeneity in subcellular muscle glycogen utilisation during exercise impacts endurance capacity in men. *J Physiol*. 2020;598(19):4271-4292.
37. Nybo L. CNS fatigue and prolonged exercise: effect of glucose supplementation. *Med Sci Sports Exerc*. 2003;35(4):589-594.
38. Carroll TJ, Taylor JL, Gandevia SC. Recovery of central and peripheral neuromuscular fatigue after exercise. *J Appl Physiol*. 2017;122(5):1068-1076.
39. Tomazin K, Morin JB, Millet GY. Etiology of neuromuscular fatigue after repeated sprints depends on exercise modality. *Int J Sports Physiol Perform*. 2017;12(7):878-885.
40. Granata C, Caruana NJ, Botella J, et al. High-intensity training induces non-stoichiometric changes in the mitochondrial proteome of human skeletal muscle without reorganisation of respiratory chain content. *Nat Commun*. 2021;12(1):7056.
41. Granata C, Oliveira RSF, Little JP, Bishop DJ. Forty high-intensity interval training sessions blunt exercise-induced changes in the nuclear protein content of PGC-1α and p53 in human skeletal muscle. *Am J Physiol Endocrinol Metab*. 2020;318(2):E224-E236.
42. McKay AKA, Stellingwerff T, Smith ES, et al. Defining training and performance caliber: a participant classification framework. *Int J Sports Physiol Perform*. 2022;17(2):317-331.
43. Jamnick NA, Botella J, Pyne DB, Bishop DJ. Manipulating graded exercise test variables affects the validity of the lactate threshold and VO_{2peak}. *PLoS One*. 2018;13(7):e0199794.
44. Borg G. Perceived exertion as an indicator of somatic stress. *Scand J Rehabil Med*. 1970;2(2):92-98.
45. Kuipers H, Verstappen F, Keizer H, Geurten P, van Kranenburg G. Variability of aerobic performance in the laboratory and its physiologic correlates. *Int J Sports Med*. 1985;6(4):197-201.
46. Fyfe JJ, Bishop DJ, Zacharewicz E, Russell AP, Stepto NK. Concurrent exercise incorporating high-intensity interval or continuous training modulates mTORC1 signaling and microRNA expression in human skeletal muscle. *Am J Physiol Regul Integr Comp Physiol*. 2016;310(11):R1297-R1311.
47. Lee MJ, Caruana NJ, Saner NJ, et al. Resistance-only and concurrent exercise induce similar myofibrillar protein synthesis rates and associated molecular responses in moderately active men before and after training. *FASEB J*. 2024;38(1):e23392.
48. Granata C, Oliveira RSF, Little JP, Renner K, Bishop DJ. Mitochondrial adaptations to high-volume exercise training are rapidly reversed after a reduction in training volume in human skeletal muscle. *FASEB J*. 2016;30(10):3413-3423.
49. Lee MJ, Ballantyne JK, Chagolla J, et al. Order of same-day concurrent training influences some indices of power development, but not strength, lean mass, or aerobic fitness in healthy, moderately-active men after 9 weeks of training. *PLoS One*. 2020;15(5):e0233134.
50. Jamnick NA, Pettitt RW, Granata C, Pyne DB, Bishop DJ. An examination and critique of current methods to determine exercise intensity. *Sports Med*. 2020;50:1729-1756.
51. Thomas DT, Erdman KA, Burke LM. American College of Sports Medicine joint position Statement. Nutrition and athletic performance. *Med Sci Sports Exerc*. 2016;48(3):543-568.
52. Nahorski SR, Rogers KJ. An enzymic fluorometric micro method for determination of glycoen. *Anal Biochem*. 1972;49(2):492-497.
53. Betik AC, Aguila J, McConell GK, McAinch AJ, Mathai ML. Tocotrienols and whey protein isolates substantially increase exercise endurance capacity in diet -induced obese male Sprague-Dawley rats. *PLoS One*. 2016;11(4):e0152562.
54. Kuang J, Yan X, Genders AJ, Granata C, Bishop DJ. An overview of technical considerations when using quantitative real-time PCR analysis of gene expression in human exercise research. *PLoS One*. 2018;13(5):e0196438.
55. Schmittgen TD, Livak KJ. Analyzing real-time PCR data by the comparative C(T) method. *Nat Protoc*. 2008;3(6):1101-1108.
56. Vandesompele J, de Preter K, Pattyn F, et al. Accurate normalization of real-time quantitative RT-PCR data by geometric averaging of multiple internal control genes. *Genome Biol*. 2002;3(7):Research0034.
57. Pfaffl MW, Tichopad A, Prgomet C, Neuvians TP. Determination of stable housekeeping genes, differentially regulated target genes and sample integrity: BestKeeper—excel-based tool using pair-wise correlations. *Biotechnol Lett*. 2004;26(6):509-515.
58. Andersen CL, Jensen JL, Orntoft TF. Normalization of real-time quantitative reverse transcription-PCR data: a model-based variance estimation approach to identify genes suited for normalization, applied to bladder and colon cancer data sets. *Cancer Res*. 2004;64(15):5245-5250.

SUPPORTING INFORMATION

Additional supporting information can be found online in the Supporting Information section at the end of this article.

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